RONS and Myokines in Skeletal Muscle Glucose Uptake

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The skeletal muscle is the largest organ in the body that performs different functions, including those related to the movement of the body such as stability, equilibrium, and locomotion; vital functions such as breathing; and those associated with the maintenance of metabolic homeostasis, in which the generation and expenditure of energy and heat production are critical. The adequate interplay of these functions leads to the maintenance of life in organisms. Glucose is essential in metabolism since it is one of the main substrates that produces ATP, the key molecule that transfers energy during chemical reactions in organisms. To produce ATP, glucose needs to be transported from the extracellular space into the cytosol of the cell. This process is called glucose uptake, and it is critical in skeletal muscle since it provides enough glucose to the cell to produce ATP and satisfy the high demand for energy of the skeletal muscle. Glucose uptake in skeletal muscle tissue is a process mainly regulated by insulin, which is a hormone synthesized in the pancreas and released into the blood stream, where it is transported until it binds to specific insulin receptors that are anchored at the plasma membrane of skeletal muscle cells.

Keywords: ROS ; RNS ; nitric oxide ; myokines ; cytokines ; glucose transport ; GLUT4

1. Introduction

Skeletal muscle is mainly composed of fibers, which are postmitotic multinuclear cells formed from the fusion of single cells, known as myoblasts [1][2]. Muscle fibers continuously generate reactive oxygen and nitrogen species (RONS), and the production of RONS may be augmented in different situations, such as during contractile activity, inflammation, regeneration, and the aging of skeletal muscle [3]. When RONS are generated in excess and the intracellular antioxidant systems are unable to neutralize these highly reactive molecules, the skeletal muscle is exposed to oxidative distress and the RONS react with cellular macromolecules, such as lipids, proteins, carbohydrates, and nucleic acids, producing irreversible changes in those molecules and compromising the viability, integrity, and function of the cells [4]. However, when the level of intracellular RONS is moderate or relatively low, i.e., oxidative eustress, some of that RONS may act as signaling molecules, mediators or second messengers, which, through reversible redox reactions with specific residues of macromolecules, may modulate and regulate cellular signaling pathways that drive different cellular processes [4]. Over the last two decades, researchers in the field of redox biology have claimed and pointed out the importance of investigation and improving our knowledge regarding the function of RONS participating as signaling molecules in different pathways and how they are involved in the modulation, regulation, and control of cellular functions. In addition to the general physiological control of glucose uptake in skeletal muscle led by insulin, it has been proposed that RONS may be involved in the regulation of glucose uptake in skeletal muscle. However, it is uncertain whether this process might be independent or dependent of the effect of insulin [5][6][7]. Consequently, there is a need to investigate the role of RONS in the glucose uptake process in skeletal muscle. This knowledge may help us to find new targets for the treatment of the impairment of glucose uptake and insulin resistance manifested in type 2 diabetes, obesity, and aging.

Skeletal muscle is considered a secretory organ and liberates different cytokines, peptides, proteins, or hormones known as myokines that may have endocrine and auto/paracrine effects [8]. Myokines, such as myostatin, irisin and IL-6, among others, induce changes in the muscle itself, as well as in other organs and tissues [9]. These peptides, proteins or hormones regulate or alter the functionality and adaptation of muscle tissue, modifying its metabolism and other functions including hypertrophy or muscular atrophy, angiogenesis, and inflammatory processes [10]. Myokines may interfere with the prevention of obesity, metabolic syndrome, and type 2 diabetes. Consequently, there is an important focus in biomedical research to evaluate the potential of myokines as new therapeutic targets [9]. It appears that either the auto/paracrine effect of different myokines or the endocrine effect of different hormones, cytokines and factors that interact with skeletal muscle may affect glucose uptake and insulin resistance. However, the molecular signaling pathways and the physiological impact of these molecules remain largely unexplored.

