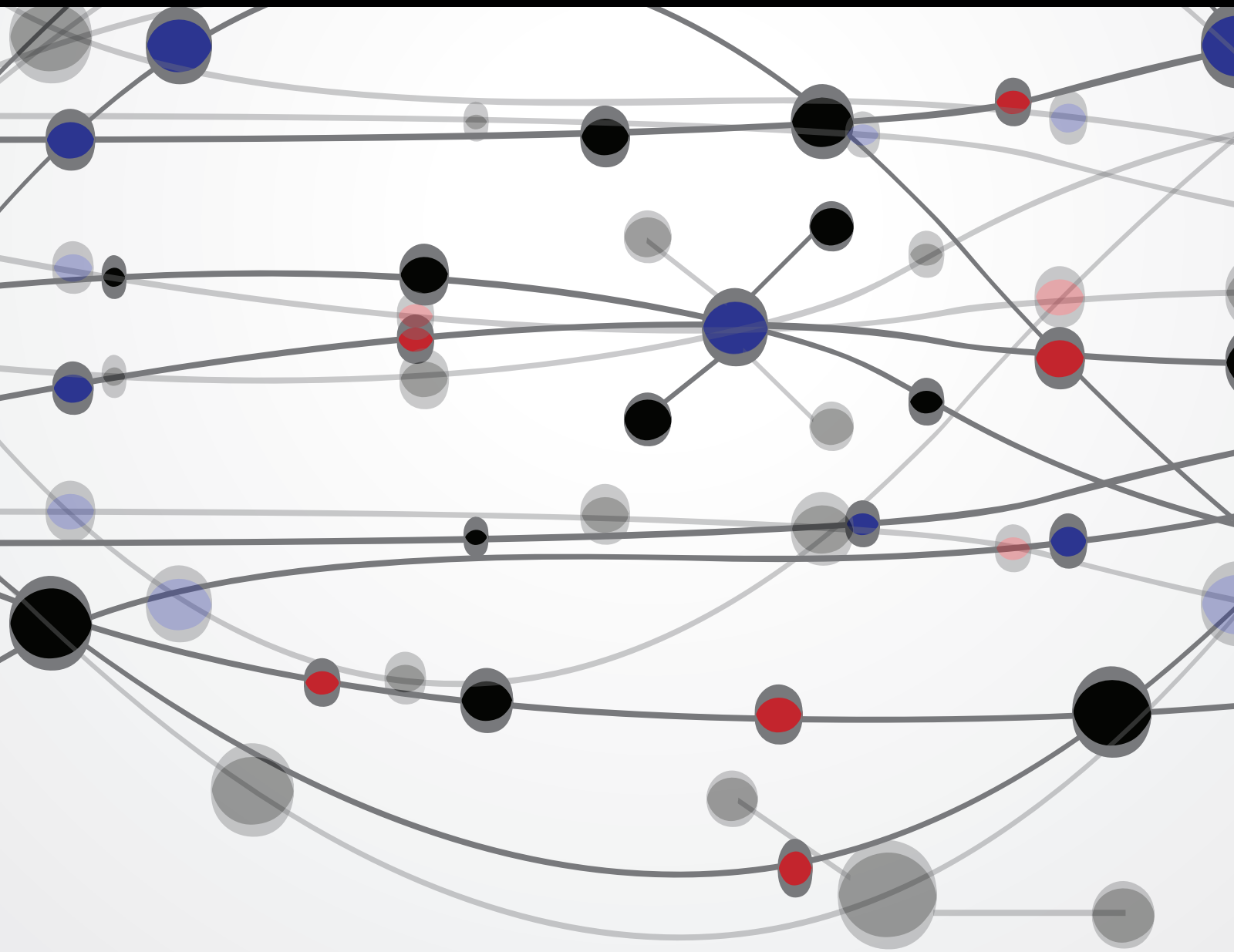
The Scientific World Journal

Skeletal Muscle Physiology

Guest Editors: Lucas Guimarães-Ferreira, Humberto Nicastro, Jacob Wilson, and Nelo Eidy Zanchi



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Skeletal Muscle Physiology

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In the beginning of the last century, muscle proteins were viewed as static structural molecules not capable of being utilized by other tissues or organs. This concept was accepted until the 30s, where Rudolf Schoenheimer presented strong evidences about the "Dynamic State of Body Constituents," which means that skeletal muscle is not only capable of contracting but also capable of releasing nitrogen derived molecules to be utilized by other organs and tissues (Guggenheim, 1991) [1]. Such concept established that skeletal muscle is a highly plastic tissue, adapting its structure and metabolism in response to diverse conditions such as contractile activity, mechanical overload, and nutrients. From this point of view several questions arise, specifically, how form follows function of skeletal muscles, as well as the synergistic role of nutrients. A large number of research groups around the world are helping to clarify this and many other questions. In particular in the last four decades, the growth in the number of publications on skeletal muscle subject is noteworthy (Figure 1).

In this special issue, the reader will be brought directly to a wide spectrum of articles regarding the skeletal muscle tissue. From France, a new hypothesis concerning how to consume amino acids and deal with catabolic conditions also is put in debate, focusing on the anabolic threshold concept (D. Dardevet et al., 2012). From the University of Tokyo, Japan, A. Wagatsuma and K. Sakuma (2013) summarize the current knowledge about the role of mitochondria as a regulator of myogenesis. From Brazil, C. O. Assumpção et al. (2013) present an extensive review on the effects of exercise-induced muscle damage on running economy in humans. Also, W. S. Dantas et al. (2013) discuss the impact of polycystic ovary syndrome on skeletal muscle tissue. A. Carsana (2013), from Italy, reviewed the documented cases of exertional rhabdomyolysis or stress-induced malignant hyperthermia and reported a possible association with RYR1 gene polymorphism. This special issue also presents original articles focusing on the cytokine response of skeletal muscle cells according its differentiation stage (M. Podbregar et al., 2013), the effects of tissue culture conditions on in vitro myogenesis (S. Hinds et al., 2013) and the differences in spontaneous physical activity between mice substrains (D. Coletti et al., 2013). All these discussions are being provided in order to generate new benefits and from athletes to debilitated populations; from basic to applied sciences. In this issue, our focus was not to restrict but, on the contrary, to be capable of proposing new hypotheses and ideas based on the current knowledge.

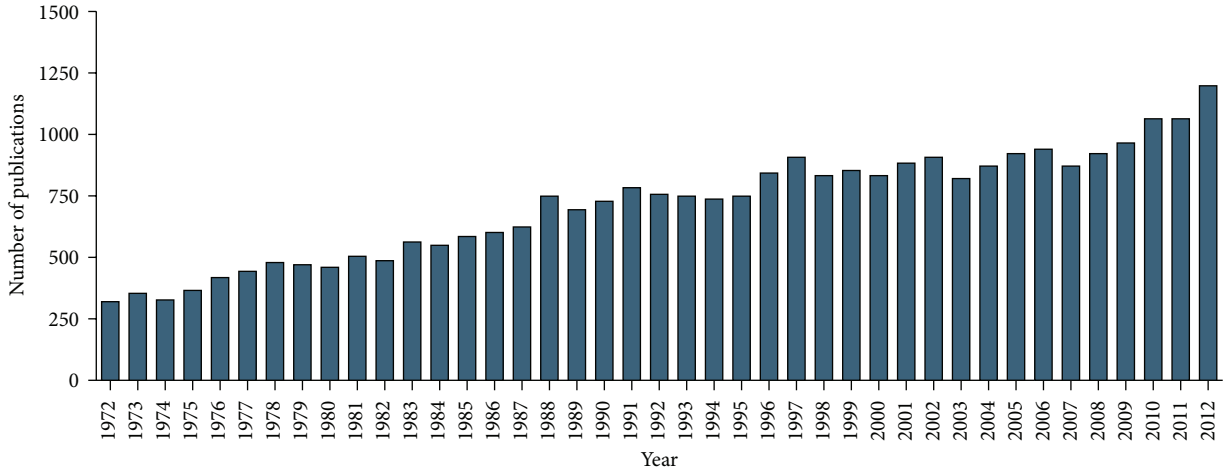

Figure 1: Number of publications in Medline database with "skeletal muscle" present on title. Data from the US National Library of Medicine National Institutes of Health (http://www.ncbi.nlm.nih.gov/pubmed?term=skeletal%20muscle [Title]).

