Ultraviolet Light-Induced Regulation of Transcription and Translation, COX-2
Expression and Noncanonical NF-κB Activation

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This dissertation titled

Ultraviolet Light-Induced Regulation of Transcription and Translation, COX-2

Expression and Noncanonical NF-κB Activation

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ABSTRACT

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Director of Dissertation: Shiyong Wu

NF-κB is a transcription factor that controls expression of genes involved in the immune and inflammatory responses as well as being a key component in the onset of cancers. In this study we provided evidence that MSK1 is responsible for a non-canonical late-phase activation of NF-κB upon UVB irradiation. Our data demonstrated that following UV irradiation, MSK1 is activated via phosphorylation at the 24 h time point coinciding with translocation of NF-κB into the nucleus. Investigations into the signaling pathways upstream of MSK1 through the use of specific inhibitors for mitogen-activated protein kinase and p38 revealed that both kinases are required for full phosphorylation during the late-phase (24 h), while p38 is paramount for phosphorylation during the early-phase (6 h). Electromobility shift assays (EMSA) showed that inhibition of MSK1 resulted in a marked reduction in NF-κB binding affinity without altering the nuclear translocation of NF-κB. Supershift EMSA implicate that the p65, but not p50, isoform of NF-κB is involved in late-phase activation in response to UVB irradiation. Together, the results of these studies shed light onto a novel pathway of MSK1 mediated late-phase activation of NF-κB in response to UV irradiation.

Exposure to UV induces a prolonged expression of COX-2 via transcriptional activation that is due in part to an increase in NF-κB activity.
While transcriptional regulation of COX-2 expression has been well studied, the role of translational regulation of COX-2 synthesis upon UV-irradiation is not yet clear. In this study, we show that the phosphorylation of the alpha subunit of eIF2α plays an important role in the regulation of COX-2 expression after UV-irradiation. Our data demonstrates 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 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 we previously reported (Wu et. Al, J. Bio. Chem, 2004). Additionally, the translational regulation of COX-2 binding protein TIAR expression is reduced in MEF^{S/S} cells but not in MEF^{A/A} cells at 24 hours post-UV. These results suggest that translation initiation plays a role in a complex and dynamic regulation of COX-2 expression. Based on our results we proposed a novel eIF2α phosphorylation-centered network for the regulation of COX-2 expression after UV irradiation.
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<td>cNOS</td>
<td>Constitutive Nitric Oxide Synthase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element Binding Protein</td>
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<tr>
<td>eIF2α</td>
<td>Eukaryotic Initiation Factor 2-Alpha</td>
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<tr>
<td>EIF2AK</td>
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<td>eNOS</td>
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<td>ERK</td>
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</tr>
<tr>
<td>H3</td>
<td>Histone 3</td>
</tr>
<tr>
<td>HuR</td>
<td>Human ELAV-like Protein</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB Kinase</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<td>MEK</td>
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<td>MSK</td>
<td>Mitogen- and Stress-Activated Protein Kinase</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
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<td>nNOS</td>
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<td>Poly ADP Ribose Polymerase</td>
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<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
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<tr>
<td>TIAR</td>
<td>T-cell-restricted Intracellular 1-related Protein</td>
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<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
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<tr>
<td>XO</td>
<td>Xanthine Oxidase</td>
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Objective: The goal of this project was to analyze cellular responses to UVR and to determine the dynamics and specific pathways responsible for regulation of COX-2 expression and noncanonical late-phase NF-κB activation.

UV light is divided into three categories: UVA (400-320 nm); UVB (320-280 nm) and UVC (280-100 nm). All three types of UV light are present in solar radiation, however the ozone layer acts to filter out essentially all UVC radiation. As with any potentially harmful substance or stimulus, it is the level of exposure that makes the toxin. Although required for synthesis of vitamin D \(^1\), excessive exposure to UV light has been reported to be responsible for approximately 90% of non-melanoma skin cancer cases \(^2\). Skin cancer is the most prevalent form of cancer with more cases being diagnosed annually than colon, lung, breast and prostate cancers combined \(^3\). In 2004 alone, over one billion dollars was spent for the treatment of skin cancers \(^4\). Because of the high occurrence of skin cancers, there has been much interest in research pertaining to the mechanisms involved in its development as well as for the development of new targeted therapeutics for its treatment.

Cellular responses to UV-induced stress span multiple signaling pathways due to the nature of UV-induced damage. Absorption of UV light directly by DNA can lead to the dimerization of adjacent thymine nucleotides leading to errors in replication \(^5\). Additionally, UV light also acts indirectly through the generation of
ROS that can induce single strand breaks in DNA and oxidize both proteins and lipids. As a result of the varying types and degrees of damage, cellular systems have developed several pathways for repair or induction of cell death. Key to many of these responses is regulation of protein expression via transcriptional and translational regulation. As such, this report will seek to investigate the mechanisms behind UV-induced-ROS mediated regulation of transcription and translation, as well as provide results from studies on the regulation of COX-2 expression and noncanonical NF-κB activation.

Of particular interest to our research group is the transcription factor NF-κB. The NF-κB family is an important group of transcription factor related proteins that was first identified as a protein capable of binding the enhancer region of κ immunoglobulin light chain in 1986. Since then, the mammalian NF-κB family has been shown to consist of the members NFKB1 (p50/p105), NFKB2 (p52, p100), cRel, RelA (p65) and RelB. These members all share a sequence of approximately 300 amino acids that is referred to as the Rel homology domain (RHD). The RHD is responsible for dimer formation as well as for the binding of NF-κB to the major groove of the Rel sequence of DNA. As a transcription factor, NF-κB plays a role in regulating over 150 genes that are involved in biological functions ranging from the immune response, to the inflammatory response and to cancer.

NF-κB is most commonly found in a homo/heterodimer configuration. Heterodimers can form between any of the members, however RelB can only form heterodimers with p50 and p52/p100. The p105 and p100 isoforms exist
as precursors to p50 and p52, respectively. The unprocessed form acts as an inhibitor to its transcription factor function that must first be removed before it can be activated. Activation is the result of ubiquitin-dependant proteasomal degradation of the inhibitory section of the peptide chain. Once this section is removed, p50 and p52 are capable of dimerization. Although all dimmers play a role in regulation of gene expression, the most abundant dimer form of the NF-κB family is the p65-p50 heterodimer.

In addition to the NF-κB family, there also exists a family of inhibitors of NF-κBs. This family consists of IκBα, IκBβ, IκBε, IκBΥ and BCL3 which all share similarity in the presence of five to seven ankyrin repeats which allow them to bind to the dimerization site of NF-κB. In the canonical pathway of NF-κB activation, NF-κB remains dormant in the cytoplasm bound to IκB, which masks a nuclear localization signal (NLS). In order for activation of NF-κB to occur, IκB must be phosphorylated and degraded. Phosphorylation of IκB is mediated by the IKK complex. This complex consists of IKKα, IKKβ and IKKγ (NEMO). IKKα and IKKβ are the kinase subunits of the complex while IKKγ serves as a regulatory subunit. Upon phosphorylation of IκB via the IKK complex, IκB is targeted by ubiquitin ligases and is polyubiquitinated. Once polyubiquitinated, IκB is proteasomally degraded which exposes the NLS on NF-κB allowing it to translocate to the nucleus of the cell and mediate regulation of gene expression. Activation of NF-κB can also be modified by phosphorylation. NF-κB contains several phosphorylation sites that include: serine-536, serine-276, serine-311 and threonine-254. Phosphorylation at one or a combination these sites has
been show to increase binding affinity to DNA, thus enhancing NF-κB activation \(^{16}\).

Although the canonical pathway of NF-κB activation is well known, this is not the sole pathway that can result in activation. A previous report by our lab investigating the late-phase activation of NF-κB in response to UVC irradiation has shown that the canonical pathway of activation is not followed during this time point \(^{21}\). In this previous report, the effects of IκB reduction during late-phase activation were analyzed. Two mouse embryo fibroblast cell lines were used in the study. One cell line contained wild type eIF2-α, designated MEF\(^{S/S}\), while the second cell line contained a mutation of serine-51 to alanine that resulted in a non-phosphorylatable eIF2-α, and was designated MEF\(^{A/A}\). eIF2-α is phosphorylated at serine-51 in response to UV, leading to a halt in global protein synthesis\(^{21-22}\). This reduction in protein synthesis leads to a decrease in IκB levels during the late-phase activation of NF-κB due to the short half-life’s of 140 minutes for complexed IκB and 40 minutes for free IκB \(^{23}\). Following irradiation in the MEF\(^{A/A}\) cell line there was no substantial reduction in the level of IκB present. Interestingly, late-phase activation via phosphorylation at serine-276 of NF-κB was not effected in the MEF\(^{A/A}\) cell line in comparison to the MEF\(^{S/S}\) cell line. Additionally, the use of ubiquitination and proteasomal inhibitors failed to rescue decreased levels of IκB in the MEF\(^{S/S}\) cell line during the late-phase of activation. These results suggested that a non-canonical IκB-independent pathway is at work during the late-phase of NF-κB activation\(^{21}\).
In order to explain this observation, an alternate pathway of activation was proposed based on phosphorylation at serine-276 of NF-κB. Since phosphorylation at this site was only observed in the nucleus of treated cells, a kinase which is located in the nucleus of the cell was required, as well as one known to target phosphorylation of serine-276. Of the kinases capable of serine-276 phosphorylation, only MSK1 was reported to be active solely in the nucleus of the cell. The studies contained within this current report confirmed MSK1 is indeed responsible for transactivation of NF-κB during the late-phase response. Additional investigations into the upstream activators of MSK1 revealed a dynamic time-dependent requirement for the kinases p38 and ERK. Combined, the results from these studies revealed a novel and dynamic signaling circuit for activation of NF-κB.