2. Reactive Oxygen and Nitrogen Species in Skeletal Muscle

Around 1954, the first report was published that indicated that skeletal muscle produces free radical intermediates [11]. Twenty years later, two studies indicated that exercise was related to the generation of products from different reactions that occur between free radicals, referred to today as RONS, and cellular biomolecules [12][13]. These findings were consolidated in the 1980s when it was demonstrated that intense exercise produces free radicals in skeletal muscle [14]. Studies in the 1990s uncovered the fact that the contractile activity of skeletal muscle produced specific reactive species. Thus, it was demonstrated that superoxide was released from the diaphragm when this muscle contracted [15], nitric oxide was released from skeletal muscle [16], and hydroxyl radicals were generated in skeletal muscle during contractions [17]. The previous studies and other derived investigations have built upon this knowledge and created the current scenario that describes the generation of reactive oxygen and nitrogen species in skeletal muscle. For more detail, the reviews of [3][18][19][20] may be consulted.

Basically, the main ROS generated in skeletal muscle cells (and cells from other tissues) are free radical species, such as superoxide anion ($O_2 \cdot -$) and hydroxyl radical (HO·), and others considered nonradical species, such as hydrogen peroxide (H_2O_2). All of these are considered reactive oxygen species (ROS) since they originate from a reduction of oxygen and the reactive atom is oxygen. In the case of reactive nitrogen species (RNS), which are reactive species generated from nitrogen, the most relevant are nitric oxide (NO·), which is a free radical species, and peroxynitrite anion (ONOO-), which is a nonradical species, although very reactive [19]. Superoxide and nitric oxide are the primary species generated in skeletal muscle fibers and lead to the formation of other secondary species such as hydrogen peroxide, hydroxyl radical and peroxynitrite, all of which may be generated in the intracellular and extracellular space, in the mitochondrial matrix, and in the mitochondrial intermembrane space. The contractile activity of skeletal muscle fibers evokes an increase in the intracellular generation of superoxide, hydrogen peroxide and nitric oxide [21][22][23][24]. In addition, contractile activity increases the production of superoxide, hydrogen peroxide, hydroxyl radicals and nitric oxide extracellularly in the muscle interstitial space [25][26]. Most of the studies indicated that the increase in ROS generation caused by contractions in skeletal muscle was attributed to the increase in mitochondrial respiration due to the high demand for energy production in the mitochondrial matrix by the electron transport chain and ATP synthase, which conduct the formation of ATP, which is the source of energy for contractile activity. Therefore, mitochondria were attributed as the main source of ROS production in skeletal muscle. However, later studies indicated a lower increase in ROS and discovered other nonmitochondrial sources for ROS generation [19]. Thus, NADPH oxidases were identified in the skeletal muscle plasma membrane [27], the sarcoplasmic reticulum [28], and T-tubules [29]. The product of NADPH oxidase activity is superoxide, and it is known from the later studies that NADPH oxidases contribute substantially to the production of superoxide and, potentially, hydrogen peroxide in skeletal muscle [30]. There are other sources of superoxide production that are less studied that may contribute to intracellular superoxide production in skeletal muscle, such as the enzyme phospholipase A2 [31]. In addition, the extracellular release of superoxide driven by a lipoxygenase was identified in skeletal muscle [32]. Another enzyme that may contribute to superoxide generation in the extracellular space of skeletal muscle is the xanthine oxidase located in the endothelial cells associated with skeletal muscle. It has been reported that this enzyme increases the superoxide concentration in the extracellular fluid of skeletal muscle during contractile activity [33].