Acknowledgment

We are pretty sure that our objective was successfully achieved. The reader will be surprised with the quality of the manuscripts as well as the diversity of skeletal muscle physiology research around the globe.

Lucas Guimarães-Ferreira
Humberto Nicastro
Jacob Wilson
Nelo Eidy Zanchi

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Review Article

Metabolic Disturbance in PCOS: Clinical and Molecular Effects on Skeletal Muscle Tissue

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Polycystic ovary syndrome is a complex hormonal disorder affecting the reproductive and metabolic systems with signs and symptoms related to anovulation, infertility, menstrual irregularity and hirsutism. Skeletal muscle plays a vital role in the peripheral glucose uptake. Since PCOS is associated with defects in the activation and pancreatic dysfunction of β-cell insulin, it is important to understand the molecular mechanisms of insulin resistance in PCOS. Studies of muscle tissue in patients with PCOS reveal defects in insulin signaling. Muscle biopsies performed during euglycemic hyperinsulinemic clamp showed a significant reduction in glucose uptake, and insulin-mediated IRS-2 increased significantly in skeletal muscle. It is recognized that the etiology of insulin resistance in PCOS is likely to be as complicated as in type 2 diabetes and it has an important role in metabolic and reproductive phenotypes of this syndrome. Thus, further evidence regarding the effect of nonpharmacological approaches (e.g., physical exercise) in skeletal muscle of women with PCOS is required for a better therapeutic approach in the management of various metabolic and reproductive problems caused by this syndrome.

1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting approximately 5–7% of women in reproductive age [1]. It was first described by Stein and Leventhal in 1935, who found an association between amenorrhea, hirsutism, and obesity with polycystic ovaries. The authors reported on bilaterally enlarged ovaries, with a thick and whitened capsule [2], multiple cysts located mainly in the subcapsular region, and a hypertrophied stroma.

Subsequently, the heterogeneity of the clinical features led to the adoption of the term "polycystic ovary syndrome." Following the introduction of new investigative techniques, such as hormone measurements by radioimmunoassay and ovarian morphology by ultrasound, the earlier diagnosis diagnosis based only on clinical and anatomical criteria was replaced by a new one which incorporates hormonal and ultrasonographic criteria [3].

Considered by the end of the last century as a disorder of the reproductive system (given the presence of menstrual disturbance and consequent infertility) and with aesthetic repercussion (given the presence hyperandrogenism, hirsutism, acne, and alopecia), nowadays the syndrome is also considered an important cardiovascular risk factor [4].

In fact, there is evidence of early impairment of the vascular system. Methods which determine the presence of subclinical atherogenesis, such as the endothelial function assessment, which measures the intima-media thickness of the carotid artery and the arterial compliance of the brachial artery were used in some studies [5]. Although not universally documented, vascular damage was observed in patients with PCOS compared with women without the syndrome. More recently, it was shown that postmenopausal patients with previous history of the syndrome have, when undergoing coronary catheterization, experienced a greater number of lesions and a worse prognosis after catheterization [6].

Table 1: Guidelines for the diagnosis of polycystic ovary syndrome.

NIH 1990[1]	Rotterdan 2003[2]	AES 2006[3]
Both criteria menstrual dysfunction	2 of the 3 criteria menstrual dysfunction	Both criteria menstrual dysfunction or polycystic ovary morphology
+	+	+
hyperandrogenemia or hyperandrogenism	hyperandrogenemia or hyperandrogenism	hyperandrogenemia or hyperandrogenism
	Polycystic ovary morphology	
+ exclusion of other causes		

Some conditions may be associated with PCOS, such as endometrial hyperplasia and carcinoma, obesity carbohydrate intolerances, type 2 diabetes, lipid metabolism disorders, hypertension and sleep apnea. Importantly, all of these conditions are associated with an increased long-term risk for cardiovascular disease. A possible link between these conditions and cardiovascular disease is insulin resistance, which is present regardless of body mass index, but potentialized by obesity [7]. It was recently documented an impaired cardiopulmonary functional capacity strictly related to insulin resistance in women with the syndrome [8]. In order to standardize the diagnosis of PCOS, various guidelines and statements have been published in recent years, resulting in the combination of the fundamental characteristics of the syndrome, that is hyperandrogenemia (increase in testosterone and/or DHEAS concentration), hyperandrogenism (hirsutism, acne, or alopecia), menstrual dysfunction, and polycystic ovarian morphology identified by ultrasound.

The three most frequent consensus are shown in Figure 1 and Table 1. A consensus on these guidelines is that PCOS is a syndrome and not a specific disease. Consequently, no single criterion can define its diagnosis, therefore it is a diagnosis of exclusion.

2. Metabolic Syndrome and PCOS

MetS is a cluster of metabolic abnormalities, primarily abdominal obesity, insulin resistance, compensatory hyperinsulinemia, impaired glucose metabolism, dyslipidemia, inflammation, endothelial dysfunction, and hypertension that currently affects approximately one out of five women in reproductive age [16]. In addition, several prospective studies have shown that MetS is associated with an increased risk for type 2 diabetes mellitus and subclinical and clinical cardiovascular diseases [14]. MetS shares many similarities with PCOS, including the frequent presence of abdominal obesity and insulin resistance [14]. PCOS is now considered as a female subtype of the metabolic syndrome, and its potential health consequences have been considered as a public-health concern (Figure 1).

The prevalence of MetS in women with PCOS largely varies, from 1.6 to 43% depending on assessed population [17–19]. The prevalence of MetS in PCOS patients was evaluated in a study conducted in the city of São Paulo (Brazil). Seventy-three women, with body mass index (BMI) of 30.4 ± 7.8 kg/m^2 and 25.0 ± 6.0 years, subdivided according to BMI, were studied retrospectively. According to the modified criteria of the Third Report of the National Cholesterol Education Program (NCEP/ATP III) for the diagnosis of MetS, which was replaced by the fasting glycemia and glycemia at 120 minutes obtained from oral glucose tolerance test, the prevalence of MetS was 85.5% in those with BMI ≥ 40 kg/m^2, 62.9% in those with BMI between 30 and 39.9 kg/m^2, 23.8% in those with overweight, BMI between 25.0 and 29.9 kg/m^2, and 0% in patients with BMI < 25 kg/m^2. In this study, the abdominal circumference greater than 88 cm was considered one of the best predictors for the MetS [19].