In addition to transcriptional regulation through factors such as NF-κB, UV irradiation also elicits a cellular response in the form of translational control. In this report we investigated the mechanisms behind translational control of COX-2, the enzyme responsible for catalyzing the synthesis of prostaglandin from arachnoid acid. Data revealed that global translational inhibition via eIF2α phosphorylation has a two-tier effect on COX-2 expression. While it reduces the translation efficiency of COX-2 mRNA at both early and late stages of UVC-, it also reduces the expression of the COX-2 mRNA binding protein TIAR that has in inhibitory effect on mRNA translation, thus promoting continued translation of COX-2 in the late stages of UVC-irradiation.
CHAPTER 2: COORDINATIVE AND DYNAMIC REGULATION OF TRANSLATION AND TRANSCRIPTION BY NITRIC OXIDE AND SUPEROXIDE UPON ULTRAVIOLET LIGHT IRRADIATION

2.1 Summary

Exposure to UV leads to a rapid elevation of NO• and superoxide $\text{O}_2$•− in irradiated cells. NO• competes with SOD for $\text{O}_2$•− to form $\text{ONOO}^-$, which increases the oxidative stress and reduces NO• bioavailability. The balance between NO• and $\text{ONOO}^-$ plays significant roles in regulation of gene expression at both the translational and transcriptional levels. In mammalian cells, three NOSs – nNOS (NOS1), iNOS (NOS2) and eNOS (NOS3) catalyze L-arginine to generate NO• in response to UV-irradiation. Depending on the type of cells and physical properties of the irradiation, the patterns of NO• and $\text{ONOO}^-$ productions can be very dynamic. The production of NO• and $\text{ONOO}^-$ leads to the phosphorylation of the alpha subunit of eIF2α by two EIF2AKs – PERK (EIF2AK3) and GCN2 (EIF2AK4). The activation of EIF2AKs and phosphorylation of eIF2α inhibit global protein synthesis, which leads to the activation of transcription factor NF-κB. Additionally, production of NO• and $\text{ONOO}^-$ generates oxidative and nitrosative stresses that induce signaling pathways that further modulate transcriptional activation. The combined effect of translational and transcriptional regulation ultimately determines aspects of cell physiology, such as growth and death.
2.2 The dynamics and mechanisms for generation of NO\(^{-}\), O\(_2\)\(^{-}\) and ONOO\(^{-}\) by UVR

2.2.1 Introduction

UVR is divided into three categories: UVA (400-320 nm); UVB (320-280 nm) and UVC (280-100 nm). While all three categories of UVR have been shown to be capable of inducing the production of NO\(^{-}\) and O\(_2\)\(^{-}\) in cells, the mechanisms and dynamics in the generation of these molecules could be very different depending on the wavelength, dose and dose-rate of UVR. UVA and UVB exist in solar light that reaches the earth’s surface and are used for studying UV-related skin damage \(^{25}\) and photo relaxation \(^{26}\). UVC is a germicidal light that is often used for studying DNA damage \(^{27}\). Due to the properties of electromagnetic radiation, the UV dose and dose rate used in generation of NO\(^{-}\) vary between categories of UV light. The efficiency for UV-induced NO\(^{-}\) production appears more correlated to dose rate and energy of photons than to total dose received by cells. Based on Planck’s Equation: 

\[
E = \frac{hc}{\lambda}
\]

(h: Planck’s constant; c: speed of light; \(\lambda\): wavelength), energy per photon ranges from 3.10-3.87 eV for UVA, 3.87-4.43 eV for UVB and 4.43-12.4 eV for UVC. Much more total energy of UVA is needed to induce NO\(^{-}\) production than total energy of UVB or UVC is needed. Common experimental dose values for UVA can range
between 1000 and 40,000 mJ/cm$^2$ while UVB can range from 5-200 mJ/cm$^2$ and UVC 1-10 mJ/cm$^2$.  

2.2.2 The dynamics of NO$^\cdot$ production after UV-irradiation

NO$^\cdot$ generation upon treatment with UVR varies between time points and cell type. An increase in NO$^\cdot$ release can be observed almost immediately following ultraviolet A, B or C irradiation. NO$^\cdot$ is released at an initial rate of 0.117 nM per sec in endothelial cells at 5 sec after UVA-irradiation. An increase of [NO$^\cdot$] is recorded at 20 sec and reached a peak at 40 sec in keratinocytes after UVB-irradiation (30 mJ/cm$^2$). The UVB-induced elevation of NO$^\cdot$ is dose-dependent in a range of 0-100 mJ/cm$^2$. UVC also induces NO$^\cdot$ release from microvessels isolated from rat skin by approximately 35% or 55% within 5 or 10 min respectively. NO$^\cdot$ generation continues to increase in a time-dependant fashion within the first 2 hours post irradiation leading to a 3-fold maximum increase. Maximal prolonged NO$^\cdot$ generation occurs between 17 and 48 hours post irradiation based on cell type and can range from 1.5-fold to 3-fold. However, by 6 hours post irradiation, NO$^\cdot$ generation has returned to near basal levels. Varying dose rates are required to elicit similar responses within various cell lines. Human keratinocytes exhibit a maximal NO$^\cdot$ release of 3-fold with a treatment of 120 mJ/cm$^2$ UVB while human endothelium cells require only 60 mJ/cm$^2$ UVB to generate a similar NO$^\cdot$ release. Treatments over these thresholds result in similar decreases in generation of NO$^\cdot$ within both cell lines.
2.2.3 The mechanisms for UV-induced elevation of NO$^\cdot$

The initial release of NO$^\cdot$ upon treatment with UVR has been found to be dependant on the activation of cNOS and on photosensitive storage molecules of NO$^\cdot$ $^{25a, 31a, 33}$. The UVR-induced production of NO$^\cdot$ is dependent on Ca$^{2+}$/calmodulin and purified eNOS was shown to produce 3 times more NO$^\cdot$ after UVR treatment. However, inhibition of NOS is not able to totally prevent NO$^\cdot$ production indicating the existence of other sources for NO$^\cdot$ production $^{25a, b}$. High performance liquid chromatography analysis of GSNO stores within mouse aortic tissues post irradiation have shown a significant decrease in concentration after UVR, correlating with an increase in NO$^\cdot$ levels and photorelaxation. These tissues also exhibit an increase in GSNO concentration when cells are treated with a solution containing 2 mM NO$^\cdot$. However, diabetic tissues with no detectable levels of GSNO still exhibit relaxation when exposed to UVR, suggesting a role for other S-nitrosothiols or other NO$^\cdot$ storage molecules $^{33b}$. Additionally, small molecules, such as $N^G$-nitro-L-arginine and NaNO$_2$ have also exhibited the ability to increase photorelaxation within rabbit corpus cavernosum. These molecules, when in solution, were shown to release NO$^\cdot$ upon stimulation with UVA $^{33a}$. Another study utilizing UVA found that nitrite stores give a low level of NO$^\cdot$ release while S-nitrosothiols are the predominant storage species for NO$^\cdot$ release. This study also found that nitrates and nitrogen-bound S-nitroso compounds had little to no effect on NO$^\cdot$ release $^{33c}$. The prolonged elevation of NO$^\cdot$ is associated with an increase in expression and activation of both cNOS
and iNOS. Control of iNOS expression is the result of both transcriptional and translational regulation following irradiation.

2.2.4 The generation of O$_2^-$ by UVR

In addition to the generation of NO$^-$ following UVR exposure, O$_2^-$ is also generated from several potential sources in cells including water photolysis, NAD(P)H oxidase, cNOS and XO. XO is one of the major sources for O$_2^-$ generation in irradiated cells. Ultraviolet radiation stimulates XO, which converts hypoxanthine to xanthine and is responsible for the production of O$_2^-$ as a byproduct. O$_2^-$ generation following UVR treatment is dependent on dose and can result in a 5 to 15-fold increase upon treatment with 100 mJ/cm$^2$ UVB depending on cell type. Release of O$_2^-$ has been shown to directly correlate in a near one-to-one fashion with XO activation. Additionally, inhibitors of XO, such as allopurinol and oxypurinol, are capable of decreasing O$_2^-$ release between 50% and 80%, which implicates XO as the major source of O$_2^-$ in response to UVR. Another potential effective generator of O$_2^-$ is uncoupled cNOS. UVR-activated NOS generates NO$^-$ from L-Arg. Since [L-Arg] is low in cells, rapid consumption of L-Arg results in a L-Arg depletion, which leads to cNOS uncoupling and increased production of O$_2^-$.

2.2.5 The formation of ONOO$^-$ upon UVR

NO$^-$ is able to compete with SOD for O$_2^-$ to form ONOO$, which is a powerful oxidant. The reaction rate for NO$^-$ and O$_2^-$ to from ONOO$^-$ is
approximately $7 \times 10^9 \, \text{M}^{-1} \, \text{sec}^{-1}$ \textsuperscript{37}. ONOO$^-$ formation rate is controlled by the diffusion rates of its reactants, thus as NO$^\cdot$ and O$_2$$^-\cdot$ generation increases in response to time point and UVR dose, ONOO$^-$ generation increases concurrently \textsuperscript{25a, 31}. Within keratinocytes, ONOO$^-$ formation reaches a level of 6-fold that of a control within 60 minutes with a treatment of 20 mJ/cm$^2$ and a maximum of an 8 to 9-fold increase in response to a UVB dose of 200 mJ/cm$^2$ \textsuperscript{25a, 31a}. Rabbit brain synaptosomes show a linear generation of ONOO$^-$ in response to UVB with a 7-fold increase observed at a dose of 100 mJ/cm$^2$ \textsuperscript{31c}. ONOO$^-$ can peroxidize lipid membranes, which results in a decrease in membrane fluidity. This decrease in fluidity has been observed in keratinocytes to correlate with a prolonged activity in a particulate form of NOS. The change in fluidity of the NOS microenvironment as an effect of lipid peroxidation results in continuation of NOS activation and therefore NO$^\cdot$ release after the initial UV response, thus serving as a positive feedback mechanism \textsuperscript{31a}. However, a study utilizing hairless mice has shown lipid peroxidation levels to decrease in response to UVB over a 6-24 hours time period. This observation has been proposed to be an effect of systemic factors not observed within cell cultures, such as release of prostaglandin, histamine and cytokines during the inflammatory response to UVR \textsuperscript{25c}.

2.2.6 The roles of NO$^\cdot$/ONOO$^-$ in regulation of apoptosis upon UV Irradiation

The release of NO$^\cdot$ after UVR has been shown to be capable of pro- or anti-apoptotic effects. However, the mechanism of NO$^\cdot$-mediated regulation of
apoptosis upon UV irradiation is still under elucidation. An elevation in [NO•] has predominately been shown to protect cells from UV-induced apoptosis 38. NO• prevents UV-induced apoptosis by inhibiting the phosphorylation of Ser-46 of p53 and impairs its nuclear import 39. NO• also inhibits apoptosis by various mechanisms including S-nitrosylation of caspases and transglutaminase 40. The level and duration of NO• elevation is a critical factor in determination of its role in regulation of apoptosis. Low doses and pretreatment with NO• have been shown to attenuate apoptosis. Low-level treatment of macrophages with NO• lead to increased levels of COX-2 expression, which resulted in an increase in cell survival 41. Additionally, early transient activation of JNK in cardiac myocytes has been shown to decrease apoptosis in response to oxidative stress 41a. However, high and prolonged levels of NO• are generally observed with the pro-apoptotic activation of JNK 42, which leads to activation of caspase-3 and modulation of apoptosis-associated proteins, such as Bcl-2, leading to increased levels of apoptosis 43. Another factor that determines the pro- or anti-apoptotic role of NO• is the level of O2•− present 28e. O2•− reacts with NO• to form ONOO−. In the presence of high level of O2•−, the bioavailability of NO• is reduced with the elevation of ONOO−, which is more reactive than NO• and O2•− and induces oxidative protein modifications including oxidation or nitration of various amino acid residues such as methionine, cysteine, tryptophan, and tyrosine 44. An elevation of ONOO− has been shown to promote apoptosis 45. Treating cells with ONOO− generator Sin-1 induced ER-stress and apoptosis 46. ONOO− can also
promote apoptosis by activating the MAPK signaling cascade and deactivating Akt signaling pathways \(^\text{47}\).

