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Regulation of neutrophil apoptosis by modulation of PKB/Akt activation

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1. ABSTRACT

The serine/threonine kinase, Akt, also known as PKB (Protein Kinase B) is one important signal transduction pathway that mediates the delay of neutrophil apoptosis caused by inflammatory mediators. Proteins controlled by the PKB/Akt pathway have been reported to prevent or reverse apoptotic-signaling pathways and regulate cell survival. In this review we discuss the role of PKB/Akt activation in the regulation of neutrophil activation during inflammation, and the importance of resolving the inflammatory response by inhibiting PKB/Akt activation and neutrophil survival. Furthermore, we introduce the concept of a dynamic Akt signal complex that is altered when an extracellular signal is initiated such that changes in protein-protein interactions within the Akt signal complex regulates Akt activity and cell survival. Various substrates of PKB/Akt as well as positive and negative regulators of PKB/Akt activation are discussed which in turn inhibit or enhance cellular survival.

2. INTRODUCTION

2.1. Neutrophil activation during inflammation

Neutrophils are immune cells and they are viable in circulation for 8 to 10 hrs. Their lifespan is prolonged to provide first line of defense during infection or inflammation. Recruitment of neutrophils to sites of infection or inflammation in diseased tissue is mediated by inflammatory mediators including chemokines (1), cytokines (2), elastase (3), metalloproteases (4), nitrosive (5), and oxidative stress (6). Neutrophils adhere to the vascular endothelium followed by diapedesis, and subsequent chemotatic migration into the diseased or infected tissue. The neutrophils play an important role in bacterial killing and elimination of microorganisms by phagocytosis. However, during inflammation uncontrolled neutrophil activation leads to excessive release of oxidants and proteases which destroy surrounding tissue. Neutrophil-induced tissue damage seen during inflammation can be controlled in part by neutrophil

apoptosis. Induction of neutrophil apoptosis inhibits the ability of neutrophils to move, release superoxide radicals, or degranulate and these apoptotic neutrophils are cleared from the inflammed site by macrophages. Uncontrolled neutrophil activation and inadequate resolution of inflammation play a critical role in tissue injury associated with the multi-organ failure syndrome of sepsis; ischemia-reperfusion injury observed in myocardial infarction, stroke, and acute tubular necrosis; and immune complex-mediated diseases. Thus, understanding how neutrophil apoptosis is controlled especially at the site of inflammation is critical.

At the site of infection, neutrophils bind and ingest invading microorganisms by phagocytosis. Bacteria secrete peptidoglycan, lipoproteins, lipoteichoic acid, lipopolysaccharide (LPS), CGP-containing DNA, and flagellin which bind to the neutrophil surface *via* pattern recognition receptors expressed on the neutrophil surface. These pattern recognition receptors include toll-like receptors (TLRs) and CD14 (7). This binding initiates a signal transduction cascade that prolongs neutrophil survival, facilitates neutrophil adhesion, phagocytosis, exocytosis or degranulation of anti-microbial proteins, and enhances release of superoxide radicals, cytokines, and chemokines, contributing to neutrophil microbicidal activity. Thus, optimal killing of bacteria, while sparing surrounding tissue, requires regulation of the presence of activated neutrophils at the site of infection.

2.2. Resolution of inflammation and infection by induction of neutrophil apoptosis

Apoptosis is an important mechanism for maintaining homeostasis of the human immune system. Apoptosis plays an important role in the normal turnover of the innate immune system. Normal turnover of aging neutrophils occurs in the absence of activation through a process known as spontaneous/constitutive apoptosis. This intrinsic ability of neutrophils to undergo apoptosis is necessary for maintaining appropriate cell numbers in circulation. Neutrophils are programmed to undergo constitutive apoptosis within hours of the time they enter into circulation. It has been demonstrated that Protein kinase B (PKB)/Akt is inactivated significantly during constitutive neutrophil apoptosis. However, inhibition of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has been shown to prevent constitutive neutrophil apoptosis by up regulating PKB/Akt activation. Thus, inactivation of PKB/Akt is a casual mediator of constitutive neutrophil apoptosis. During infection neutrophil life span is altered to combat the infection. However, continued activation of neutrophils at the site of infection can lead to inflammation. Thus, the timely removal of activated neutrophils by apoptosis, from the affected site is necessary to resolve inflammation. It was believed until recently that extravated neutrophils undergo necrosis at the inflamed site and are cleared by macrophages. It has now become apparent that neutrophils at the site of inflammation undergo apoptosis and it is this process that determines rapid clearance of intact neutrophils by macrophages (8). Neutrophil apoptosis, rather than necrosis, prevents release of toxic granule contents and tissue injury. However, inappropriate rapid rate of neutrophil apoptosis can result in inadequate removal of infectious agents, while delayed or decreased rate of apoptosis can result in tissue injury and organ failure. Thus, the ability to control neutrophil activation by apoptosis is critical to resolving inflammation. Thus enhanced survival of neutrophil during early stages of inflammation, are necessary for the clearance of bacterial pathogens, followed by a process of phagocytosis such that it initiates neutrophil apoptosis and clearance by macrophages. The rate of apoptosis is not fixed and can be regulated by various chemokines, cytokines, and chemoattrants. We reported that IL-8, GM-CSF, LTB_4, and bacterial lipopolysaccharide (LPS) delay constitutive neutrophil apoptosis through the activation of the serine/ threonine kinase, PKB/Akt (9, 10). In addition, C5a, heme, CpG motifs in bacterial DNA, *Chlamydia pneumoniae* and heat shock have also been shown to prolong neutrophil survival by regulation of PKB/Akt activation (11-15). Thus, understanding the mechanisms that regulate neutrophil apoptosis holds high significance for diseases that are classically considered inflammatory, such as rheumatoid arthritis and sepsis, and for diseases that have only recently been discovered to have an inflammatory component such as atherosclerosis and chronic kidney disease. This knowledge will allow the development of novel pharmacologic and gene based therapies to control inflammation. These pathways may apply to other cell types and these novel therapeutic approaches may extend to the regulation of PKB/Akt-mediated cellular functions and survival in other tissues and organs.

