Chronology of mitochondrial and cellular events during skeletal muscle ischemia-reperfusion

Stéphanie Paradis,1,2 Anne-Laure Charles,1,2 Alain Meyer,1,2 Anne Lejay,1,2,3 James W. Scholey,4 Nabil Chakfé,1,3 Joffrey Zoll,1,2 and Bernard Geny 1,2

1University of Strasbourg, Fédération de Médecine Translationale, EA 3072, Strasbourg, France; 2Department of Physiology and Functional Explorations, Thoracic Pathology Unit, Centre Hospitalier Régional Universitaire de Strasbourg, Strasbourg, France; 3Department of Vascular Surgery and Kidney Transplantation, Centre Hospitalier Régional Universitaire de Strasbourg, Strasbourg, France; and 4Department of Medicine and Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada

Paradis S, Charles A, Meyer A, Lejay A, Scholey JW, Chakfé N, Zoll J, Geny B. Chronology of mitochondrial and cellular events during skeletal muscle ischemia-reperfusion. Am J Physiol Cell Physiol 310: C968–C982, 2016; doi:10.1152/ajpcell.00356.2015.—Peripheral artery disease (PAD) is a common circulatory disorder of the lower limb arteries that reduces functional capacity and quality of life of patients. Despite relatively effective available treatments, PAD is a serious public health issue associated with significant morbidity and mortality. Ischemia-reperfusion (I/R) cycles during PAD are responsible for insufficient oxygen supply, mitochondriopathy, free radical production, and inflammation and lead to events that contribute to myocyte death and remote organ failure. However, the chronology of mitochondrial and cellular events during the ischemic period and at the moment of reperfusion in skeletal muscle fibers has been poorly reviewed. Thus, after a review of the basal myocyte state and normal mitochondrial biology, we discuss the physiopathology of ischemia and reperfusion at the mitochondrial and cellular levels. First we describe the chronology of the deleterious biochemical and mitochondrial mechanisms activated by I/R. Then we discuss skeletal muscle I/R injury in the muscle environment, mitochondrial dynamics, and inflammation. A better understanding of the chronology of the events underlying I/R will allow us to identify key factors in the development of this pathology and point to suitable new therapies. Emerging data on mitochondrial dynamics should help identify new molecular and therapeutic targets and develop protective strategies against PAD.

Peripheral artery disease; ischemia-reperfusion; skeletal muscle; mitochondria; oxidative stress

Peripheral artery disease (PAD) refers to a common circulatory disorder of the lower limb caused by chronic narrowing of the arteries (e.g., stenosis and occlusion) or atherosclerosis. PAD represents a broad spectrum of disease severity, ranging from asymptomatic disease to frequent pain when walking (i.e., intermittent claudication or limping) or critical limb ischemia associated with decubitus pain and/or ulcers (114, 126).

PAD is known to be associated with reduced functional capacity and quality of life. It is a major cause of limb amputation, as well as an increased risk factor for myocardial infarction, stroke, and death. The incidence of PAD varies with age, from 3–10% in young people to 15–20% in people >70 yr of age, and is asymptomatic in 40% of the cases (1), with greater prevalence among men. The major PAD risk factors, including smoking, diabetes mellitus, dyslipidemia, hypertension, and obesity, are the same as those for cardiovascular and cerebrovascular diseases (35).

Three main complementary treatment options improve the functional status and other clinical outcomes in PAD patients (54). 1) Optimization of medical therapy (i.e., pharmacotherapy) reduces the risk of cardiac ischemia, increases the distance a patient can walk, and improves the functional capacity of patients. 2) When possible, exercise training, a noninvasive and nonpharmacological therapy, improves walking ability and has protective effects in patients with PAD characterized by intermittent claudication and infrainguinal lesions (67). 3) Complementing treatment options 1 and 2, revascularization (either endovascular or open) prevents limb pain at rest and limb loss in patients with intermittent claudication who continue to have symptoms impacting their quality of life or in patients with critical limb ischemia.

However, despite relatively effective available treatments (14), PAD remains a serious public health issue associated with significant morbidity and mortality. A better understanding of physiopathology should lead to improved therapies.

The reduction or cessation of blood circulation followed by reperfusion [i.e., ischemia-reperfusion (I/R)] is the cause of local and distant alterations. I/R during PAD results in insuf-
ficient oxygen supply secondary to reduced blood flow, mito-
ochondriopathy leading to reduced energy supply, oxidative
stress (i.e., imbalance between free radical production and
elimination), and inflammation, which lead to intermittent
 Claudication, limb pain at rest, ulcers, and, potentially, limb
loss and increased morbidity/mortality (13, 39, 71, 95, 114,
162) (Fig. 1). Depending on the mass of ischemic muscle and
the duration of ischemia, these mechanisms can trigger remote
complications in organs such as the kidney, liver, heart, and
lung and lead to death (47, 200). A hypothesis explaining the
failure of these remote organs is that liberation of toxic me-
tabolites into the blood contributes to systemic inflammation
(13, 47, 63). However, PAD occurs in parallel with comorbid-
ity factors such as aging, diabetes, and hypertension, which
share common etiology and are also at the origin of several
pathological states and likely modulate local and remote effects
of I/R (33, 35). Thus, in conjunction with comorbidity factors,
local and distant sequential events that occur in skeletal muscle
fibers during ischemia and reperfusion progressively (and ir-
reversibly) impair muscle tissue to trigger death.