### 2.2.7 Conclusion

UVA, B and C light are able to immediately induce an elevation of NO\(^{-}\). The release of NO appears in two stages. In the early stage (within 6 hours post-UVR), NO\(^{-}\) is generated from L-Arg via activation of cNOS and from photosensitive NO\(^{-}\) storage molecules, such as GSNO. In the late stage (6-24 hours post-UVR), most NO\(^{-}\) is generated by iNOS due to an increase in its expression level and activity. UV irradiation also induces an immediate production of \(\text{O}_2^{-}\) from XO and uncoupled cNOS. NO\(^{-}\) rapidly reacts with \(\text{O}_2^{-}\) to form \(\text{ONOO}^{-}\), which induces oxidative and nitrosative stresses. \(\text{ONOO}^{-}\) can also induce cNOS uncoupling, resulting in an increase in the production of \(\text{O}_2^{-}\). The ratio of NO\(^{-}\)/\(\text{ONOO}^{-}\) has a significant impact in determination of growth or death rates within irradiated cells. As the bioavailability of NO\(^{-}\) decreases, the ratio of NO\(^{-}\)/\(\text{ONOO}^{-}\) lowers and cell death rates increase due to the activation of apoptotic cascades. However, this process can be interrupted by late stage production of NO\(^{-}\), which increases the ratio of NO\(^{-}\)/\(\text{ONOO}^{-}\) (Fig. 2.1).
Figure 2.1. A model for NO⁻/ONOO⁻-mediated cell growth and death upon UV irradiation.

2.3 NOS-mediated regulation of translation and transcription after UVR

2.3.1 Introduction

Eukaryotic initiation factor 2 regulates translation by forming an eIF2•GTP•Met-tRNAᵢ ternary complex, which promotes the binding of Met-tRNAᵢ to the 40S ribosome-mRNA complex at the expense of hydrolization of GTP to GDP. In order to restart the initiation cycle, the guanine exchange factor eIF2B refreshes the eIF2-GDP to eIF2-GTP. Phosphorylation on Ser-51 of eIF2α
stabilizes the eIF2-GDP-eIF2B complex preventing GDP-GTP exchange, thus halting the translational initiation process. Environmental stimuli inhibit initiation of protein synthesis by activating one or more EIF2AKs. Four protein kinases are known to phosphorylate Ser-51 in eIF2α in response to different stress stimuli: (1) the heme-regulated inhibitor kinase (HRI, EIF2AK1) that responds to heme deprivation; (2) the dsRNA-dependent protein kinase (PKR, EIF2AK2) that is activated by dsRNA produced during viral infection; (3) PERK that responds to the accumulation of unfolded proteins in the ER as well as glucose depletion; and (4) GCN2 that responds to amino acid depletion. Previous studies demonstrated that PERK and GCN2 could mediate translational inhibition in response to UV irradiation. The UV-induced activation of PERK and GCN2 also leads to NF-κB activation via the eIF2α phosphorylation pathway.

### 2.3.2 NOS-mediated phosphorylation of eIF2α upon UVR

Recently, NOS has been shown to play a key role in coordinating the activation of both PERK and GCN2 upon UVB-irradiation. The UVB-activated NOS produces NO•, which reacts with O2•⩭ to form ONOO• and activate PERK. NO• and ONOO• have been shown to directly or indirectly induce endoplasmic reticulum (ER)-stress and the UPR, which are the key inducers for PERK activation. NO• inhibits the sarcoendoplasmic reticulum pump Ca2+ ATPase 1 (SERCA 1) by a direct interaction with the pump resulting in a decrease in Ca2+ uptake in the ER. NO• production is also associated with the down regulation of SERCA mRNA synthesis, which leads to the depletion of Ca2+ stores resulting in
ER stress \(^{57}\). ONOO\(^-\) has also been implicated in the direct inhibition of the SERCA isoform 2b \(^{58}\). Depletion of Ca\(^{2+}\) stores results in improper protein folding which activates the UPR \(^{59}\). Additionally, NO\(^-\) also mediates cytokine-induced UPR. Inhibition of NO\(^-\) production reduces IL-1-induced elevation and slicing of Xbp-1 mRNA \(^{60}\), which is an indicator of the elevation of the UPR and ER-stress \(^{61}\). The UPR consists of the activation of several proteins involved in proper ER protein folding as well as specific kinases to help moderate function. PERK is one such kinase activated by the UPR and ER-stress \(^{52a, 62}\). Once activated, PERK phosphorylates eIF2\(\alpha\) at Ser-51, which globally inhibits protein translation. This helps alleviate the level of unfolded protein in the ER by reducing the level of newly synthesized protein that enters the ER for post-translational modification \(^{36, 54a, 55a, 62}\). Treatment with the NOS inhibitor N\(^G\)-Methyl-L-Arginine (LNMMA) or the GSH precursor N-Acetyl-L-Cysteine (LNAC) reduces the level of eIF2\(\alpha\) phosphorylation upon UVB-irradiation suggesting the role of NO\(^-\) and ONOO\(^-\) in UVB-induced activation of PERK \(^{36}\).

While elevation of NO\(^-\) and ONOO\(^-\) induces PERK activation, the consumption of L-Arg by NOS during NO\(^-\) production leads to L-Arg depletion and GCN2 activation. L-Arg is the only substrate for NOS mediated NO\(^-\) synthesis. Upon activation of NOS, cellular L-Arg levels begin to deplete as it is converted to NO\(^-\) and L-Citrulline. This depletion can eventually lead to L-Arg starvation \(^{63}\). In order to alleviate the demand for L-Arg in translation, L-Arg sensitive GCN2 phosphorylates eIF2\(\alpha\) leading to inhibition of protein synthesis \(^{63}\). Additionally, depletion of L-Arg leads to NOS uncoupling and the generation of
$O_2^{-}$, which can then react with NO$^-$ to generate ONOO$^-$ resulting in further oxidative and ER stress culminating in additional PERK activation. Supplementation with L-Arg has demonstrated the ability to decrease eIF2 phosphorylation, however this addition could subsequently allow for an increase in NO$^-$ and thereafter PERK activation$^{36}$. In UVB-irradiated MEF cells, treatment with the NOS inhibitor LNMMMA or supplementation with L-Arg significantly reduces phosphorylation of eIF2$\alpha$ compared to untreated UV irradiated cells. Treatment with LNAC to scavenge $O_2^{-}$ shows a significant decrease in eIF2$\alpha$ phosphorylation, indicating the importance of oxidative stress. Additionally, knockouts of PERK or GCN2 reduce or eliminate the increase of eIF2$\alpha$ phosphorylation after UVB-irradiation$^{36}$. Combined, UVB-induced activation of PERK and GCN2 plays a significant role in regulating the phosphorylation of eIF2$\alpha$.

2.3.3 Phosphorylation of eIF2$\alpha$-mediated NF-$\kappa$B activation in the early phase of UVR

UV-induced activation of NF-$\kappa$B is a postponed and prolonged process. In the late phase (12-24 hours post-UV)$^{64}$, IKK is activated and phosphorylates I$\kappa$B$\alpha$ at Ser-32 and Ser-36$^{65}$, which leads to its dissociation from the NF-$\kappa$B complex and rapid degradation through the polyubiquitin-dependent proteasomal pathway$^{66}$. The removal of I$\kappa$B exposes a nuclear localization signal on NF-$\kappa$B that allows it to be shuttled into the nucleus and activate target genes$^{67}$. 
However, this canonical pathway is not applicable to UV-induced early phase (within 12 hours) activation of NF-κB\(^64,68\). During this time UVR does not induce IKK activation nor N-terminal serine phosphorylation of IκB\(^α\)\(^64b\). However, while IKK activation is not detected above the basal level after UV-irradiation, IKK activity is still required as the IKK-targeted serine phosphorylation sites on IκB\(^α\) are critical for UV-induced NF-κB activation\(^69\).

The early phase activation of NF-κB after UVR is mediated via an eIF2\(\alpha\) phosphorylation and translational inhibition signaling pathway\(^55a, b\). In this pathway, translational inhibition due to the phosphorylation of eIF2\(\alpha\) results in decreased IκB synthesis. Reduced expression of IκB, in combination with its short half-life, decrease total IκB which allows NF-κB to exist in the uninhibited state and translocate to the nucleus where it carries out its function. The activation of eIF2\(\alpha\) kinases, PERK and GCN2, regulate the early phase activation of NF-κB. Accordingly, when a PERK or GCN2 knockout cell line was used in studies, the UV-induced eIF2\(\alpha\) phosphorylation and NF-κB activation was significantly inhibited\(^55a, b\). Analysis of NF-κB activation in a MEF cell line containing an eIF2\(\alpha\) mutant in which Ser-51 was mutated to alanine (MEF\(^A/A\)) showed similar inhibition of NF-κB\(^55c\).

While IKK activation and IκB degradation do occur in the late phase of UVR, a recent study suggests that this may not be the cause of NF-κB activation\(^21\). The reason is that the activation of NF-κB in the MEF\(^A/A\) cell line is independent of IκB depletion during the late phase of UVR\(^21,55a\). Further studies
indicate that neither ubiquitination nor proteasomal degradation have
detectable contributions to late-phase UV-induced IκBα depletion.
Phosphorylation of Ser-536 of NF-κB (p65), which is a direct target of activated
IKK\textsuperscript{70}, is not induced after UVR. However, phosphorylation levels of Ser-276 of
NF-κB (p65), which is targeted by MSK in the nucleus\textsuperscript{24a} and protein kinase A
(PKA) in the cytosol\textsuperscript{71}, have been shown to be dramatically increased in the
nucleus, but not in cytosol. These results lead to a hypothesis that UV-induced
late-phase activation of NF-κB is mediated by the MSK signaling pathway\textsuperscript{21,24a}.

2.3.4 Potential roles of NO\textsuperscript{•} and O\textsubscript{2}\textsuperscript{•−} in direct mediation of transcription

activation upon UVR

The UV-induced activation of MSKs and their downstream transcription
activators are potentially mediated by the elevation of NO\textsuperscript{•} in combination with
oxidative stress. NO\textsuperscript{•} generated from NOS has been shown to activate MSK by
activation of p38 and ERK\textsuperscript{72}. Activation of MSK via p38 is mediated by stress
generated by NO\textsuperscript{•} and oxidative stress that activates MEK3 and MEK6, the
upstream kinases responsible for the phosphorylation of p38. Once activated,
p38 translocates to the nucleus where it activates MSK via phosphorylation\textsuperscript{73}.
Additionally, ERKs are also responsible for the activation of MSK. Activation of
ERKs involve surface receptors such as tyrosine kinase and G protein coupled
receptors. These receptors elicit Ras and Raf activation, which in turn activates
the kinases MEK1 and MEK2 upstream of ERK. Activated ERK then translocates
to the nucleus and phosphorylates MSK\textsuperscript{73}. 
MSKs are responsible for the regulation of transcription factors as well as for remodeling of the chromatin structure\textsuperscript{72b}. MSKs regulate transcriptional regulation by activation of the transcription factors CREB and NF-\(\kappa\)B\textsuperscript{41b, 72b}. MSKs, along with ribosomal S6 kinase 2 (RSK2), are the key kinases responsible for the activation of CREB, which mediates transcriptional regulation of a number of genes involved in the inflammation process. Upon activation, MSK targets CREB for phosphorylation on Ser-133 conferring to it full transcriptional activation\textsuperscript{72b}. MSKs are also responsible for the activation of the transcription factor NF-\(\kappa\)B via a non-canonical pathway. Activation of NF-\(\kappa\)B via MSK1 is reliant upon the phosphorylation of NF-\(\kappa\)B on Ser-276 of the p65 subunit\textsuperscript{24a, 72b}.