3. REGULATION OF APOPTOSIS BY PKB/Akt

Protein kinase B or PKB/Akt is a Ser/Thr kinase with high homology to protein kinase A and C, and was therefore termed PKB. PKB/Akt contains a pleckstrin homology domain (PH), a kinase domain and a C-terminal tail. PKB/Akt is present in the cytosol of unstimulated cells. PKB/Akt is constitutively phosphorylated on Ser^{124} and Thr^{450} (16). Constitutive phosphorylation of PKB/Akt Ser^{124} and Thr^{450} is not required, but contributes to PKB/Akt activation as PDGF-stimulation of PKB/Akt Ser^{473}/Thr^{308}phosphorylation is reduced in NIH3T3 cells transfected with the S124AS450A mutant compared to vector transfected cells (16). Thr^{450} phosphorylation has been shown to be required for proper folding which appears to be an important first step for PKB/Akt activation. Agonist stimulated cellular activation leads to PI-3K activation and generation of PtdIns $(3,4,5)P_3$ (PIP_3) and PtdIns $(3,4)P_2$ (PIP_2). The second step of PKB/Akt activation entails translocation of PKB/Akt to the plasma membrane by binding of PI-3K dependent PIP_3 and PIP_2 to the PH domain of PKB/Akt (17). The third step of PKB/Akt activation, involves phosphorylation on 2 sites namely Thr^{308} and Ser^{473} by PDK1 and PDK2 respectively (18, 19). Thus, PI-3K activation is necessary for Akt translocation and activation. Translocation of PKB/Akt to the plasma membrane has been shown to be critical for its activation. However, recently it has been shown that cAMP-elevating agents such as PGE2 or Ado, are capable of inducing fMLP-stimulated PKB/Akt phosphorylation on

Ser^{473} and Thr^{308} in the cytosol, independent of PI-3K activation (20). PKA activation has been shown to antagonize PKB/Akt translocation to the membrane without affecting the ability of PKB/Akt to be phosphorylated on its 2 critical sites (20). In addition, PKB/Akt phosphorylation has previously been demonstrated in the absence of PI-3K activation (21-25). Activated PKB/Akt translocates to the golgi, endoplasmic reticulum, mitochondria, and nucleus (26). The mechanisms regulating these translocations are not completely understood. PKB/Akt, a key regulator of cellular apoptosis, is expressed in a variety of cell types, and is shown to inhibit both spontaneous and stress-induced neutrophil apoptosis. PKB/Akt is activated by a variety of agents including chemokines, cytokines, chemoattractants, and growth factors. Proteins controlled by the PI3K/PKB/Akt pathway have been reported to prevent or reverse apoptotic-signaling pathways and play a role in the delay of apoptosis caused by inflammatory mediators. The active form of PKB/Akt exerts its anti-apoptotic effect by phosphorylating a number of pro-apoptotic proteins, including Bax, Bad, caspase-9, Ask1, GSK beta, IKK alpha and transcription factors, CREB, NF-KB, and the forkhead transcription factors (27-36).

3.1. JNK

In contrast to PKB/Akt, JNK pathways promote apoptosis in a variety of cells. JNK exerts its pro-apoptotic effect by phosphorylating and inactivating pro-survival proteins. A cross-talk between PKB/Akt and JNK pathways has been documented. PKB/Akt can exert its anti-apoptotic effect by negatively regulating JNK signaling pathway. PKB/Akt antagonizes JNK by binding to and inactivating SEK1, an upstream activator of JNK. In turn, binding of JIP-1 (JNK interacting protein-1) to PKB/Akt, inhibits PKB/Akt activity and restores JNK activity (37-40). Thus, changes in protein-protein interactions in PKB/Akt complexes can regulate PKB/Akt activation and cell survival. Identification of these novel pathways will lead development of pharmaceutical interventions to regulate cellular functions, including apoptosis.

