Effect of Geranylgeranyl Pyrophosphate Synthase on Hypoxia/Reoxygenation-Induced Injury in Heart-Derived H9c2 Cells

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Summary
Recent studies have revealed that geranylgeranyl pyrophosphate synthase (GGPPS), a key enzyme involved in protein prenylation, plays a critical role in postnatal heart growth by regulating cardiomyocyte size. However, the role of GGPPS in myocardial ischemia/reperfusion (MIR) injury is still not clear. The objective of this work was to investigate the effect of GGPPS on MIR injury in H9c2 cells subjected to hypoxia/reoxygenation (HR) to mimic MIR. Prior to HR, the cells were transfected with GGPPS, shGGPPS, or shGFP. The results showed that cell viability was reduced, and cell injury and cell apoptosis were increased as a result of overexpression of GGPPS. Knockdown of GGPPS improved cell viability, and decreased cell injury and cell apoptosis. Furthermore, overexpression of GGPPS increased Rac1 activity and ROS generation, while GGPPS silencing decreased Rac1 activity and ROS generation. Based on these findings, we propose that the alteration of GGPPS expression changed the Rac1 activity and ROS production, and finally led to the different severity of HR-induced injury in H9c2 cells. These findings indicate that GGPPS might be a potential target in preventing H9c2 cells from HR-induced injury.

Key words: NADPH oxidases, Reactive oxygen species, Rac1

Ischemic heart disease is the most important cause of cardiac mortality and morbidity. Timely recovery of coronary flow is necessary to resuscitate the ischemic or hypoxic myocardium. However, previous studies have suggested that upon reperfusion, exposure of ischemic tissues to molecular oxygen greatly augments organ damage, a phenomenon termed "ischemia/reperfusion (IR) injury." IR results in the generation of reactive oxygen species (ROS). ROS are produced in two stages, ischemia and reperfusion, at low and high levels, respectively. The production of ROS and tissue function are related to each other. Excess ROS production in the reperfusion phase is injurious for cardiac tissue, since ROS can attack cellular components and provoke apoptosis and necrosis. NADPH oxidases are known to be the only enzyme family producing ROS as their sole and primary function. Furthermore, activation of the small GTP-binding protein Rac1 leads to NADPH oxidase activation. Moreover, studies of nonphagocytic cells have demonstrated that the production of ROS appears to require participation of Rac 1. Geranylgeranyl pyrophosphate synthase (GGPPS) is a key enzyme in the synthesis of geranylgeranyl pyrophosphate (GGPP). It catalyzes the formation of GGPP from one molecule of farnesyl diphosphate (FPP) and one molecule of isopentenyl diphosphate. GGPP mainly participates in post-translational modification for various proteins, including the Rho/Rac, Rap, and Rab families. Studies have indicated that Rac1 plays an important role in IR-induced injury. In addition, recent studies have revealed that GGPPS participates in hepatocyte apoptosis, as well as cardiomyocyte hypertrophic growth. However, the effect of GGPPS on IR injury is still unknown.

In this study, heart-derived H9c2 cells were transfected with GGPPS or shGGPPS. We investigated the effect of GGPPS on HR-induced injury and the relevant mechanisms in heart-derived H9c2 cells.

Methods
Cell culture: H9c2 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO, MO, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, MO, USA) at 37°C and 5% CO2.

Plasmid transfection: pAV-rGGPPS (GGPPS plasmid), shGGPPS (shRNA plasmid) and shGFP (blank plasmid) were purchased from Obio Technology Co. (Shanghai, China), with primer sequences as follows: pAV-rGGPPS.
Western blot analysis: Western blot analysis was performed to detect GGPPS protein. Whole cell lysates were prepared in 1x RIPA lysis buffer (Millipore, CA, USA) containing a 1x protease and phosphatase inhibitor cocktail (Thermo Scientific, MA, USA). Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, IL, USA) according to the manufacturer’s protocol. Equal volumes of lysate (50 μg) were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After 1 hour of blocking with 5% BSA in TBST at room temperature, the PVDF membranes were incubated overnight with anti-GGPPS antibody (Abcam, London, UK) diluted in 1x TBST with 5% BSA at 4°C. Membranes were then washed 3 times with 1x TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (EarthOx, CA, USA) for 1 hour at room temperature. Hybridizing bands were visualized using an ECL-HRP chemiluminescence kit (Biologic Industries, BI, Israel). Tubulin was used as an endogenous control.

