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Review

Akt signaling and its role in postnatal neovascularization

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Summary. Postnatal neovascularization has been known to be involved in not only angiogenesis but also vasculogenesis. Several lines of evidence suggest a link between neovascularization and Akt, a family member of serine/threonine protein kinases. Akt phosphorylates endothelial NO synthase (eNOS) and thereby enhances endothelial NO synthesis and influences postnatal vessel growth. Akt signaling is activated by a variety of stimuli in endothelial cells and endothelial progenitor cells (EPCs). Activation of the Akt kinase orchestrates a number of signaling pathways potentially involved in angiogenesis. Dominant negative Akt overexpression leads to functional blocking of EPC bioactivity. Because neovascularization is implicated in the pathophysiology of a number of diseases and is becoming an important therapeutic strategy for those diseases, further dissection of the Akt pathway and elucidation of the downstream effector molecules will lead to a better understanding of postnatal neovascularization and may provide avenues for the development of novel therapeutic interventions. In this review, molecular mechanisms of Akt signal pathway will be discussed with special emphasis on its role in neovascularization.

Key words: Akt, Angiogenesis, Vasculogenesis, Nitric oxide, Progenitor cell

Introduction

The Akt gene product in mice is the cellular homolog of the v-akt oncogene transduced by AKT8, an acute transforming retrovirus in mice that was originally described in 1977 (Staal et al., 1977; Bellacosa et al., 1991). It has subsequently been documented that Akt is a protein with molecular weight of 57 kD and belongs to a family of serine/threonine protein kinase. Akt shares 68% and 73% homologies with protein kinase A and protein kinase C respectively, and therefore it is also named as protein kinase B (PKB) or RAC (related to protein kinase A and protein kinase C) (Coffer and Woodgett, 1991; Konishi et al., 1995).

Akt is activated by a number of growth factors and cytokines in a phosphatidylinositol-3 kinase-dependent manner and serves as a multifunctional regulator of cell biology, glucose metabolism and protein synthesis (Franke et al., 1995; Hemmings, 1997). In this review, Akt signaling in endothelial cells and endothelial progenitor cells and its critical roles in the regulation of angiogenesis and vasculogenesis will be discussed.

The structure and expression of Akt

Akt has three isoforms in Mammalian: Akt1/PKBα, Akt2/PKBß, Akt3/PKBγ, which share high homology (Datta et al., 1999) The N-terminus of PKB contains a pleckstrin homology (PH) domain, a central kinase domain, and a glycine-rich region. The C-terminus has two regulatory phosphorylation sites at threonine and serine (Haslam et al., 1993; Datta et al., 1995). (Fig. 1).

The location of the human Akt1 gene was mapped to chromosome 14q32, proximal to the immunoglobulin-heavy-chain locus, a region frequently affected by translocations and inversions in human T-cell leukaemia /lymphoma and mixed-lineage childhood leukaemia. Akt2, on the other hand, was mapped to chromosome region $19q^{13.1}$-$q^{13.2}$ (Staal, 1987).

Akt1 and Akt2 are widely expressed in various tissues. Akt1 is most abundant in brain, heart, and lung, whereas Akt2 is predominantly expressed in skeletal muscle and embryonic brown fat. The expression of Akt3 is relatively restricted and it is expressed highly in brain and testes but low in heart, spleen, lung and skeletal muscle. All tissues contain at least one form of Akt (Altomare et al., 1998; Brodbeck et al., 1999). Their expression, however, appears to be up-regulated as cells become more terminally differentiated (Altomare et al.,

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1995) .

The activation of Akt

Akt kinase activity is induced by a number of growth factors such as VEGF, angiopoietin and cytokines such as TNF and IL-1 in a phosphatidyl-inositol-3 kinase-dependent manner (Gerber et al., 1998; Kim et al., 2000).

PI3K is a heterodimer of two subunits, catalytic subunit and regulatory subunit, with molecular weights of 110 kD (p110) and 85 kD (p85), respectively (Pons et al., 1995; Yu et al., 1998). The p85 subunit consists of several domains including the SH3 domain, two proline-rich domains (PRD), and two SH2 domains separated by the inter SH2 (iSH2) sequence. The iSH2 domain provides the interaction between the p85 and p110 subunits, and the two SH2 domains are responsible for the binding of the p85/p110 heterodimer with receptor tyrosine kinases (RTK). The p110 subunit of PI3K is homologous to protein kinases and possesses both serine/threonine protein kinase and phosphoinositide kinase activities (Fig.2).