3.2. GSK-3β

The glycogen synthase kinase 3 has two isoforms, alpha and beta (GSK-3). GSK is an unusual Ser/Thr kinase in that this kinase is normally active in cells, and is regulated by inhibition of its activity *via* phosphorylation. PKB/Akt has been shown to phosphorylate GSK-3 on Ser^{21} and Ser^{9} resulting in GSK-3 inactivation (29, 32). Recently, GSK-3-mediated phosphorylation of an anti-apoptotic Bcl-2 family member Mcl-1 resulted in Mcl-1 ubiquitination and proteasomal degradation (41). Thus, negative regulation of GSK-3 by PKB/Akt inhibits Mcl-1 phosphorylation, ubiquitination, proteasomal degradation, and increases Mcl-1 stability promoting cell survival.

3.3. Bcl-2 family of proteins

Apoptosis can be positively or negatively regulated by Bcl-2 family of proteins. Pro-apoptotic Bcl-2 family proteins include Bax, Bak, Bim, Bcl-X_S, and Bad while anti-apoptotic proteins include Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1. These proteins have all been shown to regulate apoptosis by acting at the level of the mitochondria in a variety of cell types. However, in neutrophils, their mode of action is not completely understood. Unlike Bcl-2 and Bcl-X_L, both Bax and Mcl-1 are expressed in mature neutrophils (34). In the neutrophils, Mcl-1 is expressed in the nucleus, while Bax is localized in non-nuclear membranes. Mcl-1 has a PEST sequence and other motifs in its N-terminal sequence that target it for turnover by the proteasome (42, 43). In addition, increased Mcl-1 expression is correlated to increased neutrophil viability during inflammation. Bax is present in the cytosol of healthy neutrophils. Apoptotic stimuli promote a conformational change in Bax promoting its insertion in mitochondrial membranes, where it forms oligomers, resulting in disruption of mitochondrial permeability, release of cytochrome C, and apoptotic cell death (44). PKB/Akt over-expression has been shown to inhibit Bax localization to the mitochondria (45). In addition, PKB/Akt-mediated Bax phosphorylation in neutrophils, promotes its sequestration in the cytoplasm, increasing its ability to heterodimerize with anti-apoptotic Bcl-2 family proteins including Bcl-X_L and Mcl-1 (34). PKB/Akt-mediated Bad phosphorylation results in its dissociation from Bcl-2 and allowing its binding to 14-3-3 proteins (46). Mcl-1 forms heterodimers with Bad. Thus, the anti-apoptotic activity of Mcl-1 is blocked by its association with Bad. Disruption of Mcl-1/Bad heterodimers preserves mitochondrial integrity and blocks cytochrome c release inhibiting neutrophil apoptosis.

3.4. Hexokinase and caspase activation

PKB/Akt mediated hexokinase association with mitochondria has also been shown to prevent cytochrome C release and cellular apoptosis in the presence or absence of Bak and Bax (47). Hexokinase is the first obligatory step in glucose utilization. It converts glucose to Glucose-6-phosphate which is required for its entry into glycolysis. Neutrophils rely primarily upon glycolysis for energy production to carry out all neutrophil functions. It is known that during hyperglycemia, PKB/Akt dependent regulation of energy metabolism initially promotes an adaptive response by inducing hexokinase association with the mitochondria, modulating VDAC open state, maintaining mitochondrial membrane integrity, and effectively inhibiting cellular apoptosis. However, when PKB/Akt-mediated hexokinase association with mitochondria is compromised, Bax and Bak homo-oligomerize at the outer mitochondrial membrane, resulting in the release of cytochrome C. Cytochrome C released from the mitochondria forms a complex with dATP and apoptotic protease activating factor-1 (APAF-1) that is termed the apoptosome. The apoptosome then mediates the activation of caspase-9 that, in turn, activates caspase-3 (48). PKB/Akt has been reported to phosphorylate caspase-9 on Ser196 resulting in its inactivation. Caspase-9 activation has also been associated with feedback disruption of the mitochondrial potential through cleavage of anti-apoptotic proteins Bcl-2, Bcl-Xl, and Mcl-1. A balance between PKB/Akt and caspase activity controls cell survival, as caspases can inactivate PKB/Akt and

inhibit the PKB/Akt signal transduction pathway, by cleaving off C-terminal hydrophobic motif of PKB/Akt (49, 50).