Dyslipidemia in PCOS is multifactorial and appears to be mediated by insulin resistance and androgen excess as well as environmental factors. In PCOS, a number of lipid abnormalities has been found. The most frequent is a decrease in HDL-C and an increase in triglycerides, which is a lipid pattern known to be associated with insulin resistance. Obese women with PCOS have the most atherogenic lipid profiles [20, 21]. Rocha et al. (2011) studied one hundred forty-two women with PCOS with an average BMI of 29.1 kg/m^2 and an average age of 25.12 years. According to the BMI, 30.2% were normal weight, 38.0% were overweight, and 31.6% were obese. Thirty-one eumenorrheic women matched for BMI and age, with no evidence of hyperandrogenism, were recruited as controls. The incidence of dyslipidemia in the PCOS group was twice that of the control group (76.1% versus 32.25%). The most frequent abnormalities were low HDL-C (57.6%) and high triglyceride (28.3%). HDL-C was significantly lower in all subgroups of healthy with PCOS when compared to the subgroups of healthy women, and the BMI had a significant impact on this abnormality [15] (Figure 2).

3. Impaired Glucose Tolerance and Type 2 Diabetes Mellitus in PCOS

The prevalence of insulin resistance (IR) in PCOS patients have ranged from 44 to 70% [22–26]. This wide range may be due to several factors, including the heterogeneity of the diagnostic criteria for PCOS employed in these studies [22], the genetic background among the assessed population [6], and the differences regarding the methods used for defining IR [22, 25, 26]. It has been shown that the presence of chronic anovulation associated with higher androgen levels was associated with lower insulin sensitivity and higher prevalence of cardiovascular risk factors, such as IR, impaired glucose tolerance (IGT), type 2 diabetes mellitus, and dyslipidemia [22], However the presence of two PCOS phenotypes identified according to the Rotterdam criteria—hyperandrogenism and polycystic ovaries with ovulatory cycles and anovulation and

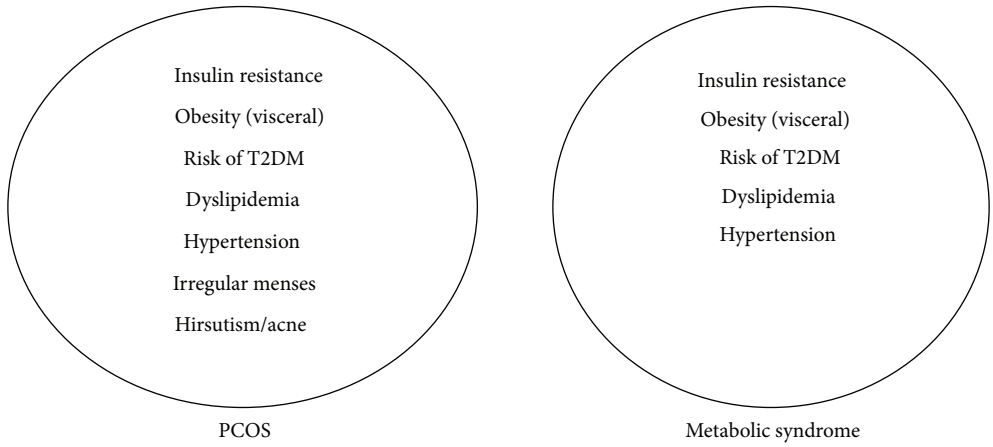

Figure 1: Common features of PCOS and the metabolic syndrome. Adapted from Tfayli and Arslanian [14].

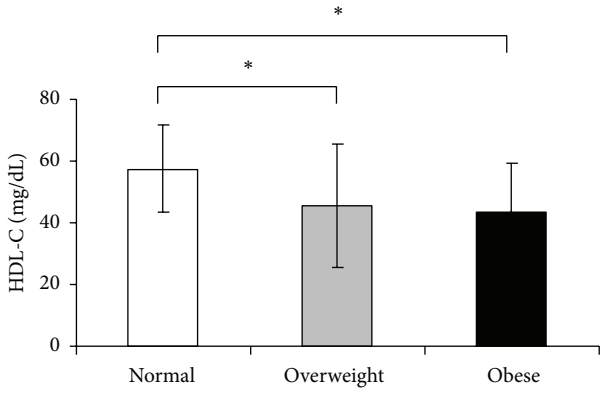

Figure 2: The serum HDL-C level (mean ± SD), according to the BMI. $^{*}P < 0.05$ [15].

polycystic ovaries without hyperandrogenism—have little or no evidence for IR using surrogate markers [22]. Regarding the ethnicity, there is evidence suggesting that insulin sensitivity may be determined by genetic factors. Goodarzi et al. [27] showed that Mexican-Americans PCOS patients have higher incidence of IR when compared with other ethnic groups.

There are several methods for detecting IR, such as the hyperinsulinemic-euglycemic clamp technique, the fasting insulin, the homeostatic model assessment of IR (HOMA-IR), the quantitative insulin sensitivity check index (QUICKI), the area under the curve of insulin, and the frequent sample IV glucose tolerance test (FSIVGTT). It is known that these methods differ with respect to their accuracy in assessing IR but no study involving PCOS has demonstrated that the incidence of IR depends on the IR assessment method [29]. Even normal weight PCOS patients may suffer from IR [30]. Nonetheless, it is known that both PCOS and obesity have an additive deleterious effects on insulin sensitivity and its metabolic complications [30–32].

Given the frequent occurrence of IR in PCOS, it is not surprising that PCOS is associated with impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM), and the syndrome is now considered to be a significant risk factor for development of T2DM [21]. Up to 35–40% of women with PCOS have IGT, and 10% develop T2DM during the third or fourth decade of life [33–35]. Moreover, epidemiologic studies indicate that the odds ratio for the development of diabetes in women with PCOS is around 2.0 after adjusting for BMI. By amplifying insulin resistance, is a confounding factor in the development of IGT and T2DM in women with PCOS, but the increasing prevalence of obesity in the population means that a further increase in the prevalence of diabetes is also expected [21].

The study conducted by Barcellos et al. (2007) showed that the prevalence of disorders of carbohydrate metabolism (i.e., impaired fasting glucose, IGT, and T2DM) in patients with PCOS, using the fasting plasma glucose (FPG) and the plasma glucose at 120 minutes after a challenge with 75 grams of glucose (G120′) in the oral glucose tolerance test. In this study, the normality criteria employed for FPG and G120′ were <100 mg/dL and <140 mg/dL, respectively. Patients were subdivided into three groups according to BMI as follows: normal BMI, overweight, and obese. Using FPG and G120′, the prevalence of IR observed in women with normal BMI, overweight and obesity were 3.7%, 13,3% and 32,2%, respectively, (Figure 3). That is, the prevalence of intolerance to carbohydrate was progressively higher with the increasing BMI, regardless of diagnostic criteria employed. One of the conclusions of this study was that all PCOS patients should be tested with oral glucose tolerance, since this method was more sensitive than FG in the detection of IR [28].

