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AIP1-mediated stress signaling in atherosclerosis and arteriosclerosis

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Abstract

AIP1 (encoded by the *DAB2IP* gene), a signaling scaffolding protein, is abundantly expressed in vascular endothelial cells (EC). While it was initially discovered as an ASK1-interacting protein, AIP1 broadly suppresses inflammatory responses triggered by cytokines and stresses such as TNF, LPS, VEGF and ER stress in EC (therefore AIP1 is an **Anti-Inflammatory Protein**). Human genome-wide association study (GWAS) has identified *DAB2IP* gene variants conferring susceptibility to cardiovascular diseases. Consistently, a global or vascular EC-specific deletion of *DAB2IP* in mice strongly enhances inflammatory responses and exacerbates atherosclerosis and graft arteriosclerosis progression in mouse models. Mechanisms for AIP1 function and regulation associated with human cardiovascular diseases need further investigations.

Keywords

AIP1; Vascular endothelial cell (EC); Inflammation; TNF; LPS; TLR (toll-like receptor); ER stress; Oxidative stress; Signaling pathway; Atherosclerosis; Arteriosclerosis; Angiogenesis; Lymphangiogenesis

Introduction

Cardiovascular disease remains the leading cause of death in the United States. It has become clear that increases in inflammatory and stress responses represent common pathogenic mechanism for cardiovascular disease (1-4). The vascular cell that normally limits the inflammation is the endothelial cell (EC). Inflammatory cytokines and stress stimuli, by activating intracellular signaling cascades, induce EC activation that is characterized by expression of proinflammatory molecules, EC dysfunction that is characterized by a reduction in amount of bioavailable nitric oxide (NO) in the vasculature, and EC apoptosis. We have previously demonstrated Apoptosis signal-regulating kinase 1

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(ASK1), a member of the MAPK kinase kinase (MAP3K) family, is critical mediator in vascular inflammation and apoptosis (5, 6). We subsequently identified AIP1 (ASK1-interacting protein-1; also DAB2IP) that is highly expressed in vascular endothelium and function as signaling adaptors that mediate stress-induced ASK1 signaling and EC phenotype changes (7-12). Surprisingly, studies from AIP1 deficient mice support that AIP1 functions as an inflammatory suppressor. Mice with a global deletion or an EC-specific deletion are viable with slightly delayed vascular development but without obvious defects in the adult vasculature (10, 13). AIP1-KO mice show dramatically enhanced inflammatory responses in ischemic hindlimb, inflammatory sponge, carotid ligation for neointimal formation, atherosclerosis and graft arteriosclerosis models (10, 12). In supporting our mouse models, a recent human genome-wide association study (GWAS) has identified a sequence variant within intron 1 of the *AIP1* (*DAB2IP*) gene conferring susceptibility to cardiovascular disease including abdominal aortic aneurysm, peripheral vascular disease, early onset of myocardial infarction and pulmonary embolism (14). Mechanistic studies suggest that AIP1 functions as an endogenous inhibitor in multiple stress signaling pathways. Although AIP1 was initially identified as an ASK1-interacting protein, it contains multiple functional domains including PH, C2, GAP at the N-terminal half while a period-like domain, a proline-rich region, a coiled-coil and leucine-zipper (CC/LZ) at its C-terminal half (7-12). While AIP1 promotes stress-induced activation of ASK1 apoptotic signaling, it suppresses TNF and TLR-induced NF- κ B, and IFN- γ -induced JAK2 signaling pathways (7-12) (Fig.1 for AIP1-regulated signaling). Therefore, AIP1-deficient mice exhibit enhanced inflammation and cell proliferation with reduced apoptosis. Here, we review our studies on the AIP1-mediated inflammatory and stress signaling pathways in the vasculature and the in vivo function in vascular inflammation.