Several experimental models have been developed to inves-
tigate PAD. Acute models have limitations, since skeletal
muscle is subjected to a single episode of ischemia followed by
a single period of reperfusion. This might be only partially
relevant in the case of PAD, which leads to chronicity because
of recurrent episodes of ischemia and reperfusion (91, 101).
Thus recent experimental models of chronic PAD have been
proposed to be more relevant to clinical PAD (91, 96). Inter-
estingly, we observed that the mechanisms involved in chronic
lower limb I/R that best reflect human pathology are very
similar to those observed in acute models (96) and human
physiopathology (149). The use of such experimental models,
therefore, remains relevant and indispensable to understand the
mechanisms underlying I/R and tissue damage leading to PAD
and to develop new therapeutic strategies. One of these strat-
egies is ischemic conditioning, which corresponds to several
brief I/R episodes before (i.e., preconditioning), during (i.e.,
perconditioning), or after (i.e., postconditioning) prolonged
ischemia. Ischemic preconditioning is frequently used in acute
experimental models and appears to be protective (88, 135,
176). Importantly, the report of Mansour et al. (108) that
remote and local ischemic preconditioning equivalently protect
skeletal muscle mitochondrial function during lower limb I/R
opens therapeutic avenues, since remote (at a distant organ, not
suffering from ischemia) ischemic preconditioning is easier to
accomplish than local preconditioning. In the context of
chronic PAD, ischemic conditioning, a natural phenomenon
that occurs during walking, must be considered a part of PAD
physiopathology. This reinforces the need to develop chronic
experimental models better mimicking the human physiopa-
thology of PAD.

The chronology of these I/R events is poorly described in the
literature. In this context, after a review of the basal state of
skeletal muscle cells and mitochondrial function, our purpose
is to review the physiopathology of I/R at the level of the
myocyte. We will focus on cellular and mitochondrial impair-
ments during ischemia and reperfusion in skeletal muscle and
on associated events (inflammation and morphological and
microvascular changes) in surrounding tissues, finally leading
to cell death.

**BASIC KNOWLEDGE OF MYOCYTES: IMPORTANCE OF MITOCHONDRIA**

Skeletal muscle is composed of excitable cells that have
been studied at rest and during contractile activity. At rest,
myocyte membrane potential ranges from $-60$ to $-90$ mV,
and intracellular pH is $\sim 7$. The pH decreases during contractile
activity (37, 64, 158).
Ca\(^{2+}\) plays a central role as the main regulatory and signaling molecule in muscle physiology. Indeed, expression of several genes, as well as muscular contraction and relaxation, is a phenomenon controlled by Ca\(^{2+}\). Free cytosolic Ca\(^{2+}\) concentration in the resting skeletal muscle cell is maintained at \(\sim\)50 nM but can reach \(\sim\)100-fold higher levels during contraction (12). Intracellular Ca\(^{2+}\) concentrations depend on the fiber type: they are more elevated in red slow-twitch (type I) fibers (oxidative metabolism) than in white fast-twitch (type II) fibers (glycolytic metabolism) (87, 105). Cellular Ca\(^{2+}\) dynamics that accompany the action potentials are characterized by specific kinetics according to the fiber phenotype: lower amplitude and longer duration in oxidative fibers and higher amplitude and shorter duration in glycolytic fibers (9, 10, 23, 141). These specific kinetics are related to sarcoplasmic reticulum (SR) isoforms of Ca\(^{2+}\)-ATPase and ryanodine receptors and to their density (156). This difference is also observed in mitochondria of these various fiber types (140).

Mitochondria are essential organelles found in nearly all eukaryotic cells. They participate in many physiological functions, such as cell differentiation, energy metabolism, Ca\(^{2+}\) signaling, and apoptosis (53, 175). Their density and localization vary according to cell type. In skeletal muscle, their number depends on the fiber type: they are more numerous in oxidative fibers (141). Mitochondria occupy a small myocyte volume. In humans, mitochondrial volume is \(\sim\)6%, \(\sim\)4.5%, and \(\sim\)2.3% in type I, IIA, and IIX fibers, respectively (165). In rats, mitochondrial volume is \(\sim\)2.2% and \(\sim\)10% in white gastrocnemius (predominantly glycolytic) and soleus (predominantly oxidative) muscles, respectively (165). Mitochondria are located around nuclei or between bundles of myofilaments (20, 190). This location allows mitochondria to communicate with one another via a complex network (100) and to participate in physiological functions by regulated fission and fusion mechanisms, termed “mitochondrial dynamics.” Although little is known about mitochondrial dynamics in skeletal muscle, mitochondrial dynamics have been implicated in mitochondrial DNA renewal, cell differentiation and development, mitochondrial respiration, and bioenergetics (46, 100, 120, 129, 142).