3.5 CREB, FOXO, NFKB

PI-3K/PKB/Akt-mediated-phosphorylation of the transcription factors CREB, FOXO/FKHRL1, and NFKB were shown to regulate neutrophil apoptosis. C5a promotes delay of neutrophil-like HL-60 cells by inducing Bcl-2 expression, which is mediated in part by PI-3K/PKB/Akt-dependent CREB activation (51). The FOXO/FKHRL1 transcription factor has been shown to regulate expression of the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) and the Fas ligand that can accelerate the rate of neutrophil apoptosis (52). PKB/Akt-mediated-phosphorylation of FOXO/FKHRL1 promotes its association with adaptor protein 14-3-3 resulting in its translocation and sequestration in the cytosol (53). Upon dephosphorylation, FOXO/FKHRL1 translocates to the nucleus and induces expression of genes that are critical for cell death, including TRAIL and Fas ligand. Recently Bonni and coworkers demonstrated that Mst1 mediates oxidative stress-induced FOXO phosphorylation on Ser^{207}, resulting in disruption of FOXO/14-3-3 association in the cytosol and leading to its nuclear localization and induction of apoptotic genes (54). Moreover, Sung-Wuk Jang and co-workers demonstrated that PKB/Akt mediated Mst1 phosphorylation inhibited Mst1-triggered FOXO nuclear localization and promoted cell survival (55). Furthermore, in the experimental models of acute lung injury and sepsis, PKB/Akt-mediated NFκB activation contributes to nuclear localization of NFKB, resulting in increased production of pro-inflammatory cytokines and exacerbation of lung injury (56). Suppression of PKB/Akt and NF-κB activation has been shown to decreases acute inflammatory processes and organ dysfunction. Therefore, modulation of PKB/Akt activation may be an appropriate therapeutic target in patients with inflammatory diseases.

4. REFGULATORS OF PKB/Akt

4.1. Tyrosine kinases

Tyrosine kinases have been shown to regulate neutrophil apoptosis by modulation of PKB/Akt activation. Several tyrosine kinases including Janus kinases (JAKs) and Src-kinases such as Lyn, Hck, anf Fgr are expressed in neutrophils (57). JAKs are activated by cytokine receptors while Src-kinases are activated by both cytokine receptors and G-protein-coupled receptors. In neutrophils, JAK2 promotes GM-CSF induced delay of neutrophil apoptosis by activating PI-3K/PKB/Akt signal transduction pathway (58). In contrast, NADPH oxidase-stimulated Src kinase Lyn has been shown to activate SH2-containing inositol 5-phosphatase (SHIP) leading to PKB/Akt inactivation and induction of neutrophil apoptosis. The exact mechanisms of tyrosine kinase mediated regulation of PKB/Akt activation and neutrophil apoptosis are unclear (59). Direct tyrosine phosphorylation of PKB/Akt has been reported (60). PKB/Akt tyrosine phosphorylation on Tyr^{315} and Tyr^{326} has been shown to be required for PKB/Akt activation induced by growth factors (60). Furthermore, PKB/Akt-Y^{474} phosphorylation has been shown to be necessary for the full activation of the kinase (61). In support of this notion, tyrosine kinase Src directly associates with PKB/Akt *via* its SH3 domain and the conserved proline rich motif (PXXP) in the C-terminal tail region of PKB/Akt. Binding between PKB/Akt and tyrosine kinase Src was shown to be necessary for PKB/Akt tyrosine phosphorylation and full PKB/Akt activation (62).

4.2. PDK1/PDK2

PKB/Akt is phosphorylated on 2 sites namely Thr^{308} and Ser^{473} for its full activation. Although the upstream kinase responsible for phosphorylating PKB/Akt Thr^{308} has been identified as PDK1, the identity of PDK2, which phosphorylates PKB/Akt Ser^{473}, has been debated for several years. Several candidate kinases have been reported to function as PDK2, including PDK1 (63). This is proposed to occur through a small C-terminal fragment of protein kinase C-related kinase-2 (PRK2), which converts PDK1 to PDK2. PKB/Akt itself has been suggested to autophosphorylate itself at Ser^{473} (64). In addition, integrin-linked kinase (65, 66), double-stranded DNA-dependent protein kinase (67), staurosporine-insensitive kinase (68), ataxia telangiectasia mutated kinase (69), and mammalian target of rapamycin (mTOR) (70) have also been identified as PDK2 kinases for PKB/Akt Ser^{473} phosphorylation. Recently we demonstrated for the first time that p38 MAPK-dependent MAPKAPK-2 or MK2 phosphorylates PKB/Akt on a critical site Ser^{473} that is required for PKB/Akt activation and neutrophil survival (71). These studies were followed by several others that also demonstrated p38-MAPK-dependent PKB/Akt activation in a variety of cell types (72-76). Recently, Baudhuin *et al* showed that lysophosphatidic acid, sphingosine-1-phosphate and platelet-derived growth factor stimulates PKB/Akt Ser^{473} phosphorylation in a MAP kinase kinase (MEK) and p38 MAPK dependent manner, while insulin stimulates PKB/Akt Ser^{473} phosphorylation independent of both MEK and p38 MAPK in HEY ovarian cancer cells (77). Thus, the requirement of p38 MAPK for PKB/Akt Ser^{473} phosphorylation seems to be stimuli specific.

