The Role of Lipid Raft-Translocation of Prohibitin in Regulation of Akt and Raf-
Protected Apoptosis of HaCaT Cells upon Ultraviolet B Irradiation

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This dissertation titled
The Role of Lipid Raft-Translocation of Prohibitin in Regulation of Akt and Raf-
Protected Apoptosis of HaCaT Cells upon Ultraviolet B Irradiation

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ABSTRACT

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The Role of Lipid Raft-Translocation of Prohibitin in Regulation of Akt and Raf-
Protected Apoptosis of HaCaT Cells upon Ultraviolet B Irradiation

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Modification of major lipid raft components, such as cholesterol and ceramide, plays a role in regulation of Ultraviolet B light (UVB)-induced apoptosis. Upon UVB irradiation, an alternation of major lipid raft components can lead to the recruitment/activation of raft-associated proteins and initiation of downstream apoptotic signaling pathways. To identify potential regulators of UVB-induced apoptosis, we used two-dimensional gel electrophoresis and mass spectrometry fingerprint analysis to identify proteins that are altered in the rafts after UVB irradiation. Our data shows that levels of several proteins, including prohibitin (PHB), were changed in lipid rafts after UVB irradiation. PHB plays a critical role in many cell functions, such as cell-cycle control, cell proliferation, senescence, development, apoptosis, and tumor suppression. However, until now, no report has identified a role for PHB in mediating apoptosis upon UVB irradiation. We now demonstrate that while total and cell surface PHB expression was not changed, the protein was enriched in lipid rafts after UVB irradiation. Reduced expression of PHB using siRNA knockdown resulted in an increase in apoptosis after UVB irradiation.

To reveal the detailed mechanism for PHB-mediated apoptosis upon UVB irradiation, we evaluate whether PHB is involved in regulation of Akt and Raf activity as well as oxidative stress in UVB-irradiated HaCaT cells. Our data shows that upon UVB irradiation PHB is translocated to the lipid rafts, which is correlated with a release of both Akt and Raf from membrane. Overexpression of Akt and/or Raf impedes UVB-induced lipid rafts translocation of PHB. Immunoprecipitation analysis indicates that UVB alters the interactions among PHB, Akt, and Raf. Reduced expression of PHB leads to a decreased phosphorylation of Akt and ERK, as well as a decreased activity of Akt, and an increased apoptosis of the cells after UVB irradiation. In addition, our data shows that the effect of LNAC on UVB-mediated apoptosis and proliferation is independent of PHB knockdown, suggesting an alternative pathway independent of UVB-induced oxidative stress. These results suggested that PHB regulates UVB-induced apoptosis of keratinocytes via a mechanism that involves detachment from Akt and Raf on the plasma membrane, and sequentially lipid rafts translocation.

DEDICATION

I would like to dedicate this work to my parents for their love and support.

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LIST OF ABBREVIATIONS

2-DE	two-dimensional electrophoresis
A-SMase	acid sphingomyelinase
ANX5	annexin V
DISC	death-inducing signaling complex
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLIP	FLICE-like inhibitory protein
GCMN	giant congenital melanocytic nevus
GSH	glutathione
HP1	heterochromatin protein 1
HRP	horseradish peroxidase
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MCM	minichromosome maintenance complex of proteins
MERRF	myoclonic epilepsy and ragged-red fibers
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
PDK1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology

PHB	prohibitin
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PMF	peptide mass fingerprinting
ROS	reactive oxygen species
SPFH	stomatin/prohibitin/flotillin/HflK/C
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
UV	ultraviolet
xIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1: BACKGROUND

Ultraviolet (UV) Light -Induced and Lipid Raft-Mediated Apoptosis

UV light

According to the American Cancer Society, every year more than 3.5 million cases of non-melanoma skin cancer and about 76,600 cases of skin cancer are reported in the United States, resulting in about 12,000 deaths in 2013. It is clear that UV light is one of the main risk factors of skin cancer. There are three types of UV light: UVA (400nm-315nm), UVB (315nm-280nm), and UVC (280nm-100nm) (Diffey 2002). UVC has the highest energy, and is the most dangerous type of UV light. However, it is filtered out of the atmosphere. So, the UV light that can reach the earth is of type UVA and UVB (see Figure 1.1). Due to its long wavelength, UVA penetrates the skin more deeply than UVB. Some studies suggest that UVA can cause the aging of the skin, and probably the initiation of skin cancer (Attard and Karran 2012; Mitchell and Fernandez 2012; Robin, Courderot-Masuyer et al. 2012; Song, Liu et al. 2012). UVB causes more damage than UVA since it has higher energy. Many scientists and clinicians believe that UVB is the main cause of various skin problems, such as sunburns, direct skin DNA damage, and, most importantly, skin cancer (Van

Laethem, Claerhout et al. 2005; Situm, Buljan et al. 2007; Kawachi, Xu et al. 2008; Dickinson, Olson et al. 2011).

Figure 1.1 Ultraviolet light.

Due to different wavelengths, UV light is divided into three different types: UVA, UVB, and UVC.

In order to discover the therapeutics against UV-induced skin cancer, numerous research projects have focused on the relationship between UV irradiation and cell apoptosis. It has been reported that UV light can lead to apoptosis through DNA damage (Iwasaki, Izawa et al. 1996; Lu, Sato et al. 1996; Souvannavong, Lemaire et al. 1997). Other reports have focused on signaling

pathways involved in UV-induced apoptosis (Charruyer, Grazide et al. 2005; Rotolo, Zhang et al. 2005; Elyassaki and Wu 2006; Charruyer, Jean et al. 2007; Singh, Schneider et al. 2009).

UV-induced apoptosis

Apoptosis, which is different from necrosis caused by damage by an external force, was first defined by Kerr as a controlled cell deletion (Kerr, Wyllie et al. 1972). During apoptosis, there is no inflammatory response or organelle swelling (Gerschenson and Rotello 1992). This is due to the flipping of phosphatidylserine, originally localized in the inner leaflet of the plasma membrane, onto the outer leaflet (Savill, Gregory et al. 2003). Apoptosis plays an important role in the regulation of cell development and growth. The process of apoptosis is characterized by the following changes: cell shrinkage, DNA fragmentation, nuclear condensation, and the formation of apoptotic bodies (Kerr, Wyllie et al. 1972; Parr, Tung et al. 1987; Tanaka, Koga et al. 1993; Jarvis, Turner et al. 1994). There are two major apoptotic pathways: one is the extrinsic pathway that is activated by death receptors, and the other is the intrinsic pathway that is triggered by various stress stimuli (Gastman 2001; Kiechle and Zhang 2002; Schultz and Harrington 2003; Zhang, Zhang et al. 2003; MacFarlane and Williams 2004). These two signaling pathways finally recruit

caspases, a highly conserved family of cysteine proteases, such as caspase -3, -7, -8, -9, -10, leading to their activation (Thornberry and Lazebnik 1998).

UV irradiation-induced apoptosis is a highly complicated process that serves as a protective mechanism to eliminate the UV-damaged cells that have the potential of malignant transformation. There are many signaling pathways involved in UV-induced apoptosis, and some signaling pathways even crosstalk with one another. One of these signaling pathways is mediated through lipid raft. The following part will focus on the characterization of lipid raft.

Lipid rafts

The plasma membrane, composed of the phospholipid bilayer, is the boundary that separates the interior of the cell from the outside environment. The lipid raft model was first proposed as a non-ionic, detergent-resistant (such as Triton X-100) eukaryotic plasma membrane fraction that was enriched in sphingolipids, cholesterol and caveolins (Rothberg, Ying et al. 1990; Brown and Rose 1992; Simons and Ikonen 1997). Caveolins, the lipid raft marker proteins, are a family of integral membrane proteins that are enriched in the specific lipid raft microdomains named caveolae (Parton 1996). Sphingolipids are one of the major classes of lipids in eukaryotic plasma membranes. Sphingomyelin is one type of sphingolipids that is present in lipid raft. Ceramide, the central building block of sphingolipids, is one of the most studied sphingolipids because it is

implicated in the mediation of stress-induced apoptosis (Obeid, Linardic et al. 1993; Verheij, Bose et al. 1996). After UVA or UVC irradiation, the acid sphingomyelinase (A-SMase), which originally localized in the cytosol of the cell, translocates onto the plasma membrane and consumes the sphingomyelin to generate ceramide (Zhang, Mattjus et al. 2001; Kashkar, Wiegmann et al. 2005; Rotolo, Zhang et al. 2005). However, more studies need to be done to explore the relationship between UVB irradiation and ceramide generation from A-SMase. The generated ceramide translocates to the outer leaflet of the membrane, where it can form ceramide-enriched platforms (Zhang, Mattjus et al. 2001; Lacour, Hammann et al. 2004; Charruyer, Grazide et al. 2005; Kashkar, Wiegmann et al. 2005; Rotolo, Zhang et al. 2005; George, Elyassaki et al. 2012). In addition, ceramide displaces sterols such as cholesterol from lipid raft, leading to the alteration of lipid raft structure and function (Megha and London 2004; Megha, Bakht et al. 2006; George, Elyassaki et al. 2012). As a result, cholesterol level increases in the early time but decreases in the late time post-UVB in M624 cells, which is critical for UVB-induced cell death (George, Elyassaki et al. 2012). However, in some cell lines, cholesterol cannot be displaced in ceramide-enriched platform (George, Elyassaki et al. 2012). Due to its ever-changing components, the lipid rafts are very dynamic, which leads to the vast variety of signaling mechanisms that can take place within them. The following part will discuss some important regulators that are involved in lipid raft-mediated apoptotic signaling pathways upon UV irradiation, in detail.

Regulators in UV-induced and lipid raft-mediated apoptosis

By trapping and clustering different signaling molecules, lipid rafts enable the cell to respond to different stimuli, UV irradiation included. George and Wu have summarized the mechanism of lipid raft activation under stimuli (see Figure 1.2) (George and Wu 2012). There are three possible mechanisms: A) upon stimuli, the components of the lipid raft have been altered and the lipid raft is modified, which will promote the activation of signaling molecules on lipid rafts; B) upon stimuli, the signaling molecules that originally localize outside of lipid raft aggregate onto the lipid raft. These aggregated molecules are activated and regulate the downstream signaling cascades; C) upon stimuli, the ceramide-enriched platforms are very hydrophobic and stable, which promotes the clustering of lipid rafts (Grassme, Jekle et al. 2001; Grassme, Bock et al. 2002; Grassme, Jendrossek et al. 2002). Furthermore, the clustering of lipid raft forming ceramide-enriched platforms often initiates the activation of different signal molecules, which mediates downstream signaling pathways, including but not limited to apoptosis.

Figure 1.2 Mechanism of lipid raft activation (George and Wu 2012).

(A) Signaling molecules localize on lipid rafts. (B) Signaling molecules localize outside of lipid rafts. (C) Signaling molecules localize on small lipid rafts.