In addition to direct regulation of transcription by the activation of transcription factors, MSKs are also involved in the remodeling of chromatin; further adding to its role in transcriptional regulation. MSKs are capable of phosphorylating H3 at Ser-10 and Ser-28\textsuperscript{72b, 74}. Both isoforms 1 and 2 of MSK have been shown to phosphorylate H3, however MSK2 has been observed to play a more crucial role in H3 modification\textsuperscript{72b}. Phosphorylation of either of these sites on H3 results in a change in conformation, as well as a change in the molecule; both of which play important roles in chromatin binding\textsuperscript{72b, 74b}. In this way, MSK is capable of regulating transcription by direct activation of transcription factors as well as modifying the availability of genes for transcription.
2.3.5 UV-induced and NO\(^{-}\)-mediated translational regulation and apoptosis

NOS mediates the UV-induced phosphorylation of eIF2\(\alpha\) through activation of both PERK and GCN2 \(^{36}\). Phosphorylation of eIF2\(\alpha\) plays roles in regulation of apoptosis due to various stimuli. MEF cells with a GCN2 knockout (MEF\(^{\text{GCN2}\,-/}\)) are more susceptible to UV-induced apoptosis \(^{22,55b}\), which is similar to the MEF\(^{\text{A/A}}\) cells in which the phosphorylation site, Ser-51, of eIF2\(\alpha\) is replaced with a non-phosphorylatable Ala (Ser-51-Ala) \(^{22}\). Additionally, PARP expression in MEF\(^{\text{A/A}}\) cells is reduced without being cleaved after UVR. In contrast, PARP is cleaved without a significant decrease in parental PARP in MEF\(^{\text{S/S}}\) cells after treatment with UVR. It appears that inhibition of eIF2\(\alpha\) phosphorylation sensitizes MEF cells to apoptosis by reducing PARP via a caspase independent signaling pathway \(^{75}\). In contrast to MEF\(^{\text{GCN2}\,-/}\) and MEF\(^{\text{A/A}}\) cells, cells with a PERK knockout, or cells transfected with a dominant negative PERK, are less sensitive to UV-induced apoptosis. Overexpression of wild-type PERK also sensitizes cells to UV-induced apoptosis without directly inducing cell death \(^{22}\). It is known that activation of PERK could be involved in pro- or anti-apoptotic cell death. PERK activation promotes reperfusion-induced apoptosis of brain cells by inducing the synthesis of activating transcription factor 4 (ATF4), which transcriptionally up-regulates C/EBP homologous protein (CHOP) expression \(^{76}\). However, PERK activation can also protect pancreatic beta cells from ER-stress induced apoptosis \(^{77}\). As such, PERK activation appears to play a pro-apoptotic role in regulation of UV-induced apoptosis.
2.3.6 Conclusion

UV-induced NOS activation and NO\(^{\cdot}\) production mediate both translational and transcriptional regulation of gene expression. UVR induced NOS activation results in a rapid generation of NO\(^{\cdot}\) from L-Arg. The consumption of L-Arg leads to a shortage of the amino acid, which activates GCN2 and induces cNOS uncoupling. Uncoupled cNOS produces O\(_2^{\cdot-}\), which is also generated by XO upon UV irradiation. In combination with NO\(^{\cdot}\) production, increased levels of O\(_2^{\cdot-}\) lead to an elevation in ONOO\(^{-}\), which generates oxidative/nitrosative stress and activates PERK. Activated PERK and GCN2 catalyze the phosphorylation of eIF2\(\alpha\), which subsequently inhibits protein synthesis. During the early phase response to irradiation, UV-induced translational inhibition leads to a reduction in I\(\kappa\)B followed by NF-\(\kappa\)B activation. The release of NO\(^{\cdot}\) upon UV irradiation also induces transcriptional activation of multiple signaling pathways. Induction of the MSK signaling pathway leads to the activation of NF-\(\kappa\)B during the late phase response to UV irradiation. NO\(^{\cdot}\), in combination with oxidative stress, activates p38 MAPK and ERK which phosphorylate MSK. Subsequently, activated MSK can then phosphorylate Ser-276 on NF-\(\kappa\)B, resulting in activation of NF-\(\kappa\)B in a mechanism independent of IKK-mediated reduction of I\(\kappa\)B (Fig. 2.2). In this fashion, NOS-mediated activation of EIF2AKs, phosphorylation of eIF2\(\alpha\) and activation of NF-\(\kappa\)B coordinately regulate apoptotic cell death in response to UV irradiation.
Figure 2.2. A model for UVR-induced and NOS-mediated eIF2α phosphorylation and NF-κB activation.
3.1 Summary

NF-κB is a transcription factor that controls expression of genes involved in the immune and inflammatory responses as well as being a key component in the onset of cancers. In this study we provided evidence that MSK1 is responsible for a non-canonical late-phase activation of NF-κB upon UVB irradiation. Our data demonstrated that following UVB irradiation, MSK1 is activated via phosphorylation at the 24 h time point coinciding with translocation of NF-κB into the nucleus. Investigations into the signaling pathways upstream of MSK1 through the use of specific inhibitors for mitogen-activated protein kinase and p38 revealed that both kinases are required for full phosphorylation during the late-phase (24 h), while p38 is paramount for phosphorylation during the early-phase (6 h). Electromobility shift assays (EMSA) showed that inhibition of MSK1 resulted in a marked reduction in NF-κB binding affinity without altering the nuclear translocation of NF-κB. Supershift EMSA implicate that the p65, but not p50, isoform of NF-κB is involved in late-phase activation in response to UVB irradiation. Together, the results of these studies shed light onto a novel pathway of MSK1 mediated late-phase activation of NF-κB in response to UVB irradiation.
NF-κB is a transcription factor that plays a crucial role in controlling expression of genes involved in the immune and inflammatory responses, proliferation regulation, as well as being a key component in the onset of a large number of cancers. Inactive NF-κB resides in the cytoplasm as a dimer bound to the inhibitor of κB (IκB). In the canonical pathway of activation IκB must first be dissociated/degraded from the NF-κB complex to allow the NF-κB dimer to translocate to the nucleus via the exposure of a nuclear localization signal, thus allowing NF-κB to bind to DNA and mediate transcription. However, a previous investigation by our lab revealed a noncanonical pathway that operates independently of IκB degradation. It was found that in response to UV irradiation, NF-κB was able to successfully translocate into the nucleus despite high expression levels of IκB in a cell line incapable of inhibiting global protein synthesis (thus allowing continued synthesis of IκB post UV irradiation). Furthermore, it was discovered that UV induced late-phase (24 h) NF-κB activation was dependent on phosphorylation of Ser-276 upon entering the nucleus. This transactivation of NF-κB via phosphorylation was hypothesized to be mediated by the activity of MSK1 based on a report that linked MSK1 to NF-κB activation in response to stimulus with tumor necrosis factor alpha (TNFα).

MSK1 is a nuclear kinase that resides downstream of the mitogen-activated protein kinases (MAPK) signaling pathway and is directly activated via
phosphorylation by p38 and ERK1/2. ERKs operate downstream of tyrosine kinase receptors and G protein-couple receptors while p38 is activated in response to cellular stresses such as osmotic fluctuations, UV irradiation and cytokine signaling. Upon their activation, both ERK and p38 translocate to the nucleus where they target MSK1. MSK1 contains two individual kinase domains that reside independently at the N- and C-terminals of the protein. Once p38/ERK have entered the nucleus they dock with MSK1 and phosphorylate the C-terminus. In turn, the C-terminus of MSK1 then autophosphorylates several sites that led to the activation of the N-terminus. The N-terminal kinase domain can then target several factors involved in transcription including activating transcription factor 1 (ATF1), CREB and Histone H3. In this study we sought to elucidate the time dependent dynamics of the upstream signaling pathway that is involved in MSK1-mediated activation of NF-κB in response to UVB irradiation. Based on our results, we proposed a novel and dynamic signaling circuit for regulation of UVB induced MSK1-mediated NF-κB activation.

3.3 Materials and Methods

Cell Cultures. JB6 Cl41 mouse epidermal skin cells were grown in MEM medium containing 100 units/ml penicillin/streptomycin (Corning Life Sciences) and 5% FBS (Atlanta Biologicals) at 37°C and 5% CO₂ in a humidified incubator. Stably transfected JB6 Cl41 cells containing a pCMV5-Flag-MSK1-A195/NH₂
terminal kinase dead vector (a generous gift from Dr. Yong Yeon Cho, The Hormel Institute, University of Minnesota) were grown under similar conditions.

**Treatments.** Cells were pretreated with either 50 µm PD 98059, 10 µm SB 203580, 10 µm H89 or a combination of the aforementioned inhibitors (Sigma-Aldrich) 1 h prior to UVB irradiation. UVB light was generated by two 15 W UVB lamps (UVP Inc.) and measured by a UVB meter (UVP Inc.) to determine intensity. Media from the cells to be treated was removed and the cells exposed to the UVB light to obtain a dose of 50 mJ/cm². After treatment the media was returned and the cells incubated until sample collection.

**Western Blot Analysis.** Following treatment, cell lysates were collected at 6 and 24 h time points post UVB irradiation. Whole cell lysates were collected using PhosphoSafe lysis buffer (Sigma-Aldrich) according to the manufacturer’s recommended protocol. Upon collection, samples were prepped and 20-30 µg of protein ran on 10% SDS-polyacrylamide gel. The samples were then transferred to a nitrocellulose membrane and blocked with 5% milk in TBS-T for 1-3hrs (depending on protein of interest) before being incubated overnight (~18hrs) with antibodies targeting p65, PARP, MSK1, Phospho-T581 MSK1 or β-actin (Santa Cruz Biotechnology). Bands were visualized using the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and exposed to radiosensitive film (HyBlot CL, Denville Scientific).

**Electromobility Shift Assay.** Cytoplasmic and nuclear lysates were prepared in a step wise fashion using Sucrose Buffer (0.32 M sucrose, 10 mM Tris HCl, 3 mM CaCl₂, 2 mM MgOAc, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT) to
collect cytoplasmic proteins and a Low Salt Buffer (20 mM HEPES, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT) and High Salt Buffer (Low Salt Buffer with 800 mM KCl and 1% NP-40) to collect nuclear proteins. NF-κB consensus sequence (Santa Cruz Biotechnology) was radiolabeled with ³²P according to the following procedure: 200 ng oligo was incubated with 150 µCi (50 pmol) [γ-³²P]-ATP (3000 Ci/mmol 10 mCi/ml, Perkin Elmer), 20 units T4 Kinase, 10X Labeling Buffer (New England Biolabs) and diH₂O (to give a total of 50 µL) for 1 h at 37° C. The probe was then purified with a QIAquick Nucleotide Removal Kit (Qiagen) according to the manufacturers protocol. The resultant solution was then diluted 15 fold (200 µL to 3000 µL) in TE buffer. For the binding reaction, 30 µg of nuclear lysates were incubated with 1 µL labeled probe, 2 µg poly-dIdC, 5X Binding Buffer (50 mM Tris HCl pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol, 1 mM DTT) and diH₂O (to give 40 µL) for 30 min at room temperature. Samples were then loaded onto a 5% non-denaturing polyacrylamide gel and ran in 0.5X TBE Buffer. Gels were dried and exposed to radiosensitive film at -80° C for 12-48 h to visualize bands. For competition tests and supershift assays, 20 ng unlabeled probe or 1 µg antibodies specific for p50/p65 NF-κB (respectively) were added to the binding reaction prior to the addition of the labeled probe.