**Mitochondria, the “Powerhouse” of the Cells**

Skeletal muscle is highly energy-dependent: 95% of the energy stores contribute to mitochondrial metabolic activity (192). This energy is provided by ATP molecules, for which storage is limited (\(\sim\)35–55 \(\mu\)mol/mg protein) (2, 64, 66).

In the resting myocyte, ATP is mainly produced in mitochondria by the oxidative phosphorylation (OXPHOS) process, after transformation of free fatty acids or glucose by \(\beta\)-oxidation, glycolysis, and the tricarboxylic acid (Krebs) cycle (192), and lactate production from metabolism is low. However, according to the fiber type and, thus, the metabolic pathway, muscle tissues have developed a specific adaptation in terms of respiratory control and energy distribution, depending on their needs. The mitochondrial oxidative capacity of glycolytic muscles is greater than that of oxidative muscles with glyceral-3-phosphate, a substrate of glycolysis. Conversely, the mitochondrial oxidative capacity of oxidative muscles is greater than that of glycolytic muscles with palmityl carnitine, a substrate of \(\beta\)-oxidation (141, 150).

Under normal circumstances, mitochondria are the most important source of ATP. Skeletal muscle can increase its ATP turnover, allowing it to transition from rest to exercise/contractile activity, where different energy-producing substrates (high-energy phosphate compounds, glucose, glycerogen, and lipids) can be oxidized (49, 66, 84, 135). The most concentrated substrates in skeletal muscle are glycogen and phosphocreatine (PCr) (192), with higher PCr levels in glycolytic muscles (26). Some authors have demonstrated that ATP content remains stable during contraction (49, 84), while others have found that it decreases (72, 106); this discrepancy may be explained by the use of different species and contraction protocols. However, all agree that there is a shift in metabolites used to synthesize ATP, with a decrease in glycogen (increase in glycolysis) and PCr [increase in creatine kinase (CK) reactions] and an accumulation of lactate in myocytes.

Energy-consuming processes are essentially localized in myofibrillar compartments, the sarcolemma, and the SR, whereas energy is produced within mitochondria or glycolytic complexes. Because of the restricted diffusion of adenosine nucleotides near the ATPases of myofilaments and SR, there are local systems for rephosphorylating ADP. The CK system is localized near or within these different compartments and efficiently controls local adenylate pools, linking energy production and utilization (185). CK isoenzymes catalyze the reversible transfer of a phosphate moiety between creatine and ATP. The mitochondrial CK isozyme, bound to the outer surface of the inner mitochondrial membrane, generates ATP by OXPHOS and transphosphorylated PCr (85). In addition, the cytosolic CK isozyme, which is structurally associated with myofibrils and SR membranes, can use PCr to rephosphorylate ADP and, thus, provide enough energy for normal contractile kinetics or SR Ca\(^{2+}\) uptake (160, 185).

**Mitochondria, an Important Source of Reactive Oxygen Species**

Reactive oxygen species (ROS) and reactive nitrogen species are free radicals produced in myocytes, endothelium, and extracellular space from peroxisomes, SR, PLA\(_2\), nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidases (XO), and nitric oxide (NO) synthases (111, 139). However, mitochondria of skeletal muscle cells are the predominant source of ROS (139). They are mainly produced by complexes I and III of the mitochondrial respiratory chain. Some research groups have shown higher ROS concentrations in mitochondria from glycolytic muscles (3, 141), whereas others have observed higher ROS concentrations in oxidative muscles (15, 115). The primary reactive species produced in myocytes are superoxide anion (O\(_2^-\)) and NO, which lead to formation of several secondary reactive species, such as H\(_2\)O\(_2\), hydroxyl radical (HO\(_\cdot\)), and peroxynitrite (7).

Low cellular production of free radicals is involved in physiological signaling and regulatory functions (7, 153). As second messengers, they modulate changes in cell and tissue homeostasis and gene expression. They are rapidly eliminated by enzymatic and nonenzymatic antioxidant systems, such as catalase, superoxide dismutase (SOD), glutathione peroxidase and...
(GPx), glutathione, thioredoxin reductase, coenzyme Q, and vitamin E, in myocytes, endothelium, and extracellular space (7, 77). Nevertheless, the activity of antioxidant enzymes, particularly GPx, is significantly lower in glycolytic than oxidative muscles (141).

Myocytes respond to contractile activity by an increase in intra- and extracellular production of free radicals (77). Indeed, in different contraction protocols, an increase in reactive species production, particularly HO and H2O2, has been observed (128, 134, 188). In parallel, myocyte antioxidant defenses are modified after contractile activity or exercise. Some researchers have found an increase in enzymatic antioxidant systems, such as SOD and catalase (82, 113), while others have demonstrated that muscle contractile activity decreases glutathione and thiol protein contents, two nonenzymatic antioxidant systems (188).

Thus mitochondria play a key role in skeletal muscle fiber physiology at rest and during contractile activity. They are essential 1) in energetic metabolism, supplying necessary energy, and 2) in cell signaling, mediating adaptive and protective processes through Ca2+ and low ROS production.