There are many membrane signaling molecules involved in UV-induced apoptosis. Some of these molecules are activated within lipid rafts, which may

be due to the modification or component alteration of lipid rafts as discussed above. To better understanding the role of lipid rafts involved in UV-induced apoptosis, the potential mechanism has been studied on some important lipid raft-associated proteins that mediate apoptosis through lipid raft activation upon UV irradiation.

CD95

CD95, also known as the Fas receptor, is a member of the tumor necrosis factor (TNF) superfamily. UVB and C induce the aggregation of CD95 molecules on the cell surface, which, in turn, induces apoptosis via recruitment of the Fas-associated death domain protein (FADD) followed by activation of the caspase-8 cascade (Rehemtulla, Hamilton et al. 1997; Aragane, Kulms et al. 1998). However, UVA-induced activation of FADD-caspase-8 death pathway is Fas receptor independent (Zhuang and Kochevar 2003). UVB induces the aggregation and activation of CD95 on lipid rafts in M624 cells (Elyassaki and Wu 2006). Additionally, UVC-induced CD95 aggregation recruits CD95 death domain-associated protein Daxx, which mediates c-Jun N-terminal kinase (JNK) activation and promotes apoptosis (Wu, Hu et al. 2002). Taken together, these data suggest that UV irradiation induces activation of CD95 in lipid rafts, leading to apoptosis.

TRAIL

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of TNF family, is expressed on the cell surface and induces apoptosis (Wiley, Schooley et al. 1995). TRAIL is localized on lipid rafts (Kabsch, Mossadegh et al. 2004; Gregory-Bass, Olatinwo et al. ; Min, Shi et al. 2009; Rossin, Derouet et al. 2009; Xu, Zhang et al. ; Li, Han et al. 2013), and A-SMase is essential for TRAIL function in lipid rafts (Li, Han et al. 2013). UVB irradiation induces TRAIL sensitivity that leads to cell apoptosis via the inhibition of the FLICE-like inhibitory protein (FLIP) expression (Zeise, Weichenthal et al. 2004), the inhibition of the X-linked inhibitor of apoptosis protein (xIAP) expression (Thayaparasingham, Kunz et al. 2009), as well as the induction of the release of the cytochrome c and Smac (a mitochondrial protein that promotes cytochrome c-dependent caspase activation) from mitochondria (Qin, Bacon et al. 2004). In addition, UVC can enhance apoptosis in acquired TRAIL-resistant cell (Kim, Park et al. 2008). However, no detailed mechanism has been shown how TRAIL mediates apoptosis upon UVC irradiation. Besides UVB and UVC, there is no direct evidence to show the relationship between UVA irradiation and TRAIL-mediated apoptosis by now. The results of these studies lead to the conclusion that UV irradiation increase TRAIL-mediated apoptosis, which is possibly related to lipid raft regulation.

PKCζ

PKCζ is an atypical isoenzyme of the protein kinase C (PKC) family of serine and threonine protein kinases (Hirai and Chida 2003). Distinct from the classical isoenzymes of PKC, PKCζ exhibits enzymatic activity that is independent of Ca^{2+} , diacylglycerols, and phorbol esters (Ways, Cook et al. 1992), consistent with its structure (Ono, Fujii et al. 1989). PKCζ can be recruited into lipid rafts and be activated (Cuschieri, Bulger et al. 2007; Shin, Yang et al. 2008). PKCζ protects the cells from UVC-induced apoptosis by inhibiting the translocation and activation of A-SMase into the lipid rafts (Charruyer, Jean et al. 2007), which leads to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Lozano, Berra et al. 1994) and inhibition of CD 95 recruitment and caspase-8 activation (Gomez-Angelats and Cidlowski 2001; Leroy, de Thonel et al. 2005). These data, taken together, suggest that PKCζ protects the cells from UV-induced apoptosis through the inhibition of lipid raft-mediated signaling pathways.

PDK1

Phosphoinositide-dependent kinase 1 (PDK1) is a serine and threonine protein kinase that phosphorylates and activates several AGC-family members (Alessi, Deak et al. 1997). PDK1 is activated in the lipid rafts of the cell (Gao, Lowry et al.). UVB induces PDK1 phosphorylation (Zykova, Zhang et al. 2005). Overexpression of wild type PDK1 protects cells from UV-induced apoptosis,

while overexpression of constitutively nuclear PDK1 cannot protect UV-induced apoptosis (Lim, Kikani et al. 2003), which indicates that the translocation of PDK1 into nucleus may diminish its role in the regulation of cell survival. It is highly possible that UV irradiation induces PDK1 translocation to lipid rafts, not to nucleus, promotes PDK1 phosphorylation and activation in lipid rafts with resultant of protection of cell apoptosis.

The membrane proteins, as mentioned above, play important roles in mediating UV-induced and lipid raft-mediated apoptosis (see Table 1.1 for summary). Dr. Wu's research group has already demonstrated that Fas was recruited to lipid raft upon UV irradiation, which is related to the induction of apoptosis (Elyassaki and Wu 2006). To further explore the lipid raft-mediated proteins involved in UV-induced apoptosis, a proteomics study on the lipid rafts has been performed. Several proteins, including prohibitin (PHB), have been identified within the lipid raft that has altered protein expression levels after UVB irradiation. However, it remains undetermined whether PHB plays a role in UVB-induced apoptosis.

Table 1.1

Summary of UV-induced and lipid raft-mediated apoptosis

Proteins	Effects	Recruited counterparts
CD95	+	FADD, Daxx, JNK, and Caspase 8
TRAIL	+	FLIP, xIAP, cytochrome c and Smac
PKC ζ	-	NF- κ B, and CD95
PDK1	-	Not determined

Footnote: “+” represents induction of apoptosis; “-” represents inhibition of apoptosis

PHB

PHBs are comprised of four conserved and ubiquitously expressed proteins, prohibitin-1 (PHB1, or PHB), as well as its homologous proteins prohibitin-2 (PHB2, also called B-cell receptor-associated protein 37 (BAP37) and repressor of estrogen receptor activity (REA)), prohibitin-3 (PHB3), and prohibitin-4 (PHB4) (Nadimpalli, Yalpani et al. 2000). Current research has focused on PHB and PHB2, and here we specifically target PHB. In 1989, PHB was first isolated as an M5 clone from a set of cDNAs in rat liver cells, and identified as an antiproliferative gene (McClung, Danner et al. 1989). At this time, M5 clone was then termed “prohibitin” since it was shown to inhibit DNA synthesis (McClung, Danner et al. 1989; Nuell, Stewart et al. 1991). Later, the

3'-UTR of PHB gene was found to regulate the tumor suppressor activity of PHB (Jupe, Liu et al. 1996; Jupe, Liu et al. 1996). Recently, PHB has been demonstrated to inhibit DNA replication by interacting with a minichromosome maintenance complex of proteins (MCM) (Rizwani, Alexandrow et al. 2009). Other than antiproliferation, tumor suppression and cell growth arrest, PHB has been suggested in maintaining other cell functions, such as cell apoptosis (Welburn and Murphy 1998), cell senescence, and cell development (McClung, Jupe et al. 1995). Previously, most research studies have found that PHB serves as an anti-apoptotic protein (Chowdhury, Branch et al. 2011). However, some groups have claimed that PHB induces apoptosis (Liu, Peck et al. 2012). By now, the role of PHB in UVB-induced apoptosis is still unknown. Before determining the anti-apoptotic or pro-apoptotic role of PHB upon UVB irradiation, the detailed introduction on its structure, distribution, and specific regulation will be given in light of the importance of PHB in maintaining cellular signaling pathways, as well as its role in diseases.

Structure

In different species from prokaryotes to eukaryotes, it has been found that PHBs and other proteins, like erlins, podocin, stomatins, and flotillins, contain the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain (also called PHB domain) (Browman, Hoegg et al. 2007). The PHB domain-containing proteins that tend to

form of oligomers, and the C-terminal region of these proteins has been suggested to be involved in the oligomerization (Langhorst, Reuter et al. 2005). Among these proteins, PHB and PHB2 have been reported to form hetero-oligomers (Langhorst, Reuter et al. 2005; Tatsuta, Model et al. 2005). The PHB domain-containing proteins are enriched within lipid rafts and mediate different cellular processes through the regulation of these lipid rafts. It has been suggested that PHB domain-containing proteins can regulate ion channels in lipid raft (Gillespie and Walker 2001; Huber, Schermer et al. 2006), as well as regulate the lipid raft-dependent endocytic pathways (Glebov, Bright et al. 2006). It remains unknown whether PHB domain-containing proteins have any other signaling functions within the lipid rafts, such as the regulation of apoptosis.

PHB is a 32 kDa protein, while PHB2 is a 34 kDa protein. PHB and PHB2 share around 50% identical amino acid residues, and more than 60% similarity at the amino acid level. As shown in Figure 1.3, besides the PHB domain, PHB contains an N-terminal transmembrane domain that forms from a hydrophobic alpha helix (Theiss, Idell et al. 2007). Compared to PHB, PHB2 contains an N-terminal hydrophobic domain that not only serves as a transmembrane function, but also regulates the signal information sorting function (Tatsuta, Model et al. 2005). The specific structure of PHB indicates its localization in the mitochondrial inner membrane as well as in the nucleus (Tatsuta, Model et al. 2005). Later studies have found that PHB also localizes in other different subcellular locations, such as plasma membrane (see Figure 1.4).

Figure 1.3 Domain structure of mammalian PHBs (Browman, Hoegg et al. 2007). Grey, transmembrane domain; Blue, SPFH domain; Brown, hydrophobic domain.

Distribution and regulation

The diverse functions of different localized PHB will be discussed in the following paragraphs in detail.

TRENDS in Molecular Medicine

Figure 1.4 Subcellular distribution of PHB (Mishra, Murphy et al. 2005).

PHB localizes in different subcellular locations: mitochondria, nucleus, and plasma membrane.

PHB in mitochondria

The best studied function of PHB is as a mitochondria chaperone protein (Coates, Nenutil et al. 2001; Tatsuta, Model et al. 2005; Gregory-Bass, Olatinwo et al. 2008; Osman, Merkwirth et al. 2009; Kathiria, Butcher et al. 2012; Zhou, Qian et al. 2012). PHB in the mitochondria stabilizes mitochondria function, such

as the maintenance of mtDNA stability and mitochondrial morphology, as well as the assembly of the respiratory-chain (Osman, Merkwirth et al. 2009). The dysfunction of the respiratory chain leads to mitochondria membrane damage and mitochondria dysfunction. It is known that reactive oxygen species (ROS) are mainly generated as a by-product of respiration in the mitochondria, and PHB protects cells from oxidative stress (Zhou, Qian et al. 2012). There are many studies on PHB to explore its role in mitochondria. For example, the expression level of PHB decreased in response to oxidative stress, while PHB overexpression can increase glutathione (GSH) level and GST π expression to decrease ROM accumulation (Theiss, Idell et al. 2007). In addition, PHB protects cardiomyocytes from oxidative stress-induced damage, which is related to cardiomyocyte death (Liu, Ren et al. 2009). Taken together, these data suggest that PHB decreases ROS produced by mitochondria and protects cells from oxidative-stress-mediated apoptosis.