Nitric oxide, the primary reactive nitrogen species (RNS), is generated enzymatically through nitric oxide synthase (NOS). NOS catalyzes the conversion of the amino acid L-arginine into L-citrulline, with cofactors NADPH and oxygen, and generates nitric oxide [19][20]. There are three NOS isoenzymes: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). nNOS is expressed in neurons and skeletal muscle, eNOS is expressed mainly in endothelial cells and in skeletal muscle with less expression, and iNOS appears to be associated with inflammatory processes that, in some circumstances, might affect the skeletal muscle [23]. Balon and Nadler were the first to identify the release of NO from skeletal muscle, which was augmented by contractile activity [16]. Other studies reported that skeletal muscle releases nitric oxide to the extracellular space, although they were uncertain which cells (i.e., skeletal muscle fibers, lymphocytes, fibroblasts, and endothelial cells) originate NO and the contribution they make to the total extracellular NO concentration [34][35]. Further work uncovered the release of NO specifically from skeletal muscle cells, such as myotubes [21][26]. In addition, it was indicated that the contractive activity of skeletal muscle produced an increase in the intracellular concentration of NO in a model of myotubes of a rat skeletal muscle origin [21]. Further studies, using the model of isolated single muscle fibers, demonstrated that contractile activity induced in fibers by electrical stimulation produced a rapid increase in the intracellular concentration of NO, which also rapidly decreased when contractile activity ceased [23]. Moreover, studies performed with a model of skeletal muscle fibers subjected to a protocol of passive elongation showed that the passive stretch applied to fibers isolated from old mice produced an increase in nitric oxide intracellular activity [36].

Hydrogen peroxide, hydroxyl radical and peroxynitrite are reactive species derived from the primary RONS, superoxide and nitric oxide, that play a relevant role in oxidative distress [37]. In skeletal muscle cells and other tissues, hydrogen peroxide is enzymatically generated in the cytosol, the mitochondrial matrix, and the extracellular space. Hydrogen peroxide is generated by the dismutation of superoxide in a reaction that is catalyzed by the enzyme superoxide dismutase (SOD). There are SOD isoenzymes that are expressed in different subcellular compartments. Manganese-SOD (Mn-SOD) is expressed in the mitochondrial matrix, copper/zinc-SOD (Cu/Zn-SOD) is expressed in the cytosol and mitochondrial intermembrane space, and there is an extracellular SOD (ec-SOD) that appears in the interstitial fluid in skeletal muscle [19][20]. Therefore, hydrogen peroxide is generated at different subcellular locations. When H_2O_2 concentration is high, it may appear oxidative distress in the cell. However, a lower H_2O_2 concentration leads to oxidative eustress, which is crucial for regulating and maintaining cellular processes through the modulation of different signaling pathways in the cell [4][37]. H_2O_2 may produce another secondary reactive species, such as hydroxyl radicals (HO·). This may occur when H_2O_2 is in the presence of divalent metals, mainly iron (Fe^{++}), and H_2O_2 is decomposed to HO· by the Fenton reaction. The hydroxyl radical is a strong oxidant that is not diffusible through membranes and reacts with every close molecule. This produces alterations in macromolecules (lipids, proteins, and nucleic acids), which may disturb cellular homeostasis and other functions [19][20]. In other words, the hydroxyl radical mainly evokes oxidative distress. Peroxynitrite (ONOO-) is produced in a reaction between nitric oxide and superoxide [38]. This reaction is three times faster compared with the reaction of superoxide dismutation that produces hydrogen peroxide. Therefore, this is the primary reaction when both superoxide and hydrogen peroxide appear close together. Peroxynitrite shows a high oxidizing power and is very reactive; moreover, it may oxidize thiol groups of peptides, damage DNA and cause the nitration of proteins [19][20]. Hence, an abundance of peroxynitrite may potentially evoke oxidative distress.