**Immunocytochemical staining.** Cells were plated into a 12-well plate containing glass slide cover slips to allow for mounting then treated with UVB irradiation and specific inhibitors as previously mentioned. Cells were then fixed with a 3.7% formaldehyde/PBS solution for 15 min, washed in PBS and
permeabilized in a solution of PBS containing 0.1% TritonX-100 (Sigma-Aldrich). Following an additional PBS rinse, cells were blocked for 1 h (2 mg/mL BSA in PBS) and incubated overnight (~18 h) at 4˚ C with primary p65 NF-κB antibody binding buffer (200 fold antibody [Santa Cruz Biotechnology] dilution in PBS containing 10 mg/ml BSA and 0.3% TritonX-100). Cells were then rinsed with PBS and incubated with FITC-conjugated secondary antibody buffer (400 fold antibody [Santa Cruz Biotechnology] dilution in PBS containing 10 mg/ml BSA and 0.3% TritonX-100) for 1 h at room temperature, rinsed with PBS and mounted on slides using VectaSheild mounting medium (Vector Laboratories, Inc.) and visualized on a Nikon Eclipse E600 microscope.

**Flow Cytometry.** Following treatment with UVB irradiation and/or specific inhibitors, cells were collected at 6 and 24 h time points by trypsinization. Cells were then washed 2x with PBS and stained by utilizing an AnnexinV-FITC Apoptosis Detection Kit II (BD Pharmingen) according to manufacturer's recommended protocol. In brief, cell pellets were resuspended in 1x Binding Buffer to which 5 µl of Propidium Iodide and AnnexinV were added. After incubation at room temp for 15 min an additional 400 µl of 1x Binding Buffer was added to the samples before being analysis for fluorescence on a FACSort flow cytometer utilizing CellQuest Pro software (BD Pharmingen).
3.4 Results

3.4.1 UVB induces MSK1 phosphorylation via p38 and ERK in a time dependent manner

Activation of MSK1 requires phosphorylation from upstream kinases p38 and/or ERK. These kinases target Thr-581 and Ser-360, which then allow MSK1 to autophosphorylate into its active form. In this study, western blot analysis was performed to determine expression levels of active (Thr-581 phosphorylated) as well as total MSK1. MSK1 was found to be phosphorylated at Thr-581 well above basal levels at both 6 and 24 h following UVB irradiation in all four cell lines tested (Fig. 3.1 lanes 2, 5, 8, 11 and 3, 6, 9, 12 vs. 1, 4, 7, 10; respectively). This phosphorylation was present at 2 fold higher levels at 6 h post UVB than at 24 h in wild type cells (Fig. 3.1 lanes 2 vs. 3 and Fig. 3.2C lane 2 vs. 5).

In order to determine the contribution of ERK and p38 upstream pathways in MSK1 phosphorylation, wild type cells were treated with specific inhibitors 1 h prior to irradiation. Treatment with inhibitor PD 98059 for MEK (upstream of ERK) showed a slight reduction in phosphorylation at 6 h post UVB (Fig. 3.2A lane 4 vs. 2) while treatment with SB 203580, a p38 inhibitor, was able to effectively block phosphorylation at 6 h (Fig. 3.2A lane 6 vs. 2). These results suggest that p38 is the predominant kinase responsible for MSK1 phosphorylation at the 6 h time point. However, at 24 h post UVB irradiation, treatment with either inhibitor resulted in a return to basal phosphorylation levels (Fig. 3.2B lanes 4 and 6 vs. 1) demonstrating a requirement for both kinases during the late-phase response.
Combined treatment with the inhibitors was also able to prevent phosphorylation of MSK1 at 24 h (Fig. 3.2C lane 7 vs. 1) but only resulted in a slight reduction at 6 h (Fig. 3.2C lane 4 vs. 2). Control treatments with either inhibitor, and without UVB, resulted in a small decrease in basal phosphorylation levels at both time points (Fig. 3.2A/B lanes 3 and 5 vs. 1), whereas combined inhibitor treatment showed a slight increase (Fig. 3.2C lanes 3 and 6 vs. 1).

Exposure to UVB also resulted in a decrease of total MSK1 expression levels at 6 h with levels remaining comparably reduced at 24 h (Fig. 3.2A/B lane 2 vs. 1 and Fig. 3.2C lanes 2 and 5 vs. 1). Interestingly, the reduction of MSK1 phosphorylation via treatment with specific inhibitors resulted in rescued levels of total MSK1 expression (Fig. 3.2A/B lanes 4 and 6 vs. 2 and Fig. 3.2C lanes 4 vs. 2 and 7 vs. 5). These findings suggest reduced levels of MSK1 expression following irradiation may rely on a phosphorylation dependent pathway of degradation.
Figure 3.1. Expression levels of MSK and phosphorylated MSK following treatment with UV in four cell lines. JB6 CL41 wild type cells, cells containing a pCMV5-Mock vector, cells containing a pCMV5-Flag-MSK1-Over Expression vector and cells containing a pCMV5-Flag-MSK1-A195/NH₂ vector for a dominant-negative N-terminal kinase dead MSK1 mutant were irradiated with 50 mJ/cm² UVB. Whole cells lysates were collected after 6 and 24 h and analyzed via western blot.
Figure 3.2. Expression levels of MSK and phosphorylated MSK following treatment with UV and specific inhibitors. A) JB6 CL41 cells were pretreated for 1 h with or without the inhibitors PD98050 (50 µM) or SB203580 (10 µM) and irradiated with 50 mJ/cm² UVB. Whole cells lysates were collected after 6 h and analyzed via western blot. B) Identical to A with the exception of lysates being
collected at 24 h. C) Cells were pretreated with both PD98050 (50 µM) and SB203580 (10 µM) and lysates collected at 6 and 24 h post irradiation.

3.4.2 Inhibition of MSK1 decreases late-phase NF-κB Binding Affinity

In order to assess NF-κB activation during the early- and late-phases following UVB irradiation, an electromobility shift EMSA was performed to determine the relative binding affinity of NF-κB to its consensus sequence. The EMSA revealed a strong DNA binding capacity of NF-κB at 24 h post UVB irradiation while binding at 6 h increased approximately 23% (not statistically significant) (Fig. 3.3A/B lanes 2 and 5 vs. 1). In addition to stimulation with UVB, cells were treated with specific inhibitors for MSK (H89), as well as its upstream activators, p38 (SB 203580) and MEK (PD 98059) in order to assess their role in activation of NF-κB. Inhibition of MSK1 resulted in a significant reduction in NF-κB binding affinity at 24 h (Fig. 3.3B lane 7 vs. 5), signifying its importance in transactivation of NF-κB in response to UVB irradiation. Inhibition of MEK also resulted in a marked reduction in binding affinity at 24 h (Fig. 3.3A lane 6 vs. 5). However, inhibition of p38 alone or combined with inhibition of MEK resulted in a marked increase in NF-κB binding affinity at 24 h (Fig. 3.3A lane 7 vs. 5 and 3.3B lane 6 vs. 5). Similar results were observed at 6 h where inhibition of p38 alone or combined with inhibition of MEK resulted in an increase in binding affinity (Fig. 3.3A lane 4 vs. 2 and Fig. 3.3B lane 3 vs. 2). Inhibition of MSK at 6 h also showed a slight increase in NF-κB binding affinity (Fig. 3.3B lane 4 vs. 2). Control
treatments with the inhibitors alone showed no increase in DNA binding capacity (data not shown).

Figure 3.3. Electromobility shift assay for NF-κB binding activity. A) JB6 CL41 cells were pretreated for 1 h with the inhibitors PD98059 (50 µM) or SB203580 (10 µM) and irradiated with 50 mJ/cm² UVB. Nuclear lysates were collected after 6 or 24 h and incubated with radiolabeled NF-κB consensus sequence. Samples
were then ran on a non-denaturing gel and visualized with exposure to radiosensitive film. B) Identical to A with the exception of samples being pretreated with a combination of PD98059 (50 µM) and SB203580 (10 µM) or H89 (10 µM).

In order to assess the involvement of p50, p65 or the p50/p65 heterodimer in response to NF-κB activation following UVB irradiation, an electromobility super-shift assay was performed. A clear band shift upon sample incubation with an antibody specific for p65 was observed at 24 h (Fig. 3.4 lane 7 vs. 6) and 6 h (over-exposure of same figure not shown). However, incubation with an antibody specific for p50 failed to elicit a shift (Fig. 3.4 lane 8 vs. 6). A cold-competition test was run to ensure that the binding observed in this, and previous EMSA studies, were specific for the NF-κB binding sequence. Results confirmed this ascertain, as bands were lost at 24 h (Fig. 3.4 lane 9 vs. 6) and 6 h (over-exposure of same figure not shown).
Figure 3.4. EMSA Supershift assay for NF-κB binding activity. JB6 CL41 cells were irradiated with 50 mJ/cm² UVB. Nuclear lysates were collected after 6 or 24 h. Samples for supershift analysis were incubated with p50 or p65 antibody before being incubated with radiolabeled NF-κB consensus sequence. A competition test was run on two samples by adding 20 ng unlabeled probe to the incubation mixture. Samples were then ran on a non-denaturing gel and visualized with exposure to radiosensitive film.

3.4.3  p38 inhibition-induced NF-κB activation and MEK inhibition-induced NF-κB inactivation are dependent on MSK1

In order to aid in our studies, we utilized a stable JB6 Cl41 cell line expressing a pCMV5-Flag-MSK1-A195/NH₂ vector for a dominant-negative N-terminal kinase dead MSK1 mutant. This cell line allowed us to analyze the upstream signaling to NF-κB in a system mostly devoid of functional MSK1.
Results from an electromobility shift analysis revealed an induction of NF-κB DNA binding affinity 24 h post-UVB irradiation (Fig. 3.5A/B lane 5 vs. 1) while binding remained near basal levels at 6 h (Fig. 3.5A/B lane 2 vs. 1). In contrast to the wild-type cell line, treatment with a single inhibitor for MEK or p38 did not affect NF-κB binding affinity (Fig. 3.5A lanes 6 and 7 vs. 5). These results indicate that p38 inhibition-induced NF-κB activation and MEK inhibition-induced NF-κB inactivation (Fig. 3.3A lanes 7 and 6 vs. 5) observed in wild type cells post irradiation are dependent on proper MSK1 function. However, upon combined treatment an increase in binding affinity was observed at 24 h post-UVB irradiation with a slight decrease occurring at 6 h (Fig. 3.5B lanes 6 and 3 vs. 5 and 2). Additionally, treatment of this cell line with a MSK inhibitor resulted in a slight increase NF-κB binding affinity at 6 h and slight reduction at 24 h post irradiation (Fig. 3.5B lanes 4 and 7 vs. 2 and 5).
Figure 3.5. Electromobility shift assay for NF-κB binding activity in ND cell line. A) Stably transfected JB6 CL41 cells containing a pCMV5-Flag-MSK1-A195/NH₂ vector for a N-terminal kinase dead MSK1 mutant were pretreated for 1 h with the inhibitors PD98059 (50 µM) or SB203580 (10 µM) and irradiated with 50 mJ/cm² UVB. Nuclear lysates were collected after 6 or 24 h and incubated with radiolabeled NF-κB consensus sequence. B) Identical to A with the exception of samples being pretreated with a combination of PD98059 (50 µM) and SB203580 (10 µM) or H89 (10 µM).
Figure 3.6. Immunohistochemical staining of NF-κB translocation. Cells were grown on glass cover slips and pretreated for 1 h with the inhibitors PD98059 (50 µM), SB203580 (10 µM), H89 (10 µM) or a combination and irradiated with 50 mJ/cm² UVB then fixed after 6 and 24 h. Fixed cells were incubated with primary
antibody for p65 then FITC-conjugated secondary antibody and visualized via fluorescence microscopy.