AIP1 is a novel member of the RAS-GAP family

Human AIP1 gene locates at 9q33.1–q33.3 and cDNA sequence spans approximately 3.5 kb with 14 exons and 13 introns. Analysis of the AIP1 promoter revealed that it is a typical TATA-less promoter containing many GC-rich sequences (15). Epigenetic regulation, such as DNA methylation, histone acetylation and histone methylation, plays a potential role in regulating AIP1 expression (15-17). AIP1 protein (1065 amino acids) has a molecular mass of 117 kD containing several potential function domains: an plekstrin homology (PH) domain, a C2 domain and a Ras-GTPase activating protein (GAP) domain in the N-terminal half; a period-like domain and a proline-rich region (7). The alignment of the GAP domain of AIP1 with other Ras-GAPs, such as GAP120, NF1, SynGAP, and nGAP, shows a high degree of amino acid homology (40% ~ 90%). AIP1 and other GAPs contain a common structural region called the GAP related domain (GRD). The GRD is the catalytic unit of these proteins, which stimulates the GTPase activity of Ras protein (18). Therefore, AIP1 is considered as a novel member of the RAS-GAP family proteins. The function of the Ras-GAP activity has been investigated. The AIP1-mediated inhibition of Ras-ERK1 signaling is required for TNF-induced ASK1 activation in EC (7) (see below). Whether or not it is critical for other signaling in EC and vascular inflammation needs to be further determined.

AIP1 is a signaling adapter molecule involved in endothelial inflammation

AIP1 mediates a balance between ASK1-JNK versus IKK-NF- κ B signaling

TNF, one of the most important pro-inflammatory cytokines (19-21), via its receptor TNFR1 activates several signaling pathways in EC to induce EC dysfunction and apoptosis (1, 22). We show that AIP1 specifically allows the TNFR1/TRADD/RIP1/TRAF2 signaling complex to activate a proapoptotic ASK1/JNK signaling pathway, while inhibiting the same complex from activating a prosurvival I κ B kinase (IKK)/NF- κ B signaling pathway (8). In resting EC, AIP1 is localized on the plasma membrane in a closed form and in a complex with TNFR1. TNF binding induces release of AIP1 from TNFR1, resulting in cytoplasmic translocation and concomitant formation of an intracellular signaling complex comprised of TRADD, RIP1, TRAF2, and AIP1. At the same time, AIP1 binds to the C-terminal domain of ASK1 and activates ASK1 by recruiting phosphatase PP2A to dephosphorylate of ASK1 at pSer967, leading to dissociation of inhibitor 14-3-3 from ASK1 (23). 14-3-3, a phosphoserine-binding molecule, binds to ASK1 specifically via Ser-967 of ASK1 and has been reported to inhibit ASK1/JNK signaling pathway and ASK1-induced apoptosis. Thereby, AIP1 mediates the activation of proapoptotic ASK1/JNK signaling pathway through the TNFR1 signaling complex and PP2A. Deletion analyses indicated that different sequences on AIP1 have distinct roles in this TNFR1 signaling pathway: A proline-rich region (amino acids 796 – 807) is critical for maintaining AIP1 in a closed form, which associates with a region of TNFR1 distinct from the death domain, the site of TNFR1 association with TRADD; A PERIOD-like domain (amino acids 591–719) of AIP1 specifically interacts with the intact RING finger of TRAF2 to enhance TNF-induced ASK1-JNK but to inhibit IKK-NF- κ B signaling (8); The N-terminus of AIP1 containing the C2 and GAP domains constitutively binds to the C-terminal domain of ASK1 and facilitates the release of 14-3-3 from ASK1 (7).

In this TNFR1/TRADD/RIP1/TRAF2 signaling system (19-21), RIP1-mediated AIP1 phosphorylation at the 14-3-3-binding site Ser-604 plays a critical role in TNF-induced ASK1-JNK/p38 signaling (9). As demonstrated by both overexpression and small interfering RNA knockdown of RIP1 in EC, RIP1 (the Ser/Thr protein kinase receptor-interacting protein) associates with the GAP domain of AIP1 and mediates TNF-induced AIP1 phosphorylation at Ser-604 and JNK/p38 activation. Furthermore, we identified homeodomain-interacting protein kinase 1 (HIPK1) as a novel signal transducer in TNF-induced ASK1-JNK/p38 activation (24, 25). TNF-induced desumoylation and cytoplasmic translocation of HIPK1 induces dissociation of ASK1 from its inhibitors 14-3-3 and thioredoxin and synergizes with AIP1 to induce ASK1-JNK/p38 activation and EC apoptosis. The mechanism by which AIP1 inhibits IKK/NF- κ B signaling is not completely known. AIP1 appears to directly bind to RIP1 and TRAF2 thus block the recruitment and activation of the IKK complex.

Vascular EC also express TNFR2, the second TNF receptor (26), which normally elicits survival and angiogenic signaling in EC. Recent studies from our group show that AIP1 also mediates TNFR2-dependent NF- κ B and JNK activation in vascular EC. We have identified the critical domains of TNFR2 involved in TNFR2-mediated NF- κ B and JNK signaling.