PHB in nucleus

Several reports demonstrate that PHB localizes and is associated with other nuclear proteins in nucleus in different cell lines. PHB colocalizes with the Rb protein (Wang, Fusaro et al. 2002), Brg-1 and Brm (Wang, Zhang et al. 2002), as well as recruited heterochromatin protein 1 (HP1) gamma to E2F-mediated proliferative promoters (Rastogi, Joshi et al. 2006), which is required for the repression of E2F-mediated transcription. Moreover, PHB colocalizes with p53

and induces p53-mediated transcription (Fusaro, Dasgupta et al. 2003). Skp2B attenuate p53 activity by interacting with PHB and by promoting PHB degradation (Chander, Halpern et al. 2010). In addition, PHB colocalizes and interacts with MCM2, MCM5, and MCM7, and inhibits DNA replication (Rizwani, Alexandrow et al. 2009). PHB has also been found to colocalize with c-myc, and c-fos (Shi, Li et al. 2009; Li, Liang et al. 2011). A recent report suggested that PHB serves as a cancer prevention target through its downregulation and cellular trafficking from nucleus to cytoplasm (Shi, Li et al. 2009). Therefore, PHB, one of the essential cell-cycle regulators, mediates signaling pathways in the nucleus, such as transcription regulation, and DNA replication. However, no direct evidence shows whether PHB in nucleus is critical in mediating cell apoptotic signaling pathways.

PHB in plasma membrane

In 1994, PHB was reported to associate with the IgM antigen receptor in B lymphocytes (Terashima, Kim et al. 1994), which suggests PHB localizes on the plasma membrane. This subcellular localization was later confirmed in other cell types or tissues (Kolonin, Saha et al. 2004; Mengwasser, Piau et al. 2004; Sharma and Qadri 2004; Rajalingam, Wunder et al. 2005; Ande and Mishra 2009; Chiu, Ho et al. 2013). By screening a phage display library, Kolonin and his colleagues have isolated a peptide motif, CKGGRAKDC (Kolonin, Saha et al. 2004), which has been found to bind to membrane protein PHB in white adipose

tissue. Another pro-apoptotic peptide, KLAKLAKKLAKLAK, which is known to disrupt the mitochondrial membrane and induce apoptosis, was used in this study. They coupled this pro-apoptotic peptide with PHB binding peptide motif, and found the occurrence of the ablation of white fat, indicating the apoptosis. These data suggests that PHB translocates from membrane to the other subcellular locations, such as the mitochondria, possibly leading to cell apoptosis. The CKGGRAKDC peptide motif has been used to detect cell surface PHB by immunofluorescence (Patel, Chatterjee et al. 2010). However, there is no direct evidence to show that PHB is a transmembrane receptor.

Recent studies reported the involvement of membrane PHB in two main cell survival signaling pathways, Raf/ERK and Phosphoinositide 3-kinase (PI3K)/Akt (Rajalingam, Wunder et al. 2005; Ande and Mishra 2009; Mishra, Ande et al. 2010; Chiu, Ho et al. 2013). Both PI3K/Akt and Raf/ERK are essential in UV-induced apoptosis. Before exploring the role of PHB in mediating PI3K/Akt and Raf/ERK signaling pathways, a general background will be given on PI3K/Akt and Raf/ERK activation, as well as the role of PI3K/Akt and Raf/ERK in UV-induced apoptosis.

Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinase (MAPK) family that regulate cellular processes (e.g., proliferation, differentiation, and cell cycle progression) by converting tyrosine phosphorylation into the serine/threonine phosphorylations of downstream elements (Boulton, Nye et al. 1991). Localized in the cytoplasm in

quiescent cells, ERKs translocate to the nucleus in response to extracellular stimuli, and then relocate to the cytoplasm via MAPK kinase-dependent active transport (Adachi, Fukuda et al. 2000). UV irradiation activates ERKs in some cell lines (Huang, Ma et al. 1999; Chen, Tang et al. 2001; Zhu, Zhang et al. 2004; Ming, Han et al. 2010), and inactivates them in others (Wang, Chen et al.). ERK activation provides a survival signal against stress-induced apoptosis (Kitagawa, Tanemura et al. 2002), including UV irradiation (Ming, Han et al.), mediating apoptosis resistance in keratinocytes with mutant keratin (Russell, Ross et al. 2010).

PI3Ks are members of a unique and conserved family of lipid kinases that mediated many cell functions like cell growth, proliferation, survival, and migration, and the activation of PI3Ks can occur through growth factor and cytokine receptors, Ras, immunoreceptors, and G-protein-coupled receptors (Wymann, Zvelebil et al. 2003). The activation of PI3K is due to the binding of its p85 regulatory subunit to tyrosine-phosphorylated IRS (Hadari, Tzahar et al. 1992). PI3K phosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3), and then induces the activation of serine/threonine kinase Akt (Banfic, Tang et al. 1998). Akt signaling is mediated through lipid rafts, as the disruption of lipid raft does not affect binding ability of PI3K to EGFR but impairs Akt phosphorylation, leading to the increased sensitivity of apoptosis in both normal HaCaT cell and transformed (A431) keratinocytes (Calay, Vind-Kezunovic et al.). PKB/Akt has multiple downstream targets that are mainly associated with metabolic and

survival responses that protect cells from apoptosis (Marte and Downward 1997). The activation of Akt mediated by PI3K promotes cell growth and survival and inhibition of apoptosis (Franke, Kaplan et al. 1997). It has been suggested that upon UV irradiation, PI3K has been activated, and Akt has been phosphorylated (Ibuki and Goto 2000) and activated (Li, Wu et al. 2006; Han, Ming et al. ; Lin, Xu et al. 2012), indicating the inhibition of apoptosis. However, it has also been reported that UV irradiation causes Akt inactivation through mediating the activation of JNK (Wang, Chen et al.). To this day, there is no conclusion to reveal the relationship between UV-mediated Akt activation and UV-induced apoptosis.

The pleckstrin homology (PH) domain of Akt facilitates Akt translocation to plasma membrane, and direct binding to PI3K-produced phospholipids (Cheng, Lindsley et al. 2005). Akt is then phosphorylated at threonine 308, followed by the fully activation of Akt through serine 473 phosphorylation (Cheng, Lindsley et al. 2005). Raf is also recruited to plasma membrane and activated via phosphorylation at serine 338 and dephosphorylation at serine 259 (Rajalingam, Wunder et al. 2005). The activation of Raf has been suggested to occur when localized on caveolin- and cholesterol-enriched membrane microdomains (Mineo, James et al. 1996; Hekman, Hamm et al. 2002). Akt directly binds to Raf and inhibits Raf by phosphorylating Raf at serine 259 (Zimmermann and Moelling 1999).

The crosstalk between Raf/ERK and PI3K/Akt pathways has been demonstrated in many reports (Myhre, Sterri et al. 2004; Merighi, Benini et al. 2006; Lin, Chang et al. 2008; Aksamitiene, Kholodenko et al. 2010; Romano, Matallanas et al. 2010; Yang, Guo et al. 2011; Ozel, Seidel et al. 2012), and PHB plays a critical role in regulating this crosstalk. PHB interacts with Raf in the caveolin-1-enriched fraction, which is essential for Raf/ERK activation (Rajalingam, Wunder et al. 2005). PHB is phosphorylated at threonine 258 by Akt (Ande and Mishra 2009), which promotes the activation of PI3K/Akt signaling. Overexpression of PHB phosphorylation at threonine 258 facilitates the activation of both PI3K/Akt and Raf/ERK signaling cascades (Chiu, Ho et al. 2013). Taken together, this evidence suggest that PHB serves as a scaffold to regulate both PI3K/Akt and Raf/ERK signaling cascades, and may play a potential role in regulating the crosstalk for other important signaling pathways. Since the dysfunction of these signaling pathways leads to many kinds of diseases, PHB may serve as a potential target for disease therapy. It is well-appreciated that UV irradiation cause a lot of skin problems. The following paragraph will focus on discussion the potential role of PHB in mediating skin dysfunction and disease.

Role of PHB in skin disease

PHB is upregulated in skin fibroblasts from patient with myoclonic epilepsy and ragged-red fibers (MERRF) syndrome (Wu, Ma et al. 2010). PHB is also

upregulated in the giant congenital melanocytic nevus (GCMN) (Kim, Kim et al. 2012) and also in melanoma cells A375 (Li, Su et al. 2013), which suggests PHB might play an important role in the melanoma formation. There is a recent clinical report that studied melanoma patients receiving UV light irradiation in a high ultraviolet index region, which shows that PHB 3'UTR genotype increases in patients with melanoma compared to controls (Francisco, Goncalves et al. 2013). However, current research has not yet found the detailed mechanism of PHB involved in melanoma formation. More studies need to be done to disclose the role of PHB in melanoma tumorigenesis before we target it as a melanoma cancer therapy.

In this dissertation, the role of PHB involved in UVB-induced human keratinocyte apoptosis has been explored. After understanding the basic role of PHB in UVB-induced apoptosis, future study will be emphasized on whether there is dysfunction of PHB in a certain type of skin cancer -- melanoma. This information will be used to focus on PHB as a potential cancer therapy target in UV-induced melanoma formation.

CHAPTER 2: LIPID RAFT-ASSOCIATED PROHIBITIN, A REGULATOR OF ULTRAVIOLET B LIGHT-INDUCED APOPTOSIS

Lipid rafts are sphingolipid- and cholesterol-enriched microdomains of the cellular membrane that are characterized by light buoyant density using density gradient centrifugation while treated with non-ionic detergent Triton X-100 at the low temperature (Rothberg, Ying et al. 1990; Brown and Rose 1992). Several apoptotic regulatory proteins, such as Fas, Ras, and $\text{TNF}\alpha$, have been shown to be recruited into lipid raft upon various stimuli (Smart, Graf et al. 1999; George and Wu 2012). UVB is one of the stimuli that alters major components of lipid rafts, which regulate apoptosis through recruiting and promoting the aggregation of Fas, formation of DISC and activation of Fas- FADD-caspase 8 cascade in skin cells (Elyassaki and Wu 2006; George, Elyassaki et al. 2012). However, the functions of other lipid rafts-associated proteins in regulation of UVB-induced apoptosis are still generally unknown. In this report, proteomic screening was used to identify lipid rafts-associated proteins in human keratinocytes HaCaT cells before and after UVB-irradiation. Three potential apoptosis regulators, ATP synthase, NADH dehydrogenase and PHB, were increased in lipid rafts after UVB irradiation. Further analysis indicated that PHB, a lipid rafts-associated protein (Fusaro, Dasgupta et al. 2003; Kim, Lee et al. 2006; Gregory-Bass, Olatinwo et al. 2008; Liu, Ren et al. 2009; Staubach, Razawi et al. 2009;

Gylfason, Knutsdottir et al. ; Lee, Nguyen et al. ; Ponce, Brea et al.), plays a role in regulation of UVB-induced apoptosis of keratinocytes.