3.4.4 MSK1-mediated NF-κB activation is independent of NF-κB nuclear translocation

In order to assess the mechanism of MSK1-mediated NF-κB activation upon UVB irradiation, translocation of NF-κB was visualized via immunohistochemical staining. Following staining, NF-κB was observed to translocate to the nucleus during the late-phase in response to UVB irradiation, but no detectable change during the early-phase was observed (Fig. 3.6). Immunostaining of the cells following treatment with UVB and specific inhibitors did not result in significant changes in translocation in comparison to the UVB-only treated cells (Fig. 3.6). These findings indicate that changes in NF-κB binding affinity following treatment with specific inhibitors are not the result modified NF-κB translocation within the nucleus of the cell. Additionally, western blot analysis of cytoplasmic and nuclear p65 was performed (Figure 3.7), however these results proved difficult to reproduce and as such our conclusions were based on our immunohistochemical staining methodology.
Figure 3.7. Western blot analysis of cytoplasmic and nuclear p65. JB6 CL41 cells were pretreated for 1 h with or without the inhibitors PD98050 (50 µM) or SB203580 (10 µM) and irradiated with 50 mJ/cm² UVB. Cytoplasmic and nuclear lysates were collected after 6 and 24 h and analyzed via western blot for p65 expression.
3.4.5 *Inhibition of MSK via H89 deceases cell viability during the late phase response*

In order to determine the physiological effect on the cells following treatment with specific inhibitors, flow cytometry was used to determine cell viability following treatments with inhibitors in conjunction with or without UVB irradiation. Results indicated that cell viability is unaffected following UVB irradiation at 6 h while it is significantly reduced 38% at 24 h (Fig. 3.8A/B). Control treatments with inhibitors alone showed statistical significance in their ability to reduce cell viability at 6 h compared to the untreated control, while the only inhibitor/s capable of a significant effect when combined with UVB irradiation was that of the PD 98050 and SB 203580 combined treatment (Fig. 3.8A). At the 24 h time point, treatment with H89 was able to significantly decrease cell viability to 56% and the combined PD 98050 and SB 203580 treatment was able to increase cell viability to 22%, in respect to the observed decrease of 38% with UVB irradiation alone (Fig. 3.8B). The only inhibitor control treatments capable of eliciting a statistically significant effect at 24 h where those containing PD 98050 (Fig. 3.8B). Additionally, control treatments with the inhibitor delivery vehicle (DMSO) did not have a statistically significant effect on cell viability (Fig. 3.8A/B). These results suggest that inhibition of MSK1, via treatment with H89, is capable of decreasing cell viability through a reduction in NF-κB activation, as well as other down stream targets.
Figure 3.8. Cell viability following treatment with UV irradiation and specific inhibitors. JB6 CL41 cells were pretreated for 1 h with or without the inhibitors PD98050 (50 µM) or SB203580 (10 µM) and irradiated with 50 mJ/cm² UVB. Cells were collected at 6 and 24 h after treatment, stained with AnnexinV-FITC and PI and their fluorescence was measured via flow cytometry. Cells were considered viable if negative for both markers. * = statistical significance of \( p \leq 0.05 \) for change in viability verified through student’s t-test.
3.5 Discussion

Misregulation of NF-κB is a hallmark amongst various forms of skin cancer. Suppression of UV-induced NF-κB activation has been suggested as a potential chemopreventive method for photocarcinogenesis. However, the mechanism of NF-κB activation in response to UV irradiation is not fully understood. In this study, we sought to elucidate the pathways involved in MSK1-mediated NF-κB activation in response to UVB irradiation and to determine the time-dependent dynamics through which this activation is achieved. Our data indicated that MSK1 in JB6 cells is strongly phosphorylated at Thr-581, which is a marker for activation, at 6 h post irradiation with lowered, but continued, activation occurring at the 24 h time point (Fig. 3.3C lanes 2 and 5 vs. 1). However, only the late-phase MSK1 activation coincides with NF-κB nuclear translocation and activation (Figs. 3.3, 3.3 and 3.6). Inhibition of MSK1 with H89 results in the inhibition of UVB-induced NF-κB activation (Fig. 3.3B lane 7 vs. 5) indicating that MSK1 mediates late-phase activation of NF-κB. In addition to H89, inhibition of MEK with PD 98059 resulted in a decrease in NF-κB binding affinity at 24 h post UVB irradiation (Fig. 3.3A lane 6 vs. 5), which correlates to the downstream decrease in MSK1 phosphorylation (Fig. 3.2B lane 4 vs. 2). Contrarily, inhibition of p38 with SB 203580 alone or combined with the inhibition of MEK, resulted in an increase in NF-κB DNA binding affinity at both 6 and 24 h post UVB irradiation (Fig. 3.3A lanes 4 and 7 vs. 2 and 5, Fig. 3.3B lanes 3 and 6.
vs. 2 and 5); suggesting that although p38 inhibition decreases MSK1 phosphorylation at both time points, additional regulatory mechanisms may be at work. In addition, our data indicated that the inhibition of NF-κB activation is not accompanied with the inhibition of nuclear translocation of NF-κB (Fig. 3.3 vs. 3.6). These results indicate that although MSK1 is highly phosphorylated at 6 h post irradiation, NF-κB has yet to translocate to nucleus and is not available for transactivation via MSK1. However, once NF-κB has been compartmentalized to the nucleus, specific inhibition of MSK1 is able to attenuate NF-κB binding affinity. Additionally, treatment of the cells with specific inhibitors failed to elicit significant changes in NF-κB translocation. Combined, these observations confirm that MSK1 is responsible for enhancing the binding affinity of NF-κB via transactivation at 24 h post irradiation.

The analysis of the upstream activators of MSK1 revealed a time-dependent requirement of p38 and ERK. Results indicated that p38 activity during the early-phase (6 h) response to UVB irradiation is a requirement as treatment with specific inhibitor SB 203580 resulted in a decrease in MSK1 phosphorylation to near basal levels (Fig. 3.2A lane 6 vs. 1). However, treatment with specific inhibitor PD 98059 for MEK (upstream of ERK) was unable to fully attenuate MSK1 phosphorylation (Fig. 3.2A lane 4 vs. 1). Combined, these results indicate that during the early-phase response signaling through p38 is paramount to MSK1 activation while signaling through ERK plays a lesser, but still required, role. This dynamic shifts during the late-phase response as inhibition of either p38 or MEK is able to decrease MSK1 phosphorylation back to
basal levels (Fig. 3.2B lanes 4 and 6 vs. 1). Combined treatment with both inhibitors during the late-phase results in a prevention of MSK1 phosphorylation, while the early-phase phosphorylation is not completely blocked. Together, these combined observations reveal a time dependent shift in the necessity of p38 and ERK signaling to MSK1 post UVB irradiation.

In order to confirm NF-κB binding levels observed through inhibition of p38 and MEK were indeed acting through MSK1, we employed a JB6 Cl41 cell line expressing a pCMV5-Flag-MSK1-A195/NH₂ vector for a dominant-negative N-terminal kinase dead MSK1 mutant. Although some endogenous MSK1 is most likely still present (as apparent in a slight decrease in NF-κB binding affinity following treatment with H89), this model allowed us to analyze NF-κB activation in a system mostly devoid of functional MSK. In contrast to the wild type cell line, treatment with either PD 98059 or SB 203580 failed to elicit a change in NF-κB binding affinity (Fig. 3.5A lanes 6 and 7 vs. 5) at 24 h. These observations confirm that p38 inhibition-induced NF-κB activation and MEK inhibition-induced NF-κB inactivation (Fig. 3.3A lanes 7 and 6 vs. 5) observed in wild type cells post irradiation are dependent on proper MSK1 function. Decreased levels of NF-κB binding following inhibition of MEK can be explained by the observed decrease in MSK1 phosphorylation (Fig. 3.2B lane 4 vs. 2), however, increased levels of NF-κB binding following p38 inhibition presents an interesting scenario as decreased MSK1 phosphorylation is observed along side increased NF-κB binding affinity. Specific inhibition of p38 could affect the activity of ERK as it has been reported that p38 can negatively regulate the function of MEK and ERK through activation
of protein phosphatase 2A. However, results from the N-terminal dead MSK1 cell line show a MSK1 dependent desensitization to p38 inhibition-induced NF-κB activation. As such, future research into the association of MSK1 with p38 will be necessary to elucidate the mechanism behind this activation. Combined treatment with p38 and MEK inhibitors showed an increase in NF-κB binding at both the early- and late-phases in the N-terminal dead MSK1 cell line (Fig. 3.5B lane 3 vs. 2 and 6 vs. 5) similar in trend of the wild type cell line. This observation can most likely be contributed to disruption of multiple sections of the complex signaling pathways upstream of NF-κB.

The combined results from this study reveal a novel and dynamic pathway for the late-phase activation of NF-κB in response to UVB irradiation. In our proposed model UVB irradiation stimulates the activation of MSK1 via phosphorylation from ERK and p38. During the early-phase response, activation of MSK1 is heavily dependent on the function of p38, as opposed to the late-phase in which p38 and ERK work in a concerted effort to phosphorylate MSK1. Although MSK1 is highly phosphorylated during the early-phase, it is not until UVB has stimulated the translocation of NF-κB into the nucleus of the cell during the late-phase that MSK1 is able to transactivate NF-κB into a fully active form (Fig. 3.9).
Figure 3.9. Proposed model of MSK-1 mediated NF-κB activation following UVB irradiation.
Exposure to UV induces a prolonged expression of COX-2. While transcriptional regulation of COX-2 expression has been well studied, the role of translational regulation of COX-2 synthesis upon UV-irradiation is not yet clear. In this report, we show that the phosphorylation of the alpha subunit of eIF2\(\alpha\) plays an important role in the regulation of COX-2 expression after UV-irradiation. Our data demonstrates 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 eIF2\(\alpha\), 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-\(\kappa\)B activation as we previously reported\(^{55a}\). Additionally, the translational regulation of COX-2 binding protein TIAR expression is reduced in MEF\(^{S/S}\) cells but not in MEF\(^{A/A}\) cells at 24 hours post-UV. These results suggest that translation initiation plays a role in a complex and dynamic regulation of COX-2 expression. Based on our results we propose a novel eIF2\(\alpha\) phosphorylation-centered network for the regulation of COX-2 expression after UV irradiation.
4.2 Introduction

COXs catalyze the rate-limiting step in the production of PG from arachnoic acid. 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. COX-2 is the inducible isoform activated, amongst others, by inflammatory cytokines, oncogenes, growth factors and UV-radiation. 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. The most well studied mechanism for transcriptional activation of UV-induced COX-2 expression is p38MAPK, which activates the CREB/ATF1 pathway. NF-κB was also shown to transcriptionally regulate COX-2 expression. However the role of NF-κB in the UV-induced COX-2 expression is still not clear.

