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Disruption of mitochondrial quality control in peripheral artery disease: new therapeutic opportunities

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Abstract

Peripheral artery disease (PAD) is a multifactorial disease initially triggered by reduced blood supply to the lower extremities due to atherosclerotic obstructions. It is considered a major public health problem worldwide, affecting over 200 million people. Management of PAD includes smoking cessation, exercise, statin therapy, antiplatelet therapy, antihypertensive therapy and surgical intervention. Although these pharmacological and non-pharmacological interventions usually increases blood flow to the ischemic limb, morbidity and mortality associated with PAD continue to increase. This scenario raises new fundamental questions regarding the contribution of intrinsic metabolic changes in the distal affected skeletal muscle to the progression of PAD. Recent evidence suggests that disruption of skeletal muscle mitochondrial quality control triggered by intermittent ischemia-reperfusion injury is associated with increased morbidity in individuals with PAD. The mitochondrial quality control machinery relies on surveillance systems that help maintaining mitochondrial homeostasis upon stress. In this review, we describe some of the most critical mechanisms responsible for the impaired skeletal muscle mitochondrial quality control in PAD. We also discuss recent findings on the central role of mitochondrial bioenergetics and quality control mechanisms including mitochondrial fusion-fission balance, turnover, oxidative stress and aldehyde metabolism in the pathophysiology of PAD, and highlight their potential as therapeutic targets.

Graphical abstract

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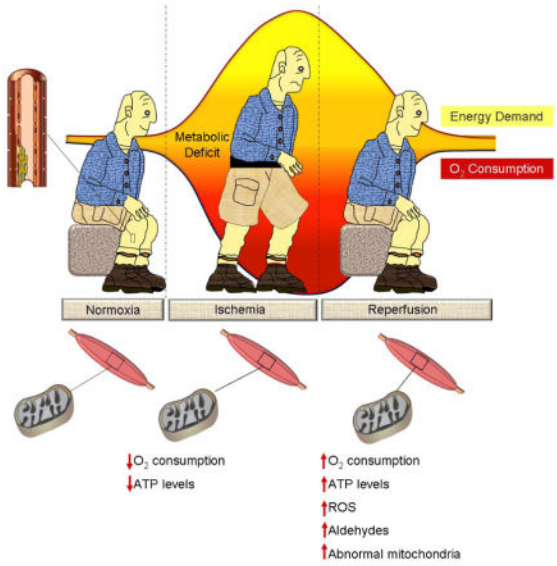

Keywords

Myocyte; mitochondrial metabolism; redox balance; 4-Hydroxynonenal; mitochondrial dynamics; mitophagy

Introduction

Peripheral artery disease (PAD) is a manifestation of atherosclerosis that results in either partial or complete obstruction of the peripheral arteries with consequent reduction of blood flow to the affected limb. PAD affects approximately 10% of individuals over the age of 65 years, and about 20% of individuals over the age of 80 years (1).

PAD has become a global burden in the 21st century. The number of individuals with PAD increased about 21% during the last decade, affecting over 200 million people in both high-income countries and low-to-middle-income countries (2). Therefore, PAD is considered one of the most harmful and morbid non-comunicable diseases worldwide. Its progression, initially triggered by reduced blood supply to lower extremities, is associated with myopathy, exercise intolerance and to limb amputation in most the extreme cases. PAD causes a pronounced reduction in both exercise performance and routinely ambulatory activities, thus causing a major decline in quality of life (3, 4). In addition to its direct effect on the ischemic limbs, PAD has a detrimental impact on the cardiovascular system; individuals with PAD have increased risk of cardiovascular events, cardiovascular disease mortality and all-cause mortality compared to the general population (5). Indeed, it is expected that about 22% of PAD patients will die from coronary or cerebrovascular disease during a 10 year period (6).

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Diagnosis and classification

Despite of its epidemological relevance as well as its negative impact in quality of life due to elevated rates of limb-related morbidity and cardiovascular events, PAD is considered an unmet clinical need. Unfortunately, most of individuals with PAD are not diagnosed accordingly in primary care practice (7, 8).

PAD can be classified into 4 categories according to symptoms: asymptomatic, atypical leg symptoms associated with exercise intolerance, intermittent claudication and critical limb-ischemia. Diagnosis of PAD is focused primarily on classical intermittent claudication symptoms, characterized by exercise-induced leg pain that is relieved by rest. Intermittent claudicaton can be detected by performing a 6-minute walk test (9) or applying different surveys such as the self-reported Walking Impairment Questionnaire or the Rose Claudication Questionnaire (10). These methods are highly sensitive in detecting intermittent claudication, the most typical symptom of PAD. However, only about 20% of individuals with PAD have signs of intermittent claudication (8). Aproximately 50% of people with PAD are asymptomatic and about 30% have exertional leg symptoms other than intermitent claudication (8, 11, 12). Therefore, other methods to detect PAD are required to overcome the frequent underdiagnosis of PAD.

PAD can be noninvasively identified in primary care practice using the ankle-brachial index (ABI), which is the ratio of Doppler recorded systolic blood pressure at the ankle to that in the arm (13, 14). It is considered a simple, accurate and reproducible test, presenting high sensitivity and specificity as compared to other methods (15). PAD is defined by ABI $\leq$0.9 (14, 16, 17). Recently, the American Heart Association (AHA) guidelines recommended that borderline ABI rate between 0.91 and 0.99 should be classified as mild stenosis (17).