Cell viability assessment: A CCK assay was used to assess cell viability. Cells were seeded into 96-well plates at a concentration of 5 × 10^3 cells/mL. After transfection and HR, 10 μL of Cell Counting Kit (CCK) (7sea biotech, Shanghai, China) mixed with 100 μL of complete medium was added into each well followed by incubation for 2 hours. Results were quantified using a microplate reader (Molecular Devices) at an absorbance of 450 nm.

Measurement of LDH or SOD: Lactate dehydrogenase (LDH) or superoxide dismutase (SOD) was examined to evaluate cell injury. Cells were seeded into 6-well plates (1 × 10^5 cells/mL). Following transfection and HR, LDH release in the medium was measured using a LDH Assay Kit (Jiancheng Bio, Shanghai, China). SOD generation in cell lysates was measured using a SOD Assay Kit (Jiancheng Co) according to the manufacturer’s protocol. Results were quantified using a microplate reader (Molecular Devices) at an absorbance of 450 nm. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, IL, USA) according to the manufacturer’s protocol.

Cell apoptosis assessment via flow cytometry: Apoptotic cell death was measured using a PE AnnexinV Apoptosis Detection Kit (BD Biosciences, NJ, USA) according to the manufacturer’s protocol. Briefly, cells were seeded into 6-well plates. After transfection and HR, the cells were washed twice with cold PBS and then resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/mL. A 100 μL aliquot of the solution was transferred to a 5 mL culture tube. Cells in each tube were stained using 5 μL of PE Annexin V mixed with 5 μL 7-AAD for 15 minutes at room temperature in the dark. To each tube 400 μL of 1 × binding buffer was added and the cells were analyzed using flow cytometry.

Activation of RhoA, Rac1, Cdc42 and Ras: RhoA, Rac1, Cdc42 or Ras activity was determined by an absorbance-based G-LISA Activation Assay Biochemistry Kit (Cytoskeleton, Denver, CO, USA) according to manufacturer’s protocol. Results were quantified using a microplate reader (Molecular Devices) at an absorbance of 490 nm. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, IL, USA) according to the manufacturer’s protocol.

Measurement of ROS: A Cellular ROS Red Fluorescence Assay Kit (Genmed Scientific Inc., Alington, TX, USA) was used to detect ROS generation according to the manufacturer’s protocol. Briefly, cells were seeded into 6-well plates. After transfection and HR, the cells were collected into a 15 mL centrifuge tube and counted using a cell counter. Cells (1 × 10^6) were transferred into a new centrifuge tube and incubated with the Cellular ROS Red Fluorescence Assay Kit working reagent for 20 minutes at 37°C for 15 minutes at room temperature. To each tube 20 μL of working reagent was added followed by incubation for 15 minutes at room temperature in the dark. To each tube 400 μL of 1 × binding buffer was added and the cells were analyzed using flow cytometry.
Figure 1. Effect of pAV-rGGPPS or shGGPPS transfection on H9c2 cells. A: Transfection efficiency of plasmid in H9c2 cells. Transfection efficiency is represented as GFP-positive cells using fluorescence microscopy. B: Transfection efficiency was measured using flow cytometry. C: Expression of GGPPS after 48 hours of transfection measured by Western blotting. Tubulin was the loading control. D: Relative expression levels of GGPPS normalized to tubulin expressed as the mean ± SME (n = 3). **P < 0.01 versus shGFP. E: RT-PCR analyses of GGPPS mRNA levels in H9c2 cells. GAPDH was the loading control. ***P < 0.001 versus shGFP. F: RT-PCR analyses of GGPPS mRNA levels in H9c2 cells. GAPDH was the loading control. ***P < 0.001 versus shGFP. CON indicates control; and HR, hypoxia/reoxygenation.

Figure 2. Effect of GGPPS on cell proliferation. Cell viability after transfection and HR. Data are presented as the mean ± SEM (n = 3). ###P < 0.001 versus CON. *P < 0.05 versus shGFP + HR. **P < 0.01 versus shGFP + HR. CON indicates control; and HR, hypoxia/reoxygenation.