Expression of COX-2 is also translationally regulated. COX-2 mRNA is locally 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. Two translational regulators of COX-2, HuR and TIAR, were shown to locally regulate the translation of COX-2 mRNA upon UVC-irradiation. HuR and TIAR, are shown to increase or decrease the translational efficiency of the bound mRNA, respectively. UV-irradiation also inhibits global protein synthesis by inducing the phosphorylation of eIF2α, which activates NF-κB translationally via translational inhibition of IκBα synthesis. However, the mechanism for
regulation of COX-2 expression via global translation inhibition is not clear. In this report, we show a mechanism of translational regulation of UVC-induced COX-2 expression both at global levels and that of RNA binding proteins.

In this study, we provide evidence that phosphorylation of eIF2α also plays an important role in the regulation of COX-2 expression after UVC-irradiation. Our results demonstrate eIF2α phosphorylation is required for UVC-induced expression of COX-2 at the transcriptional and translational levels. Without eIF2α phosphorylation, the UVC-induced transcriptional activation of COX-2 is delayed, which correlates with NF-kB activation, as previously reported. The increased translation of COX-2 is correlated to the ratio of activated HuR/TIAR. These findings elucidate a complex and dynamic role of translation initiation in regulation of COX-2 expression.

4.3 Materials and Methods

Cell culture. Wild type mouse embryo fibroblasts (MEF^{S/S}) and mutated line (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 then returned to the cultures.

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).

Quantitative real-time PCR. Total RNA was extracted from UVC-treated cells by a RNeasy Mini Kit (Qiagen). Complementary DNA was prepared from 1 μg of RNase free DNase treated RNA, using iScript cDNA Synthesis Kit (Bio-
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’: GCTGTACAAGCAGTGCAAA; 3’: CCCAAAAGATAGCATCTGGA. 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 COX-2 mRNA were normalized to the levels of β-actin mRNA in each sample.

4.4 Results

4.4.1 Inducibility of COX-2 upon UVC is eIF2α phosphorylation dependent

COX-2 expression is induced by NF-κB activation under various stimuli. Since NF-κB activation upon UVC-irradiation can be regulated by translational inhibition of IκB synthesis, we have assessed the role of translation on UVC-induced COX-2 expression. We first examined whether eIF2α phosphorylation plays a role in UVC-induced expression of COX-2. MEF cells with wild-type eIF2α (MEF^S/S) or with a Ser-51→Ala mutation at the phosphorylation site in eIF2α (MEF^A/A) were used in the experiments. The dose of 30 J/m² was selected to keep consistency with previous experiments, where this dose was used to induce both eIF2α phosphorylation and NF-κB activation. Western
blot analysis demonstrated that COX-2 expression was increased 1.5 to 2.1-fold from 4 to 12 hours (Fig. 4.1A lanes 2-4 vs. 1; Fig. 4.1B) in MEF\(^{S/S}\) cells, whereas COX-2 increased only 0.4 to 0.8-fold in the same time period in MEF\(^{A/A}\) cells after UVC-irradiation (Fig. 4.1A lanes 6-9; Fig. 4.1B). In comparison to the 12 hour time point the COX-2 levels were reduced in both cell lines at 24 hours post-UVC (Fig. 4.1A lanes 5 vs.4 and 10 vs. 9; Fig. 4.1B).
Fig. 4.1. Effect of translation on UVC-induced COX-2 expression. MEF$^{S/S}$ and MEF$^{A/A}$ cells were irradiated with UVC (30 J/m$^2$) and then were harvested at the indicated time points. (A) The COX-2 and COX-1 protein levels were detected by western blot analysis using anti-COX-2 and anti-COX-1 antibodies respectively. A western blot analysis of β-actin was used to monitor the loading of proteins. (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-UVC-irradiation for both cell lines (experiment performed by Csaba Laszlo).

To determine whether elimination of eIF2α phosphorylation specifically impacts COX-2 expression, we analyzed COX-1 expression in UVC-treated MEF^{S/S} and MEF^{A/A} cells. Our data showed that COX-1 expression levels are the same in both cell lines before and after UVC-irradiation (Fig. 4.1). These results suggest that eIF2α phosphorylation may play a dual-role in regulation of COX-2 expression upon UVC-irradiation. While translational inhibition leads to the activation of NF-κB and transcriptional activation of COX-2 expression in MEF^{S/S} cells after UVC-irradiation, maintaining a high level of active eIF2 increases translational efficiency of COX-2 in MEF^{A/A} cells with or without UVC-irradiation.

4.4.2 Translation inhibition activates COX-2 transcription

UV-irradiation inhibits protein synthesis through phosphorylation of eIF2α. However our results indicated COX-2 expression was increased in both cell lines at 4 hours post-UVC (Fig. 4.1). 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 UVC irradiated MEF^{S/S} and MEF^{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-UVC in the MEF^{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. 4.2). The increase of transcription of COX-2 (Fig. 4.2) is correlated to the activation of NF-\kappa B in the two cell lines after UVC-irradiation \textsuperscript{99}. The results suggest that the translational regulation of NF-\kappa B activation plays a role in UVC-induced expression of COX-2.

\textbf{Fig. 4.2.} UVC-induced eIF2\alpha phosphorylation up-regulates COX-2 transcription. MEF\textsuperscript{S/S} and MEF\textsuperscript{A/A} cells were irradiated with UVC (30 J/m\textsuperscript{2}). At the indicated time points, total mRNA was isolated and q-RT-PCR was used to determine the levels of COX-2 in the cells.
4.4.3 The phosphorylation of eIF2α affects the expression and activation of COX-2 mRNA-binding proteins

To further elucidate the mechanism of translational regulation of COX-2 expression, we analyzed the extent of effect of UVC-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.

Western blot analysis showed that cytoplasmic levels of HuR/TIAR were increased in both MEF\textsuperscript{S/S} and MEF\textsuperscript{A/A} cells at 4 hours post-UVC (Fig. 4.3A 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 UVC-irradiation. Interestingly, at 24 hours post-UVC, while cytoplasmic HuR was decreased in both cell lines (Fig. 4.3A lanes 3 and 6), TIAR was slightly increased above the base level in MEF\textsuperscript{S/S} cells (Fig. 4.3A lanes 3 vs. 1), and was significantly increased in MEF\textsuperscript{A/A} cells (Fig. 4.3A lanes 6 vs. 4). These results imply that translation of COX-2 could be less efficient in the late stage of UVC-irradiation due to a relatively lower HuR/TIAR ratio (Fig. 4.3A lanes 3, 6 vs. 1, 4). The results also explain how low efficiency of COX-2 translation in the presence of higher levels of mRNA (Fig. 4.2) at 24 hours post-UVC could be a combined effect of the 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 UVC-irradiation (Fig. 4.3B), which suggests that HuR expression is not correlated to eIF2α phosphorylation. In contrast to HuR, TIAR expression remained near basal levels at 4 hours post-UVC (Fig. 4.3B lanes 2, 5 vs. 1, 4), while the activity in the cytosol was increased (Fig. 4.3A 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-UVC (Fig. 4.3B lane 3, 6 vs. 2, 5), while activity is decreased in MEF<sup>S/S</sup> cells but increased in MEF<sup>A/A</sup> cells (Fig. 4.3A 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 UVC-irradiation.
Fig. 4.3. The effect of UVC on post-transcriptional regulators of COX-2. MEF<sup>S/S</sup> and MEF<sup>A/A</sup> cells were irradiated with 30 J/m<sup>2</sup> of UVC. At the indicated time points, the cytoplasmic (Panel A) and nuclear (Panel B) proteins were isolated. The proteins were subjected to western blot analysis using antibodies against HuR and TIAR. To
monitor the loading and sub-cellular contamination, expression levels of β-actin and nuclear marker hnRNP C1/C2 were also determined using western blot analysis.

4.5 Discussion

Previous studies demonstrate that the phosphorylation of eIF2α plays a role in the early stage of UVC-induced NF-κB activation. In this report have systematically analyzed the extent of the effect of eIF2α phosphorylation on COX-2 expression upon UVC-irradiation. Our results demonstrate that eIF2α phosphorylation does not only regulate COX-2 protein expression but also impacts the regulators and stabilities of COX-2 at the translational level.

The influence of eIF2α phosphorylation upon COX-2 expression was studied by using MEF^{S/S} and MEF^{A/A} cells. Our data show that the expression of COX-2 was induced much more in MEF^{S/S} cells than in MEF^{A/A} cells after UVC-irradiation (Fig. 4.1). To further determine the mechanism of UVC-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. 4.2). Although at this point we cannot quantitatively analyze the contribution of transcription and RNA stability towards the total COX-2 mRNA levels, these transcript levels can be associated with NF-κB activation patterns in the two cell-
lines after UVC-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-κB which we showed to be active during this period despite the inability of UVC to block translation \(^{97b,99}\). Since the COX-2 transcript levels and translation \(^{101}\) upon UVC-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 UVC treatment. HuR stimulates COX-2 mRNA translation, while TIAR inhibits it \(^{95a}\). Our data demonstrate that HuR expression could bypass UVC-induced and eIF2\(\alpha\) phosphorylation-mediated translation inhibition (Fig. 4.3), whereas TIAR expression was significantly inhibited at 24 hours post-UVC (Fig. 4.3B lane 3). The differential expression of HuR and TIAR altered the ratio of activated HuR/TIAR (Fig. 4.3A) and impacted the translation of COX-2 after UVC-irradiation.

Our results indicate that the UVC-induced eIF2\(\alpha\) 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 UVC-induced COX-2 expression (Fig. 4.4). We propose that activation of eIF2\(\alpha\) kinases leads to translational inhibition of I\(\kappa\)B synthesis and activation of NF-κB, which in turn induces COX-2 transcription in the early stage of UVC-irradiation. The eIF2\(\alpha\) phosphorylation has a two-tier effect. While it reduces the translation efficiency of COX-2 mRNA at both early and late stages of UVC-irradiation through its
global translational inhibition, it also reduces the expression of COX-2 mRNA binding protein TIAR, thus promoting the translation of its target in the late stages of UVC-irradiation.

*Fig. 4.4.* Proposed model for UVC induced COX-2 regulation. The molecular networks were generated by Ingenuity Pathways Analysis (Ingenuity® Systems) using Ingenuity database established on previously published data.
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APPENDIX A: DETERMINING NF-KB PHOSPHORYLATION AND ACTIVATION

A.1 Introduction

In this appendix we cover the various pitfalls occurred while attempting to determine the phosphorylation and activation levels of NF-κB following UV irradiation and how we arrived at the decision to analyze NF-κB activation as a measure of DNA binding capacity via EMSA. Since our hypothesis in chapter 3 was dependent on MSK1 mediated transactivation of NF-κB via phosphorylation of serine-276, we chose to utilize commercially available antibodies for the detection of phosphorylation levels. However, through careful observation it was determined that these antibodies did not bind specifically to phosphorylated NF-κB, but rather a protein with a molecular weight of ~85 kDa (the specific band according to manufacturer’s provided literature), whereas antibodies for total NF-κB and serine-536 phosphorylated NF-κB showed specific bands corresponding to a weight of ~70 kDa. Additionally, a later discovered publication corroborated these findings and found that the bands at ~85 and ~130 kDa were non-p65 PKA-regulated proteins, but that the antibody was specific upon isolation of p65 in an in vitro kinase assay. Based on this finding, we proceeded to IP total p65 in an attempt to purify and thusly determine ser-276 phosphorylation. However, while we were able to purify p65 and utilize greater protein levels for the analysis, we were unable to detect ser-276 phosphorylation. At this point it was
determined that direct detection of p65 modification was not a feasible means and as such we switched our focus to that of a measure of activation based upon DNA binding capacity of NF-κB. Again, a commercially available product was chosen. In this transcription factor analysis well plates coated with an oligo probe for NF-κB utilized user-provided nuclear lysate to detect relative binding levels via immunohistochemical detection methods. However, this analysis failed to produce meaningful or reproducible results. Based on the success rate of our previous attempts to determine NF-κB activation via commercially available products, we then chose to perform an EMSA with reagents prepared in-lab, from which we were able to acquire repeatable results.