4.3. PKC

Protein kinase C (PKC) isoforms comprise a family of Serine/Threonine kinases that are grouped based on their structural and biochemical properties. These include "conventional" or Ca^{2+}-dependent ("cPKCs" alpha, beta I, beta II, and gamma) which are activated by the second messenger diacylglycerol, "novel" or Ca^{2+}-independent PKCs ("nPKCs" delta, epsilon, eta, theta) which are activated by diacylglycerol and "atypical" PKCs ("aPKCs" zeta and lamda/iota) which do not require diacylglycerol for activation. The role of PKC isoforms in the regulation of PKB/Akt activation and cellular apoptosis has been described. PKC has been shown to either promote or inhibit apoptosis depending upon the cell type and the PKC isoform that is activated. Recently Bae *et al* have demonstrated that WKYMVm, a chemotactic peptide, attenuated serum deprivation-induced monocyte apoptosis by activation of PKB/Akt in a PKC alpha and beta II

isoform dependent manner (78). Additionally, GMCSF inhibited Fas ligand-induced neutrophil apoptosis by promoting cPKC-induced PKB/Akt activation (79). In contrast, PKC delta was shown to promote spontaneous neutrophil apoptosis while TNF-alpha-induced PKC zeta activation contributed to neutrophil apoptosis (80, 81). PKC zeta-mediated PP2A activation has been attributed to down regulation of PKB/Akt phosphorylation and induction of cellular apoptosis (82). Alternatively, PKC zeta-mediated PKB/Akt-Thr^{34} phosphorylation has been shown to inhibit phosphatidylinositol 3,4,5-triphosphate (PIP_3) binding to PKB/Akt-PH domain preventing its translocation and activation at the plasma membrane (83). PKC zeta also is shown to promote PKB/Akt gamma activation by acting as an adaptor for staurosporine-insensitive PDK2 enzyme that catalyzes PKB/Akt-gamma-Ser^{472} phosphorylation (84).

4.4. PTEN

Neutrophil spontaneous apoptosis is critical for the resolution of the inflammatory response. Although caspase activation mediated neutrophil apoptosis, pretreatment of neutrophils with caspase inhibitors failed to prevent spontaneous neutrophil apoptosis. In contrast, neutrophils isolated from PTEN knockout mice did not demonstrate spontaneous apoptosis as compared to neutrophils from wild type mice (85). PTEN is a phosphatidylinositol 3'-phosphatase, that converts phosphatidylinositol-3,4,5-polyphosphate (PIP_3) to phosphatidylinositol-3,4-polyphosphate (PIP_2). Depletion of PTEN leads to accumulation of PIP_3 at the plasma membrane leading to enhanced PIP_3/PKB/Akt signaling. These studies identified PTEN-mediated PKB/Akt deactivation as a causal mediator of spontaneous neutrophil apoptosis. Furthermore, chemoattractant stimulation of PTEN depleted neutrophils exhibited increased PKB/Akt phosphorylation, actin polymerization, and superoxide production. These results were not surprising as PKB/Akt activation has previously been shown to regulate neutrophil superoxide burst and chemotaxis (86-89).

4.5 SHIP

The SH2-containing inositol 5-phosphatase (SHIP) is widely expressed in hematopoietic cells. SHIP hydrolyzes PIP_3 and inositol-1,3,4,5-polyphosphate (IP_4) to generate PIP_2 and inositol-1,3,4-polyphosphate. Inhibition or depletion of SHIP results in accumulation of PIP3 and subsequently leads to PKB/Akt activation. Therefore, neutrophils and bone-marrow derived mast cells from mice lacking SHIP are less susceptible to apoptosis induced by a variety of apoptotic stimuli or by growth factor withdrawal as compared to neutrophils and mast cells from wild type mice. Strassheim *et al.* demonstrated that neutrophils from SHIP (-/-) knockout mice that were stimulated with peptidoglycan (PGN), a TLR2 ligand, had increased PKB/Akt, p38 MAPK, and ERK activation as compared to neutrophils from SHIP (+/+) transgenic mice (90). In addition, they demonstrated an increase in phosphorylation of NFKB p65 subunit and an increase in cytokine release in TLR2-stimulated SHIP (-/-) neutrophils. Increased neutrophil activation in SHIP (-/-) knockout mice, as a consequence of intratracheal PGN administration, correlated with a significant increase in interstitial lung edema causing severe lung injury as compared to neutrophils from SHIP (+/+) transgenic mice. Furthermore, neutrophil infiltration in the lungs, as measured by myeloperoxide levels, after PGN exposure, showed greater than 12 fold increase from baseline values in SHIP (-/-) mice compared to SHIP (+/+) mice. These studies identified SHIP as a negative regulator of PKB/Akt, myeloid cell survival and homeostasis. In addition, these studies demonstrated the participation of PKB/Akt signal transduction pathway in TLR2-induced pro-inflammatory pathways in neutrophils and macrophages leading to acute lung injury. Therefore, similar to PTEN, SHIP could serve as a therapeutic target to down regulate PKB/Akt activation and neutrophil survival during inflammation.