In general, the definition of symptomatic PAD relies on the presence of both typical intermitent claudication and abnormal ABI (<0.90), where asymptomatic PAD is characterized by abnormal ABI alone without limb symptoms. However, most medical practices do not routinely measure ABI. A recently published systematic review on the diagnostic and prognostic value of the ABI concluded that there is insufficient evidence supporting the accuracy of ABI as a screening tool (18). Therefore, detecting PAD in either asymptomatic individuals or those who have atypical leg symptoms remains challenging.

Mechanisms

There are convincing evidence supporting the primary pathophysiology of PAD, initially triggered by an atherosclerotic obstruction of the lower extremities with consequent decrease in blood flow (ABI<0.9). In fact, increasing blood supply to the compromised limb after surgical revascularization clearly improves symptoms and hemodynamic parameters in PAD patients with life-style limiting claudication or critical limb ischemia (19). However, this procedure is not sufficient to induce recovery of metabolic properties and functional capacity (20, 21).

PAD progression cannot be solely explained by reduced blood flow to the affected limb (22). In fact, a decline in ABI values does not necessarily reflect a worsen PAD clinical outcome

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(19, 23). Pipinos *et al.* have provided evidence using magnetic resonance spectroscopy that PAD patients with mild to moderate claudication have lower extremity dysfunctional energy metabolism independent of both blood flow and severity of vascular occlusion (24). Moreover, McDermott *et al.* demonstrated that PAD patients with similar hemodynamic dysfunction (similar ABI values) have different symptoms ranging from asymptomatic to intermittent claudication (25). Therefore, additional mechanisms rather than impaired hemodynamics might contribute to PAD pathophysiology.

Recent evidence suggests that PAD-related morbidity depends on changes in metabolic, morphologic and functional status of the skeletal muscle distal to the primary arterial obstruction (7, 22, 26, 27). Lower extremity skeletal muscles of patients with PAD present dysfunctional bioenergetics, increased fat deposition, sarcopenia, loss of strength and impaired contractility properties as compared with skeletal muscles of individuals without PAD (25, 26, 28–30). Mc Dermott *et al.* found that ABI values positively correlate with skeletal muscle cross-sectional area (31). Of interest, the same group demonstrated in another study that PAD patients with similar ABI values have different profile of skeletal muscle cross-sectional area (25). Asymptomatic PAD patients (which are less active and consequently present reduced metabolic activity) have smaller calf muscle cross-sectional area, peripheral nerve dysfunction and poorer functional performance as compared with individuals with intermittent claudication (23). These findings clearly show that despite of hemodynamic impairment, intrinsic changes in the distal affected skeletal muscle play critical role in the PAD pathophysiology.

Ischemia-reperfusion injury

The biochemical, morphological and functional properties of the skeletal muscles are likely to be affected by the phenomenon of ischemia-reperfusion, triggered by exercise-rest cycles in individual with PAD (mostly characterized in intermittent claudication patients). In healthy individuals during exercise, increased metabolic demand results in elevated oxygen uptake by the active skeletal muscle. However, in PAD patients, the atherosclerotic obstruction limits the supply of oxygen and nutrients, resulting in calf muscle ischemia. Short periods of ischemia, characterized by a bioenergetic deficit, are compensated by increased glucose uptake and switching the metabolism to glycolysis. However, sustained oxygen and nutrients deprivation results in irreversible damage to the affected skeletal muscle. Thus, rest [and consequent reperfusion of the ischemic limb], although a prerequisite for reestablishing homeostasis, may induce reperfusion injury. Such ischemia and reperfusion injury has been well characterized in cardiac tissue (32–34). However, its impact on skeletal muscle pathophysiology is still not fully understood.

A single bout of ischemia-reperfusion is sufficient to induces necrosis, myofiber derangement and reduces metabolic activity in human skeletal muscle in a time-dependent manner (35). Recently, it was demonstrated that acute ischemia-reperfusion injury reduces mitochondrial respiratory rates in permeabilized skinned muscle fibers isolated from rats (36). Therefore, it is expected that repeated cycles of ischemia and reperfusion should negatively affect skeletal muscle mitochondrial bioenergetics and thus contributing to impaired lower extremity functioning in individuals with PAD (Figure 1).

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Mitochondrial dysfunction in PAD

Mitochondria are double membrane organelles with their own DNA that use redox reactions to generate an electrochemical gradient required for ATP synthesis through oxidative phosphorylation (37). These organelles are critical in maintaining metabolite transport and ion homeostasis in most eukaryotic cells (38). Mitochondria are also considered key players in the control of both redox signaling (physiological process) and oxidative stress (pathological process), since they are the main source of reactive oxygen species (ROS) and have the highest intracellular antioxidant capacity (34). Therefore, the maintainance of mitochondrial function and integrity is critical for human health (39).