IL, USA) according to the manufacturer’s protocol. NADPH oxidase activity was evaluated spectrophotometrically using an NADPH Oxidase Activity Assay kit (GMS 50095.2 v.A; Genmed Scientific Inc.,) according to the manufacturer’s protocol.

Statistical analyses: Significant differences were determined using SPSS16.0 software. All data are represented as the mean ± SEM. For direct comparison of differences between two groups, Student’s t-test was used. Analysis of variance (ANOVA) in the case of serial measurements, followed by the multiple-comparison Bonferroni t-test, was used to assess differences among groups. A P < 0.05 was considered significant. All experiments were repeated 3 times.

Results

Pav-rGGPPS and shGGPPS altered GGPPS expression of H9c2 cells: Approximately 50–55% of cultured H9c2 cells were transfected by pAV-rGGPPS, shGGPPS, or shGFP (Figure 1A and 1B). Compared with the shGFP group, GGPPS protein was highly expressed in the pAV-rGGPPS group and was down-regulated in the shGGPPS group (Figure 1C and 1D). RT-PCR results showed that GGPPS mRNA was highly increased in the pAV-rGGPPS group and markedly reduced in the shRNA group (Figure 1E).

Overexpression of GGPPS attenuated cell viability and GGPPS silencing improved cell viability: To investigate the effect of GGPPS on cell viability after HR in H9c2 cells, pAV-rGGPPS, shGGPPS, or shGFP was used to transfect H9c2 cells. Cell viability was quantified using
Figure 3. Effect of GGPPS on cell injury after HR in H9c2 cells. A: Level of LDH release in medium after 48 hours of transfection, following 8 hours of hypoxia and 4 hours of reoxygenation. Data are expressed as the mean ± SEM (n = 3). ***P < 0.001 versus CON. **P < 0.01 versus shGFPP + HR. ***P < 0.001 versus shGFPP + HR. B: Level of SOD in cell lysates after 48 hours of transfection, following 8 hours of hypoxia and 4 hours of reoxygenation. Data are expressed as the mean ± SEM (n = 3). ***P < 0.001 versus CON. ***P < 0.001 versus CON. *P < 0.05 versus shGFPP + HR. CON indicates control; and HR, hypoxia/reoxygenation.

Figure 4. Effect of GGPPS on H9c2 cell apoptosis after HR. A: Apoptotic cell death in total number of cells after 48 hours of transfection, following 8 hours of hypoxia and 4 hours of reoxygenation. B: Apoptotic cell death in total number of cells expressed as the mean ± SEM (n = 4). ***P < 0.001 versus CON. *P < 0.05 versus shGFPP + HR. **P < 0.01 versus shGFPP. CON indicates control; and HR, hypoxia/reoxygenation.
CCK assay after HR. We found that overexpression of GGPPS reduced the viability and GGPPS silencing increased the viability in H9c2 cells after HR (Figure 2).

**Overexpression of GGPPS increased cell injury and GGPPS silencing decreased cell injury:** LDH and SOD levels were measured to assess cell injury after HR. The results indicated that LDH release in the medium significantly increased in the pAV-rGGPPS group and decreased in the shGGPPS group (Figure 3A). SOD generation was significantly reduced in the pAV-rGGPPS group and elevated in the shGGPPS group (Figure 3B).

**Overexpression of GGPPS increased HR-induced apoptosis and GGPPS silencing reduced HR-induced apoptosis in H9c2 cells:** As shown in Figure 4, after HR, the level of apoptosis (both early and late-stage) of H9c2 cells in the shGFP group was 10.26 ± 0.66%. The transfection of GGPPS elevated the apoptotic cell ratio to 13.75 ± 0.64%. In the shGGPPS group, the apoptotic cell ratio was decreased to 7.8 ± 0.47%.