4.6. CTMP (Carboxyl-Terminal Modulator Protein)

A yeast two-hybrid analysis identified a novel 27 kDa protein as an PKB/Akt binding protein. Due to its ability to directly bind to the COOH-terminus of PKB/Akt this novel protein was named as carboxyl-terminal modulatory protein or CTMP (91). CTMP binding to PKB/Akt at the plasma membrane inhibited PKB/Akt activation by preventing its phosphorylation by upstream kinases. In addition, pervanadate, insulin, and IGF-1 stimulation of PKB/Akt activation, was inhibited by CTMP. Subsequently PKB/Akt-mediated inhibition of c-fos promoter activity was reversed by CTMP over-expression in HEK293 cells. Moreover, PKB/Akt-mediated GSK-3 beta phosphorylation was also reduced in the presence of CTMP. Depleting CTMP from HEK-293 cells with antisense-CTMP induced sustained endogenous PKB/Akt activation indicating that CTMP acts as a negative regulator of PKB/Akt *in vivo*. CTMP overexpression resulted in phenotypic regression of v-PKB/Akt-transformed (PKB/Akt8) cells to wild type and inhibited tumorigenic potential of PKB/Akt8 cells thus, confirming CTMP's role as a negative regulator of PKB/Akt. While PTEN and SHIP inhibit PKB/Akt activation indirectly by reducing the amounts of PIP_3 pools at the cell membrane, Maira *et al* suggested that CTMP inhibits PKB/Akt activity by directly binding to the C-terminal tail of PKB/Akt and preventing its phosphorylation and activation by its upstream kinases (91). In support of Maira *et al* studies, Chae *et al* demonstrated the ability of exogenously expressed CTMP to inhibit growth factor-induced PKB/Akt activation and subsequent trafficking of functional calcium activated potassium (K (ca)) channels (92). Furthermore, Knobbe *et al* observed reduced CTMP mRNA levels in 40% primary tissues and 67% of glioma cell lines with concomitant increase in PKB/Akt activation (93). However, these studies have been recently contradicted by Ono *et al* which demonstrate that CTMP is a positive regulator of PKB/Akt (94). These differences could be attributed to different cell lines used and dose response effects of various stimulants utilized in the two studies.

4.7. Heat shock protein 27

Another protein controlled by the PI3K/PKB/Akt pathway is the small molecular weight chaperone protein Hsp27, a member of the heat shock proteins expressed after

environmental stress. Hsp27 is phosphorylated by PKB/Akt and has been shown to inhibit neutrophil apoptosis (19, 71). Hsp27 exists in two distinct quaternary states. When cells are subjected to thermal stress, Hsp27 forms oligomeric structures of approximately 400-800 kDa. These oligomeric structures act as molecular chaperones to prevent the aggregation of denatured proteins. When phosphorylated on Ser-15, -78, and -82 the large oligomers dissociate into dimers and monomers. Hsp27 dimers and monomers have been associated with binding of the apoptosome, stabilization of cytoskeleton, and binding of mediators of Fas-dependent apoptosis (95, 96). When over-expressed in tumor cells, Hsp27 increases tumorigenicity (97, 98) and protects against apoptotic cell death triggered by various stimuli, including hyperthermia, oxidative stress, staurosporine, ligation of the Fas/Apo-1/CD95 death receptor, and cytotoxic drugs (99-101). These stimuli also induce Hsp27 expression, providing an example how pro-apoptotic stimuli, delivered below a certain threshold level, can elicit protective responses. One possible mechanism of Hsp27 anti-apoptotic activity results from its function as a molecular chaperone. Hsp27 binds to and inactivates the pro-apoptotic molecules caspase-3, caspase-9, and cytochrome c (102-105). Hsp27-mediated suppression of Bid translocation to the mitochondria correlates with an inhibition of cytochrome C release (106). Hsp27 and Hsp70 appear to suppress apoptosis by binding to and inhibiting Daxx and Ask-1 (95, 99, 107). Hsp27 has also been shown to promote survival pathways by modulating IKK complex stability and activity. Parcellier *et al* demonstrated that Hsp27 can mediate NF-KB activation and cell survival by promoting the proteasomal degradation of polyuiquitinated IKB (108). Of note, a number of key regulators of apoptosis including Bid (109) Bcl-2 (110), and Bim (111, 112) are regulated by ubiquitination and proteasomal degradation. The role of Hsps in modulating the turn over of apoptosis relevant proteins by regulating the proteasome has not been investigated.

The first indication that Hsp27 might regulate PKB/Akt activity was reported by Konishi *et al*, who demonstrated an association of Hsp27 and PKB/Akt in COS-7 cells (22). Subsequently, we demonstrated, for the first time, that PKB/Akt exists in a signaling complex with p38 mitogen activated protein kinase (p38 MAPK), MAPK-activated protein kinase-2 (MK2) and heat shock protein 27 (Hsp27) in human neutrophils (19), and Hsp27 directly interacts with PKB/Akt through its acidic linker region (113). The physical association of Hsp27 with PKB/Akt was shown to be a critical determinant of neutrophil survival, as removal of Hsp27 from the PKB/Akt signal module prevented PKB/Akt activation and resulted in accelerated PMN apoptosis (71). We also showed previously that MK2, in addition to phosphorylating Hsp27, acts as PDK2 for PKB/Akt in human neutrophils, phosphorylating Ser^{473} (19). We recently demonstrated that Hsp27 scaffolds MK2 to Akt promoting MK2-medited Akt Ser^{473} phosphorylation and neutrophil survival.