Concomitant to RONS generation, cells are equipped with an antioxidant system that neutralizes the activity of reactive molecules. Therefore, there is a balance between the production and elimination of RONS to maintain the level of RONS at a steady state, facilitating homeostasis and cellular function. A moderate increase in the level of ROS is critical to trigger and modulate cellular signaling pathways that control several cellular functions. As mentioned above, this state is referred to as oxidative eustress [4]. However, when the level of RONS is elevated, if there is an excess of RONS being produced or the antioxidant system is unable to neutralize the RONS produced, then these reactive species interact with and oxidize other molecules (lipids, proteins, DNA, and carbohydrates), causing oxidative damage to them and possibly affecting their normal functions, which, ultimately, could compromise cellular homeostasis and viability. This state, already mentioned above, is currently referred to as oxidative distress in redox biology [4], in place of the classical term of oxidative stress.

The cellular antioxidant system is constituted of both an enzymatic and nonenzymatic system. The main antioxidant enzymes are superoxide dismutase (SOD), which catalyzes the conversion of superoxide anions into hydrogen peroxide; glutathione peroxidase (GPx), which neutralizes hydrogen peroxide to convert it to water and appears at different subcellular locations, particularly the mitochondria and cytosol; and catalase (CAT), which transforms hydrogen peroxide to water. In addition, there are accessory proteins that protect cells against oxidation, such as peroxiredoxin (PRX), glutaredoxin (GRX), and thioredoxin reductase (TRX). For more details, other comprehensive reviews in the field, i.e., [19], may be consulted. The nonenzymatic antioxidant system of the cell involves compounds such as glutathione, vitamins C and E, carotenoids, polyphenols, alpha-lipoic acid, coenzyme Q10, uric acid and bilirubin. These compounds are involved in thiol–disulfide exchange reactions and play a role in maintaining the redox balance in the cell [3][19]. Glutathione (GSH) is the most abundant nonenzymatic antioxidant in the cell, and it plays an important role due to its high reductive power. Glutathione reacts with different RONS, transferring protons and neutralizing these reactive species. Furthermore, glutathione is the substrate for the antioxidant enzyme GPx, and participates in the recycling of other antioxidant enzymes, such as TRX and GRX. Moreover, glutathione leads to the reduction of other antioxidants such as vitamins E and C [19]. Glutathione is critical in contractile activity and in the adaptation of skeletal muscle to exercise. This has been an important matter of investigation in the field of redox biology. It is out of the scope here, but readers may refer to other comprehensive key publications that describe the crucial role of glutathione in the redox biology of skeletal muscle. i.e., [39][40][41].

To recapitulate, RONS are generated in skeletal muscle at rest and during contractile activity. A high level of RONS might cause the excessive oxidation of macromolecules, which leads to cellular damage and dysfunction. However, a moderate RONS level is essential to modulate redox signaling processes that regulate cellular homeostasis and adaptation. The term RONS refers to several specific species. Some of them are mainly prone to induce oxidative distress, such as hydroxyl radicals and peroxynitrite. However, other RONS appear to be responsible for oxidative eustress and, currently, they are considered to be signaling molecules. These include nitric oxide and hydrogen peroxide, and even superoxide, which is the precursor to hydrogen peroxide. Over the last two decades, the focus of research in this area has been centered on nitric oxide, in addition to other important roles of NO in physiology and pharmacology, and especially on

hydrogen peroxide. Different studies indicate that hydrogen peroxide has a crucial role in redox signaling pathways that regulate cellular homeostasis and other functions. Consequently, there is great interest in the field of redox biology and the knowledge continues to grow, although there is still much to discover.