4. Insulin Signaling in Skeletal Muscle with PCOS Women

Skeletal muscle plays a pivotal role in the peripheral glucose uptake. In healthy subjects with normal weight, almost one-third of the ingested glucose is taken up by the liver after a meal whilst almost two-third is taken up by skeletal muscle through insulin-dependent mechanisms. After the glucose

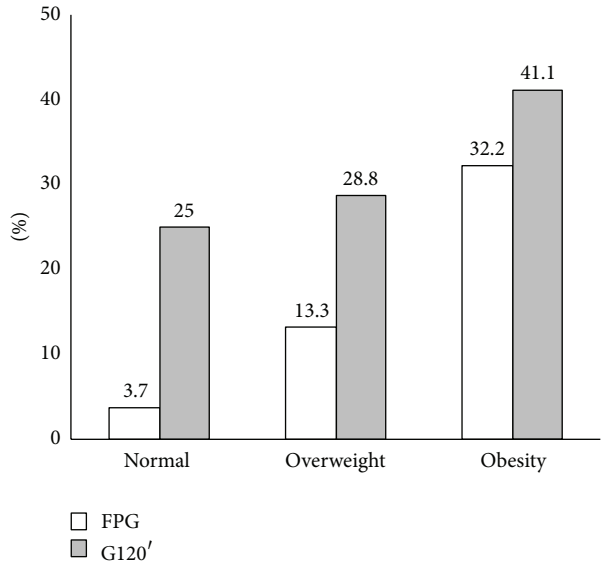

Figure 3: Prevalence of disorders of carbohydrate metabolism in patients with PCOS according to the BMI [28].

intake, the increase in plasma glucose stimulates insulin secretion via pancreatic beta cells. Increased insulin resulting from increased plasma glucose suppresses lipolysis decreasing the rate of lipid oxidation [36]. Simultaneously, insulin stimulates glucose uptake by skeletal muscle, increasing the glucose outflow, and by activation of enzymes related to glucose oxidation in this site [37].

The cellular events that initiate the crosstalk between insulin and its receptors are present in the specific surface of skeletal muscle cells. The insulin receptor consists of two subunits (α and β) linked by disulfide bonds lying in the extracellular environment sarcoplasmic membrane. The binding of insulin with its receptor leads to phosphorylation of the β-subunit in several tyrosine residues as the insulin receptor has kinase activity [39]. However, due to the hydrophilic characteristic of the glucose molecule, it does not diffuse through the lipid layer of cell membrane. Therefore, it is necessary a membrane transporter to make possible the uptake of glucose by the cell. In humans, these proteins constitute a family of transporters (GLUT) [39]. GLUT-4 express is the major transporter in skeletal muscle, activated (and translocated) to the surface of the cellular membrane in response to insulin and exercise [40–42]. The GLUT-4 translocation is stimulated by insulin in skeletal muscle and the reduced speed-determining step in the glycogen synthesis are observed in T2DM patients [43]. While evidence suggests impairment in the GLUT-4 translocation in patients with T2DM, the total GLUT-4 content is not reduced in the skeletal muscle of type 2 diabetic patients [43]. Therefore, the uptake of glucose into skeletal muscle in insulin-resistant individuals can be partially explained by defects in insulin signaling in the GLUT-4 translocation [44]. An overview of the insulin signaling pathways regulating glucose transport can be seen in Figure 4.

Since PCOS is associated with defects in insulin activation and β-cell pancreatic dysfunction [45], the interest in the molecular mechanisms underlying the insulin resistance in PCOS has increased. Insulin resistance in the skeletal muscle is a major risk factor for the development of T2DM in women with PCOS [46]. For instance, Dunaif et al. (1995) studied skeletal muscle tissue of obese and lean PCOS and and reported an excessive serine phosphorylation (Ser^{312}) of insulin receptor in cultured human muscle cells and fibroblasts [47]. However, Corbould et al. (2005) did not confirm these previous findings in cultured skeletal muscle of obese women with PCOS, showing a decrease in insulin sensitivity in cultured muscle cells from women with PCOS, but normal basal phosphorylation levels as well as normal phosphorylation of tyrosine β-subunit of the insulin receptor after stimulation with insulin [48].

Muscle biopsies performed during hyperinsulinemic euglycemic clamp showed that a significant reduction in glucose uptake mediated by insulin and IRS-2 significantly increased in skeletal muscle. In the basal period, the activity of IRS-1-associated phosphoinositide 3-kinase (PI3k) was shown to be normal, but insulin-mediated activity of IRS-1-associated PI3k was significantly reduced [49]. The increased expression of IRS-2 protein in skeletal muscle in women with PCOS may be interpreted as a potential compensatory mechanism of the decreased insulin sensitivity. Yet, the attenuated insulin sensitivity (as assessed by the hyperinsulinemic euglycemic clamp) suggests that protein expression of IRS-2-associated PI3k cannot compensate this decreased sensitivity [45]. Evidence of defects in the post-receptor insulin signaling has been shown *in vivo* in women with PCOS. The basal phosphorylation levels of Akt at Ser^{473} and Thr $residues^{308}$are not altered in women with PCOS women compared with controls [50]. However, when the group of women with PCOS was subjected to an euglycemic hyperinsulinemic clamp, phosphorylation at both residues was attenuated independently of obesity [51]. The total amount of protein TBC1D4 (also known as AS160) in skeletal muscle of women with PCOS is not different at baseline compared to control women. Nonetheless, the phosphorylation of TBC1D4 in women with PCOS undergoing biopsies hyperinsulinemic euglycemic clamp was attenuated compared to control women [52].

Several pharmacological options for attenuating IR are available. Thiazolidinediones (TZDs) are agonists of the peroxisome proliferator-activated receptor (PPAR γ). Pioglitazone (one of the main representatives drugs of this class) exerts its effect through mechanisms related to the expression of genes involved in mitochondrial biogenesis, insulin signal transduction, and glucose and lipid metabolism [53]. The PPAR γ is abundantly expressed in adipose tissue, and to a lesser extent, in liver and muscle tissue [54]. Women with PCOS treated with pioglitazone (30 mg per day) showed improved insulin sensitivity and a decreased insulin secretion [55]. The molecular mechanisms of the beneficial action of TZDs in skeletal muscle tissue are not fully understood, but they may include increased insulin receptor downstream signaling [56] and improved the uptake and oxidation of free fatty acids [57]. Treatment with TZDs is also associated with increased activity of AMP-activated protein kinase (AMPK)

