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Gα12 IN MEMBRANE TRAFFICKING IN ENDOTHELIAL CELLS. A.V. Andreeva, M.A. Kutuzov, R. Vaiškunaite, T.A. Voyno-Yasenetskaya, Department of Pharmacology, University of Illinois at Chicago, Chicago, IL.

Proper functioning of intracellular membrane trafficking in endothelial cells is indispensable for maintaining endothelial barrier, as well as for regulated secretion of a variety of pro- and anticoagulants and inflammatory proteins. While the involvement of several heterotrimeric G proteins in regulation of membrane trafficking has been documented, there is only one work published on the possible role of Gα12 in this respect in PC12 cells, where Gα12 has been reported to inhibit exocytosis. In the present study, we addressed possible roles of Gα12 in membrane trafficking in endothelial cells, using human umbilical vein endothelial cells (HUVEC) as a model. Using confocal microscopy, ECIS measurements, and velocity sedimentation assays, we examined the effects of overexpression of wild-type or constitutively active Gα12, as well as siRNA-mediated depletion of endogenous Gα12, on several markers in resting or thrombin-stimulated HUVEC. Our data indicate that Gα12 has stimulatory effect on exocytosis in HUVEC. Moreover, we were able to detect macromolecular complexes of Gα12 with transported proteins, suggesting its direct involvement in membrane trafficking.

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ADIPOSITY AND INSULIN GROWTH FACTOR–DEPENDENT SIGNALING IN TPA-INDUCED MOUSE MODELS. B. Assaad,[1] H. Kakaji,[1] T. Quinn,[1] A. Molteni,[1] W. Wang,[2] L. Xie,[2] D. Vasques,[2] P. Ouyang,[2] H. Doan,[2] B. Herndon,[1] [1]University of Missouri-Kansas City School of Medicine, Kansas City, MO; [2]Kansas State University, Manhattan, KS.

Caloric restriction is associated with increased longevity and cancer resistance in many species. Recent work on mice with selective loss of insulin signaling—only in adipose tissue—suggested that reduced adiposity, not food restriction, extended longevity (Science 2003;299:572). To determine if this finding also applied to cancer prevention, three groups of SENCAR mice were treated with TPA following 10 weeks pretreatment: (a) ad libitum fed, sedentary; (b) exercise with pair feeding at the same amount as group a; and (c) 20% calorie restriction. Body weight and percent fat were significantly decreased in groups b and c after 10 weeks. Dorsal skin was shaved and topically treated with 3.2 nmol TPA, and tissue was harvested at necropsy 2 hours later. Subcutaneous fat in skin sections was evaluated by immunohistochemistry for two pathways of insulin/insulin growth factor (IGF-1)-dependent cellular signaling, PI3K and H-ras. Tissue was graded for staining density using computer standards under guidance of a pathologist without knowledge of tissue treatment. H-ras staining in TPA-treated subcutaneous fat was significantly different between groups: sedentary (a) vs exercise (b) was significant ($p = .02$) and sedentary (a) vs dietary restriction (c) was highly significant ($p < .001$). There were no significant differences in staining of PI3K in subcutaneous fat in any group. We conclude that the subcutaneous fat in SENCAR mice treated with TPA shows important decreases in the upstream IGF- MAPK pathway with either exercise or calorie restriction, not seen with downstream PI3K.

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A NOVEL ROLE OF SPHINGOSINE KINASE 1 IN THE DE NOVO BIOSYNTHESIS OF DIHYDROSPHINGOSINE-1-PHOSPHATE IN MAMMALIAN CELLS. E. Berdyshev,[1] I. Gorshkova,[1] P. Usatyuk,[1] Y. Zhao,[1] B. Saatian,[2] W. Hubbard,[2] V. Natarajan,[1] [1]Department of Medicine, The University of Chicago, Chicago, IL; [2]The Johns Hopkins University, Baltimore, MD.