PHYSIOPATHOLOGY OF I/R IN SKELETAL MUSCLE

Lower limb ischemia resulting from PAD complications is defined as oxygen and nutrient deprivation following reduced blood flow, leading to cell impairment. Reperfusion allows blood to recirculate in ischemic tissues but is paradoxically associated with further damage. I/R of the lower limb impairs the entire local muscle environment (endothelial and muscle cells, vessels, and nerves) via complex processes, leading to loss of muscle function and failure of remote organs.

In contrast to the myocardium, where lesions are generally irreversible after 20–40 min of ischemia (80, 157), skeletal muscle seems relatively tolerant to ischemia, with the degree of injury directly related to the severity and duration of ischemia (138). In rats, Belkin et al. (11) showed that the first muscle injuries appear at 3 h of ischemia, while severe injury appears at 4–6 h. Martou et al. (110) reported that 3–4 h of hypoxia followed by 2 h of normoxia is needed to significantly alter human skeletal muscle. Other parameters, such as temperature, presence or absence of collateral flow, and fiber type, influence the tolerance of skeletal muscle for ischemia (138, 193, 198).

In mice, the vastus muscle (predominantly glycolytic) is more vulnerable to ischemia than the soleus muscle (predominantly oxidative) (25). This difference in vulnerability has also been observed between the gastrocnemius (predominantly glycolytic) and soleus (predominantly oxidative) muscles in rats (193). We have proposed that reduced antioxidant capacity in the gastrocnemius might explain the greater sensitivity to ischemia (27, 50).

Regardless of fiber type, I/R induces locally histomorphological and biochemical changes in myocytes that lead to cell death and systemic injuries. PAD-induced I/R leads to important myopathic (necrosis, phagocytosis, central nuclei, and fibrosis) and neurogenic (myofibrillar degeneration) changes (147). It also modifies the cellular organelle structure, particularly mitochondria, leading to functional alterations and oxidative damage (145, 146).

Chronology of Events at the Level of Skeletal Muscle Fiber

Cellular and mitochondrial events during ischemia. ADENOSINE TRIPHOSPHATE DEPLETION, INTRACELLULAR PH DECREASE, AND CYTOSOLIC CALCIUM OVERLOAD. During ischemia, the nutrient- and oxygen-deprived blood supply cannot meet the energy demands of muscles, leading to numerous ionic and metabolic changes. The low ATP reserve is used predominantly to maintain membrane potential and ion compartmentalization, but because of oxygen deprivation, mitochondrial OXPHOS and the electron transport chain are inhibited, and the mitochondrial membrane potential decreases (26, 102). Brandão et al. (17) showed in isolated rat mitochondria that respiration and membrane potential are altered after 5 h of ischemia. More specifically, mitochondrial activity of complexes I, II, and IV is altered during prolonged ischemia (176, 177). This leads to a fall in ATP synthesis and a rise in inorganic phosphate (Pi) and adenine nucleotide concentrations (135, 192). After 2-3 h of ischemia, ADP is catabolized into hypoxanthine and xanthine (18, 135), substrates that will contribute to ROS production during reperfusion. Chouchani et al. (30) demonstrated in the heart that succinate is another universal metabolite produced during ischemia by reversal of succinate dehydrogenase, which accumulates in cells and contributes to deleterious effects at the moment of reperfusion. To continue to produce ATP, anaerobic metabolism and PCr pathways are activated. In skeletal muscle, ATP falls at a very low rate during the first 3 h, when PCR and glycogen reserves are large. After 3 h of ischemia, the ATP store declines rapidly until 6–7 h, when exhaustive depletions of ATP, PCr, and glycogen occur (correlated with almost complete skeletal muscle death) (Fig. 2) (92, 123, 181, 183, 192). These changes in metabolism lead to accumulation of NAD, lactate, and H+, acidifying the intra- and extracellular environments (Fig. 2) (47, 64, 109, 195) and inhibiting glycolysis (66, 154). Harris et al. (70) showed that lactate production is continuous during skeletal muscle ischemia (up to 6 h) (70). This accumulation was observed after 4 h of anoxia by Vezzoli et al. (191). Noll et al. (125) showed local lactate accumulation after 2 h of ischemia in mice. Na+/H+ exchangers are activated to restore pH. Different ionic exchangers of the sarclemma (Na+/K+-ATPases and Ca2+-ATPases) are inhibited by the low ATP concentration, inducing an increase in cytosolic Na+. The mechanism of Na+-Ca2+ antiporters is reversed in an attempt to restore the cytosolic Na+ concentration, which leads to the accumulation of cytosolic Ca2+ (47, 74). In addition, SR Ca2+-ATPases are impaired, while Ca2+ continues to be extruded from the SR to the cytosol (83, 177). This Ca2+ accumulation can cause irreversible damage to cell integrity by degrading cellular enzymes such as phospholipases, lysozymes, proteinases, and nucleases, contributing to inflammation and cell death by necrosis and apoptosis (38, 63, 178).