3. Myokines Involved in Glucose Uptake in Skeletal Muscle

Skeletal muscle is receiving increasing attention as an endocrine organ due to its release of peptides or proteins, referred to as myokines, that may influence the metabolism of virtually every organ in the body, including the muscle itself. Myokines present a new paradigm for understanding how muscles communicate with other organs [42][43]. However, special attention has also been given to the auto/paracrine effects of myokines within skeletal muscle affecting muscle functions [44]. This view, originally addressed over three decades ago, suggests that proteins and other peptides produced, expressed, and released by muscle fibers after exercise exert autocrine, paracrine, or endocrine effects through actions on their receptors [45]. However, a myokine may be secreted independently of muscle contractions [46]. It is worthy of note that myokines may represent potential therapeutic targets to combat obesity and associated metabolic disorders such as insulin resistance and T2D. Myokines produced by muscles during contraction may improve insulin sensitivity and glucose oxidation via autocrine actions [47]. A secretome-based analysis of the human myocyte culture medium has revealed more than 600 myokines to date [48]. However, most of these myokines are still insufficiently characterized. In addition, the signaling pathways of certain myokines are altered in the skeletal muscle of patients with T2D [49]. Taken together, this suggests that myokine secretion is an important factor contributing to the development of muscle metabolic defects in T2D. However, whether these myokines and their effects influence both glucose uptake and GLUT4 translocation remains largely unexplored. Thus, an update on the myokines that have been identified in association with glucose uptake and insulin resistance is presented.

Fibroblast growth factor 21 (FGF21) has emerged as a promising therapeutic agent for the treatment of obesity and T2D [50]. FGF21 is a protein preferentially expressed in the liver, but it has also been described as a myokine since its expression and secretion are regulated by insulin and Akt activation [51]. This peptide hormone is secreted by several organs and can act on multiple tissues to regulate energy homeostasis [52][53]. FGF21 has been proposed as a novel metabolic regulator given its ability to normalize glucose and lipid metabolism and prevent the development of obesity and diabetes [50]. Recently, it has been reported that FGF21 regulates glucose uptake through a mechanism mediated by GLUT4 and that is dependent on atypical PKC-ζ- in skeletal muscle [54]. FGF21 gene therapy in animals receiving a long-term high-fat-diet feeding or in *ob/ob* mice showed marked reductions in body weight, adipose tissue hypertrophy and inflammation, hepatic steatosis inflammation and fibrosis, and insulin resistance due to the higher expression of FGF21 [55].

Irisin is a myokine that is secreted after exercise and is associated with increased energy expenditure because of its ability to stimulate the browning of white adipose tissue [56]. In skeletal muscle, it has been proposed that irisin stimulates glucose uptake after the activation of AMPK in L6 myotubes [57]. Decreased irisin secretion contributes to muscle insulin resistance, which was observed in high-fat-diet mice when the insulin action was significantly inhibited [58][59]. In addition, irisin reverses insulin resistance in C2C12 muscle cells via the p38-MAPK-PGC-1α pathway and enhances mitochondrial function [60]. Irisin improves fatty acid oxidation and glucose utilization in T2D by regulating the AMPK signaling pathway [61].

The identification of IL-6 as a myokine has created much interest around its acting as a metabolic regulator molecule [62]. However, the elevation of systemic IL-6, often in obesity and metabolic syndrome, and the role of IL-6 in metabolic disease remains controversial [62]. After exercise, IL-6 plasma levels rise because of the increased local production in muscle, and this increase may enhance substrate metabolism and whole-body glucose homeostasis [63][64][65]. Acute IL-6 exposure increases glucose metabolism in resting human skeletal muscle without changing insulin-stimulated glucose transport and insulin signaling [66]. On the contrary, it has been reported that IL-6 administration increases insulin sensitivity in vitro in muscle via the AMP-activated protein kinase [47]. Interestingly, IL-6 induces lipolysis and free fatty acid release from adipocytes and skeletal muscle [67]. In addition, low-grade systemic inflammation is one of the earliest and main pathological events that might lead to the development of insulin resistance [68]. Therefore, IL-6 may exert both pro- and anti-inflammatory effects and may even promote muscle anabolism or catabolism depending on the target structure, the predominant cytokine environment, and the mode of release [69].