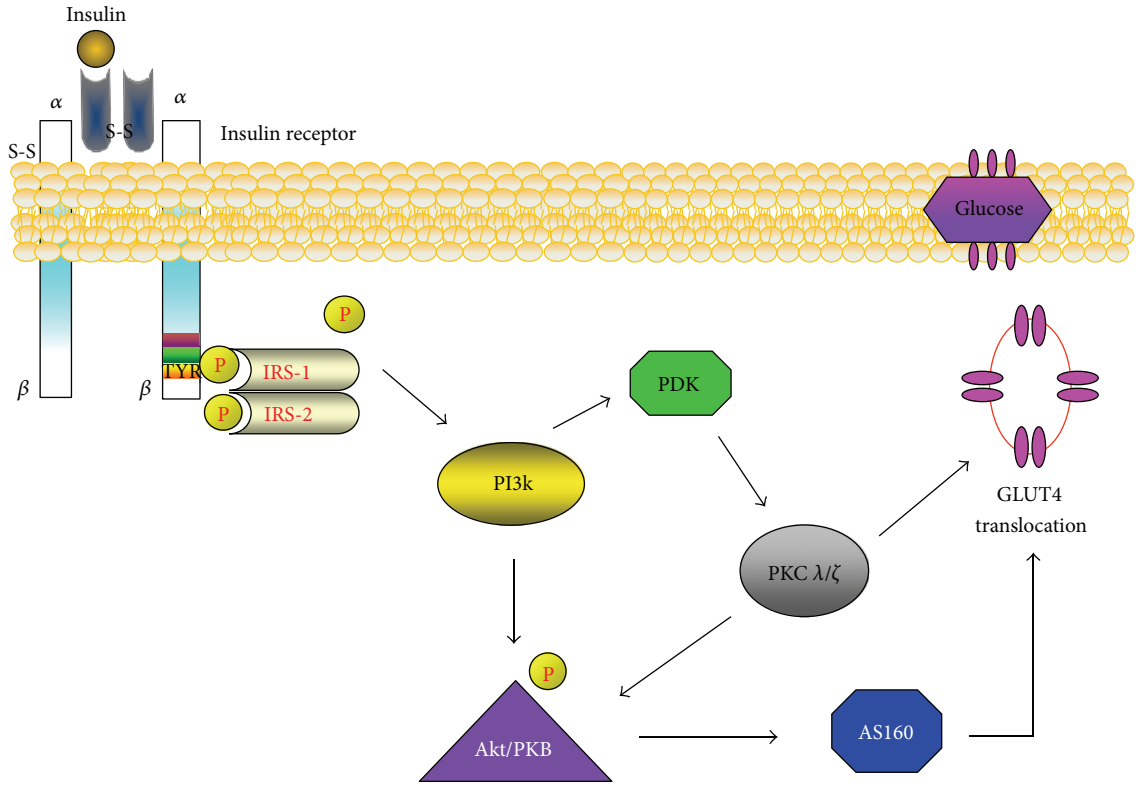

Figure 4: In brief, the insulin binds with its membrane receptor which has intrinsic tyrosine kinase activity, triggers a signaling cascade to downstream substrates resulting in glucose transport. Subsequently, tyrosine phosphorylated IRS (IRS-1/2) recruits signaling molecules incluinding phosphoinositide 3-kinase (PI3k). After a activation of PI3k a complex formation ofphosphatidylinositol-3,4,5-trisphosphate (PI3P) that serves as regulator of phosphoinositide-dependent kinase (PDK) which was later shown to activate others prototypes proteins kinase (e.g., PKC). With this, the protein Akt is activated and propagates the hormonal signal to activate protein AS160 (GTPase activating protein of 160 kDa), which in turn sensitizes the glucose transporter in skeletal muscle (GLUT-4) to the translocation process to the lipid membrane to glucose uptake [38].

and PPAR γ coactivator-1-α (PGC-1-α) in skeletal muscle [58].

In conclusion, it is recognized that the etiology of IR in PCOS is likely to be as elusive as in type 2 diabetes. Indeed, IR plays plays a major role in the metabolic and reproductive phenotypes of this syndrome. Insulin signaling in PCOS women may be as a result of the interaction of genetic and environmental factors that are specific to PCOS or T2DM [59]. Further studies on the effect of pharmacological and non-pharmacological approaches (e.g., physical exercise) in skeletal muscle of women with PCOS are of therapeutic relevance in this syndrome.

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Research Article

Substrains of Inbred Mice Differ in Their Physical Activity as a Behavior

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Recent studies strengthen the belief that physical activity as a behavior has a genetic basis. Screening wheel-running behavior in inbred mouse strains highlighted differences among strains, showing that even very limited genetic differences deeply affect mouse behavior. We extended this observation to substrains of the same inbred mouse strain, that is, BALB/c mice. We found that only a minority of the population of one of these substrains, the BALB/c J, performs spontaneous physical activity. In addition, the runners of this substrain cover a significantly smaller distance than the average runners of two other substrains, namely, the BALB/c ByJ and the BALB/c AnNCrl. The latter shows a striking level of voluntary activity, with the average distance run/day reaching up to about 12 kilometers. These runners are not outstanders, but they represent the majority of the population, with important scientific and economic fallouts to be taken into account during experimental planning. Spontaneous activity persists in pathological conditions, such as cancer-associated cachexia. This important amount of physical activity results in a minor muscle adaptation to endurance exercise over a three-week period; indeed, only a nonsignificant increase in NADH transferase+ fibers occurs in this time frame.

1. Introduction

Exercise adaptations result from a coordinated response of multiple organ systems, including cardiovascular, pulmonary, endocrine-metabolic, immunologic, and skeletal muscle, recently reviewed by Boveris and Navarro [1], by Freidenreich and Volek [2], and by Perrino et al. [3]. Exercise training has been suggested as a promising countermeasure to prevent several disease states and as a rehabilitation tool aimed to restore both muscle strength and endurance, depending on the type of exercise [4]. Regular resistance exercise combined with adequate protein intake to maintain muscle mass is proposed to counteract sarcopenic obesity in an aging global population, a major public health challenge [5]. For all the above, rodent models of caloric intake and exercise are widely used [6] and novel molecular mechanisms underlying the effects of physical activity have been recently brought to light [7, 8]. Nonetheless, the anatomy and physiology of rodents differ significantly from those of humans. While it appears clear that *Homo sapiens* has evolved to support the svelte phenotype of an endurance runner [9], a better understanding of similarities and differences between human and animal models is becoming of paramount importance for translating discoveries in preclinical models to clinical settings.

The two main types of contractile activity that are classified as low muscular tension development over an extended duration, or high-tension generation of limited duration, are characteristic of endurance and resistance exercise, respectively. The aforementioned adaptive responses at the whole body and cellular and molecular levels depend on the mode of

exercise performed [10]. For instance, increased strength [11–13], power [14], muscle cross-sectional area [15–17], RNA, and protein content [18] typically occur following resistance exercise training. Aerobic, endurance exercise training has been shown to enhance exercise capacity [19], augment maximal oxygen consumption [20], increase oxidative enzymes [21], and elevate mitochondrial content [22].

Several protocols of exercise training were developed for rodent models to mimic either resistance or endurance exercise. For instance, to climb a vertical ladder as a mode of progressive resistance exercise has been used for rats [23]. Recently, a very interesting equipment and system of resistance exercise, based on squat-type exercise for rodents, with control of training variables, has been validated [24]. The latter is based on a conditioning system composed of sound, light, and feeding devices, thus being not necessary to impose fasting or electric shock for the animal to perform the task proposed. Endurance exercise is based on more standardized protocols, basically running. The intensity-controlled treadmill exercise represents a well-characterized model of endurance exercise [25]. Slope and velocity of treadmill can be regulated and the animals are hosted in an enclosed chamber with a shock grid for motivating mice to run. One of its major advantages is the possibility of increasing time-wise exercise intensity, thus allowing the researcher to submit rodents to specific training programs. One of the drawbacks of treadmill is the fact that it may induce stress in the mice due to environmental, nonphysiological conditions. On the contrary, spontaneous exercise is often the favored type of exercise for experimental purposes since it is physiologic: it is performed at will, mostly during the nighttime; it mimics natural behavior, such as intermittent locomotion, typical of wildtype rodents; finally, it has been shown that such a voluntary activity is repeatable and stable within individual mice [26]. Hosting the mice in wheel-equipped cages, in which they exercise at will, classically induces such a spontaneous physical activity. A drawback of this approach is a certain degree of inter and intrapopulation variability, which makes absolutely necessary to individually monitor running activity by tachometers.

Small genetic differences may have a great influence on behavioral phenotypes [27]. Thus, the genetic background of different substrains should be carefully chosen, equated, and considered in the interpretation of mutant behavioral phenotypes. To this purpose, Knab et al. assessed the repeatability of a commonly used maximal exercise endurance treadmill test as well as voluntary physical activity measured by wheel running in mice: they found no significant differences in exercise endurance between different cohorts of BALB/c J and DBA/2 J mice indicating strains overall generally test the same [26]. Both strains are inbred mice; that is, populations that are nearly identical to each other in genotype due to long inbreeding. The usual procedure is mating of brother-sister pairs for 20 generations, which will result in lines that are roughly 98% genetically identical. Indeed, inbred strains of animals are frequently used in laboratories for experiments where for reproducibility of conclusions, all the test animals should be as similar as possible.

