REVIEW

This paper is dedicated to the 70th anniversary of the founding of Physiologia Bohemoslovaca (currently Physiological Research)

Molecular Basis of the Effect of Atorvastatin Pre-treatment on Stem Cell Therapy in Chronic Ischemic Diseases – Critical Limb Ischemia

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Summary

Autologous stem cell therapy is the most promising alternative treatment in patients with chronic ischemic diseases, including ischemic heart disease and critical limb ischemia, which are characterized by poor prognosis related to serious impair of quality of life, high risk of cardiovascular events and mortality rates. However, one of the most serious shortcomings of stem cell transplantation are low survival after transplantation to the site of injury, as large number of stem cells are lost within 24 hours after delivery. Multiple studies suggest that combination of lipid-lowering drugs, statins, and stem cell transplantation might improve therapeutic efficacy in regenerative medicine. Statins are inhibitors of HMG-CoA reductase and belong to recommended therapy in all patients suffering from critical limb ischemia. Statins possess non-lipid effects which involve improvement of endothelial function, decrease of vascular inflammation and oxidative stress, anti-cancer and stem cell modulation capacities. These non-lipid effects are explained by inhibition of mevalonate synthesis *via* blocking isoprenoid intermediates synthesis, such as farnesylpyrophospate and geranylgeranylpyrophospate and result in modulation of the PI3K/Akt pathway. Moreover, statin-mediated microRNA regulation may contribute to the pleiotropic functions. MicroRNA interplay in gene regulatory network of IGF/Akt pathway may be of special significance for the treatment of critical limb ischemia. We assume further studies are needed for detailed analysis of statin interactions with microRNA at the molecular level and their link to PI3K/Akt and IGF/Akt pathway in stem cells, which are currently the most promising treatment strategy used in chronic ischemic diseases.

Key words

Atorvastatin • Chronic ischemic diseases • Mesenchymal stem cells • microRNA

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Stem cells and chronic ischemic diseases

Ischemia or ischaemia is defined as an inadequate blood supply to a local area following blockage of the blood vessels, resulting in oxygen shortage. Chronic ischemic diseases include ischemic heart disease caused by coronary artery narrowing and critical limb ischemia (CLI). CLI is the most advanced stage of peripheral artery disease (PAD), defined by chronic ischemic rest pain, ischemic ulcerations, or

ischemic gangrene as a result of compromised blood flow to the affected extremity. Approximately 40 % of patients with CLI are ineligible for surgical or endovascular revascularization (no-option CLI patients) with 1-year mortality rate from 10 % to 40 % and 25 % 1-year amputation rate (Becker *et al.* 2011, Hassanshahi *et al.* 2019). Chronic ischemic diseases are characterized by macrovascular alterations accompanied by microvascular abnormalities, involving endothelial dysfunction mediated by inflammation, oxidative stress and ischemia (Vemulapalli *et al.* 2015). Despite the modern development of therapeutic techniques and methods, the treatment of no-option CLI patients is still very limited, among which stem cell transplantation appears to be one of the most promising alternative option. Stem cells are a class of undifferentiated cells with unique self-renewal capacity and the ability to differentiate into various cell types (Hassanshahi *et al.* 2019).

Several studies showed stem cell therapies in the treatment of no-option CLI patients reduce the rate of major amputations and pain and improve walk distance, perfusion and transcutaneous oxygen pressure (Compagna *et al.* 2015). A major shortcoming of the treatment is poor survival of transplanted bone marrow derived mesenchymal stem cells. Most mesenchymal stem cells (MSCs) are lost within 24 hours after cell-based treatment and only 15 % of them are able to survive for 3 months. Therefore, protection of the implanted cells from acute death in ischemic injury is of major importance for clinical applications. Multiple studies reported that combination of lipid-lowering drugs, statins, recommended in prevention of coronary heart disease, with stem cell transplantation might improve therapeutic efficacy in regenerative medicine (Gorabi *et al.* 2020).