5. NEW MODEL FOR PKB/Akt ACTIVATION

Based on our data and reports by other investigators, we propose a new model for PKB/Akt activation illustrated in Figure 1. In this model, inactive PKB/Akt exists in the cytosol in a complex with positive regulators including p38 MAPK, MK2, via Hsp27. By acting as a scaffolding protein, Hsp27 maintains the association of MK2, and p38 MAPK with PKB/Akt at the acidic linker region. PI3-K-generated phosphoinositides lead to translocation of PKB/Akt complexes to the plasma membrane and stimulate PDK1 and p38 MAPK activity. Binding of the PKB/Akt PH domain to phosphoinositides produces a conformational change that brings MK2 in close proximity with Ser^{473}. MK2 activation by p38 MAPK results in Ser^{473} phosphorylation, forming a docking site for PDK1 (114). Active PDK1 then binds to PKB/Akt and phosphorylates Thr^{308}, resulting in full activation of PKB/Akt. In contrast, disruption of PKB/Akt-Hsp27 interaction prior to cellular stimulation would result in recruitment of apoptosis regulating proteins or PKB/Akt-negative regulators or dissociation of PKB/Akt-upstream activators from the PKB/Akt signal complex. These changes in protein-protein interactions would result in induction of cellular apoptosis. Alterations in protein-protein interactions may be promoted by post-translational modifications such as phosphorylation or ubiquitination. Understanding the complex interactions between PKB/Akt and its regulators, during neutrophil activation, is critical for the development of novel therapeutic approaches to control the inflammatory response.

6. CONCLUSIONS

Research from various laboratories has established the presence of multiprotein signal complexes that regulate signal transduction pathways in cells. We have demonstrated that under control conditions, Akt is in a complex with p38 MAPK, MK2, and Hsp27. In fMLP-stimulated PMNs, Hsp27 dissociated from Akt while p38 MAPK and MK2 continued to associate with Akt. In addition, disruption of Akt-Hsp27 interaction promoted neutrophil apoptosis. Akt-Hsp27 disruption induced changes in protein-protein interactions with in the Akt signal complex such that certain pro-survival proteins dissociated from Akt while certain pro-death promoting proteins were recruited into the complex (data not shown). Furthermore, we demonstrated that Hsp27 protein acts a scaffolding protein by bringing Akt upstream activators in close proximity to Akt. These studies suggested to us that the Akt signal complex is not static but rather a dynamic complex, which is regulated when a certain signal is initiated. Regulation of PKB/Akt activation and function has been studied over the past several years. Up-regulation or down-regulation of PKB/Akt signaling has been shown to contribute to a variety of diseases, making it an important target for further investigation. A role of PKB/Akt in regulating a variety of neutrophil functions including superoxide burst, chemotaxis, and apoptosis has been established. However, the role of a wide variety of