BALB/c are an inbred strain of mice distributed globally and are among the most widely used inbred strains. The founding animals of the strain (the "Bagg albino") were obtained by Halsey J. Bagg of Memorial Hospital, NY, from a mouse dealer in Ohio in 1913. By 1935, the animals were in the possession of Muller's student, George Davis Snell, who moved them to The Jackson Laboratory. This stock provided the basis of all the BALB/c substrains that are now in use around the world. BALB/c ByJ (Jackson mice, donated to Jackson labs by Bailey J., in 1974) was separated from the BALB/c J strain in 1935. BALB/c ByJ mice have the advantage of better reproductive performance and less aggressiveness than the BALB/c J substrain and pose many other differences with the J substrain. Between the fifties and seventies, a third substrain got separated from the above-mentioned first two substrains, that is, the J and the ByJ: the Charles River AnNCrl (to Andervont in 1935 to NIH in 1951 from Andervont at F72 to Charles River in 1974 from NIH).

The three BALB/c substrains have been kept separated over decades and could have diverged to such an extent to develop sufficient genetic differences to account for behavioral differences among substrains, while remaining homogeneous within the same population. Mice may significantly differ for what concerns their physical activity as a behavior, which is of pivotal importance for the reproducibility and significance of studies exploiting exercise models. These differences may appear easily accountable when dealing with animals of different sexes or strains. However, we wondered whether even very fine differences (such as those distinguishing murine substrains of a single inbred strain) are able to determine significant behavioral differences. For this reason, we compared the physical activity behavior of the AnNCrl, the ByJ, and the J BALB/c mice and found striking differences concerning their willingness to run when hosted in wheel-equipped cages. Our findings have important experimental consequences with relevant economical and scientific fallouts.

2. Materials and Methods

2.1. Mice. Mice were generously provided by Janvier (Le Genest Saint Isle, St Berthevin Cedex, France). Throughout the study we used 7-week-old BALB/c mice of the following substrains: AnNCrl, ByJ, and J. We used a total of 21, 12, and 12 female mice of the substrains AnNCrl, ByJ, and J, respectively. Mice were allowed to settle in the animal facility for one day and then transferred to wheel equipped cages. Cachexia was induced by subcutaneous grafting, using a trocar of a $0.5\,mm^3$ fragment of colon carcinoma (C26, obtained from the National Cancer Institute) in the dorsal region as previously described [28]. Mice were hosted in standard conditions with day/night cycles of 12 hours and food *ad libitum*. Mice were treated in strict accordance to the guidelines of the Institutional Animal Care and Use Committee and to national and European legislation, throughout the experiments.

2.2. Cages. Cages were purchased from Animal Care System (Centennal, CO). The wheels, structured as a circular open ladder with a diameter of 15 cm, were purchased as wheels for rodents in general customer pet shops. The tachometers, either model DC-4 or DC-9, were purchased from Decathlon. Readings were recorded every morning, before 10 am. Sporadic events of day running activity were observed. A Kleenex was introduced into the cage as material for the construction of a nest, with the aim to reduce stress due to being isolated (one animal per cage).

2.3. Tissue Immunohistochemical Analysis. NADH transferase staining was performed as described previously [28]. Morphometric analysis was performed on type IIB (low NADH transferase activity, glycolytic), type IIA/X (medium NADH transferase activity, intermediate), and type I (high NADH transferase activity, oxidative) fibers separately. For each muscle, the whole muscle cross-section was analyzed to calculate the percentage of each fiber type by using ImageJ 1.41 (freeware developed by Dr. W. Rasband at NIH, and available at http://rsb.info.nih.gov/ij/). The fibers with a medium and a high content in mitochondria were pooled and collectively considered as NADH transferase+ fibers, that is, oxidative. Photomicrographs were obtained by means of an Axioskop 2 plus system (Zeiss, Oberkochen, GE) or a Leica Leitz DMRB microscope fitted with a DFC300FX camera (Leica, Wetzlar, Germany).

2.4. Statistical Analysis. One-way or two-way analysis of variance (ANOVA) was used for one or two variate analysis, respectively. Either the Tukey LSD test or Student's t-test was used for the *post hoc* comparisons between specific groups, as indicated. The significance levels for these tests were set at a $P < 0.05$ or $P < 0.01$, as specified. Point and interval were estimated at the 95% confidence level. Statistical analyses were performed by using VassarStats, the website for statistical computation freely available at http://vassarstats.net/.

3. Results

Mice of three BALB/c substrains, that is, the AnNCrl, the ByJ, and the J, were obtained by the same vendor and hosted at the same time in the same animal facility. Each wheel-equipped cage was used for a single mouse, whose spontaneous wheel running activity was recorded by a commercial tachometer. The distance run over a period of four days was recorded daily; the average Km/day over such period of time was considered to minimize daily variations. Mice clearly divide into two populations of runners and nonrunners, the latter totally ignoring the wheel as such and showing no interest in wheel running at all. The threshold to define a runner was set to 1 Km of distance run on the wheel over the 4-day period of time. Several rounds of independent experiments, involving 6 mice for each of the three substrains, were repeated and the percentage of runners for each substrain in each experiment was assessed. In this way, an average percentage of runners in a given experiment and its associated SEM were calculated as a function of the substrain. We found that the runners were 85.1 ± 3.4%, 72.0 ± 3.0%, and 38.2 ± 7.7% of the AnNCrl, the ByJ, and the J, respectively (Figure 1(a)). The 95% confidence intervals were found to be 76.9–93.2%, 59.1–84.9%, and 18.5–57%, respectively. One-way ANOVA ($F = 21.61$; $P < 0.0001$) demonstrated a significant dependence of the percentage of runners on the substrain, with the BALB/c J running showing a significantly lower number of runners as compared to the other two substrains by Tukey's HSD *post hoc* test. Considering only the population of runners, we then assessed the distance run daily on the wheel by representatives of the three substrains. We found that the mice run 5.0 ± 0.3 Km/d, 4.7 ± 1.4 Km/d, and 3.7 ± 0.6 Km/d for the AnNCrl, the ByJ, and the J substrain, respectively (Figure 1(b)). The 95% confidence intervals were found to be 4.1–5.8 Km/d, 3.3–6.1 Km/d, and 2.2–5.0 Km/d, respectively. While one-way ANOVA ($F = 1.88$; $P = 0.167$) failed to demonstrate a dependence of the observed trend in the daily run distance on the basis of the substrain, we tentatively used the Student's t-test to statistically explore the difference shown by the J substrain and found that is significantly lower as compared to the AnNCrl substrain. In summary, we observed that the majority of the BALB/c J mice do not spontaneously run on a wheel, differently from the BALB/c AnNCrl and the BALB/c ByJ, the vast majority of which is willing to run. Moreover, even when the BALB/c J do run, they cover on average a smaller distance as compared to the BALB/c AnNCrl and the BALB/c ByJ mice. We concluded that the BALB/c AnNCrl is the best substrain for studies involving spontaneous physical activity, such as wheel running. For this reason, this substrain has been used throughout the rest of the study.