Specifically, the C-terminal 59 residues (aa380–439) containing the TRAF2- and Bmx-binding sites are essential for NF- κ B activation; the adjacent domain II (aa289–338) and domain III (aa338–380) are critical for its association with the JNK-specific signaling molecule AIP1, providing the molecular basis for its role in JNK activation. Interestingly, we found that the TRAF2/Bmx-binding sites appear to be important in maintaining TNFR2 on the plasma membrane, leading to NF- κ B but not JNK activation. However, deletion of this sequence causes TNFR2 intracellular localization and forms a complex with AIP1 via its serine/threonine-rich sequences, leading to JNK activation. Therefore, both internalization and AIP1 association of TNFR2 are required for TNFR2-dependent JNK and apoptotic signaling (27).

AIP1 is essential for transducing the IRE1-mediated ER stress response

Environmental stresses, including oxidative stress (also known as oxy stress), endoplasmic reticulum (ER) stress, metabolic stress, and genotoxic stress, trigger endothelial inflammation and cell dysfunction (1-4). In response to ER stress, there are two important and distinct signaling pathways are triggered: protein kinase activated by ds RNA (PKR)-like ER kinase (PERK)-CHOP1 axis and inositol-requiring enzyme-1 (IRE1)-JNK/XBP-1 axis. The ER stress induced IRE1-JNK/XBP-1 axis, but not the PERK-CHOP1 axis, is blunted in AIP1-KO cells. Specifically, ER stress-induced activation of IRE1 and synthesis of XBP-1 were blunted in AIP1-KO EC. In contrast, phosphorylation of PERK and induction of CHOP expression are still activated in AIP1-KO cells. ER stress strongly induces an association of AIP1 with IRE1 and the PH domain of AIP1 is critical for AIP1-mediated IRE1 activation as demonstrated by reconstitution of AIP1-KO cells with AIP1 or AIP1 mutant with a deletion of the PH domain. Moreover, AIP1 facilitates IRE1 dimerization, a prerequisite step for activation of IRE1 and the subsequent downstream events. Thus, AIP1, a TNF signaling component, may directly regulate IRE1 activity, and specifically involve in ER stress-induced IRE1-JNK/XBP-1 axis without effects on the PERK-CHOP pathway.

ER stress also induces an association of TRAF2 with IRE1 and AIP1. It has been shown that IRE1 recruits TRAF2, which in turn activates ASK1-JNK cascade in response to ER stress. Interestingly, IRE1-TRAF2 complex is not detected in AIP1-KO cells, suggesting that the AIP1 is crucial for ER stress-induced activation of IRE1 and formation of the IRE1-TRAF2 complex. While the kinase activity of IRE1 is critical for IRE1-TRAF2 interaction, the AIP1-IRE1 interaction is independent of the kinase activity of IRE1 (28). It needs to be further determined the localization of the IRE1-AIP1 complex and investigated the exact mechanism by which AIP1 mediates IRE1 dimerization and activation.

AIP1 functions as Arf6-GAP to negatively regulate TLR4 signaling

TLR4 is unique among all the Toll-like receptors because it can utilize the sorting adaptor TLR/IL1R-domain-containing adaptor protein (TIRAP) and mediate activation of both the MyD88-dependent NF- κ B and MAPK cascades as well as the MyD88-independent IRF-3 signaling pathway (29). The ability of TIRAP to function as a sorting adaptor is dependent on its amino-terminal localization domain, which interacts with plasma membrane-localized phosphatidylinositol-4,5 bisphosphate (PIP2) (30, 31). Mutations in the PIP2 binding