Apelin is a peptide secreted from various tissues that has been classically characterized as an adipokine. Moreover, it has been described as a myokine that improves glucose metabolism and shows antidiabetic properties [70][71]. Apelin knockout mice are insulin resistant, a condition that can be reversed after apelin treatment [72]. It has been reported that both short- and long-term apelin treatments improve insulin sensitivity in obese and insulin-resistant mice, mainly due to the increase

in glucose uptake in skeletal muscle that was observed in [72][73]. Using in vivo and in vitro pharmacological and genetic approaches, the involvement of eNOS, AMP-activated protein kinase, and Akt was reported in apelin-stimulated glucose uptake in the soleus muscle [73]. In C2C12 myotubes, apelin increased glucose uptake and Akt phosphorylation [72]. Apelin expression is induced by exercise signaling pathways and secreted in vitro in human primary myotubes [74]. However, it has recently been reported that exercise-induced insulin sensitization occurs independently of plasma apelin changes [75]. Interestingly, apelin treatment increases complete fatty acid oxidation, mitochondrial oxidative capacity, and biogenesis in the muscle of insulin-resistant mice. This suggests that the improvement in the insulin sensitivity triggered by apelin might be secondary to the decrease in adiposity, and it might be due to a direct action in skeletal muscle [76].

Myostatin is a myokine of the TGF-β superfamily expressed in both embryonic and adult skeletal muscle that regulates muscle mass and function, producing muscle atrophy [77]. Serum myostatin is upregulated in obesity and correlates with insulin resistance in humans [78]. The overexpression of myostatin in mice causes insulin resistance [79] whereas anti-myostatin antibodies prevent obesity [80] by stimulating fatty acid oxidation and increasing energy expenditure [81]. In addition, it has been reported that myostatin inhibits glucose uptake via the suppression of insulin-dependent and -independent signaling pathways in skeletal muscle [82]. Myostatin inhibits low basal glucose uptake, insulin-induced IRS-1 tyrosine (Tyr495) phosphorylation, and both the expression and activation of PI3K, along with diminishing Akt phosphorylation, which leads to a reduction in insulin-induced GLUT4 membrane translocation and glucose uptake [82]. In addition, this myokine decreases AMPK activity, which is accompanied by reduced GLUT4 gene expression and glucose uptake [82]. Moreover, it has been reported that myostatin regulates glucose metabolism via the AMPK pathway by promoting glucose consumption and glucose uptake, increasing glycolysis, and inhibiting glycogen synthesis in skeletal muscle cells [83].

Musclin is a factor secreted by skeletal muscle and is a potent regulator of glucose metabolism [84]. In humans, an increase in the circulating musclin level has been reported in diagnosed T2D patients [85] and those with metabolic syndrome [86]. In the latter, a positive correlation was found between both altered insulinemia and glycemia and a body composition profile with high visceral fat and lean mass [86]. The musclin-induced impairment of insulin-stimulated glucose uptake in skeletal muscle is related to Akt inhibition and PPARγ/LXRα in mice [87] and causes endoplasmic reticulum stress in rats [88]. Recently, it was found that a reduction in muscle-derived musclin production through chronic resistance exercise was involved in improving insulin resistance in rats with T2D [89]. Exercise training improves lipid metabolism and insulin sensitivity by upregulating GLUT4 and downregulating musclin in skeletal muscle [90].

Brain-derived neurotrophic factor (BDNF) is a member of the nerve growth factor family that is generated mainly by the brain [91], but is also secreted by skeletal muscle in response to contractions, and enhances fat oxidation via the activation of the AMP-activated protein kinase [92]. During short-duration aerobic exercise, immediately after a short-duration high-intensity exercise to exhaustion, there is a transient augmentation of serum BDNF concentration in humans [93]. Low levels of BDNF accompanying impaired glucose metabolism in T2D patients have been reported [94]. The repetitive subcutaneous or intracerebroventricular administration of BDNF ameliorates glucose metabolism by enhancing the glucose utilization in muscle in *db/db* mice [95]. Interestingly, a peripheral BDNF treatment promotes GLUT4 protein expression as well as hypophagia in skeletal muscle [96].

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