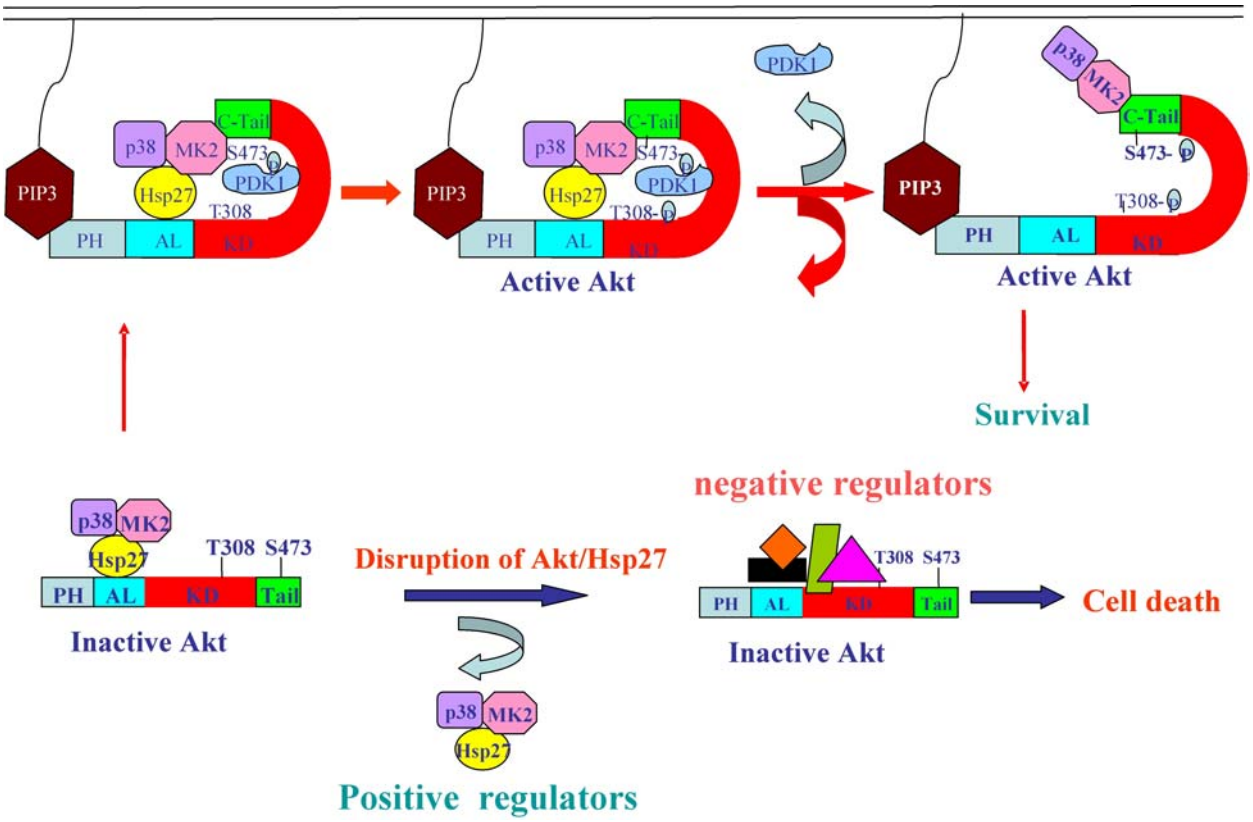

Figure 1. Hypothetical Model of Akt activation. In the unstimulated cells, Hsp27 mediates p38 MAPK and MK2 interaction with Akt. Disruption of Akt-Hsp27 interaction in the basal state prevents Akt association with its positive regulators (+ve) or upstream activators namely, p38 MAPK and MK2. Akt-Hsp27 disruption promotes recruitment of negative regulators (-ve) of Akt to the Akt signal complex. This leads to loss Akt activation and induces neutrophil apoptosis. On the other hand, if Akt-Hsp27 interaction is intact, then upon ligand stimulation, Akt translocates to the membrane and is fully activated by phosphorylation on Thr308 and Ser473 by PDK1 and PDK2 respectively. Activated Akt induces Hsp27 phosphorylation and its subsequent dissociation from Akt. Dissociation of phosphorylated Hsp27 from Akt, after Akt activation, does not affect Akt activity or neutrophil apoptosis.

Akt substrates in regulating these neutrophil functions has not yet been elucidated. Therefore, future studies should be targeted at identifying Akt substrates that regulate neutrophil functions. Modulation of these PKB/Akt substrates localization and/or activity may serve as appropriate therapeutic targets in patients with inflammatory diseases.

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Abbreviations: Hsp27: Heat Shock Protein 27; JNK: c-Jun NH_2 terminal kinase; JIP-1: JNK interacting protein-1; SEK1: Jun kinase kinase or SAPK/Erk kinase or MKK4; MK2: MAP kinase-activated protein kinase-2; MAPK: mitogen activated protein kinase; JAK-2: Janus kinase 2; mTOR: mammalian target of rapamycin; PKA: Protein kinase A; PKB/Akt: Protein Kinase B; PKC: Protein kinase C; PRK2: C-terminal fragment of protein kinase C-related kinase-2; PTEN: Phosphatase and Tensin homologue deleted on chromosome 10; PH domain: Pleckstrin Homology domain; PI3K: phosphatidylinositol 3-kinase; PDK1/2: phosphoinositide-dependent kinase 1 and 2; PIP2:

PtdIns (3,4)P_2; PIP3: PtdIns (3,4,5)P_2; K (Ca): Calcium activated potassium channel; Ask-1: apoptosis signal-regulating kinase (ASK)1-interacting protein 1); Bcl-2: B-cell lymphoma protein 2; VDAC: voltage dependent anion channel; APAF-1: Apoptotic protease-activating factor 1; CTMP: carboxyl-terminal modulatory protein; SHIP: SH2-containing inositol 5-phosphatase; Fas/Apo-1/CD95: receptor for Fas ligand; TLR2: toll-like receptor 2; PGN: peptidoglycan; PEST sequence: a peptide sequence which is rich in proline (**P**), glutamic acid (**E**), serine (**S**), and threonine (**T**); GSK beta: glycogen synthase kinase beta; IKK alpha: I kappa B kinase alpha; CREB: cAMP response element-binding protein; NFKappaB: nuclear factor-kappa B; Mcl-1: myeloid cell leukemia sequence 1; IL-8: interleukin 8; GM-CSF: granulocyte monocyte colony stimulating factor; LPS: lipopolysaccharide; LTB4: Leukotriene B4; C5a: complement activation factor; FOXO/FKHRL1: forkhead transcription factor; TNF: tumor necrosis factor; TRAIL: TNF-related apoptosis inducing ligand; Mst-1: macrophage stimulating protein 1.

Key Words: PKB/Akt, Hsp27, Neutrophils, Inflammation, p38 MAPK, MK2, PI-3K, PDK1, PDK2, Apoptosis, Review

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