In unstimulated cells, Akt protein exists in cytoplasm and two regulatory phosphorylation sites are in an unphosphorylated state. Upon growth factor stimulation, RTK undergoes self-phosphorylation. Phosphorylated tyrosine residues interact with SH2 domains of the P85 subunit of PI3K, which leads to activation of the P110 subunit (Fruman et al., 1998). Activated P110 subunit phosphorylates PtdIns, generating PtdIns (3) P, PtdIans (3,4) P2 and PtdIns (3,4,5) P3 (Carpenter et al., 1996). Akt is then sequentially phosphorylated at T308 and S473 by upstream kinases, PDK1 and PDK2, to yield a fully activated kinase (Hemmings,1997). PDK1 has been isolated and well characterized, but the identity of PDK2 is still controversial. PDK1 is only active in the presence of lipid vesicles containing $PI(3,4,5)P_3$ or $PI(3,4)P_2$ and has therefore been termed 3-phosphoinositide-dependent protein kinase-1 (Downward, 1998). Fully activated Akt becomes available to phosphorylate its downstream target molecules such as Bad, forkhead, GSK3 and endothelial nitric oxide synthase (eNOS). Then Akt is dephosphorylated and inactivated by protein phosphatases such as protein phosphatase 2A (PP2A) (Andjelkovic et al., 1996). (Fig. 3).

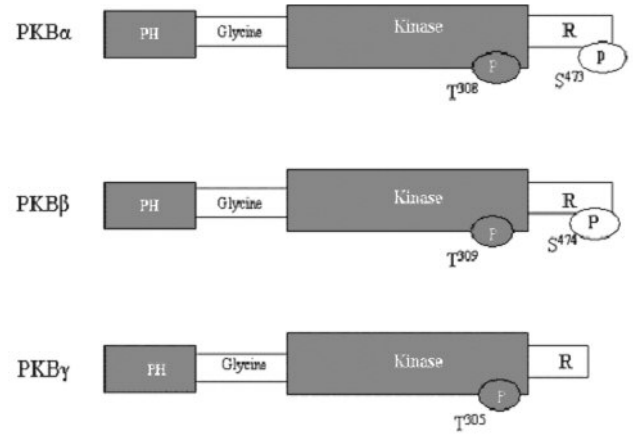

Fig. 1. Schematic structure of PKB. The N-terminus of PKB contains a PH domain and glycine-rich region. The C-terminus has a regulatory role and shows similarity to protein kinase C isoforms. PKBγ lacks the most C-terminal serine phosphorylation site owing to truncation.

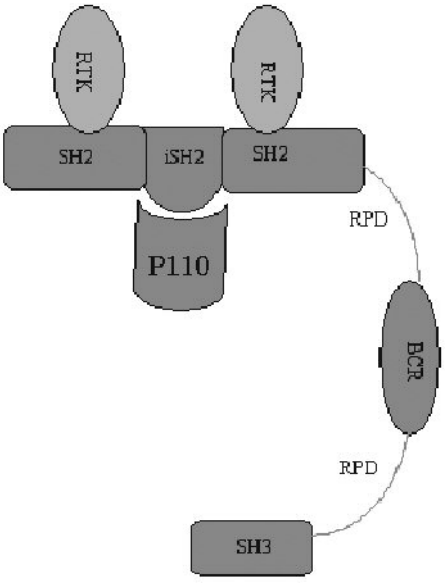

Fig. 2. Schematic structure of the p85/p110-heterodimer. The regulatory p85 subunit consists of an SH3-domain, two PRDs, separated by a BCR-homologous domain (breakpoint clusterregion), and two SH2-domains, separated by the iSH2 (inter SH2) sequence, which is responsible for binding with the p110 subunit.

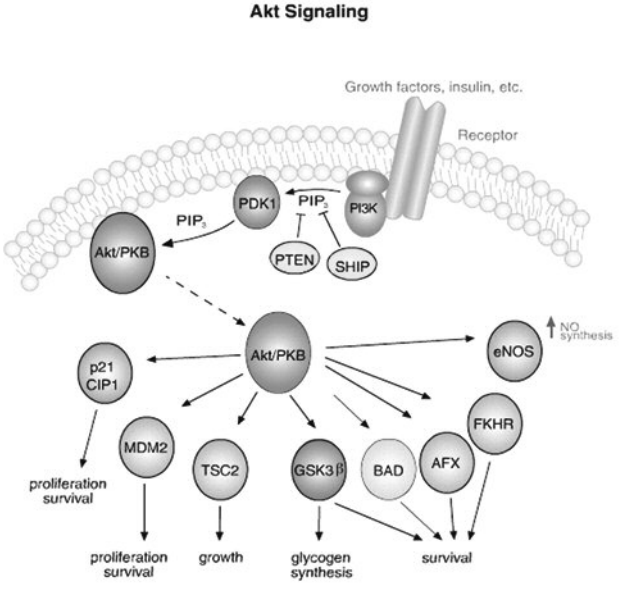

Fig. 3. Mechanism of Akt activation and partial list of down-stream molecules. Akt is activated by growth factors or cytokines in a PI3K-dependent manner, and phosphorylation of two residues by PDK is required for its full activation.