**GGPPS altered the Rac1 activation:** Protein geranylgeranylation is necessary for the activation of small GTPases. In this experiment, it was shown that Rac1 activation was significantly increased in the pAV-rGGPPS group and decreased in the shGGPPS group (Figure 5A) compared to the shGFP group. However, our findings did not show significant changes in Ras activity (Figure 5B). Also, the RhoA and Cdc42 activity were not significantly changed (Figure 5C and 5D). Thus, the results suggested a direct relationship between the GGPPS gene and Rac1. This implied that GGPPS was involved in HR-induced injury through Rac1.

**Overexpression of GGPPS increased ROS generation and GGPPS silencing decreased ROS generation:** The production of ROS and tissue function are related to each other. Studies of nonphagocytic cells have demonstrated that the production of ROS appears to require the participation of the small GTP-binding protein Rac1. Therefore, the effects of GGPPS on ROS generation in HR were studied. Figure 6 shows the measurement of intracellular ROS induced HR treatment in cultured H9c2 cells. As assessed by dihydroethidium bromide (DHE) fluorescence, the ROS positive cells as well as the fluorescence levels were increased as a result of overexpression of GGPPS, and decreased in the GGPPS silencing group.

**Overexpression of GGPPS increased NADPH oxidase activity and GGPPS silencing decreased NADPH oxidase activity:** NADPH oxidase activity was measured to evaluate the generation of ROS. NADPH oxidase activity was significantly increased in the pAV-rGGPPS group and decreased in the shGGPPS group (Figure 7) compared to the shGFP group.
Discussion

Recovery of oxygen supply to hypoxic cardiomyocytes can cause necrotic and apoptotic cell death. IR results in the generation of reactive oxygen species (ROS), and oxidative stress arising from excessive production of reactive oxygen species has long been associated with IR injury, and this pathological process can be mimicked when cultured cells are subjected to HR challenge. A recent study indicated that the IL-1 signaling pathway participates in the process of ROS production through the recruitment and activation of neutrophils. The MAPK signaling pathway plays an important role in the pathogenesis of IR injury. Other studies have demonstrated that inhibition of ROS production or scavenging of ROS offers protection against tissue injury incurred during ischemia/reperfusion. Moreover, other studies have aimed at inhibiting the source(s) responsible for the production of ROS. Studies have demonstrated that inhibition of Rac1 protects IR-induced injury by decreasing the production of ROS. GGPP is necessary for the activation of small GTPases including Rac1. We designed this study to investigate the effect of GGPPS on HR-induced injury in vitro, and to explore its possible mechanism.

GGPPS is a key enzyme in the mevalonate pathway. GGPPS participates in the synthesis of GGPP which is necessary for protein geranylgeranylation and activation of small GTPases such as RhoA and Rac1. Recent studies have revealed that GGPPS participates in hepatocyte apoptosis, as well as cardiomyocyte hypertrophic growth. We speculate that GGPPS has an influence on MIR-induced injury in cardiomyocytes. In order to verify this hypothesis, we used heart-derived H9c2 cells which...
were subjected to 8 hours of hypoxia following 4 hours of reoxygenation to mimic MIR. PAV-rGGPPS or shGGPPS was transfected into H9c2 cells to achieve the objective of overexpression of GGPPS or GGPPS silencing. Cell viability, cell injury, and cell apoptosis were detected. Both cell viability and cell apoptosis can reflect the effect of the intervention on cells directly. Our results showed that GGPPS silencing improved cell viability and decreased cell injury and apoptotic cell death, while overexpression of GGPPS had an opposite effect. According to the results, we can conclude that knockdown of GGPPS has a protective effect on cell viability and increases cell apoptosis.

The Rho family of small GTP-binding proteins, including the Rho, Rac, and Cdc42 subfamilies, regulates cytoskeletal function in many aspects. Studies have indicated that Rac1 plays an important role in IR-induced injury, and conditional cardiac-specific overexpression of RhoA in mice protected the heart against IR-induced injury. Another study has suggested that inhibition of Rhokinase protected the mouse heart against IR-induced injury. To further characterize the effect of GGPPS on HR injury, small GTP-binding proteins related to HR injury such as Ras, RhoA, Rac1, and Cdc42 were examined. The results showed that Rac1 activity was significantly improved in the pAV-rGGPPS group and reduced in the shGGPPS group. There was no significant difference in basal Rac1 activity between the groups. The reason for this phenomenon is that HR is an inducing factor which stimulates the joining of the 20-carbon geranylgeranyl group (GGPP) to protein-cysteines (Rac1) at or near their carboxy-termini that is catalyzed by protein geranylgeranyltransferase-I (GGTase-I). There was no significant difference compared with the shGFP group for other small GTP-binding proteins. This may be explained in terms of the alteration of GGPPS expression changed the Rac1 activation.