Despite of increased knowledge regarding the involvement of mitochondrial bioenergetics and ROS generation in cardiac ischemia and reperfusion injury, less is known about the impact of mitochondrial metabolism on skeletal muscle during repeated cycles of ischemia-reperfusion injury in PAD. Changes in skeletal muscle oxidative metabolism has been demonstrated in PAD patients using non-invasive nuclear magnetic resonancy spectroscopy (24, 40). Skeletal muscle from individuals with PAD display slowed phosphocreatine and ADP recovery after mild exercise as compared to control population (41, 42). Moreover, experiments using near-infrared spectroscopy provide evidence that skeletal muscle hemoglobin desaturation is impaired at the onset of exercise in PAD patients as compared to controls (43, 44). This pattern of reduced metabolic kinetics in the affected limb suggests a scenario where dysfunctional mitochondria do not generate enough ATP to match skeletal muscle metabolic demand during exercise, resulting in intrinsic bioenergetic deficit and the consequent abortion of exercise (Figure 1). Decreased oxygen uptake kinetics in PAD might also be explained, at least in part, by reduced oxygen availibility in the ischemic tissue due to atherosclerotic obstructions (41, 45).

Another reliable approach to detect the impact of ischemia-reperfusion on skeletal muscle metabolism in PAD is measuring mitochondrial respiratory rates in the affected skeletal muscle. Pre-clinical studies using an *in vivo* mouse model of hindlimb ischemia demonstrate that either permeabilized skinned muscle fibers or mitochondria isolated from ischemic limb (mainly skeletal muscle myocytes) display reduced oxygen consumption as compared with contralateral control limb (28, 46, 47). Moreover, changes in mitochondrial bioenergetics are accompanied by oxidative stress, increased mitochondrial mass, decreased cross-sectional area and myofiber derangement in the ischemic limb (28). These findings suggest that mitochondrial metabolism, morphology and oxidative stress in skeletal muscle are affected by hindlimb ischemia-reperfusion injury in rodents. However, the involvement of mitochondrial dysfunction in the establishment and/or progression of PAD is still unknown.

There are also direct evidence of defective mitochondrial metabolism in skeletal muscle from individuals with PAD. Lower extremity skeletal muscle biopsies (mainly myocytes) from PAD patients with symptoms of intermittent claudication display changes in mitochondrial electron transport chain characterized by reduced complexes I and III activities (48). It is known that during ischemia-reperfusion injury, complexes I and III are the main source of ROS in cardiac and liver tissues (34, 49). However, it is not known whether PAD patients with intermittent claudication present increased ROS levels in the

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affected skeletal muscle. Future studies characterizing the skeletal muscle mitochondrial redox balance in this PAD population are needed.

Disruption of mitochondrial metabolism is also present in advanced PAD. Patients undergoing lower extremity surgery due to critical limb ischemia present compromised electron transport chain function, reduced activity of antioxidant enzymes (*e.g.*, superoxide dismutase, catalase and glutathione peroxidase) and elevated protein carbonyls in the affected skeletal muscle (mainly myocytes) compared to non-PAD patients (26). Of interest, reduced skeletal muscle mitochondrial capacity of ATP generation along with exacerbated oxidative stress are positively associated with the duration of PAD symptoms (26).

Overall, studies using either non-invasive (nuclear magnetic resonancy spectroscopy) or invasive (skeletal muscle biopsies) approaches provide evidence that impaired mitochondrial bioenergetics in the affected skeletal muscle is a hallmark of PAD in patients with either intermittent claudication or critical limb ischemia. Unfortunately, there is no data available showing the profile of mitochondrial bioenergetics and redox state in lower extremity skeletal muscle from asymptomatic PAD patients, the largest PAD population (50). These individuals display severe myopathy (*i.e.,* slower calf muscle area and higher calf muscle percent fat) and reduced physical performance (*i.e.,* slower six minute walk test) compared to symptomatic PAD (25). The mechanisms related to increased morbidity in asymptomatic PAD are still unknown.

Skeletal muscle microenvironment

Skeletal muscle is a multicellular environment mainly composed of myofibers [excitable myocytes with contractile properties], resident progenitor cells [i.e. satellite and endothelial progenitor cells and pericytes), fibroblasts, endothelial cells, vessels and nerves. In general, this microenvironment responds positively to short periods of ischemia by triggering skeletal muscle satellite cell proliferation and self-renewing (51–54) as well as activation of endothelial progenitor cells and angiogenesis (55). The impact of short periods of ischemia on pericytes [myogenic precursors located at the skeletal muscle microenvironment (56, 57)] is still unknown.

Despite the positive impact of short periods of ischemia on skeletal muscle, repeated cycles of ischemia-reperfusion injury impairs the capacity of skeletal muscle microenvironment to recruit/activate resident cells capable of inducing local angiogenesis and myofiber regeneration through a multifaceted process that involves both circulating and local components (57–60). Recent evidence demonstrates that therapies capable of coordinately improving local skeletal muscle angiogenesis and myogenesis have a better impact on tissue regeneration during hindlimb ischemia in rodents (61, 62). However, dissecting the contribution of specific population of cells located at the skeletal muscle microenvironment to PAD establishment and/or progression remains a challenge.

Overall, it is expected that therapies capable of improving PAD pathophysiology should affect the whole skeletal muscle microenvironment [not only myocytes or endothelial cells] in order to increase its regenerative capacity. One possibility is tackling the excessive

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generation/release of reactive molecules in different skeletal muscle cell types as well as counteracting their autocrine and paracrine effects during ischemia-reperfusion injury. Considering that most of skeletal muscle cells are directly or indirectly affected by changes in mitochondrial morphology and electrochemical properties as well as elevated oxidative stress imposed by ischemia-reperfusion condition, it is expected that therapies capable of improving mitochondrial quality control and redox balance will positively impact PAD prognosis by affecting every single cell at the skeletal muscle microenvironment (Table 1).