While activation of PI3K appears to induce PKB universally, the reverse does not hold. For example, heat-shock-mediated activation of PKB is insensitive to inhibition by wortmannin, an inhibitor of PI3K, in some cell types (Konishi et al., 1996). Furthermore, it has been shown in 293 cells that pharmacological reagents that elevate cAMP levels, such as forskolin, can activate PKB in a PI3K-independent manner (Sable et al., 1997). However, insulin and forskolin were observed to have synergistic effects on PKB activation, suggesting alternative mechanisms (Moule et al., 1997). Thus it appears that, while activation of PI3K is the major factor regulating activation of PKB, several alternative mechanisms may exist that are utilized by specific stimuli.

Regulation eNOS activity by Akt

VEGF stimulates NO release from endothelial cells, furthermore, VEGF-induced increase of NO release is attenuated by PI3K inhibitors, wortmannin and LY294002 (Gerber et al., 1998). Synthesis of NO has been shown to be tightly regulated by a family of isoenzymes, nitric oxide synthase (NOS), which share in common the property of converting arginine to citrulline, yielding free NO (Knowles and Moncada, 1994). Three distinct isoforms of NOS have been identified with different location and regulation: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The eNOS is the membrane-bound isoform that was first found in vascular endothelial cells and regulated by the level of intracellular calcium. Calcium binds to calmodulin, and this complex associates with eNOS to cause the enzyme activation (Ostermann et al., 1994). Subsequently, it is demonstrated that VEGF-induced NO increase is mediated by Akt. Akt phosphorylates eNOS at Ser1177, leading to a persistent activation of calcium-independent eNOS (Papapetropoulos et al., 1997; Fulton et al., 1999). It is also reported that production of NO in response to fluid shear stress in cultured endothelial cells is controlled by Akt-dependent phosphorylation of eNOS (Dimmeler et al., 1999). Studies in intact animals have shown that overexpression of constitutively active Akt in the vascular endothelium increases resting diameter and blood flow, whereas transduction of dominant-negative Akt attenuates endothelium-dependent vasodilation induced by acetylcholine, demonstrating that Akt acts as a regulator of vasomotor tone in vivo (Luo et al.,2000).

The oxidized low-density lipoprotein (ox-LDL) inhibits endothelial cell migration toward VEGF by dephosphorylation of Akt and inactivation of eNOS (Chavakis et al., 2001). And hyperglycemia has been shown to lead to the glycosylation of the Akt phosphorylation site in eNOS, resulting in an inhibition of eNOS activity (Du et al., 2001).

It has also been shown that eNOS interacts with heat shock protein 90 (Hsp90) on stimulation with VEGF or shear stress, and this interaction enhances eNOS activity(Garcia-Cardena et al., 1998). Interestingly, Akt also interacts with Hsp90 on stimulation and this interaction enhances Akt enzymatic activity, suggesting that Hsp90 may serve as a scaffold protein for the efficient phosphorylation of eNOS by Akt (Sato et al.,2000) (Fig 4).

Postnatal neovascularization by angiogenesis and vasculogenesis

The vasculature is established by two successive steps: angiogenesis and vasculogenesis (Han and Liu, 1999). Vasculogenesis is the process of in situ differentiation of endothelial cells from a mesodermally-derived multipotent precursor, the hemangioblast (Risau and Flamme, 1995). In contrast, angiogenesis is involved in proliferation, migration and remodeling of preexisting endothelial cells and recruitment of periendothelial supporting cells, such as smooth muscle cells and pericytes to provide maintenance and modulation of the vessel (Risau, 1997).