Actions of statin in treatment

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins are principal drugs used in primary and secondary prevention of coronary heart disease. They inhibit the HMG-CoA reductase activity, the limiting enzyme in cholesterol synthesis in liver. Moreover, clinical trials have shown that statins have additional cholesterol-independent effects which involve improvement of endothelial function, decrease of vascular inflammation and oxidative stress, anti-cancer and stem cell modulation capacities (Xu *et al.* 2013). These non-lipid effects could be mediated by inhibition of mevalonate synthesis *via* blocking isoprenoid intermediates synthesis, such as farnesylpyrophospate and geranylgeranylpyrophospate. Isoprenoid intermediates serve as important posttranslational lipid attachments for intracellular signalling molecules such as the Rho GTPases (Rikitake and Liao 2005).

For clinical application were tested nine statins, which differ in their tissue permeability and metabolism. Lipophilic statins, like atorvastatin are widely distributed by passive diffusion to different tissues and cell types, including MSCs. In vivo, atorvastatin is metabolized by cytochrome P450 3A4 to ortho and para hydroxyl derivatives, with approximately 70 % of the circulating inhibitory activity attributed to the biologically active metabolites (Lennernas 2003). Cytochrome P450 expression occurs mainly in hepatocyte, and in addition its presence was also show in MSC isolated from bone marrow cells (Chiang *et al.* 2014). Therefore, we suggest that the effect of atorvastatin on the outcome of stem cell therapy is mediated by the molecular effect of atorvastatin on the population of MSCs present in mononuclear bone marrow derived cells.

According to European Society of Cardiology guidelines on the diagnosis and treatment of PAD statins belong to the recommended therapy for all patients with PAD. It was shown that statins cause reductions in all-cause mortality and cardiovascular events (Aboyans *et al.* 2017). The resulting improvement in therapeutic outcomes of regenerative medicine suggests benefit from combination of statin therapy and stem cell transplantation. The use of statin in combination with MSCs in the context of cellular therapy appears promising in also pre-clinical studies. Some pre-clinical studies combined statin therapy with cell transplantation using MSC or endothelial progenitor cells (EPCs). The study of Park *et al.* focused on the effect of statin therapy and MSC infusion in hindlimb ischemia in mice model. It reported improved outcomes, involving greater incorporation of transplanted cells into the size of injury, improved perfusion in affected limb and decreased apoptosis in ischemic muscle (Park *et al.* 2016). Additionally, different analysis evaluated results from pre-clinical studies involving animals with acute myocardial infarction treated with MSCs or atorvastatin and MSCs together. They found atorvastatin exerts protective effects on the myocardium undergoing infarction and reperfusion injury in connection with MSCs transplantation (Dai *et al.* 2015). Clinical studies also analyzed statin therapy impact on CAD, using atorvastatin or rosuvastatin. They reported an increase in circulating EPCs levels after 5 days of statin therapy and greater

numbers of colony-forming units after 6 months of treatment. They confirmed a reduction in wound infections, reduction in incidence of atrial fibrillation or other complications after following cardiac surgery (Park *et al.* 2016).

Molecular basis of statins effects

According to current knowledge, statins could affect MSC fate by several signalling pathways. They could influence MSCs differentiation, proliferation, angiogenic potential, survival, migration and homing capacities (Zhang *et al.* 2018). The main effect of statins is mediated by reduction in an early step of cholesterol synthesis, thus also inhibit the synthesis of isoprenoids such as farnesylpyrophoshate and geranylgeranyl-pyrophosphate. They are essential for posttranslational lipid attachments of intracellular signalling molecules like Rho GTPases family. GTPases function is regulated by their membrane localization, prenylation and post-translational modification, which is impaired by statins. It affects Rho GTPases stability and activity, and is responsible for regulation cytoskeleton organization, cell adhesion, migration, and others. HMG-CoA inhibitors prevent endothelial dysfunction by RhoA inhibition, which is crucial for stabilization of mRNA endothelial NO synthase, and results in endothelial-dependent relaxation. Furthermore, RhoA and Rho-kinases inhibition increases mRNA endothelial NO synthase expression and activation and causes endothelial-dependent relaxation. Moreover, statins maintain NO availability by reducing its degradation by free radical molecules. Rho-kinases also inhibit serine/threonine kinase Akt phosphorylation and its activity, which is reversed by statins. Thereafter Akt can phosphorylate endothelial NO synthase on serine 1179 and enhances its ability to generate NO (Gelosa *et al.* 2007). Through these statin effects on isoprenoid intermediates, we suggest that atorvastatin affects MSCs mainly through the phosphatidyl inositol 3-kinase (PI3K)/Akt signalling pathway (Fig. 1).