Recent evidence demonstrates that changing mitochondrial metabolism is a critical step in skeletal muscle regeneration (63, 64). In fact, increasing mitochondrial number, oxidative capacity and redox balance is important to induce skeletal muscle satellite cell proliferation and differentiation (65–67) as well as angiogenesis (68, 69). However, there is no much evidence showing the effectiveness of mitochondrial-specific therapies on the multicellular skeletal muscle microenvironment during PAD. The following section will describe the impact of mitochondrial quality control on skeletal muscle homeostasis during PAD, highlighting some pre-clinical studies using mitochondrial-target therapies in PAD. These studies focused mainly on skeletal muscle myocytes and endothelial cells.

Impaired mitochondrial quality control in PAD

Mitochondria fusion-fission balance and clearance

Recently, Thompson *et al.,* demonstrated that mitochondrial content (measured by citrate synthase activity) in the skeletal muscle (mainly myocytes) is a predictor of mortality rate in PAD patients (70). Of interest, a U-shaped relationship between gastrocnemius citrate synthase activity and mortality was found in that study. Patients in both lowest and highest tertile of citrate synthase activity had higher five-year mortality rate compared to middle tertile. This pattern of change in mitochondrial mass along with reduced respiratory rates is well described in hearts subjected to ischemia-reperfusion injury and reflect a disruption of mitochondrial fission-fusion balance and/or mitophagy (71, 72) (Figure 2).

Mitochondrial fission-fusion balance [a process mediated by large guanosine triphosphatases, GTPases] and mitophagy [autophagy-mediated mitochondrial removal] are part of mitochondrial quality control machinery and play critical role in controlling mitochondrial bioenergetics, morphology, number and clearance (73–75) (Figure 2). Mitochondrial fission is triggered by dynamin-related protein 1 (Drp1) (75, 76). Upon activation, Drp1 translocates to the outer mitochondrial membrane, binds to acessory proteins and form spirals that constrict both outer and inner membrane (75, 77, 78). Mitochondrial fusion is mediated by other large GTPases, mitofusins 1 and 2 (mfn1, mfn2; outer-membrane fusion) and Opa1 (Optic atrophy 1; inner-membrane fusion) (74, 75). Loss of mitochondrial fission-fusion balance is associated with several pathologies triggered by ischemia-reperfusion injury, including acute kidney injury, myocardial infarction and stroke (32, 79–81). Indeed, recent evidence demonstrates that restablishing mitochondrial fission-fusion balance protects these tissues against ischemia-reperfusion injury (32, 79–81). Therefore, decreasing mitochondrial ability to fuse and divide is not only a marker of stress but it plays critical role in ischemia-reperfusion injury (72).

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Despite the well-known impact of mitochondrial fission-fusion balance and mitophagy on cardiac ischemia-reperfusion injury, there has been no direct demonstration of their potential role into ischemic myopathy seen in PAD. Emerging evidence supports that mitochondrial fission-fusion balance is crucial in skeletal muscle physiology. In general, skeletal muscle myocytes have three spatially distinct population of mitochondria: subsarcolemal, intermyofibrillar and perinuclear (82, 83). Intermyofibrillar mitochondria form elongated networks that regularly share matrix content through fission-fusion events (82, 83). Mitochondrial fusion rates are different according to skeletal muscle fiber types. Oxidative muscle fibers (I and IIa) have elongated mitochondria and higher rates of mitochondria fusion compared to glycolitic fiber types (84). The switch of a glycolitic fiber to an oxidative one requires mitochondrial fusion and elongation in a process that is dependent on Mfn1 and Mfn2 (84). *In vivo* silencing of Mfn1 or Opa1 (but not Mfn2) reduces mitochondrial fusion rate in skeletal muscle myocytes (83). Chen *et al.,* showed that conditional deletion of Mfn1 and Mfn2 in skeletal muscle myocytes results in a massive accumulation of fragmented mitochondria between myofibrils, increased mitochondrial myopathy and lethality in rodents (85).

Accumulation of fragmented mitochondria also occurs by Drp1 activation or after disruption of mitochondria clearance (mitophagy) in skeletal muscle myocytes (86, 87). Both conditions are sufficient to induces muscle loss in rodents. Masiero *et al.,* demonstrated that genetic disruption of autophagy through muscle-specific deletion of Atg7 (a critical autophagy gene) results in skeletal muscle myocytes atrophy, accumulation of big abnormal mitochondria and excessive oxidative stress in mice (88). Moreover, inhibition of autophagy exacerbates denervation- and fasting-induced myopathy (87) in muscle-specific Atg7 null mice. These findings strongly suggest that disruption of mitochondrial quality control mechanisms contributes to skeletal muscle pathophysiology. Finally, genetic perturbation of mitochondrial fusion-fission balance in skeletal muscle myocytes results in accumulation of point mutations and deletions in the mitochondrial genome, which precedes physiological abnormalities in mice (85). Of interest, individuals with PAD present a 17-fold increased frequency of mitochondrial DNA deletion mutation in the affected skeletal muscle compared to controls (89, 90). These patients also display abnormal accumulation of dysfunctional mitochondria (27, 70).