ROS produced during reperfusion can initiate a series of cellular events that eventually lead to inflammation, necrosis, and/or apoptosis. Activation of the small GTP-binding protein rac1 leads to the activation of NADPH oxidases, which is known to be the only enzyme family producing reactive oxygen species (ROS) as their sole and primary function. Also, it has been reported that the production of ROS appears to require the participation of the small GTP-binding protein rac1. Thus, we detected ROS generation to explore whether it participates in HR-induced injury in H9c2 cells. Our study showed that the generation of ROS was significantly increased in the pAV-rGGPPS group during HR, and was significantly reduced in the shGGPPS group. Thus, we speculated that the effect of GGPPS on HR injury was associated with ROS generation which was influenced by Rac1 (Figure 8).

In conclusion, we provide here important experimental evidence that overexpression of GGPPS aggravates HR-induced injury in heart-derived H9c2 cells, and GGPPS silencing protects heart-derived H9c2 cells against HR-induced injury. These are accompanied by the alteration of Rac1 activity and ROS generation. These findings indicate that GGPPS might be a potential target in preventing H9c2 cells from HR-induced injury. Further studies are required to investigate the effect of GGPPS in primary cardiomyocytes and in vivo.

Disclosures

Conflicts of interest: Authors have no conflict of interest to declare.