Materials And Methods

Cell culture

The HaCaT cells (human keratinocyte cell line, kindly provided by Dr. Nihal Ahamad, University of Wisconsin, Madison, WI) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Manassas, VA) supplemented with 1% penicillin-streptomycin (Mediatech, Manassas, VA) and 10% fetal bovine serum (FBS, Denville Scientific, Metuchen, NJ). The cells were cultured at 37 °C with 5% CO₂.

UVB irradiation

UVB light (290–320 nm) was generated from two 15 W UVB tubes (UVP, Upland, CA). The intensity of UVB light was measured using a UVX digital radiometer (UVP, Upland, CA). The cells were irradiated with 50 mJ/cm² of UVB light. The culture medium was replaced with PBS during exposure and was added back to the culture plates after UVB treatment. The cells were then cultured until harvesting.

Lysis of cells

Cells were collected, pelleted, and lysed by Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl, 0.1% SDS) containing protease inhibitor cocktail (Calbiochem, La Jolla, CA). The cell lysate was incubated on ice for 15 min and centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatant was collected and protein concentration was determined by Bio-Rad Dc Protein Assay kit (Bio-Rad, Hercules, CA).

Isolation of lipid rafts

Lipid rafts fractions were isolated using Optiprep™ (Sigma, St. Louis, MO) density gradient (40% - 5%) as previously reported (George, Wu et al.) with modification. Briefly, the cells were collected, pelleted, and frozen at -80 °C overnight. Then the cells were lysed with cold 0.1% Triton X-100 membrane raft isolation buffer (50 mM Tris-HCl, PH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1% Triton X-100, and the protease inhibitor cocktail). Equal volume of the lysates were applied to a discontinuous gradient (40% - 5%) and centrifuged using a SW60 rotor (Beckman Coulter, Brea, CA) at 31,300 rpm for 5 h at 4 °C. Five 0.8 mL fractions were collected from each gradient for future two-dimensional electrophoresis (2-DE) or immunoblotting analysis.

2-DE and quantification of proteins

Equal amounts of proteins from each lipid rafts fractions of the density gradient were mixed with five volumes of modified HEPES buffer (25 mM HEPES, PH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and the protease inhibitor cocktail) and ultracentrifuged at 35,000 rpm for 30 min in SW60 rotor (Beckman Coulter, Brea, CA) at 4 °C. The pelleted lipid rafts-proteins complex was resuspended in 2-DE sample buffer [9 M urea, 2 M thiourea, 100 mM DTT, 2% CHAPS (w/v), 60 mM n-octyl β -D-glucopyranoside, 2% biolytes, and the protease inhibitor cocktail]. The proteins were then loaded onto a 17 cm immobile pH gradient gel (IPG) strip with a linear pI range of 3-10 (Bio-Rad, Hercules, CA) and separated in a Bio-Rad Protean IEF Cell for 22 h for the first dimension. The stripes were washed in the equilibration buffer [37.5 mM Tris/HCl, PH 8.8, 7 M Urea, 2% SDS (w/v), and 20% glycerol (v/v)] and cut from each side by 4.5 cm to get the final gel with the pI range of 5-8. To separate the proteins by mass, SDS-PAGE was performed as the second dimension. After electrophoresis, the gels were fixed overnight at 4 °C, and stained with Sypro Orange (1:5000) (Molecular Probes, Eugene, OR). Gels were then scanned using Pharos FX plus laser-scanner (Bio-Rad, Hercules, CA) and analyzed using QuantityOne software (Bio-Rad, Hercules, CA).

Protein identification

Proteins of interest on 2D gel were cut out and sent to Protea Biosciences, Inc. (Morgantown, WV) for sequencing. Briefly, the proteins were in-gel protein digested, and then the protein fragments were analyzed using LC-MALDI TOF-TOF MS/MS mass spectrometry, followed by the peptide mass fingerprinting (PMF).

Western blot analysis

The proteins were boiled in 5-fold Laemmli buffer (0.25 M Tris-HCl, pH 6.8, 0.5 M DTT, 10% SDS, 50% glycerol and 0.03% bromophenol blue), separated on SDS-PAGE, and transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk (w/v) in TBST buffer for 30 min at room temperature and then incubated with primary antibodies against PHB (sc-28259) and Caveolin-1 (sc-894) (Santa Cruz Biotechnology, Santa Cruz, CA), and β -actin (A5316) (Sigma, St. Louis, MO) at 4 °C overnight. After washing with TBST, the membrane was incubated with corresponding secondary horseradish peroxidase (HRP)-conjugated antibodies for 30 min at room temperature. The signals were developed using West Pico Supersignal chemilluminiscent substrate (Pierce, Rockford, IL).

PHB silencing using RNA interference

Cells were seeded in a six well tissue culture plate in antibiotic-free DMEM with 10% FBS and incubated at 37 °C with 5% CO₂ until 60-80% confluent. The medium was then replaced with a transfection mixture (500 µL), containing scrambled siRNA (60 nM, sc-37007) or PHB siRNA (60 nM, sc-37629), siRNA Transfection Medium and siRNA Transfection Reagent, prepared according to manufacture's protocol (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were then cultured at 37 °C with 5% CO₂ for 6 h before antibiotic-free DMEM with 20% FBS (500 µL) was added to each well for overnight incubation. The transfection reagent was then replaced with DMEM with 10% FBS and incubated for another 18-24 h before harvesting.

Apoptosis assay

The apoptotic cell death was determined using a fluorescein isothiocyanate (FITC)-conjugated annexin V (ANX5)/propidium iodide (PI) apoptosis detection kit following the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ). Briefly, the cells were suspended in annexin V binding buffer [10 mM Hepes/NaOH (PH 7.4), 140 mM NaCl, 2.5 mM CaCl₂] at 1×10⁶ cells/mL. The cell suspension (100 µL) was mixed with 5 µL FITC-ANX5 and 5 µL PI. After incubating in the dark for 15 min, the cells were combined with 400

μ L ANX5 binding buffer (BD Biosciences, Franklin Lakes, NJ) and apoptotic death was analyzed using a FACSort Flow Cytometer (BD Science, Franklin Lakes, NJ) equipped with CellQuest software (BD Science, Franklin Lakes, NJ). 1×10^5 cells were collected and analyzed.

Statistics

Student's t-test was used to analyze the statistic significance of data. $p < 0.05$ was considered the statistical significant.

Results

2-DE analysis of lipid rafts-associated proteins before and after UVB irradiation

UVB irradiation induces a dynamic alternation of the major components of lipid raft and rafts-associated proteins, which plays important roles in regulation of apoptosis (Elyassaki and Wu 2006; George, Elyassaki et al. 2012). To further identify proteins that are involved in UVB-induced and lipid rafts-mediated apoptosis, we analyzed protein rearrangement in lipid rafts of HaCaT cells upon UVB irradiation using gradient centrifugation followed by 2-DE analysis. To ensure the isolation of lipid rafts-associated proteins, the presence of caveolin-1, a protein marker present in lipid rafts (Pike 2006), was analyzed in each fraction

of the density gradient using western blot analysis. Our data showed that caveolin-1 was predominantly located in Fraction 2 of the density gradient (see Figure 2.1). The levels of caveolin-1 in Fraction 2 (see Figure 2.1) and the levels of total protein in each fraction (see Figure 2.2) were not significantly changed after UVB irradiation, which agreed with our previous observation (Elyassaki and Wu 2006).

Figure 2.1 The lipid rafts distribution in density gradient in HaCaT cells before and after UVB irradiation.

HaCaT cells were treated or not treated with UVB (50 mJ/cm²). At 3 h post-UVB, lipid raft from equal amount of cells were isolated using Optiprep™ density gradient ultracentrifugation (1.5 mL 5% / 1.5 mL 30% / 1.0 mL 40%). Equal amount of proteins from each fraction were used for the blot. Western blot analysis of Caveolin-1 as a lipid raft marker was performed in each fraction.

Figure 2.2 The protein distribution in each fraction in HaCaT cells before and after UVB irradiation.

HaCaT cells were treated or not treated with UVB (50 mJ/cm²). At 3 h post-UVB, lipid rafts from equal amount of cells were isolated using Optiprep™ density gradient ultracentrifugation (1.5 mL 5% / 1.5 mL 30% / 1.0 mL 40%). The data represents the means±SD of 5 independent experiments.

After confirming the isolation of lipid rafts, equal amounts of proteins from Fractions 2 and 3 were subjected to 2DE analysis. A total of thirteen spots (three on non-UVB treated HaCaT cells, and ten on UVB treated HaCaT cells) were excised, trypsinized, and analyzed by mass spectrometry (see Figure 2.3). By using Combined MS and MS/MS and searching the database of Swissprot, we finally identified six lipid rafts-associated proteins in HaCaT cells whose presence is altered upon UVB irradiation (see Table 2.1). Among these six identified proteins, three are keratins, which are abundant in keratinocytes and can be

further enriched after UVB irradiation (Kinouchi, Takahashi et al. 2002). The three keratins were shown in seven positions on the 2-DE gel, which agrees with previous report indicating that a keratin can appear at multiple positions on 2-DE gel (Huang, Elmets et al. 2005). Another two proteins, ATP synthase and NADH dehydrogenase, were found to be increased in lipid rafts upon UVB irradiation. These two proteins mainly exist in mitochondria and function in regulating redox reactions and energy production; however, it has been previously reported that mitochondrial proteins have often been found in lipid rafts in different cell lines and animal organs (Kim, Lee et al. 2006; Kim, Kim et al. 2009; Staubach, Razawi et al. 2009). PHB, another identified protein in the lipid rafts factions, was ubiquitously expressed and also identified in lipid rafts in different cell types and animals (Kim, Lee et al. 2006; Staubach, Razawi et al. 2009; Gylfason, Knutsdottir et al. ; Ponce, Brea et al.). PHB plays a critical role in regulation of many cell functions, such as cell proliferation (McClung, Danner et al. 1989), cell apoptosis (Welburn and Murphy 1998), cell-cycle control (Roskams, Friedman et al. 1993), cell senescence, development, and tumor suppression (McClung, Jupe et al. 1995). Thus, we further analyzed the lipid rafts translocation of PHB and its role in regulation of apoptosis after UVB irradiation.

Figure 2.3 2-DE analysis of lipid rafts-associated proteins in HaCaT cells before and after UVB irradiation.

HaCaT cells were treated or not treated with UVB (50 mJ/cm²). At 3 h post-UVB, lipid rafts from equal amount of cells were isolated using Optiprep™ density gradient ultracentrifugation (1.5 mL 5% / 1.5 mL 30% / 1.0 mL 40%). Equal amount of proteins in the same fraction (2 or 3) before and after UVB irradiation were subjected to the analysis.