Considering the critical role of mitochondrial quality control in skeletal muscle biology as well as its involvement in mitochondrial remodeling and turnover during ischemia-reperfusion injury in other tissues (*e.g.*, heart, brain and kidney), it is expected that mitochondrial quality control machinery plays important role also in PAD pathophysiology and might explain, at least in part, the impaired bioenergetics and increased mitochondrial mass reported in PAD. In a recent study, we found using transmission electron microscopy that hindlimb ischemia causes a heterogeneus mitochondrial distribution and remodeling in skeletal muscle myocytes from mice (91). There is an accumulation of smaller mitochondria at the subsarcolema four weeks after hindlimb ischemia surgery as compared to control (sham) animals (Figure 3). Moreover, intermyofibrillar mitochondria are either elongated/clustered close to myofiber derangements or smaller/sparse in intact areas. These findings suggest a disruption of mitochondrial morphology and connectivity after hindlimb ischemia. Future studies focusing on a better understanding of skeletal muscle mitochondrial network,

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fission-fusion balance and mitophagy during ischemia-reperfusion injury as well as their contribution to PAD pathophysiology are needed.

Mitochondrial detoxifying systems

Another crucial mitochondrial quality control mechanism in skeletal muscle relies to detoxyfing systems that provide a critical protection from damaging agents generated both endogenously and exogenously (Figure 2). Mitochondrial antioxidant systems provide quality control mechanisms against excessive generation of reactive oxygen species (ROS) (34, 73). Both enzymatic (including manganese superoxide dismutase, catalase, peroxiredoxin, thioredoxin, thioredoxin reductase and gluthatione peroxidase) and non-enzymatic (gluthatione) antioxidants are important to counteract excessive generation of ROS at the mitochondria, maintaining them at nanomolar levels [a concentration where these reactive species positively regulate intracellular processes (37)]. Disruption of mitochondrial redox balance due to exacerbated production of ROS and/or insufficient antioxidant capacity has been extensively characterized in skeletal muscle myocytes from both humans and rodents during chronic stress conditions (92–94). Mitochondrial oxidative stress is a critical factor of skeletal muscle pathophysiology rather than simply a marker of stress. In fact, sustained treatment of a skeletal muscle disuse rodent model with a mitochondrial-target antioxidant peptide counteracts mitochondrial oxidative stress and protects against skeletal muscle atrophy (95, 96).

Because lower limb ischemia-reperfusion injury in PAD results in oxidative stress and mitochondrial dysfunction in both human and rodents (28, 42), therapies that improve mitochondrial redox balance should protect skeletal muscle against ischemia-reperfusion injury and improve patients life quality. Recently, pre-clinical studies provide evidence that reduction of mitochondrial oxidative stress protects skeletal muscle (mainly myocytes) against hindlimb ischemia (46, 47). Ryan *et al.,* found that mitochondrial target overexpression of catalase [an enzyme that converts hydrogen peroxide to water and oxygen] improves skeletal muscle morphology, contractility and mitochondrial bioenergetics in obese C57BL/6 mice underwent hindlimb ischemia (47). These skeletal muscle improvements were seen without significant changes in the affected tissue perfusion. This study demonstrates that improving mitochondrial redox balance is critical to protect skeletal muscle against ischemia-reperfusion injury in rodents.

Another paper from the same group demonstrated that sustained treatment using a mitochondrial-target antioxidant peptide increases limb blood flow, skeletal muscle regeneration and mitochondrial function in BALB/c mice subjected to hindlimb ischemia (46). The individual impact of mitochondrial target-antioxidant peptide on skeletal muscle and endothelium cells was validated in culture. Peptide admnistration protected both primary myotubes and HUVEC (human umbilical vein endothelial cells) mitochondrial bioenergetics against hypoxia-reoxygenation stress (46). Both studies demonstrate that improving mitochondrial redox balance is critical to protect skeletal muscle environment against ischemia-reperfusion injury in rodents.

Recent findings revealed that not only elevated ROS are critical to ischemic tissues, but that accumulation of biogenic toxic aldehydes also plays a key role in ischemia-reperfusion

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injury. 4-hydroxy-2-nonenal (4-HNE), a secondary end-product of lipid peroxidation, negatively affects mitochondrial function during ischemic events (33, 97, 98). 4-HNE is a highly reactive aldehyde that irreversibly forms protein adducts *via* Michael addition or Schiff base reaction (99). Excessive 4-HNE adducts formation has been previously reported in ischemic hearts from both humans and rodents (100) and was associated with contractility dysfunction and impaired mitochondrial bioenergetics (33, 97, 98). Increased 4-HNE-modified proteins have also been reported in gastrocnemius muscle biopsies from individual with PAD and rodents subjected to hindlimb ischemia (28, 101, 102). Therefore, accumulation of 4-HNE appears to be a hallmark of ischemia-reperfusion injury in both cardiac and skeletal muscles.