Sphingosine kinases 1 and 2 (SK1 and SK2) generate sphingosine-1-phosphate (S1P), a potent endogenous lipid mediator. Using a highly selective and sensitive LC-MS/MS approach, here we show that SK1 overexpression, but not SK2, in different primary cells and cultured cell lines results in predominant up-regulation of the synthesis of dihydrosphingosine-1-phosphate (DHS1P) compared to S1P. Stable isotope pulse-labeling experiments in conjunction with LC-MS/MS quantitation of different sphingolipids demonstrated strong interference of overexpressed SK1 with the de novo sphingolipid biosynthesis by up-regulating the influx of L-serine into sphingolipids and by phosphorylating a major portion of the newly formed dihydrosphingosine to DHS1P. As a result of SK1 overexpression, migration and Ca^{2+} response of human pulmonary artery endothelial cells (HPAEC) to stimulation with external S1P, but not thrombin, were strongly impaired. Furthermore, infection of human bronchial epithelial cells with RSV A-2 virus increased SK1-mediated synthesis of DHS1P and S1P, whereas TNF-α enhanced only S1P production in HPAEC. These findings uncover a new functional role for SK1, which can target de novo sphingoid base metabolic flow and deviate it from the generation of ceramides toward the synthesis of DHS1P, the S1P homolog and ceramide signaling counterpart.

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RHO-SPECIFIC NUCLEOTIDE EXCHANGE FACTOR GEF-H1 MEDIATES AGONIST-INDUCED LUNG ENDOTHELIAL BARRIER DYSFUNCTION. A.A. Birukova, D. Adyshev, B. Gorshkov, K.G. Birukov, A.D. Verin, Department of Medicine, The University of Chicago, Chicago, IL.

Rationale: Endothelial cell (EC) permeability is precisely controlled by cytoskeletal elements (actin filaments, microtubules, intermediate filaments). We have recently shown that the edemagenic agonist thrombin caused partial microtubule (MT) disassembly, which was linked to activation of small GTPase Rho, Rho-mediated actin remodeling, cell contraction, and EC barrier dysfunction. In this study we tested the hypothesis that MT-associated Rho-specific GEF-H1 may mediate Rho-dependent EC barrier dysfunction associated with partial MT disassembly. **Methods:** Endogenous GEF-H1 was depleted using siRNA technique. Functional GEF-H1 mutants were introduced to EC using transient transfection and nucleofection techniques. EC permeability changes were assessed by measurements of transendothelial electrical resistance. EC monolayer integrity and cytoskeletal remodeling were evaluated by immunofluorescent analysis of MLC phosphorylation and actin rearrangement. MLC and MYPT1 phosphorylation induced by thrombin (0.02 U/mL) or MT-depolymerizing agent nocodazole (0.2 μM) was detected by immunobloting with phosphospecific antibodies. **Results:** Depletion of GEF-H1 or expression of dominant negative GEF-H1 mutant significantly attenuated thrombin- and nocodazole-induced permeability increase and actin stress fiber formation associated with increased phosphorylation of Rho-Rho-kinase targets MLC and MYPT1. In contrast, expression of wild-type or activated GEF-H1 mutants dramatically enhanced thrombin and nocodazole effects on stress fiber formation and cell retraction. **Conclusions:** These results demonstrate for the first time a role for the GEF-H1 in the Rho activation induced by MT disassembly and suggest GEF-H1 as a key molecule involved in crosstalk between MT and actin cytoskeleton in agonist-induced Rho-dependent EC barrier regulation.

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ANTI-INFLAMMATORY EFFECTS BY DIFFERENT GROUPS OF OXIDIZED PHOSPHOLIPIDS. A.A. Birukova, S. Chatchavalvanich, D.O. Burdette, K.G. Birukov, Department of Medicine, The University of Chicago, Chicago, IL.

Purpose of Study: Increased levels of oxidized phospholipids generated by oxidation of shed cell membranes have been recently detected in the local circulation and related to the development of acute lung injury and cardiac ischemia. Previous studies have demonstrated that oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) enhances basal endothelial cell (EC) barrier properties. This study characterized anti-inflammatory effects and barrier regulation in human pulmonary artery EC induced by OxPAPC-like products containing choline (OxPAPC), serine (OxPAPS), ethanolamine (OxPAPE), and glycerophosphate (OxPAPA) head groups. **Methods:** Barrier-protective properties and anti-inflammatory effects of oxidized phospholipids were characterized using in vitro models of thrombin- and LPS-induced barrier dysfunction. EC permeability changes were assessed by measurements of transendothelial electrical resistance (TER). EC monolayer integrity and cytoskeletal remodeling were evaluated by immunofluorescent analysis of actin rearrangement. Activation of small GTPases was measured by in vitro pull down assays. **Summary:** All four groups, OxPAPC, OxPAPA, OxPAPS, and OxPAPE, caused sustained concentration-dependent increases in TER accompanied by unique remodeling of actin cytoskeleton characterized by zip-like actin projections and formation of intercollated peripheral actin structures. Remarkably, oxidized phospholipids significantly enhanced recovery phase in thrombin-challenged EC monolayers, which was assessed by TER measurements and immunofluorescent analysis of paracellular gap formation. In addition, EC pretreatment with oxidized phospholipids had protective effect against thrombin- and LPS-induced permeability monitored by TER measurements. Moreover, oxidized phospholipids attenuated thrombin-induced small GTPase Rho activation and myosin light chain phosphorylation, which was accompanied by increased levels of activated Rac and Cdc42. **Conclusions:** These results demonstrate for the first time barrier-protective effects elicited by different groups of cell membrane–derived oxidized phospholipids associated with acute lung or cardiac injury and suggest a role for phospholipid polar headgroups in modulation of barrier-protective and anti-inflammatory properties of oxidized phospholipids.