Table 2.1

Lipid rafts-associated proteins (human) identified by mass spectrometry

Sample	1	2	3,5,7,9	4,10	6	8
Protein Accession	PHB	ATP5H	K1C14	K1C19	NDUS8	K1C17
Protein Mass	29786	18480	51589	44065	23690	48076
Protein Description	Prohibitin	ATP synthase D Chain	Keratin (Cytokeratin-14)	Keratin (Cytokeratin-19)	NADH dehydrogenase iron-sulfur protein 8	Keratin (Cytokeratin-17)

Translocation of PHB after UVB irradiation

To avoid the separation of PHB in two lipid rafts fractions (see Figure 2.1), we modified the discontinuous density gradient from 5% (1.5 mL)-30% (1.5 mL)-40% (1.0 mL) to 5% (1.2 mL)-30% (1.6 mL)-40% (1.2 mL) so that the lipid rafts will be collected in fraction 2 only (see Figure 2.4). We also modified the time for sample collection from 3 h to 6 h post-UVB as a more significant increase of PHB was detected in lipid rafts at that time (see Figure 2.4).

Figure 2.4 Redistribution of PHB in HaCaT cells after UVB irradiation.

HaCaT cells were treated or not treated with UVB (50 mJ/cm²). At 6 h post-UVB, the cells were lysed and lipid rafts from equal amount of proteins were isolated using Optiprep™ density gradient ultracentrifugation (1.2 mL 5% / 1.6 mL 30% / 1.2 mL 40%). Western blot analysis of PHB in each fraction. 30 µL of fractions (1, 3-5), 15 µL of fractions (2) and 3 µL of total lysates were subjected to the analysis.

A semi-quantitative analysis of the western blot (see Figure 2.4) revealed that the amount of PHB in lipid rafts fraction in non-UVB irradiation group was 45% of total PHB, while the amount of PHB in lipid rafts fraction in UVB irradiation group is 73% of total PHB (see Figure 2.5). However, the total PHB level stayed the same after UVB irradiation (see Figure 2.4). These results indicate that PHB expression level was not affected by UVB irradiation, but UVB irradiation did affect PHB translocation from non-rafts fractions into the lipid rafts fraction.

Figure 2.5 The intensity of PHB band in each fraction in HaCaT cells.

The intensity of PHB in Figure 2.4 was analyzed using ImageJ (Version 1.45, NIH). The data represents the average of three independent experiments.

The role of PHB in regulation of UVB-induced apoptosis

PHB can protect cells from oxidative stress-induced cell injury and apoptosis (Fusaro, Dasgupta et al. 2003; Theiss, Idell et al. 2007; Gregory-Bass, Olatinwo et al. 2008; Lee, Nguyen et al. ; Sripathi, He et al. 2011). Since oxidative stress plays a critical role in UVB-induced cell death (Wang, Liu et al. 2010; Wu, Wang et al. 2010), we determined whether PHB also played a role in regulation of UVB-induced apoptosis. PHB siRNA was used to reduce the expression of PHB in HaCaT cells. Our data show that while the expression of PHB was not affected by scrambled siRNA, it was significantly reduced by PHB siRNA in the presence or absence of UVB irradiation (see Figure 2.6). The reduced expression of PHB did not have a statistically significant effect on cell apoptosis ($9.7 \pm 2.7\%$) without UVB irradiation (see Figure 2.7). However, the

cells transfected with PHB siRNA were more sensitive to UVB-induced apoptosis ($52.7 \pm 8.0\%$) compared to the cells that were transfected with scrambled siRNA ($37.1 \pm 0.8\%$)(see Figure 2.7). These results suggest that PHB plays a protective role in UVB-induced skin cell apoptosis.

Figure 2.6 The expression of PHB in HaCaT cells.

HaCaT cells were transiently transfected with scrambled siRNA or PHB siRNA. The cells without transfection were used as control. At 40 h post-transfection, the cells were UVB-irradiated (50 mJ/cm^2). Western blot analysis was performed to detect PHB and β -actin.

Figure 2.7 The effect of PHB in UVB-induced apoptosis in HaCaT cells.

HaCaT cells were transiently transfected with scrambled siRNA or PHB siRNA. The cells without transfection were used as control. At 40 h post-transfection, the cells were UVB-irradiated (50 mJ/cm²). The cell apoptosis was analyzed at 6 h post-UVB using AXV-FITC/PI double staining followed flow cytometry analysis. The data represents the means $\pm$ SD of 3 independent experiments. *: $p < 0.05$.

Discussion

Lipid rafts-associated proteins play important roles in regulation of cell apoptosis (George, Elyassaki et al. 2012). UVB irradiation alters the cholesterol level in lipid rafts, which leads to the aggregation of the rafts-associated protein Fas and induces apoptosis (Elyassaki and Wu 2006; George, Wu et al. 2010; George, Elyassaki et al. 2012). To further analyze the role of lipid raft in mediating UVB-induced skin cell apoptosis, lipid rafts-associated proteins were isolated and determined before and after UVB irradiation using proteomic

screening, which has been widely applied in identification and characterization of lipid rafts-associated proteins (Kim, Kim et al. 2009; Staubach, Razawi et al. 2009; Zhai, Strom et al. 2009; Gylfason, Knutsdottir et al. ; Yang, Di Vizio et al.). Among the seven identified proteins, ATP synthase, NADH dehydrogenase and prohibitin, were previously suggested to be involved in regulation of apoptosis in different cells under various stimuli (Kataoka, Kubota et al. 1997; Almeida and Bolanos 2001; Fusaro, Wang et al. 2002; Shchepina, Pletjushkina et al. 2002; Chen, Chen et al. 2005; Ghosh and Girigoswami 2008; Gregory-Bass, Olatinwo et al. 2008; Zhu, Zhai et al. 2010; Wang, Ma et al. 2011). ATP synthase and NADH dehydrogenase were found in lipid rafts (Kim, Lee et al. 2006), but their function is mainly to protect cells from oxidative injury by regulating mitochondrial electron transport chain-regulated redox status (Almeida and Bolanos 2001; Del Carlo and Loeser 2002; Pronobesh, Dagagi et al. 2008; Garcia, Han et al. 2010). While maintenance of a healthy redox status is critical for protecting cells from UVB-induced apoptosis, the role of the increased lipid rafts-association of ATP synthase and NADH dehydrogenase upon UVB irradiation (see Figure 2.3, Table 2.1) remains to be determined. However, our data might suggest that the UVB-induced translocation of ATP synthase from mitochondria to lipid rafts increases extracellular ATP production, which can cause cell apoptosis as previously reported (Zheng, Zychlinsky et al. 1991; Schulze-Lohoff, Hugo et al. 1998). The lipid rafts translocation of ATP synthase and NADH dehydrogenase might also

lead to a reduction of the two proteins in mitochondria, where they play critical roles in reducing ROS production and protecting cells from oxidative stress-a .

While PHB is also a mitochondria-associated protein, it also plays a critical role in cell signaling pathways outside of the mitochondria, such as Raf-ERK (Rajalingam, Wunder et al. 2005) and PI3K/Akt signaling cascades (Ande and Mishra 2009). The potential involvement of PHB in UVB-induced apoptosis is reported here for the first time. Our data showed that the cellular distribution of PHB was significantly altered after UVB irradiation (see Figure 2.4). While the expression of PHB was not significantly changed (see Figure 2.6, Lane 4 vs. 1), the lipid rafts-associated PHB was increased after UVB irradiation (see Figure 2.4, Fraction 2, UVB vs. Control). Reducing the expression of PHB using PHB siRNA increased the number of apoptotic cells after UVB irradiation, but had little effect on cells without the irradiation (see Figure 2.7). These results indicate that PHB is translocated to lipid rafts and protects cells from apoptosis upon UVB irradiation. However, the role of lipid rafts translocation of PHB in UVB-induced apoptosis remains undetermined. Based on previous reports, which suggested that PHB serves as the scaffold in regulating Raf-ERK and PI3K/Akt anti-apoptotic signaling cascades (Mishra, Ande et al. 2010), our results suggest one potential mechanism for such regulation: translocation of PHB into the rafts frees Akt and Raf from PHB to form the Akt-Raf complex, which results in loss of their anti-apoptotic function. However, we cannot rule out that PHB regulates UVB-induced apoptosis via mitochondrial signaling pathways (Gregory-Bass, Olatinwo

et al. 2008; Chowdhury, Branch et al. 2011; Sato, Murata et al. 2011). Portions of this chapter have been published in *Experimental Dermatology*. Next chapter, we will focus on the detailed mechanism how PHB regulate UVB-induced apoptosis.

CHAPTER 3: THE INVOLVEMENT OF PROHIBITIN IN AKT AND RAF CO-REGULATED APOPTOSIS OF HACAT KERATINOCYTES UPON UVB IRRADIATION

Lipid rafts play a role in regulation of UVB-induced apoptosis of skin cells (Elyassaki and Wu 2006; George and Wu 2012; Wu and Wu 2012). By changing major components upon stimuli, lipid rafts regulate apoptotic signaling pathways by recruiting as well as inducing aggregation and activation of lipid raft-associated proteins, including but not limited to membrane receptors (such as Fas, CD5, and CD20), kinases (such as Akt, JNK, Src, and PKC), and calcium channels (Rotolo, Zhang et al. 2005; Charruyer, Jean et al. 2007; Calay, Vind-Kezunovic et al. 2010; George and Wu 2012). Our recent report indicated that PHB translocates onto lipid rafts upon UVB irradiation (Wu and Wu 2012). Our recent report also indicated that reduced expression of PHB enhanced UVB-induced apoptosis (Wu and Wu 2012). However, it remains unknown on the detailed mechanism of lipid rafts-translocation of PHB in regulation of UVB-induced apoptosis.

Recent studies have found that PHB is essential in the activation of both PI3K/Akt and Raf/ERK signaling cascades (Rajalingam, Wunder et al. 2005; Ande and Mishra 2009; Chiu, Ho et al. 2013). PHB interacts with and is phosphorylated at threonine 258 by Akt (Han, McGonigal et al. 2008), which was suggested to be critical for the activation of Akt signaling cascade (Ande and

Mishra 2009). PHB regulates Raf activity via two mechanisms: (1) PHB interacts with Raf, which promotes Raf membrane localization and activation (Rajalingam, Wunder et al. 2005); and (2) PHB may compete with Raf in binding and being phosphorylated by Akt (Mishra, Ande et al. 2010). Both PI3K/Akt and Raf/ERK signaling cascades are main cell survival signaling pathways mediating UVB-induced apoptosis in skin cells (Decraene, Van Laethem et al. 2004; Claerhout, Decraene et al. 2007; Saegusa, Hsu et al. 2008; Jang, Koo et al. 2013).

PHB also prevents cells from oxidative stress by increasing GSH and GST π level, as well as decreasing ROS level (Theiss, Idell et al. 2007; Lee, Nguyen et al. 2010; Zhou, Qian et al. 2012). Oxidative damage is also related to skin cell apoptosis upon UVB irradiation (Afaq, Syed et al. 2007; Kawachi, Xu et al. 2008; Piao, Hyun et al. 2012; Liu, Otkur et al. 2013; Yin, Li et al. 2013).

However, it is not known if lipid rafts translocation of PHB regulated UVB-induced apoptosis is mediated by PI3K/Akt and Raf/ERK signaling cascades. In this report, we reveal a novel mechanism that lipid rafts-translocation of PHB mediates UVB-induced apoptosis via alternating interactions and localization of PHB, Akt and Raf, which is oxidative stress-independent.