REACTIVE OXYGEN SPECIES PRODUCTION. In parallel, ischemia participates in production of ROS, mainly O2·- and H2O2 (5, 159, 168). Few studies have examined the effects of ischemia alone in skeletal muscle by measuring either ROS production directly or oxidative stress products. Guillot et al. (61) showed biphasic ROS production: during ischemia and during reperfusion. Kocman et al. (88) demonstrated an increase in malondialdehyde, an oxidative stress marker, after ischemia. During ischemia, xanthine dehydrogenase, which is found in the microvascular endothelial cells of skeletal muscle, is converted to XO (189), which, in turn, catalyzes the conversion of

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hypoxanthine to xanthine by producing $O_2^{-}$. Thus the primary sources of ROS during ischemia seem to be XO and mitochondrial complexes I and III (8, 154, 184). Skeletal muscle contains 10–15% of the body’s iron stores, mainly in mitochondria and myoglobin (7). The progressive reduction of proteins with an iron-sulfur cluster during prolonged ischemia releases iron ions, which participate in ROS production by the Fenton reaction at the moment of reperfusion. All these free radicals contribute to membrane injury and permeabilization, nonfunctional protein formation, and DNA mutations (139).

A decrease in nonenzymatic antioxidants during ischemia makes cells more vulnerable to oxidative stress (204). However, to our knowledge, no data concerning the enzymatic antioxidant systems in skeletal muscle are available. In other organs such as the heart, kidney, and brain, enzymatic and nonenzymatic antioxidant activities are significantly decreased during ischemia (42, 69, 78).

Mitochondrial permeability transition pore formation. The mitochondrial permeability transition pore (mPTP) is a nonselective multiprotein channel located in the inner mitochondrial membrane that is permeable to $\leq$1.5-kDa solutes. It plays an important role in I/R-induced damage and is well studied in the heart, but not in skeletal muscle. The exact composition of the mPTP remains uncertain, although several proteins seem to be involved in its formation and regulation (adenine nucleotide translocase, phosphate carrier, F0F1-ATP synthase, cyclophilin D, voltage-dependent anion channel, translocator protein, hexokinase II, Bcl-2 family members, glycogen synthase kinase-3B, and PKCe) (65, 122, 130).

The mPTP is regulated by various cell factors, such as ROS, Ca$^{2+}$, ATP, and P1 concentrations, pH, and membrane potential (121). Griffiths and Halestrap (58) showed that mPTP remains closed during cardiac ischemia. After the biochemical changes that occur during ischemia (increase in ROS, Ca$^{2+}$, and P1 levels and decrease in ATP content and membrane potential), the mPTP was primed but remained closed because of the low intracellular pH. More recently, Seidlmaier et al. (168) demonstrated that cardiac ischemia is associated with transient mPTP opening to protect cells from Ca$^{2+}$ accumulation and ROS production (Fig. 3).

In summary, skeletal muscle ischemia is associated with a number of deleterious consequences, which are shown chronologically in Fig. 4A. There is a gradual depletion of intracellular energy stores together with an accumulation of products from glycolysis: H$^+$ and Ca$^{2+}$. Mitochondria are particularly affected; there are changes in OXPHOS and ROS generation. Cell damage is likely irreversible after ~4 h of ischemia. Because 7 h of ischemia causes the death of almost all the muscle tissue, reperfusion is necessary and should be initiated promptly to preserve skeletal muscle.

Cellular and mitochondrial events during reperfusion. Reperfusion, which is necessary to stop ischemic injury, is an additional source of cell damage, termed “reperfusion injury.” Jennings et al. (80, 81) were the first to show the deleterious effects of reperfusion on the myocardium. In skeletal muscle, as in other organs, reperfusion injury can be fatal, damaging cells and organelles such as mitochondria by different mechanisms (83).

Oxygen paradox. A high oxygen supply at the beginning of reperfusion is the primary cause of reperfusion injury and myocyte death by generation of excessive quantities of ROS. At the cell surface, hypoxanthine produced during ischemia is converted to uric acid by XO in the presence of oxygen molecules (133), producing $O_2^{-}$ and H$_2$O$_2$ and participating in local and systemic inflammatory processes in vascular cells. Therefore, contrary to hindlimb ischemia, with few sources of ROS, several potential sources are activated at the moment of reperfusion and generate a massive burst of free radicals with a greater variety of reactive molecules (mainly $O_2^{-}$, H$_2$O$_2$, HO, NO, and peroxynitrite) (63, 83,
184). This was the case in the extracellular environment and endothelial and muscle cells (47, 192). At the onset of reperfusion, mitochondria damaged during ischemia are no longer able to function efficiently. Complex I is reversed following the accumulation of succinate during ischemia (30), and mitochondrial complexes I and III are impaired and produce O$_2^-$ and H$_2$O$_2$. It is known that mitochondrial ROS production is a self-amplified process, termed “ROS-induced ROS release.” The possible mechanisms described in the literature are 1) a mitochondrial membrane depolarization that triggers mPTP opening and 2) opening of a mitochondrial inner membrane anion channel (57, 205). Other potential important sources of free radical generation at reperfusion include NADPH oxidases, NO synthases, and XO (8, 57, 83, 86, 98). However, even if some reports suggest that mitochondria could not be a predominant source of ROS (76), activation of these processes seems to require an initial burst of mitochondrial ROS and contributes to secondary tissue damage and inflammation (31).