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PHORBOL ESTER–INDUCED ENDOTHELIAL CYTOSKELETAL REMODELING. N.V. Bogatcheva, A. Birukova, T. Borbiev, I. Kolosova, F. Liu, J.G.N. Garcia, A.D. Verin, Department of Medicine, The University of Chicago, Chicago, IL.

We have previously shown that treatment of bovine endothelial cell monolayers with phorbol myristate acetate leads to the thinning of cortical actin ring and rearrangement of the cytoskeleton into a grid-like structure, concomitant with the loss of endothelial barrier function. Here we demonstrate that phorbol myristate acetate induces both myosin and caldesmon redistribution from cortical ring into the grid-like network. However, the initial step of actin and myosin redistribution is not followed by caldesmon. Coimmunoprecipitation experiments revealed that short-term (5 minutes) treatment with phorbol ester leads to the weakening of caldesmon ability to bind actin and myosin. Prolonged incubation with phorbol myristate acetate, however, strengthens caldesmon complexes with actin and myosin, which correlates with the grid-like actin network formation. Phorbol ester stimulation leads to an immediate increase in caldesmon Ser/Thr phosphorylation. This process occurs at sites distinct from the sites specific for ERK1/2 phosphorylation and correlates with caldesmon dissociation from the actomyosin complex. Inhibition of ERK-kinase MEK fails to abolish grid-like structure formation, although reducing weakening of the cortical actin ring, whereas inhibition of protein kinase C reverses phorbol ester–induced cytoskeletal rearrangement. Our results suggest that protein kinase C–dependent phosphorylation of caldesmon is involved in phorbol ester–mediated complex cytoskeletal changes leading to the endothelial cell barrier compromise.

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FACTOR IXa-ATIII COMPLEX FORMATION IS INHIBITED BY PROTAMINE SULFATE. A.S. Brecher, A.R. Moon, Department of Chemistry, Bowling Green State University, Bowling Green, OH.

This laboratory has studied the effect of 1 and 5 μg protamine sulfate (PS) (0.2 and 1.0 nmol) upon the formation of covalent complexes between (excess) factor IXa (5 μg) and ATIII (1.92 μg) under three sets of conditions: preincubation of IXa with PS for 15 minutes prior to the addition of ATIII; preincubation of ATIII with PS for 15 minutes prior to the addition of IXa; and mixing each of the reagents within 1-minute intervals. Complex formation was detected by SDS-PAGE and quantitated. A major band for the enzyme was observed at about 45 kDa, and a minor degradation product band of the enzyme was seen at about 31 kDa. A single ATIII band of about 55 kDa was also noted. Upon mixing enzyme with inhibitor, two bands of complexes were observed, at approximately 115 kDa and 100 kDa. It was suggested that the 100 kDa band was a degradation product of the 115 kDa band. Upon preincubation of IXa, ATIII, or both with PS prior to addition of ATIII, Ixa, or buffer to the mixtures, it was noted in all three cases, and in the presence of both levels of PS, that there was a decrease in the amounts of 115 kDa and 100 kDa complexes observable. The decrease was greater in the presence of 5 μg, suggesting inhibition of complex formation. Concomitantly, with the decrease in complex formation, an increase in unreacted ATIII was noted. At the same time a decrease in free IXa was observed commensurate with an increase in the IXa fragment. The latter corresponds most likely to a promotion of autolysis of factor IXa by the presence of PS, in agreement with other proteolytic stimulations reported from this laboratory upon addition of PS to other coagulation factors.