A.2 Materials and Methods

**Cell Cultures.** JB6 Cl41 mouse epidermal skin cells were grown in MEM medium containing 100 units/ml penicillin/streptomycin (Corning Life Sciences) and 5% FBS (Atlanta Biologicals) at 37°C and 5% CO₂ in a humidified incubator. Stably transfected JB6 Cl41 cells containing a pCMV5-Flag-MSK1-A195/NH₂ terminal kinase dead vector (a generous gift from Dr. Yong Yeon Cho, The Hormel Institute, University of Minnesota) were grown under similar conditions.

**UVB Irradiation.** UVB light was generated by two 15 W UVB lamps (UVP Inc.) and measured by a UVB meter (UVP Inc.) to determine intensity. Media from the cells to be treated was removed and the cells exposed to the UVB light
to obtain a dose of 50 mJ/cm². After treatment the media was returned and the cells incubated until sample collection.

Isolation of cytoplasmic and nuclear proteins and whole cell lysates. Cytoplasmic and nuclear lysates were prepared in a step wise fashion using a NE-PER ® reagents kit according to manufacturers recommended protocol (Thermo Scientific) or through the use of a Sucrose Buffer (0.32 M sucrose, 10 mM Tris HCl, 3 mM CaCl₂, 2 mM MgOAc, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT) to collect cytoplasmic proteins and a Low Salt Buffer (20 mM HEPES, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT) and High Salt Buffer (Low Salt Buffer with 800 mM KCl and 1% NP-40) to collect nuclear proteins. Whole cell lysates were collected using PhosphoSafe lysis buffer (Sigma-Aldrich) according to the manufacturer’s recommended protocol.

Western Blot Analysis. Following treatment, cell lysates were collected at 6 and 24 h time points post UVB irradiation. Upon collection, samples were prepped and 20-30 µg of protein ran on 10% SDS-polyacrylamide gel. The samples were then transferred to a nitrocellulose membrane and blocked with 5% milk in TBS-T for 1-3 h (depending on protein of interest) before being incubated overnight (~18hrs) with antibodies targeting p65, p65 Phospho Ser-276, p65 Phospho Ser-536, MSK1, β-actin, or PARP (Santa Cruz Biotechnology and Cell Signaling). Bands were visualized using the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and exposed to radiosensitive film (HyBlot CL, Denville Scientific).
**Immunoprecipitation.** Whole cell or nuclear lysates were prepared as described above. A.) 100-200 µg of protein was incubated overnight (~16 hr) with 1-10 µg of primary p65 antibody at 4° C with light agitation. Following overnight incubation, 100 µl protein A/G agarose bead slurry was added and incubated an additional 3 hr with agitation. B.) 100-200 ug of protein was incubated overnight (~16 hr) with 20 µl anti-p65 conjugated agarose bead slurry. The beads were then separated by centrifugation at 5,000 g for 5 minutes and the remaining solution was decanted. The beads were then washed 3x with PBS and centrifuged as described above. The beads were then boiled with 1x protein loading buffer (5x; 10% w/v SDS, 20% glycerol, 0.2 M Tris-HCl pH 6.8, 0.05% bromophenolblue) and analyzed via western blot.

**Transcription factor assay.** Nuclear lysates were analyzed via a Transcription Factor Kit for NF-κB p50 and p65 (Thermo Scientific) according to manufactures recommended protocols. In brief, 10-20 µg of nuclear proteins were incubated with 1x NF-κB binding buffer and poly-dIdC for 1 h at room temperature in oligo coated wells. The wells were then decanted and washed 3x. 100 µl diluted primary antibody (1:1,000) was then added to each well and incubated for 1 h at room temp, washed 3x and then incubated with 100 µl diluted HRP-conjugated secondary antibody (1:500). Following a final wash, 500 µl Luminol/Enhancer Solution and Stable Peroxide Solution were added to each well and chemiluminescence determined by a SpectraMax M2 Multi-Mode Plate reader (Molecular Devices).
A.3 Results

A.3.1 The specific band for phospho-ser-276 p65 is at a higher molecular weight than p65 and phospho-ser-536

Western blot analysis of wild type and MSK1 N-dead cells was employed to determine expression and modification of p65 following UVB irradiation. Results revealed detection of p65 at a molecular weight of ~70 kDa in both cell lines (Fig. A.2). Additionally, analysis of ser-536 phosphorylated p65 also corresponded to a specific band occurring at ~70 kDa (Fig. A.2). However, detection of ser-276 phosphorylated p65 results in two bands at a weight of ~85 and ~150 kDa (Figure A.2). According to data supplied by Cell Signaling on the anti-Phospho-ser-276 antibody, the specific band for this protein occurs at ~80 kDa (Fig. A.1) corresponding to the band we detected at ~85 kDa in both our cell lines. This size discrepancy raised concerns for if the band was indeed ser-276 phosphorylated p65 we would expect the band to occur at ~70 kDa, just as for those of total p65 and ser-536 phosphorylated p65 did, or for the total p65 antibody to reveal bands at both ~70 and ~85 kDa.
Figure A.1. Manufacturer's data showing phosho-ser-276 p65 specific band.

Western blot analysis of extracts from NIH/3T3 cells, untreated or treated with calyculin A (50 nM) for 10 minutes, using phosho-ser-276 p65 antibody.
Figure A.2. Western blot analysis of p65 and phospho-p65. JB6 Ci41 wild type cells and cells containing a pCMV5-Flag-MSK1-A195/NH₂ vector for a dominant-negative N-terminal kinase dead MSK1 mutant were treated with 50 mJ/cm² UVB. Whole cells lysates were collected after 6 and 24 h and then analyzed via western blot for the expression of p65, phospho-ser-276 p65 and phospho-ser-536 p65.
A.3.2 Western blot analysis of immunoprecipitated p65 is unable to detect ser-276 phosphorylation

Total p65 was immunoprecipitated in an attempt to avoid detection of the predominate nonspecific bands that occur from use of the phospho-ser-276 antibody. Results from western blot analysis confirmed that p65 was indeed isolated following the IP and that the sensitivity for detection was increased in comparison to amount of protein that could loaded into the lanes from whole-cell lysates (Fig A.3 lanes 4-6 vs. 1-3). However, even with the increased amount of p65 present in the IP samples we were unable to detect ser-276 phosphorylation at ~70 kDa (Fig 4.3 lanes 4-6). We also probed with an MSK1 specific antibody to see if it physically interacts with NF-κB and could be co-immunoprecipitated, however, we were unable to detect any MSK1 present in our IP samples (lanes 4-6 vs. 1-3).
**Figure A.3.** Western blot analysis of immunoprecipitated p65. JB6 Cl41 wild type cells were treated with 50 mJ/cm² UVB. Immunoprecipitation of p65 was then performed on whole cells lysates collected after 6 and 24 h and then analyzed via western blot for the expression of p65, phospho-ser-276 p65 and MSK1.
A.3.3 Detection of p65 binding affinity via chemiluminescence detection

yields low signal and statistically insignificant results

Due to the inability to detect NF-κB transactivation via phosphorylation we sought to determine NF-κB activation as a function of its binding affinity following UVB irradiation through the use of a transcription factor assay kit utilizing NF-κB consensus sequence oligo coated well plates and antibody-chemiluminescent detection methods. However this kit failed to produce signals significantly larger than negative controls devoid of nuclear lysates (Fig. A.4).

Figure A.4. NF-κB transcription factor kit results. JB6 Cl41 wild type cells were treated with 50 mJ/cm² UVB. Nuclear lysates were collected after 6 and 24 h and then analyzed via a transcription factor assay kit. Data is reported as raw values.
or normalized to the control after subtraction of the background signal. Error bars represent fold activation +/- standard deviation.

A.4 Discussion

Commercially available antibodies for ser-276-phosphorylated p65 are grown against a synthetic peptide that mimics the structure of the site surrounding phosphorylated ser-276. This allows for the development of an antibody against a protein that would otherwise be unstable in the species employed while still generating an antibody specific for the target of interest. However, data presented in this appendix, as well as a study by Spooren et al. suggest that antibodies generated from this synthetic peptide do not selectively bind phospho-ser-276 in the presence of a mixture of proteins. Western blot analysis of phospho-ser-276 p65 demonstrated a specific band occurring at a weight of ~85 kDa that corresponds with the weight the manufacturer indicates as being the specific band (Fig. A.1 and A.2). However, when probed for total p65, a specific band of only ~70 kDa is observed when two would be expected if the band at ~85 kDa was indeed p65. While it is possible that phosphorylation could disrupt the epitope for total p65, the use of antibodies specific to several p65 epitopes down the length of the protein confirm these findings (data not shown). Additionally, probing for phosphor-ser-536 revealed a specific band of ~70 kDa (Fig. A.2), which rules out the possibility of an additional phosphate
group (95 Da) being responsible for a shift of ~15 kDa. Charge density on the phosphor-ser-276 p65 should also have little effect on a denaturing polyacrylamide gel as once the protein has been denatured, SDS surrounds the backbone of the protein imparting a large negative charge on the molecule.

Although the phospho-ser-276 antibody does not bind to p65 specifically in the presence of a mixture of proteins, Spooren at al. did find that the antibody is indeed capable of binding its site of design upon purification and in vitro phosphorylation of p65. As such, we hypothesized that by purifying total p65 via immunoprecipitation we should then be able detect ser-276 phosphorylation. Results from western blot analysis revealed that we were able to isolate p65 and were able to increase the total amount present on the membrane compared to what could be loaded on the gel from whole cell lysates alone (Fig. A.3 lanes 4-6 vs 1-3). However, we were unable to detect any phosphorylation at ser-276 in our immunoprecipitated samples (Fig. A.3 lanes 4-5). Antibodies for several p65 epitopes were used in the IP procedure to similar effect (data not shown). Additionally, attempts to pull down phosphor-ser-276 p65 through IP and detect levels with total p65 antibodies were met with no success (data not shown). We were also interested in seeing if we could detect a physical interaction between MSK1 and p65 through coimmunoprecipitation; however, we were unable to detect an interaction (Figure A.3 lanes 4-6).

Due to the lack of an adequate detection method for post-translational modification of p65 we switched our focus to try and determine the DNA binding capacity, and thus activation, of NF-κB. In order to achieve this we utilized a
Transcription Factor Assay Kit for p50 and p65 NF-κB (Thermo Scientific) that utilized well plates coated with NF-κB consensus sequence and chemiluminescent detection of bound NF-κB. However this kit failed to generate strong signals and reproducible results. In fact, background levels of negative controls (no cell lysates) wells gave signals that ranged from 50-100% of the intensity of wells loaded with samples. As such, it was determined that this test did not possess the sensitivity needed for detection of NF-κB activation (Fig. A.4). From here it was decided that an EMSA with radiolabeled probe would give us the desired sensitivity we needed to determine activation.