Well described statin mediated action is the increase of vascular endothelial growth factor (VEGF) expression, crucial for angiogenesis process. VEGF binds to tyrosine kinase receptor located on cell membrane and activate PI3K/Akt pathway. As a result, Akt phosphorylation modulates diverse cell processes including MSC differentiation into EPCs and activates signalling pathways involved in vasculogenesis. Multiple studies reported that besides VEGF statins (simvastatin, atorvastatin, rosuvastatin) also enhance the expression of several other growth factors, including basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1) and hepatocyte growth factor (HGF) (Gorabi *et al.* 2020).

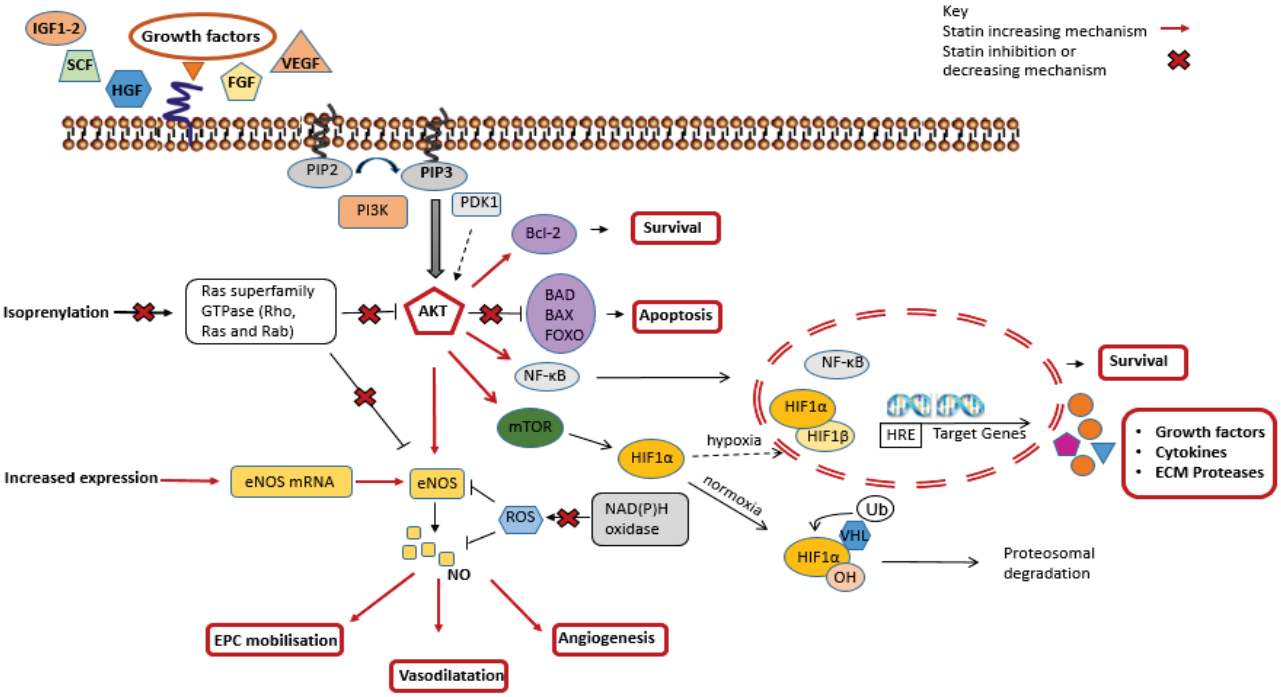

Fig. 1. Schematic representation of possible atorvastatin effects in PI3K/Akt pathway (modified Samakova et al. 2019). FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HRE, hormone response element; IGF, insulin-like growth factor; PDK1, phospoinositide-depedent protein kinase-1; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-bisphosphate; SCF, stem cell factor; VEGF, vascular endothelial growth factor.

On the other hand, it has been reported that the concentrations of VEGF and hypoxia inducible factor (HIF) may be related to process like myocardial remodelling and angiogenesis. HIF is a transcription factor responding to the reduction or hypoxia in the cell environment. Activation of HIF-1 signalling cascade mediates the effects of hypoxia on the cells and allows the cells to differentiate continuously and promote the formation of blood vessels. VEGF is the main downstream target gene of HIF-1α, able to promote neovascularization and adaptation of cells to hypoxic environment. Study performed by Dulak and Józkowicz (2005) adds more to the understanding of the diverse effect of statins in ischemia processes. They studied the effect of atorvastatin combined with conventional treatment drugs for acute myocardial infarction on rats. The results showed that the concentration of HIF-1 and VEGF in the serum of rats with acute myocardial infarction were significantly higher than that of normal rats, suggesting their involvement in the occurrence of myocardial infarctions. By comparison of HIF-1 and VEGF levels in the routine therapy group and study group with atorvastatin showed higher concentration of HIF-1 and VEGF levels in the study group, suggesting that the combination of atorvastatin and routine therapy increased the concentrations of HIF and VEGF.