A family of detoxification enzymes, called aldehyde dehydrogenases (ALDH), is responsible for the clearance of aldehydes arisen both endogenously and exogenously. Among 19 functional ALDH genes, the mitochondrial aldehyde dehydrogenase 2 (ALDH2) has emerged as a key enzyme in protecting against mitochondrial dysfunction and oxidative stress (33, 98, 103, 104). ALDH2, a tetrameric enzyme located in the mitochondrial matrix, is responsible for catalyzing the convergence of the highly reactive 4-HNE and short-chain aliphatic aldehydes into inactive acids (103, 105). Recent evidence demonstrates that impaired ALDH2 activity and increased aldehydic load contribute to ischemia-reperfusion injury. ALDH2 knockout mice are more susceptible to ischemic injury (106). Conversely, overexpression of ALDH2 attenuates cardiac ischemia reperfusion injury in mice (106). Moreover, selective ALDH2 activation using a small molecule (Alda-1) protects against cardiac ischemia-reperfusion injury and heart failure (97, 103). The ALDH2-mediated cardioprotection involves improved mitochondrial bioenergetics and reduced both aldehydic overload and oxidative stress (33, 97). This scenario provides evidence that ALDH2-mediated removal of toxic aldehydes is a critical mitochondrial quality control mechanism thus protecting against cardiac ischemia-reperfusion injury (73). These findings raise important questions regarding of ALDH2 contribution to skeletal muscle ischemia-reperfusion injury seen in individuals with PAD.

Recent evidence suggests that ALDH2 is involved in hindlimb ischemia in rodents. ALDH2 knockout mice subjected to hindlimb ischemia display slower recovery of limb perfusion and exacerbated myopathy compared to wild-type mice (107). Reduced blood perfusion in ALDH2 knockout mice was related to impaired angiogenesis (107). The impact of ALDH2 and aldehydic overload on intrinsic skeletal muscle changes in both PAD patients and hindlimb ischemia in rodents remains to be elucidated. We recently demonstrated that mice carrying a loss-of-function point mutation in ALDH2 display severe hindlimb ischemia-induced myopathy compared to wild-type mice (91). Moreover, sustained pharmacological ALDH2 activation using Alda-1 rescues skeletal muscle dysfunction seen in wild-type and ALDH2 deficiency mice underwent hindlimb ischemia (91).

The aforementioned ALDH2 deficiency [which is associated with worsen ischemic injury] is caused by a single substitution of glutamate to lysine at position 487 (E487K) of the enzyme that exerts a dominant negative effect. The ALDH2 E487K mutation is the most frequent variant in humans, present in approximately 35% of East Asians [about 560 million people (103)]. The most common phenotype related to ALDH2 E487K mutation is the facial

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flushing and increased heart rate triggered by alcohol consumption due to its inability to metabolize acetaldehyde, the first oxidized metabolic product of ethanol (108, 109). Of interest, ALDH2 deficiency has been strongly associated with a higher risk for cardiovascular diseases (110). However, the association between ALDH2 deficiency and PAD has not been studied yet.

Overall, impaired ALDH2 activity due to either genetic variation or oxidative stress results in aldehydic overload, which contributes to exacerbated hindlimb ischemia-associated myopathy. Therefore, the beneficial effect of pharmacological ALDH2 activation is associated with a better aldehyde clearance in the affected skeletal muscle. This scenario suggests that activation of ALDH2 can provide therapeutic effects by treating skeletal muscle changes in the affected limb during PAD. Further studies are required to test this hypothesis directly.

Aldehyde dehydrogenase activity has also been used to sort stem cells with increased regenerative capacity. Pre-clinical and clinical studies have demonstrated that a population of bone marrow mononuclear cells with high levels of aldehyde dehydrogenase, referred to as ALDH bright cells ($ALDH^{br}$, mainly ALDH1A1) displays elevated angiogenic capacity (111, 112). Transplantation of human bone marrow cells purified by high aldehyde dehydrogenase activity into immune-deficient mice subjected to hindlimb ischemia recovers perfusion and increases blood vessel density in the affected limb compared to recipients injected with unpurified nucleated cells (111). Moreover, autologous therapy with bone marrow-derived aldehyde dehydrogenase bright cells appears safe and tends to increase ABI values in patients with critical limb ischemia (112). Bone marrow mononuclear cells with high levels of aldehyde dehydrogenase also present high myogenic capacity (113), which may contribute to faster skeletal muscle recovery. Overall, these preliminary findings using aldehyde dehydrogenase bright cells as a cellular therapy against critical limb ischemia are positive. However, placebo-controlled studies are required to determine its effectiveness as a treatment for PAD.

Summary and conclusion

As discussed here, PAD is a degenerative disease with a profound impact on patient's quality of life and mortality. In this review, we examined the evidence from human studies and from animal studies. Much of what we knew about PAD therapies stemmed from a vascular disease perspective. However, more recent research indicates that intrinsic metabolic changes in the skeletal muscles of the affected limb triggered by intermittent ischemia-reperfusion injury are associated with PAD. This may suggest that PAD should be classified as a myopathy. Many ischemic diseases are characterized by biochemical, morphological and functional changes in the affected organ *per se*. In fact, targeting biochemical changes using pharmacological therapies is sufficient to protect the heart against ischemia-reperfusion injury and its degenerative consequences (39). Accordingly, are metabolic changes in the affected skeletal muscle benign or do they actually contribute to PAD pathophysiology?

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It seems that intrinsic metabolic changes in the affected skeletal muscle are critical to PAD pathophysiology. From symptomatic PAD patients with intermittent claudication to critical limb ischemia, reducing mitochondrial dysfunction and oxidative stress in the skeletal muscle appears to correspond to a better outcome. The contribution of mitochondrial quality control to PAD is better seen in animal studies where improving mitochondrial redox balance or increasing aldehyde clearance in the mitochondria is sufficient to protect against hindlimb ischemia.