It was initially thought that vasculogenesis was restricted to embryonic development, whereas angiogenesis was the only process involved in neovascularization in adults. However, mature ECs are terminally differentiated cells with a low proliferative potential, and their capacity to substitute damaged endothelium is limited. Therefore, endothelial repair may need the support of other cell types. Recent studies provide increasing evidence that postnatal neovascularization does not rely exclusively on angiogenesis, but also involves bone marrow -derived circulating endothelial progenitor cells (Asahara et al., 1999; Kalka et al., 2000). Accumulating evidence indicates that peripheral blood of adults contains a unique subtype of circulating, bone marrow-derived cells with properties similar to those of embryonic angioblasts (Hatzopoulos et al., 1998). These cells have the potential to proliferate and differentiate into mature ECs. Therefore, they were termed endothelial progenitor cells (EPCs). Recently, two types of EPCs in a source of

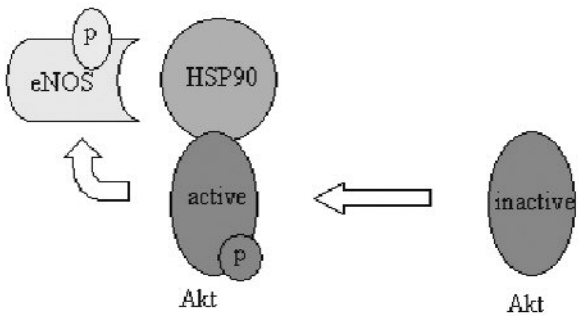

Fig. 4. Schematic illustration of the Akt-eNOS interaction. Activated Akt and eNOS also associate with Hsp90. Hsp90 is believed to function as a scaffold protein for activation of eNOS by Akt-mediated phosphorylation.

human peripheral blood have been reported (Hur et al., 2004), with different morphology, proliferation rates, survival behaviors and different gene expression profiles, leading to different roles in vasculogenesis. The early EPCs contribute to vasculogenesis mainly by secreting the angiogenic cytokines that help recruit resident mature endothelial cells and induce their proliferation and survival, whereas late EPCs enhance vasculogenesis by providing a sufficient number of endothelial cells based on their high proliferation potency. These findings suggest that vasculogenesis may also be a key component in postnatal neovascularization.

EPC and its role in postnatal neovascularization

EPCs were first isolated and described by Asahara (Peichev et al., 2000). Recently it was discovered that 3 markers characterize the functional early EPCs, CD133, CD34, and the vascular endothelial growth factor receptor-2 (VEGFR-2), termed also kinase insert domain receptor (KDR) or fetal liver kinase (Flk-1), all of which are shared by hematopoietic stem cells (HSCs) (Gehling et al.,2000; Quirici et al., 2001). Furthermore, EPCs express a variety of markers typical for the endothelial lineage, including platelet endothelial cell adhesion molecule-1(CD31), VE-cadherin (CD144), von Willebrand factor (vWF) and eNOS (Kaushal et al., 2001; Reyes et al., 2002). EPCs are mobilized from bone marrow into the circulation in response to tissue ischemia and cytokine stimulation (Takahashi et al., 1999). On the other hand, administration of vascular endothelial growth factor (VEGF) has been shown to increase the number of differentiated EPCs in vitro and to augment their incorporation into foci of neovascularization in vivo (Asahara et al., 1999). A pilot clinical study provided evidence that the number of circulating EPCs increased following VEGF gene transfer in patients with severe peripheral vascular disease(Waguro et al., 2002).

Recent studies in animals and patients suggest the transplantation of EPCs ameliorates the function of ischemic organs possibly by both induction and modulation of vasculogenesis and angiogenesis in areas with reduced oxygen supply or by stimulating the reendothelialization of injured blood vessels (Murohara, 2001; Zhang et al., 2002). Therapeutic application of EPCs was attempted in a murine model of hindlimb ischemia in our and other labs (Kalka et al., 2000; Yang et al., 2004). One day following operative excision of one femoral artery, athymic nude mice received an injection of culture-expanded human EPCs. The ratio of ischemic /normal blood flow at Day 28 in EPC-transplanted mice had been significantly improved in comparison with control mice. Histological evaluation of skeletal muscle sections retrieved from the ischemic hindlimbs showed that capillary density was markedly increased in EPC-transplanted mice.

In patients with limb ischemia, the significant improvement of the function of the ischemic limbs observed after autologous transplantation of bone marrow cells has been suggested to be the result of both CD34 positive EPCs and growth factors, released from the CD34 negative bone marrow fraction(Tateishi-Yuyama et al., 2002; Huang et al., 2004). Other clinical studies described the capacity of autologous bone marrow–derived cells or ex vivo expanded autologous EPCs for the repair of human myocardium after infarction (Kawamato et al., 2001; Strauer et al.,2002). Of particular importance might be the local application of progenitor cell by intra-coronary infusion after myocardial infarction. This specific treatment may facilitate the homing of the progenitor cells to the ischemic tissue and may help to avoid the potential risk for enhanced but unwanted vasculogenesis systematically or in tumor tissue.