Materials and Methods

Cell culture

The HaCaT cells (human keratinocyte cell line, kindly provided by Dr. Nihal Ahamad, University of Wisconsin, Madison, WI) and H1299 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Manassas, VA) supplemented with 1% penicillin-streptomycin (Mediatech, Manassas, VA) and 10% fetal bovine serum (FBS, Denville Scientific, Metuchen, NJ). The cells were cultured at 37 °C with 5% CO₂.

UVB irradiation

UVB light (290–320 nm) was generated from 15 W UVB tubes (UVP, Upland, CA). The intensity of UVB light was measured using a UVX digital radiometer (UVP, Upland, CA). The cells were irradiated with 50 mJ/cm² or 5 mJ/cm² of UVB light. The culture medium was replaced with PBS during exposure and was added back to the culture plates after UVB treatment. The cells were then cultured until harvesting.

Isolation of lipid rafts

Lipid rafts fractions were isolated using Optiprep™ (Sigma, St. Louis, MO) density gradient (40% - 5%) as previously reported (Wu and Wu 2012). Briefly, the cells were collected, cross-linked with DTSSP, pelleted, and frozen at -80 °C overnight. Then the cells were lysed with cold 0.1% Triton X-100 membrane raft isolation buffer (50 mM Tris-HCl, PH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1% Triton X-100, and the protease inhibitor cocktail). Equal volume of the lysates were applied to a discontinuous gradient (40% - 5%) and centrifuged using a SW60 rotor (Beckman Coulter, Brea, CA) at 31,300 rpm for 5 h at 4 °C. Five 0.8 mL fractions were collected from each gradient for future western blotting analysis.

Isolation of plasma membrane proteins

Plasma membrane extracts were prepared using Mem-PER™ plus membrane protein extraction kit (Pierce, Rockford, IL). Extraction of plasma membrane and cytosolic proteins was performed according to the manufacturer's protocol. Na⁺/K⁺ - ATPase and GAPDH were used as markers of the plasma membrane and cytosolic proteins, respectively.

Lysis of cells

Cells were collected, pelleted, and lysed by Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl, 0.1% SDS) containing protease inhibitor cocktail (Calbiochem, La Jolla, CA). The cell lysate was incubated on ice for 15 min and centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatant was collected and protein concentration was determined by Bio-Rad Dc Protein Assay kit (Bio-Rad, Hercules, CA).

Western blotting analysis

The proteins were boiled in 5-fold Laemmli buffer (0.25 M Tris-HCl, pH 6.8, 0.5 M DTT, 10% SDS, 50% glycerol and 0.03% bromophenol blue), separated on SDS-PAGE, and transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk (w/v) in TBST buffer for 30 min at room temperature and then incubated with primary antibodies against PHB (sc-28259), Akt-1 (sc-55523), ERK (sc-135900), caspase-3 (sc-7148), Na⁺/K⁺ - ATPase (sc-21712), GAPDH (sc-25778) and Caveolin-1 (sc-894) (Santa Cruz Biotechnology, Santa Cruz, CA), p-Akt (9271s), p-ERK (4377), and PARP (9542) (Cell Signaling, Danvers, MA), and β -actin (A5316) (Sigma, St. Louis, MO) at 4 °C overnight. After washing with TBST, the membrane was incubated with corresponding secondary horseradish peroxidase (HRP)-conjugated antibodies for 30 min at

room temperature. The signals were developed using West Pico Supersignal chemilluminescence substrate (Pierce, Rockford, IL).

Immunoprecipitation

The cell lysate (150 μ L) was incubated with 5 μ L primary antibody against PHB (sc-28259), Raf-1 (sc-133), Akt-1 (sc-55523), p-Thr (sc-5267), p-Ser (sc-81514), p-Tyr (sc-7020) (Santa Cruz Biotechnology, Santa Cruz, CA) with gentle rocking overnight at 4 °C. Protein A/G agarose beads (Pierce, Rockford, IL) were added and the cell lysate was incubated with gentle rocking for another 30 min at 4 °C. The cell lysate-bead mixture was centrifuged for 30 sec at 1,000 rpm in a benchtop centrifuge (Eppendorf, Hamburg, Germany) and washed three times with 700 μ L 1X TBST at 4°C. The lysate-bead mixture was boiled in 5-fold Laemmli buffer, and then separated on SDS-PAGE. The samples were analyzed by western blotting.

Akt kinase activity

The cell lysates were prepared using Akt kinase assay kit (9840) (Cell Signaling, Danvers, MA). The Akt kinase assay was performed according to the manufacturer's protocol.

Flow cytometry

Cells were collected and pelleted by centrifugation, washed twice with PBS, fixed with 4% formaldehyde for 10 min and cooled on ice for 1 min. Cells were then blocked in PBS containing 1% BSA for 30 min. Cells were incubated with antibody against PHB (ab75766) (Abcam, Cambridge, MA) or the isotype control anti-rabbit IgG, for 1 h at room temperature, washed twice with PBS, and incubated with FITC-conjugated secondary antibodies for 1 h. Cells were washed, and resuspended in PBS for cell surface PHB expression analysis on a FACSort analyzer (BD Biosciences, Franklin Lakes, NJ).

Immunofluorescence microscopy

Cells were incubated with 1 µg/mL FITC-conjugated cholera toxin β -subunit (Sigma, St. Louis, MO) for 15 min at 4 °C, and washed with PBS. Cells were fixed with 4% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 30 min, and then blocked in PBS containing 1% BSA for 30 min. Cells were incubated with antibody against PHB (ab75766) (Abcam, Cambridge, MA), Raf-1 (sc-133), and Akt-1 (sc-55523) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, washed twice with PBS, and then incubated with DyLight 549 secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h. Cells were washed with PBS, mounted on slides with Vectashield mounting

medium (Vector Laboratories, Burlingame, CA), and analyzed by fluorescence microscopy (Nikon ECLIPS E600, Melville, NY).

PHB silencing using the RNA interference method

Cells were seeded in a 6-well tissue culture plate in antibiotic-free medium until 60-80% confluent. The medium was then replaced with 0.5 mL transfection mixture, containing either scrambled siRNA (60 nM, sc-37007) or PHB siRNA (60 nM, sc-37629), siRNA Transfection Medium and siRNA Transfection Reagent, prepared according to manufacture's protocol (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were first incubated at 37 °C with 5% CO₂ for 6 h and then incubated in 1.0 mL antibiotic-free DMEM with 20% FBS overnight. The cells were incubated for another 18-24 h with newly replaced DMEM with 10% FBS before harvesting.

Generation of stable PHB shRNA cell line

HaCaT cells were transfected with pLKO.1-Neo-CMV-tGFP vectors containing PHB shRNA or scramble control (Sigma, St. Louis, MO) using Lipofectamine LTX and PLUS following the manufacture's protocol (Invitrogen, Carlsbad, CA). The transfected cells were maintained for 2 weeks in DMEM with

G418. Stable cell lines were generated by isolating single cell-derived colonies. The PHB expression in each cell line was determined by western blot.

Akt and Raf overexpression and transient transfection

Total RNAs from HaCaT cells were extracted using RNeasy Mini kit (Qiagen, Germantown, MD) and reverse-transcribed into first-strand DNA using oligo(dT)₂₀ (Invitrogen, Carlsbad, CA). The full-length human Akt and Raf cDNA was prepared by PCR using the following primers:

Akt primer F: 5'-ATGCGGATCCCCACCATGAGCGACGTGGCTATTGTGAAG-3';

Akt primer R: 5'-ATGCTCTAGATCAGGCCGTGCTGCTGGC-3';

Raf primer F: 5'-ATGCGGATCCCCACCATGGAGCACATACAGGGAGCTT-3';

Raf primer R: 5'-ATGCTCTAGACTAGAAGACAGGCAGCCTCG-3').

The amplified Akt or Raf cDNA fragments were ligated into the BamH1-Xba1 sites of pcDNA6/V5-His (Invitrogen, Carlsbad, CA). Raf and Akt overexpression plasmids were purified using MidiPrep (Qiagen, Germantown, MD), followed by gene sequencing at Genomics Facility (Ohio University, Athens, OH). The expression vector(s) were transiently transfected into H1299 cells using Lipofectamine 2000 following the manufacture's protocol (Invitrogen, Carlsbad, CA).

Clonogenic assay

Cells were seeded in a 6-well tissue culture plate and incubated at 37 °C with 5% CO₂ for 6 d. The cells were washed with PBS twice and fix in cold methanol for 10 min at -20 °C. The fixed cells were stained by 1% crystal violet in 25% methanol for another 10 min, and then washed with ddH₂O. The colonies with a size more than 0.5 mm were counted.

ROS detection assay

Cells were incubated with 100 µL CM-H₂DCFDA dye (5 µM) (Invitrogen, Carlsbad, CA) for 1 h before treatment and then incubated in PBS for another 5 min. ROS level was determined by reading the fluorescence excitation/emission at 495/525 nm.

Alamar Blue assay

Cells were incubated in alamarBlue (Invitrogen, Carlsbad, CA) for 2 h and read the fluorescence excitation/emission at 570/595 nm.

Statistics

Student's t-test was used to analyze the statistic significance of data. $p < 0.05$ was considered the statistical significant.

Results

The correlation between lipid raft-association of PHB and translocation of Akt and Raf after UVB irradiation

As PHB serves as a scaffold in regulating Akt and Raf signaling (Mishra, Ande et al. 2010), we examined whether UVB-induced lipid raft-translocation of PHB is correlated with a subcellular redistribution of Akt and Raf. Our data indicated that accompanied with the translocation of PHB into fraction 2 (lipid raft fraction), Akt and Raf translocated out of fraction 3 (non-raft membrane fraction) in HaCaT cells at 6 h post UVB irradiation (50 mJ/cm^2) (see Figure 3.1A). Further analysis using immunofluorescence staining showed that while PHB distributed homogenously in the unirradiated cells, it was aggregated and co-localized with GM1, a marker of lipid raft, in the HaCaT cells at 6 h post UVB irradiation (50 mJ/cm^2) (see Figure 3.1B).

Figure 3.1 PHB translocation in HaCaT cells after UVB irradiation.

HaCaT cells were irradiated with UVB (50 mJ/cm²) and harvested at 6 h. Panel A: PHB, Akt, Raf, and Caveolin-1 in each fraction of the gradient were assessed by western blotting analysis. 30 μ L of fractions (1, 3-5), and 15 μ L of fractions (2) were subjected to the analysis. Panel B: The cells were incubated with FITC-conjugated CTB, followed by fixation, permeabilization, and development for PHB fluorescence detection. The arrow: PHB colocalization with lipid raft (yellow). Scale bar, 10 μ m.

Our data also showed that the increased association of PHB in lipid raft was due to translocation but not induced expression of PHB since total PHB protein level remained almost the same within 24 h after UVB irradiation (see

Figure 3.2), and cell surface PHB protein level remained almost unchanged 6 h after UVB irradiation (see Figure 3.3).

Figure 3.2 Total PHB expression in HaCaT cells after UVB irradiation.