But, why is there such central role for mitochondria in PAD? Mitochondria are not only the powerhouse of the cell. They are also strategic intracellular nodes and multi-effector players that affect a wide range of signaling pathways that ultimately regulates cellular functioning and viability (114). Therefore, maintaining mitochondrial integrity through activation of quality control-related surveillance systems in the affected skeletal muscles and perhaps also in the surrounding tissues may be critical to counteract the intermittent ischemia-reperfusion injury seen in the affected skeletal muscle in PAD patients.

As last note, improving mitochondrial quality control might be critical to enhance the effectiveness of current therapies in PAD such as exercise. Exercise is a well-known and very effective intervention, boosting up mitochondrial bioenergetics in both health and disease (115). However, it should be performed at a moderate to high intensity to have a significant impact in biochemical and functional capacities (116). Considering the exercise limitations in PAD patients with intermittent claudication, exercise training programs are usually performed at low intensity. Therefore, their effectiveness as a therapy is only marginal (117). We believe that the optimal management of asymptomatic and symptomatic PAD patients to significantly improve their exercise performance [and maximize its positive impact in quality of life] requires a combination of pharmacological therapies that improve the functional capacity of the affected skeletal muscle (22). We suggest that such pharmacological interventions should be focused on boosting mitochondrial quality control mechanisms to improve mitochondrial fusion-fission balance, mitochondrial turnover, reduced oxidative stress and/or aldehydic overload; better mitochondrial function should be an optimal strategy to improve the adjuvant effect of exercise on PAD-related morbidity and mortality.

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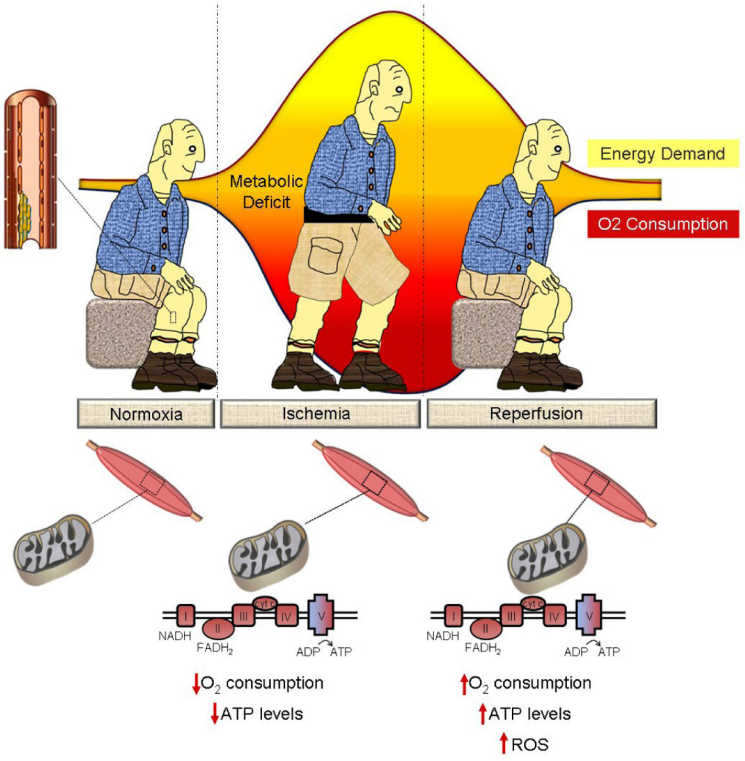

Figure 1.

Schematic panel showing the process of ischemia-reperfusion injury triggered by exercise-rest cycles in PAD patients with intermittent claudication. During exercise, there is an increased skeletal muscle energy demand. However, limited limb energy transferring due to impaired blood flow and mitochondrial dysfunction causes a metabolic deficit and consequent exercise cessation. After cessation of exercise [and consequent reperfusion of the ischemic limb] mitochondria-mediated energy transferring is reestablished. However, intrinsic changes occurred during repeated cycles of ischemia-reperfusion injury cause mitochondrial dysfunction and accumulation of reactive oxygen species, therefore, contributing to a progressive metabolic deficit and skeletal muscle degeneration in PAD.

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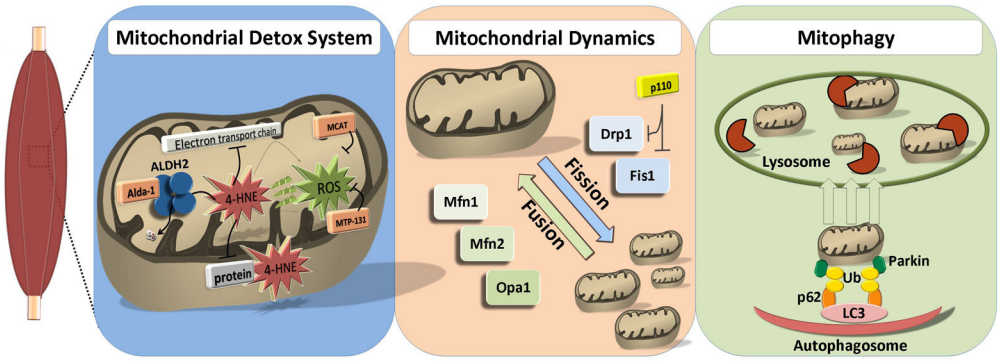

Figure 2.