References

1. Granger DN, Korthuis RJ. Physiologic mechanisms of post-ischemic tissue injury. Annu Rev Physiol 1995; 57: 311-32.
2. Verma S, Fedak PW, Weisel RD, et al. Fundamentals of reperfusion injury for the clinical cardiologist. Circulation 2002; 105: 2332-6.
3. Downey JM. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. Annu Rev Physiol 1990; 52: 487-504.
4. McCord JM, Roy RS, Schaffer SW. Free radicals and myocardial ischemia. The role of xanthine oxidase. Adv Myocardiol 1985; 5: 183-9.
5. Jaeschke H, Smith CV, Mitchell JR. Hypoxic damage generates reactive oxygen species in isolated perfused rat liver. Biochem Biophys Res Commun 1988; 150: 568-74.
6. Chan PH. Role of oxidants in ischemic brain damage. Stroke 1996; 27: 1124-9.
7. Kevin LG, Novalija E, Stowe DF. Reactive oxygen species as mediators of cardiac injury and protection: the relevance to anesthesia practice. Anesth Analg 2005; 101: 1275-87.
8. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 2008; 88: 581-609.
9. Landry WD, Cotter TG. ROS signalling, NADPH oxidases and cancer. Biochem Soc Trans 2014; 42: 934-8.
10. Garrido-Urbani S, Jaquet V, Inhof BA. ROS and NADPH oxidase: key regulators of tumor vascularisation. Med Sci (Paris) 2014; 30: 415-21.
11. Pick E. Role of the Rho GTPase Rac in the activation of the phagocyte NADPH oxidase: outsourcing to a key task. Small GTPases 2014; 5: e27952.
12. Sulciner DJ, Irani K, Yu ZX, Ferrans VJ, Goldschmidt-Clermont P, Finkel T. rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. Mol Cell Biol 1996; 16: 7115-21.
13. Sundaesran M, Yu ZX, Ferrans VJ, et al. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. Biochem J 1996; 318: 379-82.
14. Kim KS, Takeda K, Sethi R, et al. Protection from reoxygenation injury by inhibition of rac1. J Clin Invest 1998; 101: 1821-6.
15. Ozaki M, Deshpande SS, Angkekow P, et al. Inhibition of the Rac1 GTPase protects against nonlethal ischemia/reperfusion-induced necrosis and apoptosis in vivo. FASEB J 2000; 14: 418-29.
16. Kuzuguchi T, Morita Y, Sagami I, Sagami H, Ogura K. Human geranylgeranyl diphasphate synthase. cDNA cloning and expression. J Biol Chem 1999; 274: 5888-94.
17. Park HJ, Kong D, Iruela-Arispe L, Begley U, Tang D, Galper JB. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. Circ Res 2002; 91: 143-50.
18. Connor AM, Berger S, Narendran A, Keystone EC. Inhibition of protein geranylgeranylation induces apoptosis in synovial fibroblasts. Arthritis Res Ther 2006; 8: R94.
19. Gesi M, Pellegrini A, Soldani P, et al. Ultrastructural and biochemical evidence of apoptosis induced by a novel inhibitor of protein geranylgeranylation in human MIA PaCa-2 pancreatic cancer cells. Ultrastruct Pathol 1998; 22: 253-61.
20. Chen WB, Lai SS, Yu DC, et al. GGPPS deficiency aggravates CC14-induced liver injury by inducing hepatocyte apoptosis. FEBS Lett 2015; 589: 1119-26.
21. Xu N, Guan S, Chen Z, et al. The alteration of protein prenylation induces cardiomyocyte hypertrophy through Rheb-mTORC1 signalling and leads to chronic heart failure. J Pathol 2015; 235: 672-85.
22. Li X, Heinzle FR, Boengler K, Schulz R, Heusch G. Role of connexin 43 in ischemic preconditioning does not involve intercellular communication through gap junctions. J Mol Cell Cardiol 2004; 36: 161-3.
23. Zhao M, Sun L, Yu XJ, et al. Acetylcholine mediates AMPK-dependent autophagic cytoprotection in H9c2 cells during hypoxia/reoxygenation injury. Cell Physiol Biochem 2013; 32: 601-13.
24. Robin E, Guzy RD, Loor G, et al. Oxidant stress during simulated ischemia primes cardiomyocytes for cell death during reperfusion. J Biol Chem 2007; 282: 19133-43.
25. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? J Clin Invest 1985; 76: 1713-9.
26. Ambrosio G, Zweier JL, Dulio C, et al. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J Biol Chem 1993; 268: 18532-41.
27. Kim JS, Ohshima S, Pediaditakis P, Lemasters JJ. Nitric oxide protects rat hepatocytes against reperfusion injury mediated by the mitochondrial permeability transition. Hepatology 2004; 39: 1533-43.
28. Huang J, Li Y, Zhang J, Liu Y, Lu Q. The growth hormone secretagogue hexarelin protects rat cardiomyocytes from in vivo ischemia/reperfusion injury through interleukin-1 signaling pathway. Int Heart J 2017; 58: 257-63.
29. Wang G, Cui J, Guo Y, Wang Y, Kang L, Liu L. Cyclosporin A protects H9c2 cells against chemical hypoxia-induced injury via inhibition of MAPK signaling pathway. Int Heart J 2016; 57: 483-9.
30. Hearse DJ, Manning AS, Downey JM, Yellon DM. Xanthine oxidase: a critical mediator of myocardial injury during ischemia and reperfusion? Acta Physiol Scand Suppl 1986; 548: 65-78.
31. Muller MJ, Vollmar B, Friedl HP, Menger MD. Xanthine oxidase and superoxide radicals in portal triad crossclamping-induced microvascular reperfusion injury of the liver. Free Radic Biol Med 1996; 21: 189-97.
32. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 1994; 10: 31-54.
33. Xiang SY, Vanhoutte D, Del RD, et al. RhoA protects the mouse heart against ischemia/reperfusion injury. J Clin Invest 2011; 121: 3269-76.
34. Bao W, Hu E, Tao L, et al. Inhibition of Rho-kinase protects the heart against ischemia/reperfusion injury. Cardiovasc Res 2004; 61: 548-58.
35. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem 1996; 65: 241-69.
36. Cursio R, Gugenheim J, Ricci JE, et al. A caspase inhibitor fully protects rats against lethal normothermic liver ischemia by inhibition of liver apoptosis. FASEB J 1999; 13: 253-61.
37. Atalla SL, Toledo-Pereyra LH, MacKenzie GH, Cederna JP. Influence of oxygen-derived free radical scavengers on ischemic livers. Transplantation 1985; 40: 584-90.
38. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994; 94: 1621-8.