Akt and Postnatal neovascularization

Akt plays a central role in promoting the survival of a wide range of cell types (Zhan and Han, 2004). Furthermore, several lines of evidence suggest a link between Akt and neovascularization. Akt has recently been shown to phosphorylate eNOS, leading to a persistent activation (Luo et al., 2000). A large body of literature indicates an essential role of endothelial NO for postnatal neovascularization (Papapetropoulos et al., 1997; Murohara et al., 1998). Most convincingly, eNOS knockout animals are characterized by an impaired angiogenesis in response to ischemia or VEGF administration (Waguro et al., 2002). Mechanistically, Akt stimulation may enhance endothelial NO synthesis and thereby influence the long-term regulation of vessel growth. Furthermore, additional downstream substrates of Akt may be involved as well. The inhibition of the proapoptotic proteins Bad or caspase-9 in addition to the enhanced endothelial NO synthesis, which also inhibits endothelial cell apoptosis, may block apoptosis at several stages in the apoptosis-signaling cascade. The question remains as to what the importance of the Akt pathway is for endothelial cell migration and proliferation, which are prerequisites for angiogenesis. Several in vitro studies indicate that endothelial cell migration also depends on NO (Niori et al., 1998; Murohara et al., 1999). Moreover, recent experiments suggest that at least the VEGF-induced endothelial cell migration requires the activation of Akt (Asahara et al., 1999). Therefore, these studies indeed provide evidence that Akt-dependent NO synthesis contributes to endothelial cell migration. The downstream effector pathways, by which NO mediates its effects, are less clear but may involve cGMP and integrin-linked signal transduction processes (Giancotti and Rouslahti, 1999). Very recently, Akt was shown to inhibit the Raf-MEK-ERK kinase cascade by Akt-dependent phosphorylation of Raf, which regulated cell proliferation (Rommel et al., 1999; Zimmermann and Moelling, 1999). Taken together, activation of the Akt kinase orchestrates a number of signaling pathways potentially involved in

angiogenesis. The multiple downstream substrates of Akt not only converge to prevent the induction of apoptosis but may also interfere with numerous biological functions of the endothelial monolayer, which contribute to vascular remodeling and vessel integrity during the angiogenic process.

In contrast to mature ECs, little is known about the regulation of the EPC biology by Akt. The role of Akt signaling on EPCs is first suggested by the observation that simvastatin rapidly activates Akt in EPCs, enhancing proliferative and migratory activities and cell survival. Furthermore, dominant negative Akt overexpression leads to functional blocking of EPC bioactivity (Levadot et al., 2001). This finding establishes that HMG-CoA reductase inhibitors augment mobilization of bone marrow-derived EPCs through stimulation of the Akt signaling pathway. Subsequently, it is not only demonstrated that Akt regulates differentiation of EPCs, but also identified Akt as a target to modify EPCs kinetics (Dimmeler et al., 2001). Besides statins, erythropoietin (EPO) increased viability of EPCs in the tube formation assay. Furthermore, EPO activates the intracellular Akt pathway in EPCs, permitting the conclusion that EPO is a potent regulator of EPC proliferation and differentiation via the Akt signaling pathway (Bahlmann et al., 2003). Interleukin-18 binding protein (IL-18BP), a major anti-inflammatory protein, stimulates ischemia-induced neovascularization in association with an activation of VEGF/Akt signaling and an increase in EPC mobilization and differentiation (Mallat et al., 2002). Another study shows that estrogen can augment EPC mobilization into sites of neovascularization in adult organs under pathological conditions. And this process appears to require eNOS (Iwakura et al., 2003). However, Imanishi et al have shown that ox-LDL inhibits VEGF -induced EPC differentiation through an effect on the Akt pathway, inducing Akt dephosphorylation (Imanishi et al., 2003).

Concluding remarks

In summary, Akt signaling is activated by a variety of stimuli in ECs and EPCs, and then regulates multiple critical steps in angiogenesis and vasculogenesis. This signaling pathway also regulates cardiovascular homeostasis and vessel integrity at least in part by controlling NO synthesis. Neovascularization is implicated in the pathophysiology of a number of diseases and is an important therapeutic strategy for those diseases. Therefore, further dissection of the Akt pathway and elucidation of the downstream effector molecules will lead to a better understanding of blood vessel growth and may provide avenues for the development of novel therapeutic interventions.

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