With the aim to assess whether the observed wheel running activity displayed features of exercise training and whether the 5 Km/day represented the upper limit of physical activity for BALB/c mice, we recorded the kinetics of mouse wheel running over almost three weeks. For this set of experiments, we decided to use the AnNCrl substrain of BALB/c mice, since these behaved as the most active mice. We remarked that mouse running behavior is biphasic, with a first week spent to familiarize with the wheel, with an outstanding (for the size of the animals), yet moderate, daily distance if compared to the second and third week of activity, in which the daily distance covered by the mice reaches a plateau that it is more than twice the initial Km/d (corresponding to more than 11 Km/days, Figure 2). In this context, we also wondered whether mice were able to perform voluntary physical activity in pathological conditions, such as cancer-induced cachexia [29]. Thus, we recorded the daily running activity of C26 colon carcinoma-bearing mice, which develop a progressive and severe form of muscle wasting associated to weakness and fatigue [28]. C26-bearing mice run about the same Km/d as controls in the first week of activity, when the tumor size is still negligible; however, they do not show the same progressive increase in the distance run on the wheel as the controls in the second week of activity. With the disease progression and overt cachexia, they keep running for about 7 Km/d, which is a striking amount of exercise, considering that tumor-bearing mice lose about 25% of the body weight in three

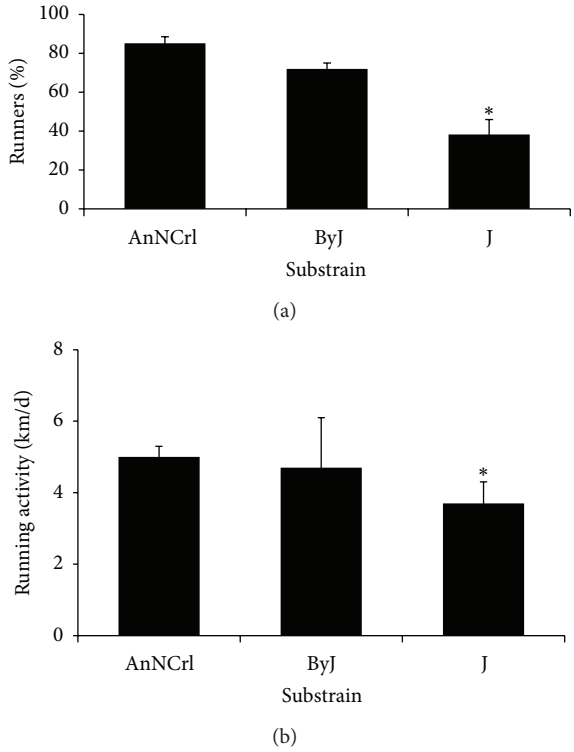

Figure 1: Different mouse substrains show differential physical activity as a behavior. Seven-week-old female BALB/c mice, belonging to three different substrains as indicated, were individually placed in wheel equipped cages. The running behavior (a) and the daily distance covered (b) were recorded for four days. The average ± SEM of at least three independent experiments, each one performed at least in quadruplicate, is shown. BALB/c J mice run significantly less than the other two substrains and the majority of this population do not show at all interest for wheel running. (a) $^{*}P < 0.01$ by Tukey HSD test versus AnNCrl or versus ByJ. (b) $^{*}P < 0.05$ by Sutdent's t-test versus AnNCrl.

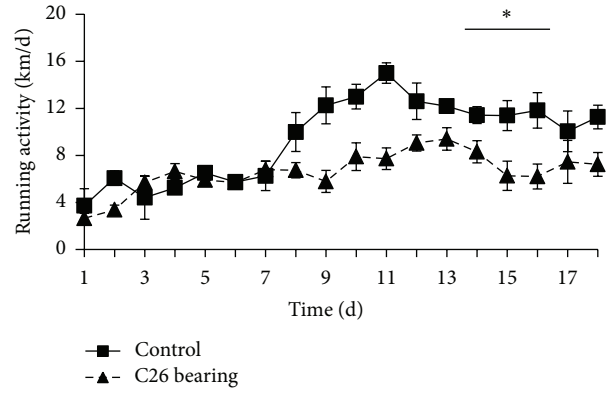

Figure 2: Kinetics of physical activity in healthy and C26-bearing BALB/c AnNCrl mice. Seven-week-old female BALB/c AnNCrl mice were individually placed in wheel equipped cages. At the same time a group (C26 bearing) was subcutaneously transplanted with the C26 colon carcinoma to induce muscle wasting. The running distance was daily recorded and averaged among replicates from at least three independent experiments. Both healthy and diseased mice do exercise, even though C26-bearing mice run for up to 6 Km/day, while healthy mice increased the daily distance run on the wheel to 11 Km/day. $^{*}P < 0.01$ by Tukey's HSD test versus C26-bearing mice.

weeks. Two-way ANOVA calculated over the last four days of the recordings (i.e., from day 15 to day 18) shows that only the presence of the tumor significantly affects the running behavior, with no interference with time ($F = 19.74$; $P < 0.0001$; Tukey's HSD $P < 0.01$ for control versus C26 bearing). These data indicate that the BALB/c AnNCrl mice not only spontaneously run several Km per day but also have the tendency to progressively increase the distance covered on the wheel. Mice bearing a tumor, a condition which deeply affects muscle mass and function, are still capable of performing a significant amount of physical activity, albeit to a lesser extent than healthy mice. This indicated that wheel running could be exploited as a model for endurance exercise intervention in pathological settings in rodents.

Endurance training activates metabolic pathways and remodeling in skeletal muscle. A prominent feature of this type of exercise is the stimulation of Krebs' cycle and the mitochondriogenesis in muscle fibers. Since in BALB/c AnNCrl mice we observed a spontaneous, yet significant, increase in physical activity during the second week of permanence in wheel-equipped cages, we hypothesized that the increased performance was associated to metabolic changes making the musculature adapted to endurance exercise. Thus, we performed a histochemical analysis of the skeletal muscle of BALB/c AnNCrl mice following two different periods of wheel running, that is, five and nineteen days, to monitor phenomena of fiber conversion to a more oxidative phenotype upon exercise. By NADH transferase staining on the *Tibialis anterior* (TA) muscle (Figures 3(a) and 3(b)), we highlighted the fibers rich in mitochondria (oxidative and intermediate fibers, typically corresponding to type I and IIA or X). The TA was chosen for its mixed population of fiber types (all types are represented), which appeared particularly suitable for studying shifts in fiber type. While we observed an increase in the number of NADH transferase+ fibers from 64 ± 5% to 72 ± 3% following nineteen days of exercise, one-way ANOVA ($F = 2.39$; $P = 0.162$) showed the lack of a statistically significant effect by exercise on this parameter (Figure 3(c)).

4. Discussion

We have shown that three substrains of the same inbred mice, namely, the BALB/c AnNCrl, ByJ, and J, display striking differences in their behavior concerning spontaneous physical activity. This phenomenon was observed in spite of the fact that the three substrains display remarkable genetic similarities, exemplified by the absence of histocompatibility barriers. The fact that the vast majority of the AnNCrl and, to a lesser extent, of the ByJ mice run for several Km per day distinguishes these two substrains from the J mice. To our knowledge, this is the first paper on significant behavioral differences among substrains of the same inbred