HaCaT cells were irradiated with UVB (50 mJ/cm²). Total amount of PHB in different time-points (0-24h) post-UVB was assessed by western blotting analysis.

Figure 3.3 Cell surface PHB expression in HaCaT cells after UVB irradiation. HaCaT cells were irradiated with UVB (50 mJ/cm²). After 6 h post-UVB, the cell surface expression of PHB was immunofluorescently labeled and detected by flow cytometry.

Akt and Raf as the regulators in UVB-induced lipid raft-translocation of PHB

Since lipid-raft-translocation of PHB appeared to be related to its binding to Akt and Raf, we determine whether overexpression of Akt, Raf or Akt plus Raf has an effect on the PHB membrane redistribution. H1299 cell line was used in this study for its high transfection efficiency. Our data showed that Akt and/or Raf were overexpressed in both unirradiated and UVB-irradiated H1299 cells (see Figure 3.4, Lanes 2-4 and 6-8 vs. 1 and 5). Further analysis using immunofluorescence staining showed that in H1299 cells, Akt and/or Raf were overexpressed highly in the cells, including plasma membrane and cytosol (see Figure 3.5). The fluorescence microscopic images revealed that PHB homogenously distributed without UVB irradiation and co-localized with GM1 in

H1299 cells transfected with empty vector at 6 h post UVB irradiation (50 mJ/cm²) (see Figure 3.6, Vector), which indicated that PHB behaved similarly in H1299 cells and HaCaT cells (see Figure 3.6 vs. 3.1B). In contrast, UVB has no effect on GM1 localization of PHB in the cells overexpress Raf, Akt or both (see Figure 3.6). Interestingly while overexpression of Akt or Akt+Raf inhibited the lipid raft translocation of PHB after UVB irradiation, overexpression of Raf appeared to promote the cell membrane translocation of PHB without UVB irradiation (see Figure 3.6). These results suggested that lipid raft-translocation of PHB might due to an increased affinity between lipid raft and PHB; or a decreased affinity among PHB, Akt and Raf upon UVB irradiation.

Figure 3.4 Western blot analysis of Akt and Raf overexpression in H1299 cells. H1299 cells were transfected with Akt and/or Raf expression plasmid. Empty plasmid was transfected into H1299 cells as a control. The cells were lysed at 6 h post-UVB (50 mJ/cm²) and the expression of Akt, Raf, and β-actin were assessed by western blotting analysis.

Figure 3.5 Immunofluorescence detection of Akt and Raf overexpression in H1299 cells.

H1299 cells were transfected with Akt and/or Raf expression plasmid. Empty plasmid was transfected into H1299 cells as a control. The expressions of Akt and Raf in the transfected cells were detected by immunofluorescent staining.

Figure 3.6 Effects of Akt and Raf overexpression on PHB translocation in H1299 cells.

H1299 cells were transfected with Akt and/or Raf expression plasmid. Empty plasmid was transfected into H1299 cells as a control. The expression of CTB and PHB in the transfected cells with or without UVB (50 mJ/cm²) were detected by immunofluorescent staining. The arrow: PHB colocalization with lipid raft. Scale bars, 20 μ m.

PHB, Akt, and Raf interactions after UVB irradiation

To further analyze the affinities among PHB, Akt and Raf, we analyzed the interaction between Akt-PHB, Raf-PHB and Akt-Raf after UVB irradiation using immunoprecipitation. Our data showed that the interaction between Akt-PHB decreased at 6 h post-UVB (50 mJ/cm²), while the interaction between Akt-Raf increased upon UVB irradiation (see Figure 3.7). The interaction between Raf-PHB was not significantly changed post-UVB (see Figure 3.7). Since UVB induces Akt phosphorylation and activation, we determined the role of Akt activation in regulating the binding of PHB with Akt by analyzing the Akt phosphorylation in different subcellular localization after UVB irradiation. GAPDH and Na⁺/K⁺-ATPase were used as markers of cytosolic protein and plasma membrane associated protein respectively. Western blotting analysis revealed that p-Akt increased in cytosolic fraction, but decreased in plasma membrane fraction at 6 h post UVB (50 mJ/cm²) (see Figure 3.8). These results suggested that p-Akt might have a weaker affinity than Akt in binding to PHB, and thus detached from PHB and sequentially freed from plasma membrane to cytosol.

Figure 3.7 PHB interactions in HaCaT cells upon UVB irradiation.

HaCaT cells were irradiated with UVB (50 mJ/cm²). At 6 h post-UVB, cells were lysed and corresponding antibodies were added. PHB, Akt, and Raf interactions were analyzed by immunoprecipitation followed by western blot analysis.

Figure 3.8 Akt phosphorylation in HaCaT cells upon UVB irradiation.

HaCaT cells were irradiated with UVB (50 mJ/cm²). At 6 h post-UVB, the levels of p-Akt, Akt, ATPase, and GAPDH on plasma membrane or in cytosol were assessed by western blotting analysis.

Since activated Akt can phosphorylate PHB at Thr258 (Chiu, Ho et al. 2013), we determined whether UVB-induced Akt phosphorylation is correlated to PHB Thr phosphorylation. Our data indicated that total Thr phosphorylation on PHB was slightly decreased at 6 h post-UVB, which correlated with the detachment and translocation of PHB from Akt from membrane into the lipid raft (see Figure 3.9). In addition to Thr phosphorylation, we also analyzed Ser and Tyr phosphorylation on PHB as they are also associated with PHB activity (Mishra, Ande et al. 2010). Our data showed that while the Ser phosphorylation on PHB was not significantly changed, Tyr phosphorylation was also slightly decreased at 6 h post-UVB (see Figure 3.9). These results suggested that the lipid rafts-translocation of PHB might be associated with PHB dephosphorylation at certain amino acid residues and thus alter the interaction among PHB, Akt and Raf.

Figure 3.9 PHB phosphorylation in HaCaT cells upon UVB irradiation.

HaCaT cells were irradiated with UVB (50 mJ/cm²). At 6 h post-UVB, the phosphorylated threonine, serine, and tyrosine residues on immunoprecipitated PHB was determined by western blot analysis.

Regulation of apoptosis by PHB knockdown after UVB irradiation

We previously reported that reduced expression of PHB enhances UVB-induced apoptosis (Wu and Wu 2012). To further determine the signaling pathways involved in PHB-mediated UVB-induced cell apoptosis, we examined the effect of PHB knockdown on phosphorylation of Akt and ERK (a downstream target activated by Raf). Our data showed that UVB induced phosphorylation of Akt and ERK in cells treated with scrambled or PHB siRNA (see Figure 3.10, lane 5 vs. lane 2, lane 6 vs. lane 3). Comparing with scrambled siRNA, treating cells with PHB siRNA slightly increased Akt phosphorylation post-UVB (see Figure 3.10, lane 6 vs. lane 5), while it decreased the inducibility of Akt phosphorylation by UVB (see Figure 3.10, lane 6/3 vs. lane 5/2). Our data also showed that treating cells with PHB siRNA inhibited UVB-induced ERK phosphorylation (see Figure 3.10, lane 6 vs. lane 5). Western blotting analysis also revealed that 6 h post UVB irradiation (50 mJ/cm²), phosphorylation of Akt and ERK increased in control cell line, but decreased in stable PHB shRNA knockdown cell line (see Figure 3.13, lane 3 vs. lane 1, lane 7 vs. lane 5). However, treating cells with PHB shRNA decreased the inducibility of both Akt and ERK phosphorylation by UVB (see Figure 3.13, lane 7/5 vs. lane 3/1), indicating that both Akt and Raf signaling were inhibited with PHB silencing upon UVB irradiation. In addition, Akt kinase activity assay showed that p-GSK-3 α/β increased in HaCaT cells, but decreased in stable PHB shRNA knockdown cell

line (HaCaT^{PHB-KD}) at 6 h post-UVB (50 mJ/cm²) (see Figure 3.14), indicating that Akt kinase activity was regulated by PHB upon UVB irradiation. These findings and previous report, taken together, proved that PHB silencing enhances UVB-induced cell apoptosis by inhibiting Akt and Raf signaling.

Figure 3.10 The role of PHB in UVB-induced apoptosis in HaCaT cells.

HaCaT cells were transiently transfected with scrambled siRNA or PHB siRNA. The cells without transfection were used as control. The levels of PHB, p-ERK, ERK, p-Akt, Akt, and β-actin in the transfected cells with or without UVB (50 mJ/cm², 6 h) were assessed by western blotting analysis.

PHB protects against oxidative stress in many cells (Coates, Nenutil et al. 2001; Theiss, Idell et al. 2007; Liu, Ren et al. 2009; Lee, Nguyen et al. 2010; Kathiria, Butcher et al. 2012; Zhou, Qian et al. 2012). Before investigating whether ROS is involved in PHB-mediated and UVB-induced apoptosis, we established HaCaT^{PHB-KD} cell lines. We isolated 14 single cell-derived colonies, and 5 of them never grew back. Then we examined PHB knockdown

effectiveness in those 9 established cell lines. Only P1, P5, P10 cell lines showed PHB knockdown compared to scrambled control cell line (see Figure 3.11). Interestingly, P8 and P13 cell lines even showed higher PHB expression level than control (see Figure 3.11). For the following experiments using HaCaT^{PHB-KD} cell lines, we choose P5 cell line. Next, we treated cells with LNAC to reduce UVB-induced ROS. In both scrambled control HaCaT and HaCaT^{PHB-KD} cells, the ROS level increased immediately at 6 h post-UVB (50 mJ/cm²), and LNAC inhibited UVB-induced ROS significantly (see Figure 3.12). While at 6 h post UVB irradiation, in scrambled control cells, the ROS level did not increased significantly, and LNAC partially inhibited ROS level; in HaCaT^{PHB-KD} cells, the ROS level increased significantly, and LNAC inhibited UVB-induced ROS significantly (see Figure 3.12). Taken together, these results reveal that UVB induces early ROS generation significantly, while the increase of ROS generation is less at 6 h post-UVB, and LNAC inhibits UVB-induced ROS generation in both control HaCaT and HaCaT^{PHB-KD} cells.

Figure 3.11 Generation of PHB knockdown stable HaCaT cell lines.

HaCaT cells were stably transfected with scrambled shRNA (control) or PHB shRNA (PHB knockdown). PHB expression was examined by western blotting analysis in these single colony-derived cell lines. Control cell line: C1; PHB knockdown cell lines: P1, P5, P7-P13.

Figure 3.12 ROS generation in UVB-induced PHB knockdown HaCaT cells.

HaCaT cells were stably transfected with scrambled shRNA (control) or PHB shRNA (PHB knockdown). ROS level was analyzed by ROS detection assay and standardized by AlamarBlue assay. HaCaT cells were pre-incubated with LNAC (10 mM) for 1 h, and were then irradiated with UVB (50 mJ/cm²). The UVB-irradiated cells that were analyzed immediately were defined as 0 h post UVB-irradiated cells, while the UVB-irradiated cells that were analyzed 6 h post UVB were defined as 6 h post UVB-irradiated cells. ROS level was assessed by ROS detection assay. Cell proliferation was determined by alamarBlue assay. The data represents the means $\pm$ SD of 3 independent experiments. * p < 0.05, control cells vs. PHB knockdown cells; ** p < 0.05, control cells vs. UVB-irradiated cells.