Interestingly, an important role in the physiology of MSCs is played by the IGF1 / IGF2 system, the association of which with statins as well as microRNAs is not fully elucidated. IGF-1 levels in adults are regulated by the bond of growth hormone to its hepatic receptor and stimulation of expression and release of IGF-1 peptide into circulation. At the molecular level, insulin-like growth factors (IGFs; IGF-1 and IGF-2) are two small polypeptides that regulate stem cell survival, self-renewal and differentiation (Youssef *et al.* 2017). IGF-1 shares more than 60 % sequence homology with IGF-2. They both act *via* the IGF-1 receptor (IGF-1R) which has 10-fold higher affinity to IGF-1, compared to IGF-2. The IGF-1R is a tetramer consisting of extracellular 2 α-chains and 2 transmembrane β-chains, including an intracellular tyrosine kinase, which mediates its biologic effects (Higashi *et al.* 2015). Randomized controlled trial by Bergen *et al.* evaluated the effect of atorvastatin treatment on IGF-1 and insulin-like growth factor binding protein levels (Bergen *et al.* 2016). Twenty patients with type 1 diabetes received placebo or 80 mg atorvastatin for two months. They found atorvastatin treatment was associated with overall reduction in IGF-1 levels, which may indicate negative link to vascular health.

A few studies researched the effects of IGF-1 on MSCs differentiation potential, migratory capacity, and growth (Feng *et al.* 2014, Xinaris *et al.* 2013; Zhou *et al.* 2016). MSCs isolated from bone marrow express and secrete both IGF-1 and IGF-2 in vitro. Ectopic IGF-1 expression in MSCs was linked with proliferation and decrease of apoptosis (Hu *et al.* 2008). In contrast, IGF-2 appeared to be involved in promoting MSC pluripotency and self-renewal capacities. IGF-2 secreted by differentiated autologous fibroblast-like cells in response to basic FGF, was required for the maintenance of human embryonic stem cell pluripotency and self-renewal through IGF-1R signalling. Study with MSC isolated from human dental pulp confirmed that IGF-1R was required for MSC multipotency (Lee *et al.* 2016). The effect of statin, specifically simvastatin was investigated in relation to cell viability and IGF-1 signalling in differentiating C2C12 mouse myoblast cells. Authors concluded that simvastatin decreases IGF-1 signalling *via* regulation of post-translational modification of IGF-1R beta chains (Ogura *et al.* 2007).