domain of TIRAP, or depletion of cellular PIP2, can abrogate both the recruitment of TIRAP to the plasma membrane and its capacity to induce cytokine production in response to LPS (32). The cellular level of PIP2 is dynamically regulated. In mammalian cells, the major pathway of PIP2 synthesis is phosphorylation of PI 4-phosphate (PI4P) by the type I PI 4-phosphate 5-kinase (PIP5K) family members. Regulation of PIP5K activity is critical for PIP2-dependent TLR4 signaling. Arf6 (ADP-ribosylation factor 6), a small GTPase, is the best characterized activator of PIP5K; and most importantly, Arf6 facilitates the recruitment of the PIP2 binding adaptor protein TIRAP to the plasma membrane (33). Recently, we found AIP1, initially identified as a member of Ras-GAP family protein in our laboratory, is a novel Arf6 GAP that can promote Arf6-GTP hydrolysis both in vitro and in vivo. Deletion of AIP1 from mouse EC can enhance cellular PIP2 levels, in turn enhancing LPS-induced NF- κ B and MAPK signaling. Conversely, overexpression of AIP1 in EC strongly reduces the PIP2 level and blunts the TLR4-MyD88-dependent signaling. Furthermore, deletion of the GAP domain on AIP1 results in a loss of its ability to mediate the inhibition of Arf6- and TLR4-induced signaling events. Thus, AIP1 suppresses LPS induced endothelial inflammation not by directly binding to any of the TLR4-TIRAP-MyD88 complex components, but in a GAP activity-dependent manner (11). Interestingly, AIP1 also limits TLR2 ligand (Pam3CSK4)-induced activations of the MAPK and NF- κ B signaling pathways in EC (11), suggesting that AIP1 may directly interact with TLR2 signaling molecules as shown in the TNF signaling.

AIP1 inhibits vascular inflammation in adult tissues

AIP1 mediates inflammatory angiogenesis

Angiogenesis, the process of new blood vessel formation, is involved in many physiological and pathological settings such as inflammatory, ischemia, diabetes, atherosclerosis, and cancer (34). The VEGFs and their receptors have been shown to be critical in regulating vessel formation in both normal physiological and pathological processes. In neonatal retina, skin and mesentery, AIP1-deficient mice exhibit reduced angiogenesis with less sprouting and fewer branches and show delayed developmental lymphangiogenesis. The reduced angiogenesis and lymphangiogenesis in AIP1-deficient (AIP1-KO) mice correlate with reduced expression in VEGFR3. Mechanistically, AIP1, by directly binding to VEGFR3, it enhances VEGFR3 endocytosis and stability (13). In contrast, AIP1 functions as an endogenous inhibitor in VEGFR2-mediated inflammatory angiogenesis in a hindlimb ischemic model and a sponge granuloma model (10). The enhanced angiogenesis is associated with increased VEGFR2 signaling. Mechanistic studies suggest that AIP1 via its C2 domain and proline-rich domain associate with phosphor-VEGFR2 and PI3K, respectively, leading to attenuation of the VEGFR2-PI3K angiogenic signaling. However, the Ras-GAP activity of AIP1 is not required for its inhibitory effect on VEGFR2 signaling (10).

AIP1 suppresses the progression of inflammation-induced atherosclerosis and graft arteriosclerosis

EC activation, dysfunction, and vascular inflammation occur when the endothelium is exposed to various insults such as pro-inflammatory cytokines, hyperglycemia,

hypertension, and hyperlipidemia. These insults lead to the pathogenesis of a range of disease states, including atherosclerosis. We used AIP1-KO mice to examine its effect on atherosclerotic lesions in an apolipoprotein E-null (ApoE-KO) mouse model of atherosclerosis. Although AIP1 deletion has no effects on body weight, serum cholesterol and triglyceride levels, and lipoprotein profiles in hyperlipidemic ApoE-KO mice, AIP1 deletion significantly augment inflammatory responses, EC dysfunction, macrophage infiltration and atherosclerotic lesion expansion in the aorta and innominate artery (35). Mechanistic studies suggest that AIP1 expression in macrophages is very low compared with aortic EC. Therefore, AIP1 deletion in EC, but not in macrophage, enhances atherosclerotic lesions in an apolipoprotein E-null (ApoE-KO), suggesting that vascular EC are responsible for the development of atherosclerosis. This is consistent with our in vitro observations that AIP1 regulates cellular signaling in EC induced by proinflammatory mediators TNF, IL-1 β and TLR ligands. AIP1-KO EC significantly enhances oxidized low-density lipoprotein-induced nuclear factor- κ B signaling, gene expression of inflammatory molecules, and monocyte adhesion (Fig.1). Our study has revealed an important role of AIP1 in limiting inflammation, EC dysfunction, and atherosclerosis (35). Of note, AIP1 deletion has similar effects on oxLDL and TLR2/4 signaling in EC suggest a shared function of AIP1 in these pathways. One possible explanation is that oxLDL utilizes TLR2/4 to activate NF- κ B and JNK pathways in vascular EC (36). OxLDL also utilizes unique receptor(s) to induce intracellular signaling in EC. It has been shown that lectin-like oxLDL receptor-1 (LOX-1), a membrane protein structurally distinct from scavenger receptors on endothelial surfaces, is involved in oxLDL-induced NF- κ B activation, endothelial dysfunction and atherogenesis (37-39). It will be interesting to investigate if and how AIP1 plays a role in LOX-1-mediated signaling.