The possibility that these different sources do not produce ROS separately but, rather, together comes to light. Indeed, ROS produced by one enzymatic source could activate another source and enhance ROS production (57). Interactions between NADPH oxidases and mitochondria or between NO synthases and XO have been reported, but not in skeletal muscle (34, 36, 40, 43, 155).

This burst of ROS cannot be eliminated, because the antioxidant defenses (at least nonenzymatic) are also altered by ischemia. Indeed, excessive ROS production and a decrease in antioxidant systems, such as glutathione, GPx, SOD, and catalases, after reperfusion have been demonstrated (28, 61, 107, 151, 174, 178, 179). Decreases in SOD2, catalases, and GPx have been verified in patients with PAD (146, 148).

Oxidative stress induces nonspecific changes in lipids via lipoperoxidation, proteins via nitration and oxidation, and DNA via base and sugar mutations (139). All these modifications are responsible for protein, enzyme, and receptor dysfunctions, membrane rigidity and permeabilization, and cell death (48, 59, 62, 192). For example, ROS may directly release mitochondrial endonuclease G or apoptosis-inducing factor, two proteins that promote DNA fragmentation and apoptosis in skeletal muscle (45). Also, cardioperoxidation may impair mitochondrial complex activities, release cytochrome c, and be responsible for apoptosis and mPTP opening (97, 172).

**CALCIUM PARADOX.** With reoxygenation, the respiratory chain of undamaged mitochondria produces ATP and the mitochondrial membrane potential is recovered. However, in cells subjected to ischemia, reperfusion further alters the activity of mitochondrial complexes I–IV as described in experimental models and PAD (28, 108, 146, 147, 176, 179). The impaired function decreases ATP synthesis (Fig. 2) (2, 92, 135, 181) and produces excessive ROS, which affects the membrane channel, including ATP-dependent and -independent exchangers, and further increases cytosolic Ca$^{2+}$ concentration (18, 87, 184). To attempt to reduce this Ca$^{2+}$ accumulation, mitochondria take up Ca$^{2+}$ via the membrane potential-dependent Ca$^{2+}$ uniporter (112). However, the elevated cytosolic Ca$^{2+}$ concentration activates phospholipases and proteases and interacts with other cell compounds. Membrane receptors, enzymes, and ion channels are affected, leading to cell membrane degradation, decreased cell viability, and cell death (55, 194).

**PH PARADOX.** Osmotically active molecules accumulate in the extracellular environment during ischemia and are eliminated by blood recirculation, generating an osmotic gradient between...
**A ISCHEMIA**

1. **↓ Oxygen and nutrients**
2. **↓ Aerobic and anaerobic glycolysis**
   - **↓ Phosphocreatine**
3. **↑ Lactates**
   - **↑ [H⁺]**
4. **↓ pH**
   - **↑ [Na⁺]**
   - **↑ [Ca²⁺]**
5. **↓ Cell integrity**

**Blood circulation**

**Hypoxanthine Xanthine**

**Myocyte**

**ATP synthase reverse**

**mPTP closed**

**Sarcoplasmic reticulum**

**Mitochondria**

**Uniporter**

**ATPase ATPase**

**Na⁺**

**K⁺**

**Ca²⁺**

**H⁺**

**[Ca²⁺]**

**[ADP] + [Pi]**

**[Na⁺]**

**ROS**

**Uncoupled and dysfunctional respiratory chain**

**Altered antioxidant system**

**Oxygen and nutrients**

**Cell death**

**Cell damages**

**Inflammation**

**Blood circulation**

**Hypoxanthine Xanthine**

**Local & systemic inflammation**

**Cell death**

**Microvascular changes**

**Uncoupled and dysfunctional respiratory chain**

**Altered antioxidant system**

**Cell death**

**Inflammation**

**Cell death**

**Blood circulation**

**Hypoxanthine Xanthine**

**Local & systemic inflammation**

**Cell death**

**Microvascular changes**

**Uncoupled and dysfunctional respiratory chain**

**Altered antioxidant system**

**Cell death**

**Inflammation**

**Cell death**

**Blood circulation**

**Hypoxanthine Xanthine**

**Local & systemic inflammation**

**Cell death**

**Microvascular changes**

**Uncoupled and dysfunctional respiratory chain**

**Altered antioxidant system**

**Cell death**

**Inflammation**

**Cell death**
the extra- and intracellular environments (Fig. 2). This leads to water uptake, swelling, and breakup of cells (192). Lactate, 
H⁺, and precursors of adenine nucleotide metabolism are also washed out (66, 133, 135). Extra- and intracellular pH are rapidly restored via activation of several exchangers at the beginning of reperfusion (37, 118), inducing lethal cell injury through mPTP opening (168). Indeed, this rapid regularization of acidosis plays an essential role in reperfusion injury. Maintaining a low intracellular pH at the onset of reperfusion delays mPTP opening and is cardioprotective (32, 73).

**MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING.** Opening of the mPTP at the onset of reperfusion occurs due to ROS, pH, Ca²⁺, ATP, and membrane potential (Fig. 3). This opening leads to mitochondrial OXPHOS uncoupling, resulting in further ATP depletion, membrane potential depolarization, and water entry into the mitochondrial matrix. The mitochondrial swelling permeabilizes and ruptures the mitochondrial membranes and leads to cell death. Apoptosis is related to the release of proapoptotic factors (cytochrome c and apoptosis-inducing factor) from the mitochondrial intermembrane space and activation of caspase cascade. Necrosis results from the activation of proteases, caspases, and phospholipases and inflammation (122, 178). Specific inhibition of mPTP opening by cyclosporine A in skeletal muscle partly protects mitochondrial functions and limits deleterious effects of reperfusion, demonstrating the essential role of mPTP in I/R injury (151). However, cyclosporine A protection is lost in aged animals (152).

In summary, even if reperfusion is essential to skeletal muscle survival, numerous deleterious events occur in muscular fibers and mitochondria following reperfusion. Excessive ROS production, Ca²⁺ overload, mitochondrial dysfunction, and mPTP opening lead to myocyte death, as detailed chronologically in Fig. 4B.

**Phenomena Associated with Skeletal Muscle Injuries Following I/R.**

**Morphological changes in the entire muscle environment.** Muscle cell structural changes and morphological lesions were identified during ischemia, with the degree of severity increasing with the duration of ischemia and during reperfusion, when alterations increase (22, 88, 96, 118, 182). Hindlimb ischemia alters the fiber structure and begins to disorganize and degenerate myocytes. Fibers are atrophic, have smaller diameters, and are cytoplasmically heterogeneous, and the morphology of their mitochondrial cristae is modified (13, 22). Ischemia also progressively alters the microcirculation and endothelial cells. Vascular permeability increases following complete disjunction of adjacent endothelial cells, and endothelial cells develop interspersed edema. Furthermore, erythrocytic, thrombotic, and leukocytic interactions develop in the microcirculation after ≥4 h of ischemia (13).

Reperfusion further degenerates fibers that are highly disorganized and hypercontracted, with clusters of mitochondria and SR swelling. In the microcirculation, red blood cells are compacted and deform and break the endothelium, which damages the local environment (13, 22). Cytosolic enzymes are released into the blood circulation, and ROS are produced by XO through O₁ oxygen molecules, which mediate prominent thrombotic interactions, characterized by an increase in platelets (13, and 2) leukocytic interactions, characterized by white blood cell adherence, infiltration, and activation (see below). Muscle tissue shows edema, inflammation, and necrosis, contributing to the no-reflow phenomenon (13, 63). Necrosis still develops 72 h after reperfusion, whereas edema progressively disappears. Severe inflammatory infiltrates are observed at 24 h of reperfusion and decrease at 72 h, when regeneration phenomena were observed (22).

**Impairments in mitochondrial dynamics.** Mitochondria are dynamic and motile organelles that continuously collide, fuse, or divide (197). Emerging data suggest that changes in mitochondrial dynamics occur in response to acute I/R and are involved in deleterious effects, modifying mitochondrial morphology and functions (19, 131). Although mechanisms are unclear, a high cytosolic Ca²⁺ concentration and an imbalance between fission and fusion proteins seem to be involved. Indeed, several research groups have demonstrated in cardiomyocytes that simulated I/R reduces fusion protein expression [optic atrophy-1 (OPA-1) and mitofusin 2] and increases fission protein expression [dynamin-related peptide 1 (DRP-1) and mitochondrial fission protein 1], which modify mitochondrial morphology (fragmentation and changes in cristae) and motility, alter mitochondrial functions, and favor mPTP opening and cell death (16, 29, 79, 132, 187). Ong et al. (132) showed that simulated I/R in HL-1 cells increases mitochondrial fission through DRP-1 and that transfection of HL-1 cells with fusion proteins or with a dominant-negative mutant form of DRP-1 protects cells from I/R damage. Varanita et al. (187) demonstrated that mild OPA-1 overexpression in transgenic mice protects from muscular atrophy and I/R damage in the heart and brain. These protections imply the preservation of mitochondrial morphology in OPA-1 transgenic mice, as mitochondria are longer in diaphragm muscle and cristae are tighter in the heart. Furthermore, recent work has demonstrated that protection against cardiac I/R injury implies inhibition of mitochondrial fission, either directly by inhibiting fission machinery (41) or via remote ischemic preconditioning (24). Mitochondrial dynamics in skeletal muscle have been studied during exercise (44), but not during ischemic preconditioning (24). This study raises an important point about acute experimental models of prolonged ischemia, where animals are anes-the-
tized and immobile: the contractile inactivation could be a confounding factor and contribute to I/R injury via alterations in mitochondrial dynamics.