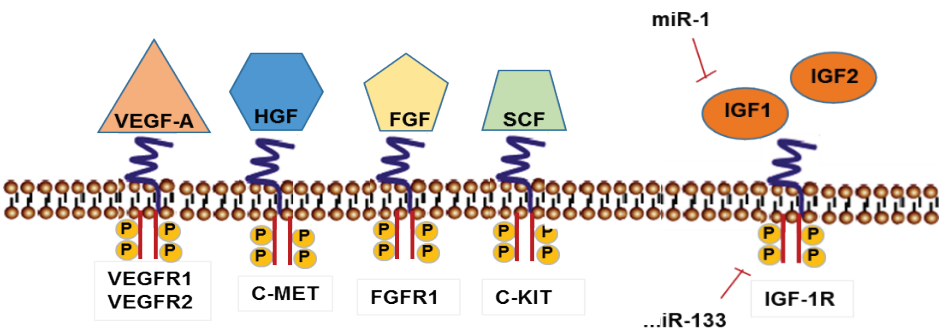

Fig. 2. Ligands and receptors involved in the activation of PI3K/Akt pathway. C-KIT, stem cell growth factor receptor; C-MET, hepatocyte growth factor receptor; FGF, fibroblast growth factor; FGFR1, fibroblast growth factor receptor 1; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor 1 receptor; miR, microRNA; SCF, stem cell factor; VEGF-A, vascular endothelial growth factor A; VEGFR1/2, vascular endothelial growth factor receptor ½.

Potential microRNA involvement in statins various effects

Moreover IGF1 and IGF2 network is modulated by microRNA, sequence specific regulators of post-transcriptional gene expression (Fig. 2). Evidence available so far demonstrates various mechanism by which microRNAs down-regulate their target mRNA and revealed their essential roles. MicroRNAs profoundly impact gene regulatory networks, specifically IGF-1/IGF-2 signalling pathway in several ways (Hu *et al.* 2013). One of the best studied processes involving microRNA effects on IGF1/Akt pathway is myogenesis. The expression levels of miR-1 and miR-133 increase during myogenesis and decrease in skeletal muscle hypertrophy. MiR-1 and miR-133 negatively regulate the IGF-1/Akt signalling pathway by targeting its positive regulators including IGF-1 and IGF-1R (Hitachi and Tsuchida 2014). It has been suggested that the beneficial effect of statins can be potentially explained by direct effect on selected mRNA levels, however another possible mechanism, microRNA post-transcriptional control of gene expression levels, needs to be elucidated.

Conclusion

Novel promising therapeutic strategies for the treatment of chronic ischemic diseases are constantly trying to increase the process of neoangiogenesis and neovascularization in the affected ischemic area. However, little is known about the benefit of long-term pharmacological pre-treatment of these patients and its effect on cell-based therapies. According to European Society of Cardiology guidelines on the diagnosis and treatment of PAD, statin belongs to recommended therapy in all patients (Aboyans *et al.* 2017). Inhibitors of HMC-CoA reductase have been widely used as hypolipidemic drugs for prevention of cardiovascular disease and have maintained a favorable safety profile for many years. It has been previously shown that statins can act through the PI3K/Akt pathway with protective effects for MSCs that could increase therapeutic outcome of stem cell transplantation in no-option CLI patients. In this review, we strongly suspect that statins affect PI3K/Akt pathway in stem cells directly through isoprenoid intermediates and indirectly through interactions with microRNA. However, further studies are needed for detailed analysis of statin interplay with microRNA at the molecular level and their link to PI3K/Akt and IGF/Akt pathway in stem cells, which are currently the most promising treatment strategy used in chronic ischemic diseases.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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