Schematic panel showing the most critical systems involved in the mitochondrial quality control in PAD. Mitochondrial Detox System: Mitochondrial dysfunction, accumulation of reative oxygen species (ROS) and consequent generation of 4-hydroxynonenal (4-HNE) in the affected skeletal muscle contribute to the PAD pathophysiology. Therapies targeting mitochondria (Alda-1, MTP-131 and catalase overexpression) improves mitochondrial detox system and provide a better PAD outcome. Mitochondrial Dynamics: In general, large GTPases regulate mitochondrial fission (Drp1 and Fis1) and fusion (mfn1, mfn2 and Opa1). The peptide p110 inhibits mitochondrial fragmentation and ROS production.. Mitophagy: Damaged mitochondria can be sequestered by autophagosomes. The autophagosomes then fuse with lysosomes to degrade sequestered mitochondria.

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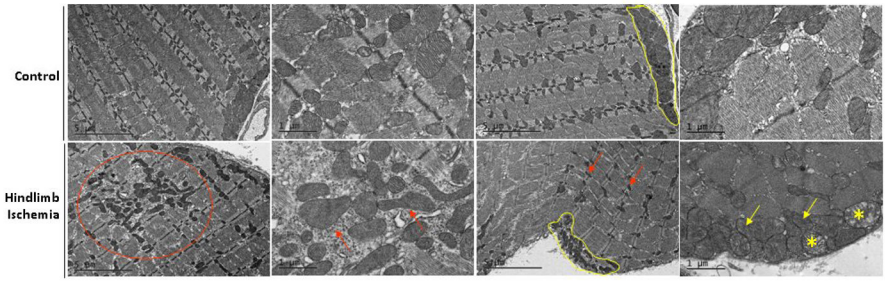

Figure 3.

Representative transmission electron microscopy (TEM) images of plantaris muscle four weeks after *sham* (control, upper panels) or hindlimb ischemia surgeries (lower panels) in mice. Circle and arrows in red indicate intermyofibrillar mitochondria. Circle and arrows in yellow indicate subsarcolemmal mitochondria. Asterisks indicate swollen mitochondria. Note that intermyofibrillar mitochondria from mice subjected to hindlimb ischemia are either elongated/clustered close to myofiber derangements (red circle) or smaller/sparse in intact areas. There is also accumulation of smaller mitochondria at subsarcolema four weeks after hindlimb ischemia surgery. These findings depict a disruption of mitochondrial morphology and connectivity after hindlimb ischemia.

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Table 1

Impact of PAD interventions on skeletal muscle

Intervention	Approved agents	Main target	Main response	Impact on skeletal muscle	Reference
Available therapies in clinics					
Lipid-lowering therapy	Statins	Liver	Decreases cholesterol biosynthesis	Unknown	(118, 119)
	Fibric acid agents	Multiple	Decreases triglyceride and LDL cholesterol levels	Unknown	(120)
Antihypertensive therapy	ACE inhibitors	Multiple	Decreases blood pressure	Unknown	(121–123)
Diabetes Mellitus treatment	Metformin	Multiple	Decreases blood glucose levels	Unknown	(124)
Antithrombotic therapy	Aspirin	Blood cells	Inhibits platelet aggregation	Unknown	(125)
	Clopidogrel	Blood cells	Inhibits blood clots	Unknown	(126)
	Dipyridamole	Endothelium and blood cells	Inhibits platelet aggregation	Unknown	(125)
	Vorapaxar	Blood cells	Inhibits platelet aggregation	Unknown	(127)
	Cilostazol	Endothelium and blood cells	Inhibits platelet aggregation and increases arterial vasodilatation	Improves exercise capacity	(128)
	Pentoxifylline	Endothelium and blood cells	Reduces blood viscosity and inhibits platelet aggregation	Improves exercise capacity (weak)	(129)
	Naftidrofuryl	Endothelium	Vasodilator and inhibits platelet aggregation	Improves exercise capacity (weak)	(130)
Smoking cessation therapy	Varenicline	CNS	Reduces craving symptoms	Unknown	(131)
	Bupropion	CNS	Reduces craving symptoms	Unknown	(132)
	Nicotine gum, patch, inhaler	CNS	Reduces craving symptoms	Unknown	(133)
Exercise training		Multiple	Increases muscle perfusion, metabolism and peripheral nerve function	Improves exercise capacity	(134)

Intervention	Candidate	Main target	Main response	Impact on skeletal muscle	Reference
Potential new therapeutic approaches (pre-clinical studies)					
Mitochondrial fusion-fission balance	P110 peptide	Skeletal muscle microenvironment (mitochondria)	Inhibits mitochondrial fragmentation and ROS production	Unknown	(32, 79)
Mitochondrial detoxifying systems-Redox balance	MTP-131	Skeletal muscle microenvironment (mitochondria)	Improves mitochondrial redox balance and function	Improves angiogenic and myogenic capacity	(46)

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Intervention	Candidate	Main target	Main response	Impact on skeletal muscle	Reference
Mitochondrial detoxifying systems-Aldehydes clearance	Alda-1	Skeletal muscle microenvironment (mitochondria)	Improves mitochondrial metabolism through better clearance of reactive aldehydes	Improves myogenic capacity and running performance	(33, 91, 97, 103)
Bone Marrow Therapy (Phase 2 clinical trial)	ALDHbr cells injection	Skeletal muscle microenvironment	Improves angiogenic and myogenic capacity	Improves angiogenic and myogenic capacity	(112)