To determine the role of ROS in UVB-induced apoptosis in HaCaT^{PHB-KD} cells, we examined the cleavage of PARP, a substrate of caspase-3. Our data showed that the cleavage of PARP was increased in HaCaT^{PHB-KD} cells compared to control HaCaT cells at 6 h post-UVB (50 mJ/cm²) (see Figure 3.13, lane 7 vs. lane 3), indicating PHB knockdown enhances UVB-induced apoptosis. Treatment with LNAC resulted in a decrease in p-Akt and p-ERK, but an increase in UVB-induced PARP cleavage (see Figure 3.13, lane 4 vs. lane 3, lane 8 vs. lane 7). However PHB knockdown induced PARP cleavage (see Figure 3.13, 2.0 in lane 8 to lane 7 vs. 1.9 in lane 4 to lane 3), stayed almost the same upon UVB irradiation, indicating LNAC treatment does not affect PHB knockdown-enhanced UVB-mediated apoptosis. To confirming Akt phosphorylation mediates UVB-induced apoptosis, we treated cells with Akt kinase inhibitor (Akt I), and examined the cleavage of caspase-3 with or without UVB irradiation (50 mJ/cm²). Treatment with Akt I resulted in a decrease in Akt phosphorylation, but an increase in UVB-induced caspase-3 cleavage (see Figure 3.15), confirming that Akt phosphorylation protects cell from UVB-induced apoptosis. Clonogenic assay showed both PHB knockdown and UVB irradiation (5 mJ/cm²) inhibited cell survival and proliferation (see Figure 3.16). However, LNAC treatment did not affect cell survival and proliferation rate (see Figure 3.16). These findings, on the whole, proved that LNAC treatment inhibits UVB-induced ROS generation as well as Akt and ERK phosphorylation as a result of an increase in UVB-induced apoptosis in early phase, which is independent of PHB-mediated cell signaling,

while does not have an impact on UVB-inhibited cell survival and proliferation in late phase.

Figure 3.13 The role of LNAC in UVB-induced and PHB-mediated cell apoptosis in HaCaT cells.

HaCaT cells were stably transfected with scrambled shRNA (control) or PHB shRNA (PHB knockdown). The cells were pre-incubated with LNAC (10 mM) for 1 h, and then were irradiated with UVB (50 mJ/cm²). Cells were incubated with LNAC until the assays were performed. At 6 h post-UVB, the amounts of PHB, PARP, and β-actin were assessed by western blotting analysis.

Figure 3.14 The effect of UVB irradiation on Akt kinase activity in PHB knockdown HaCaT cells.

HaCaT cells were stably transfected with scrambled shRNA (control) or PHB shRNA (PHB knockdown). Phospho-Akt in the cells treated or not treated with UVB (50 mJ/cm², 6 h) was immunoprecipitated by immobilized p-Akt primary antibody and incubated with GSK 3 fusion protein. Then the phosphorylation of GSK-3α/β was detected by western blotting analysis.

Figure 3.15 The effect of Akt phosphorylation inhibition on UVB-induced apoptosis in HaCaT cells.

The levels of p-Akt, Akt, caspase-3, and β -actin in Akt I (1 μ M) and/ or UVB (50 mJ/cm², 6 h) treated cells were assessed by western blotting analysis.

Figure 3.16 The role of LNAC in UVB-induced and PHB-mediated cell proliferation in HaCaT cells.

HaCaT cells were stably transfected with scrambled shRNA (control) or PHB shRNA (PHB knockdown). The cells were pre-treated with LNAC (1 mM or 10 mM) for 1 h, and then were irradiated with UVB (5 mJ/cm²). Survival and proliferation of the cells treated or not treated with UVB (5 mJ/cm²) were assessed by clonogenic assay. The data represents the means $\pm$ SD of 3 independent experiments. * $p < 0.05$, control cells vs. PHB knockdown cells; ^ $p < 0.05$, non-UVB-irradiated cells vs. UVB-irradiated cells.

Discussion

Our previous report indicated that PHB-mediated apoptosis is correlated with a lipid rafts-translocation of PHB upon UVB irradiation (Wu and Wu 2012), suggesting a potential novel mechanism of PHB in regulation of apoptotic pathway. In this report, we explore the role of lipid rafts translocation of PHB in regulation of Akt and Raf-mediated apoptosis upon UVB irradiation. Our results indicated that the lipid raft-translocation of PHB was accompanied with the

detachment of Akt and Raf from non-raft membrane upon UVB irradiation (see Figure 3.1A). The freed Akt and Raf are likely to be released into cytosol from the membrane since we didn't detect any Akt and Raf in the lipid rafts fraction (see Figure 3.1A). However, we didn't detect an increased level of Akt or Raf in cytosol either, which could be due to that Akt and Raf are mainly reside in cytosol and a slight increase of them translocated from membrane might not be able to be detected. The detachment of PHB from Akt was also indicated by the reduced total Thr phosphorylation on PHB (see Figure 3.9) as previous reports indicated that Akt phosphorylates PHB at Thr 258 (Han, McGonigal et al. 2008).

The lipid raft-translocation of PHB can be due to a reduced affinity of PHB to Akt and Raf since overexpression of Akt and/or Raf, which increased Akt and Raf in both cytosol and plasma membrane, can retain PHB on plasma membrane post-UVB (see Figure 3.6). The affinity of PHB to Akt and Raf appears to be regulated by the activation of Akt and Raf. Our result indicated that the phosphorylation of Akt, an indication of Akt activation, was reduced in plasma membrane (see Figure 3.8) when PHB dissociated from Akt (see Figure 3.7). This agrees with previous report that PHB-Akt interaction is critical for the activation of Akt signaling cascade (Han, McGonigal et al. 2008; Ande and Mishra 2009; Mishra, Ande et al. 2010). While we don't have data to show that Raf activation on the membrane post-UVB, we showed that Raf detached from membrane (see Figure 3.1A), which can lead to Raf activation as suggested in previous report (Rajalingam, Wunder et al. 2005). The increased

phosphorylation of ERK (see Figure 3.10) also supported that Raf was activated after detached from PHB. Moreover, we show that the interaction between Akt and Raf was increased upon UVB irradiation (see Figure 3.7), which could be a result of the release of Akt and Raf from PHB on the membrane and allow them to bind to each other in cytosol as previously reported (Zimmermann and Moelling 1999; Mishra, Ande et al. 2010).

Our previous report showed that reduced expression of PHB enhance UVB-induced apoptosis (Wu and Wu 2012). Since PHB mediates the activation of Akt and Raf on membrane upon UVB as showed above, we determined whether PHB protects cells from apoptosis via activating PHB-dependent Akt and Raf pathways after UVB irradiation. Our data showed that PHB knockdown inhibited the phosphorylation of Akt and ERK (see Figure 3.10), as well as inhibited the Akt kinase activity (see Figure 3.14) upon UVB irradiation. The PHB knockdown-inhibited Akt and Raf activation is correlated with apoptosis after UVB irradiation as we previously reported (Wu and Wu 2012). These results and previous report indicate that PHB is essential for Akt and Raf activation on membrane, which plays a critical role in protecting cells from UVB-induced apoptosis.

We demonstrate a novel PHB signaling pathway in UVB-induced apoptosis. Previous studies suggest that PHB also mediates intrinsic apoptotic pathway, as is demonstrated that PHB protects cells from oxidative stress in mitochondria (Coates, Nenutil et al. 2001; Liu, Ren et al. 2009; Lee, Nguyen et al. 2010; Bang, Bae et al. 2011). Our data showed that treatment with LNAC did not

change PHB-knockdown-enhanced apoptosis (see Figure 3.13), as well as cell growth arrest and death (see Figure 3.16) upon UVB irradiation, suggesting PHB-mediated and UVB-induced apoptosis is oxidative stress independent.

Based on all this evidence, we propose a model (see Figure 3.17) that UVB irradiation induces activation of PHB-associated Akt and Raf on plasma membrane, which will detach from PHB and sequentially be released to cytosol to regulate downstream anti-apoptotic signaling pathway. While the freed PHB on plasma membrane translocates onto lipid rafts, the activated Akt and Raf in cytosol will gradually form Akt-Raf dimer and deactivated.

Figure 3.17 Proposed model for UVB-induced PHB-mediated apoptosis.

CHAPTER 4: FUTURE DIRECTIONS

The translocation of PHB into lipid rafts is a feature occurring in the early phase of a long-term process of UVB-induced and PHB-mediated apoptosis, as detailed in this dissertation. In the future, the role of PHB in the late phase of UVB irradiation-triggered events needs to be resolved. Two important issues arise here: 1) Is there any location alteration of PHB in the late phase after UVB irradiation? 2) What is the biological function of PHB in the late phase after UVB irradiation? Our current data have shown that UVB irradiation induces PHB translocation into lipid rafts in the early phase. More research needs to be done to determine the PHB location in late phase upon UVB irradiation. In addition, we need to reveal the signaling pathways involved in the late phase of UVB-mediated PHB signaling pathways. We have revealed that Akt and Raf will form a complex in the early phase, however, whether PHB regulates the Akt and Raf association or dissociation in late phase remains unknown. It is highly possible that PHB still regulates Akt and Raf signaling pathways, or that PHB regulates other signaling pathways. Moreover, it remains unknown whether PHB mediates apoptosis or other cell functions in the late phase after UVB irradiation. The dynamic location and function of PHB will reveal the detailed function of PHB involved in UVB-mediated skin cell signaling transduction.

Another issue would be to explore the effect on UVB-induced apoptosis via PHB translocation inhibition. Our preliminary data have already

demonstrated that Akt and/or Raf overexpression impede PHB translocation onto lipid rafts. An alternative way to retain PHB and Akt binding is the Thr 258 mutation on PHB to a negatively charged Asp258 to mimic phosphorylation. Likewise, PHB and Raf binding sites needs to be determined and retained in future work. Moreover, since the PHB binding peptide motif CKGGRAKDC has been identified, we could design a peptide that also retains PHB binding to Akt and/or Raf. After retaining the PHB binding to Akt and/or Raf, further research needs to be done to determine its effect on the translocation of PHB onto lipid rafts. Therefore another question arises: what is the biological function of PHB translocation inhibition? Perhaps after inhibiting PHB translocation into lipid rafts, the cells will be more resistant to UVB-induced apoptosis.

Knowledge of the role of PHB upon UVB irradiation could be applied to clinical settings. In this dissertation, we have studied how PHB mediates UVB-induced apoptosis in normal keratinocytes. The UVB-related skin cancers, like melanoma, probably have some aberrant signaling pathways compared to normal skin, leading to unregulated cell growth. The screening of different melanoma cells must be done to determine whether the deficient PHB translocation exists in certain types of melanoma cells. Once the possible dysfunction of PHB is verified in melanoma skin cancer, it may result in the discovery of new therapeutic targets for abnormal PHB action related to UVB-induced melanoma generation.

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