Local and systemic inflammation. Ischemia induces numerous modifications of cellular molecules that pave the way for inflammation following reperfusion. Damage-associated molecular patterns (DAMPs), which are endogenous molecules derived from damaged cells, are implied in the inflammatory response following ischemia. They notably include high-mobility group box 1 and ATP (75, 136, 164, 196). Mitochondria altered by different cellular stresses and necrosis also release mitochondrial DNA, which could be involved in I/R-induced inflammation (102, 127, 143, 203). In several organs, DAMPs are ligands of Toll-like receptors (TLR), which participate in inflammation through the release of cytokines, chemokines, and interferons during the pathogenesis of ischemic injury. It has been suggested that TLRs are also implied in the skeletal muscle inflammatory response (52, 60, 94, 99), and, for the first time, Patel et al. (136) recently showed that some of these TLRs are upregulated in muscle biopsies of patients with critical limb ischemia, the most severe form of PAD. Other molecules secreted or expressed during ischemia participate in inflammation. Nonmuscle myosin heavy chain type II, a stress-induced self-antigen, is expressed on the cell surface (201). Chemokines are locally secreted (169, 186), and adhesion receptors, such as E-selectin and ICAM-1, are expressed on the surface of endothelial cells (51, 117).

At reperfusion, in association with muscle and endothelial cell injuries listed above and adhesion molecules expressed at the cell surface during ischemia, ROS produced via XO in endothelial cells elicit an intense proinflammatory response that increases muscle damage (21, 63, 89, 116). Indeed, ROS participate in the elaboration of chemoattractant stimuli and expression and/or activation of adhesion molecules to allow the interaction between neutrophils and endothelial cells. Leukocyte recruitment involves a complex series of events requiring several adhesive molecules (for review see Refs. 13, 56, 63, and 90). Briefly, leukocytes, particularly neutrophils, attracted by chemokines accumulate in the microcirculation during reperfusion (21, 163, 167, 202) and express adhesion molecules, such as CD11/CD18, at their surface. In the presence of adhesion molecules, such as E-selectin, P-selectin, and ICAM-1, at the endothelial cell surface, neutrophils migrate into the extravascular space through endothelial adhesion receptors, where they are activated via sensing of DAMPs by innate immune receptors (62, 161). Once activated, neutrophils also produce ROS through NADPH oxidases, and this burst of ROS causes further cell injuries such as lipid peroxidation (56, 63). Leukocyte extravasation in the reperfused muscle is highly correlated with muscle injury (166). Neutrophils are among the first cells recruited to the inflammatory site, at 1 h after reperfusion. In association with other phenomena, such as complement and coagulation activation (4, 61), they provoke muscle cell lysis and necrosis through their myeloperoxidase.

Fig. 5. Schematic overview of the chronology of myocyte ischemia-reperfusion injury. Ischemia triggers deleterious events that become irreversible after 3 h and induce total myocyte death at ∼7 h. Necessary, but late, reperfusion rapidly induces mitochondrial permeability transition pore (mPTP) opening and inflammation, causing further myocyte death and remote organ failure. ROS, reactive oxygen species.
activity (124, 171) and contribute to the no-reflow phenomenon (62, 63), increasing I/R-induced tissue damage. Macrophages reach the ischemic site 48–72 h after reperfusion (68, 170). Their exact role remains unclear, since they may be implicated in both I/R-induced tissue damage and recovery; Hammers et al. (68) identified two prominent macrophage populations (inflammatory and anti-inflammatory) at 3 and 5 days of reperfusion. Noncellular factors, especially natural IgM, also mediate reperfusion injury by recognizing non-muscle myosin heavy chain type II expressed during ischemia and activating the complement system (6, 201).

Succinate could also participate in inflammation during I/R. Indeed, Chouchani et al. (30) demonstrated in the heart and other organs that accumulation of succinate during ischemia leads to deleterious effects through ROS production by the mitochondrial respiratory chain and can participate in inflammation. Recent evidence supports the idea that this metabolite acts as a proinflammatory signal via direct (i.e., stabilizing hypoxia-inducible factor-1α and favoring inflammation via IL-1β) and indirect (i.e., producing ROS for stabilizing hypoxia-inducible factor-1α) mechanisms (119).

Depending on the skeletal muscle mass subjected to I/R and the duration of ischemia, systemic inflammation and remote organ failure can occur following an increase in enzymes and cellular molecules in the systemic circulation, a phenomenon known as “crush syndrome” (173). Several proinflammatory cytokines produced during I/R, especially TNFα and IL-1, can mediate lung failure (167). A high level of K+ leads to cardiac dysfunction, whereas the release of myoglobin causes renal failure (47). Other factors are implied in multiple organ lesions after hindlimb I/R (199, 200).

CONCLUSION

Skeletal muscle I/R injury is an important clinical problem in PAD that, if not promptly treated, results in significant morbidity and mortality. I/R injury begins with biochemical and morphological changes, and mitochondria play a crucial role, as they are at the crossroads of energy production, cell signaling, oxidative stress, and cell death. Mitochondrial damage can lead to cell death, contribute to inflammation, and elicit changes in remote organs (Fig. 5). Numerous pharmacological and surgical interventions have improved patient recovery and clinical outcome. However, knowledge of mechanisms responsible for I/R and their chronology will allow the development of new therapies. Emerging data on mitochondrial dynamics should identify new therapeutic targets and develop protective strategies against PAD.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.P. and A.-L.C. prepared the figures; S.P. drafted the manuscript; S.P., A.-L.C., A.M., A.L., J.W.S., N.C., J.Z., and B.G. approved the final version of the manuscript.

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