AIP1 suppresses the progression of graft arteriosclerosis

The proliferation of vascular smooth muscle cell (VSMC) within the intima is another critical component of atherosclerosis pathogenesis(40). Our study show that AIP1, which highly expressed in both ECs and VSMCs, functions as a negative regulator in IFN- γ dependent VSMC proliferation and progression of graft arteriosclerosis, a disease process shared similar pathogenesis and mechanism as atherosclerosis (41, 42). Specifically, graft arteriosclerosis, the major cause of late cardiac allograft failure, Is characterized by a diffuse, concentric arterial intimal hyperplasia composed of infiltrating host T cells, macrophages and predominantly graft-derived smooth muscle-like cells that proliferate and elaborate extracellular matrix, resulting in luminal obstruction and allograft ischemia. IFN- γ , a proinflammatory cytokine produced by effector T cells, is a critical mediator for smooth muscle-like cell proliferation. Our group has developed two new mouse graft arteriosclerosis models, characterized by VSMC accumulation and intimal expansion, to assess the effect of AIP1 on IFN- γ driven smooth muscle cell proliferation (12, 43-45). In our mouse models, IFN- γ signaling played a critical role for in graft arteriosclerosis progression. Importantly, AIP1 deletion augments IFN- γ responses in cultured aorta and isolated aortic VSMCs, and enhances IFN- γ -dependent VSMC migration and proliferation, two critical steps in neointima formation. Mechanistically, AIP1 functions as a brake in IFN- γ -induced arteriosclerosis, in part by downregulating IFN- γ -JAK2-STAT1/3-dependent migratory and proliferative signaling in VSMCs. In response to IFN- γ , AIP1, via its intact N-terminal

domains, directly binds to JAK2 and inhibits its activity (12). It has been shown previously that JAK-STAT signaling can be negatively regulated through three main mechanisms: the dephosphorylation of JAKs or STATs by various protein tyrosine phosphatases; the inactivation of JAKs by the suppressor of cytokine signaling (SOCS) family of proteins; and the inhibition of the transcriptional activity of STATs by protein inhibitor of STAT (PIAS) proteins. AIP1 acts in a similar manner as the SOCS family (SOCS1). Recently, we have also demonstrated that SOCS1, like AIP1, attenuates vascular inflammation and attenuates graft arteriosclerosis in the mouse models (12, 44, 45). Whether AIP1 and SOCS1 function synergistically in the inhibition of IFN- γ signaling needs to be further investigated.

Conclusions

In supporting our mouse model studies, a recent genome-wide association study (GWAS) performed on 1,292 individuals with abdominal aortic aneurysm (AAA) and 30,503 controls, has identified a sequence variant in the *AIP1* gene (rs7025486[A]) to be strongly associated with several vascular diseases (14, 46). In tests for association with specific vascular diseases, results show that AIP1 is associated with AAA, early-onset myocardial infarction (MI), venous thrombo-embolism, peripheral arterial disease (PAD), but not with intracranial aneurysm or ischemic stroke. More notably, this association is independent on classical risk factors for arterial and venous diseases - that is, smoking, lipid levels, obesity, type 2 diabetes and hypertension (14, 46). It needs to be determined how this sequence variant in the intron affects the *AIP1* gene expression. It has been recently noticed that there are at least 6 transcripts of the human *AIP1* gene, generating either by different promoters or alternatively splicing. The six transcripts produce at least 4 AIP1 protein isoforms, and the studies involved in this review have been all based on the first AIP1 isoform. Future studies are required to understand the regulation and function of these AIP1 isoforms in vascular inflammation and disease progression.

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Fig.1. AIP1 limits atherogenic signaling in EC

Proinflammatory stimuli LPS and atherogenic stimuli oxLDL induce NF-κB signaling in EC, which drives expression of adhesion molecules on EC, mediating interactions of monocytes with EC during the initiation of atherosclerosis. AIP1 deletion in vascular EC augments LPS/TLR4 and oxLDL-induced NF-κB and JNK signaling, gene expression of adhesion molecules, chemokines and monocyte adhesion, leading augmented atherosclerosis progression.