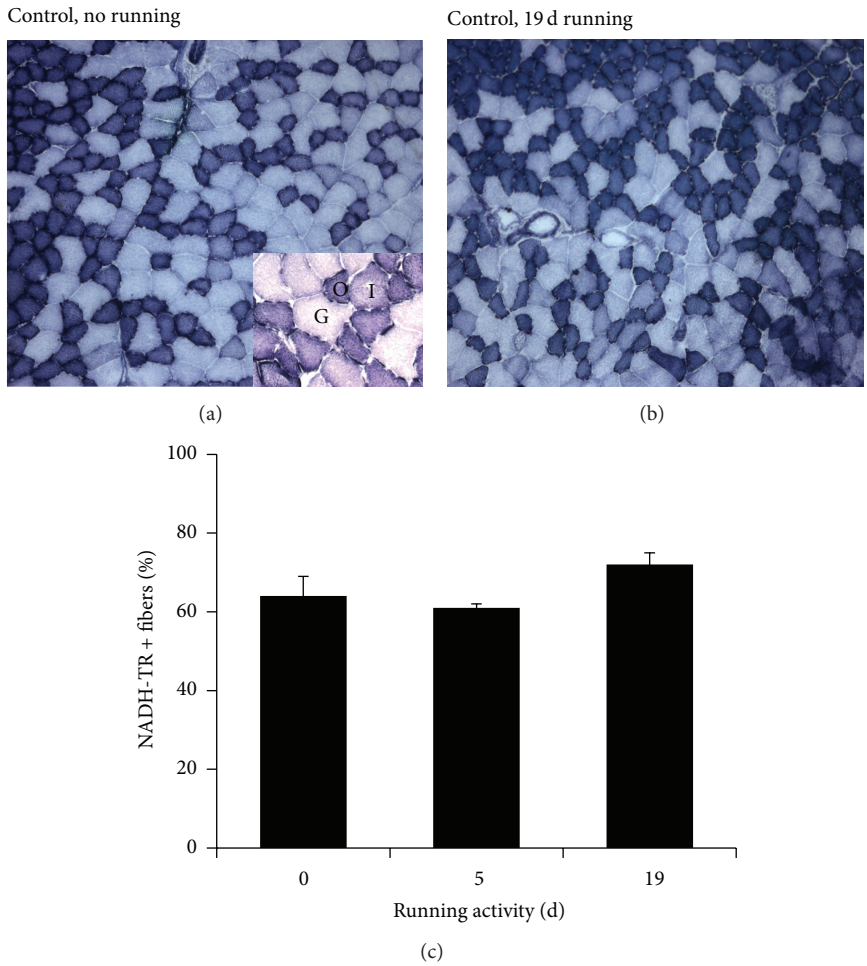

Figure 3: Muscle metabolic adaptations to wheel running in BALB/c AnNCrl mice. Histochemistry for NADH transferase activity highlights oxidative (O), intermediate (I), and glycolytic (G) fibers; that is, muscle fibers that are rich (O or I) and poor in mitochondrial content, respectively. The inset at higher magnification allows to differentiate among the three types of fibers. O + I fibers were collectively considered as rich in NADH transferase (*NADH-TR + fibers*). Representative photomicrographs of the TA of (a) control, nonexercised mouse and (b) a mouse following nineteen days of exercise. The percentage of NADH-TR+ fibers was quantified in replicate, after no (*0*), five (5), or nineteen (*19*) days of wheel running.

mouse strain. Researchers planning to perform experiments requiring wheel running should be aware of these unexpected findings. In fact, the selection of the J substrain, which is less prone to wheel running, would determine a very little amount of exercise performed by a limited number of mice. Interestingly, the J substrain is the cheapest (at least with the vendor used for this study) however, if a relevant number of animals are excluded from a study since they do not exercise, the costs must be recalculated. Researchers that, for an experimental reason, need to use the J substrain and that absolutely require that they exercise by wheel running may consider selecting the mice in preliminary experiments according to their running behavior to sort the runners from the nonrunners in advance. Otherwise, researchers interested in using BALB/c mice for exercise-related experiments may simply want to choose either the AnNCrl or the ByJ substrain.

The idea that different mice strain behave differently about wheel running is not new, but, again, our study shows that even very fine differences (such as those distinguishing substrains) are able to determine significant behavioral differences. Several studies associated genetic influence with physical activity, but animal studies were often conducted with only one sex or a limited number of strains, thus reducing the genomic coverage and generality of the result that Lightfoot et al. clearly showed that physical activity as a behavior has a genetic basis [30]. Their results suggest that potential genetic mechanisms arising from traditional noncoding regions of the genome may be involved in regulation of physical activity [30]. Of course, other studies clearly show that mouse substrains differ for several features other than physical activity as a behavior. For instance, it has been shown that the genetic background of the four different mouse substrains affects their vulnerability to cope with environmental challenges, such as exposure to novelty; the authors consistently suggest considering substrain-specific guidelines and protocols, taking

the substrain-specific adaptive capabilities into account [31]. We totally agree with the authors of this study. Another intriguing study showed that two substrains of BALB/c mice, the BALB/cByJ and the BALB/cAnNCr, are resistant and susceptible, respectively, to Theiler's murine encephalomyelitis, a virus-induced demyelinating disease [32]. The fact that the two substrains are histocompatible makes them a nice model for studying mechanisms of virus infection, since they permit the transfer of cells between naturally resistant and naturally susceptible mice in the absence of immunodepression. Similarly, one could foresee the possibility of satellite cell grafts among different BALB/c substrains to verify whether fine genetic differences are responsible for differential muscle stem cell features, such as their capability of being engrafted into regenerating muscles; one could even wonder whether satellite cells from spontaneous runners could donate to the derived muscle fibers intrinsic mechanical or contractile properties more suitable for running.

Our study is in agreement with previous results showing that mice spontaneously run for 5 to 10 Km per day [30]. This incredible level of voluntary activity is an important fact to keep in mind and represents an outstanding feat for such a diminutive species. It has been stated that "such distances covered daily by us much larger humans would probably cure most of the epidemic diseases facing the world, including obesity and type 2 diabetes" [33].

Running is associated with distinct metabolic adaptations of the skeletal muscle [34, 35]. In particular, it has been reported that voluntary running exercise induces a steady increase in the percentage of NADH transferase-positive fibers in the TA muscle, which was significant after 4 weeks of voluntary exercise [36]. We obtained very similar results in terms of exercise effect on the percentage of NADH transferase+ fibers, with the exception that we failed to demonstrate the significancy of such an effect. This may be due to several differences between the two studies: the mouse strain (BALB/c, C57/Bl6), the sex (female, male), the distance (5 Km/day, 6.8 Km/day on average), and the time frame (3 weeks, 4 weeks); each of these differences could be sufficient to explain why we could not observe a statistically significant increase in the oxidative fibers of exercise mice. The observed trend is consistent with an increased demand of muscle oxidative capacity suggesting that endurance exercise invariably affects muscle metabolism by favoring the oxidative muscle fiber phenotype.

Finally, it should be noted that the therapeutics and ergogenic effects of controlled exercised as opposed to spontaneous exercise may differ significantly in rodents. Intriguingly, the two types of exercise may have very different outputs depending on the targeted organ. For example, voluntary activity causes a more evident plastic changes in the hippocampal formation of rat than that one induced by forced exercise [37].

5. Conclusion

Recognizing the proven benefits of exercise training on health outcomes and the trend towards increasing inactivity at the population level has made recommending exercise a directive of paramount importance. In parallel, studies on organismal and muscle-specific adaptations to increased physical activity steadily increase over time, as shown by the trend in PubMed citations with the keywords exercise and endurance/resistance. With such a proliferation of animal and experimental models dedicated to exercise, it is important to clearly define the major features of the experimental models used in a given study and to be very formal in assessing to which extent generalization of the results can be driven. We report here that three substrains of the same inbred mouse strain, the BALB/c, display significant differences in physical activity as a behavior. We propose that not only the strain of mice used but also the substrain must be clearly specified and chosen consciously, since the differences in spontaneous physical activity between substrains can impact exercise-induced muscle adaptations.

Acknowledgments

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Research Article

Improved Tissue Culture Conditions for Engineered Skeletal Muscle Sheets

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The potential clinical utility of engineered muscle is currently restricted by limited *in vitro* capacity of expanded muscle precursor cells to fuse and form mature myofibers. The purpose of this study was to use isotropic skeletal muscle sheets to explore the impact of (1) fibroblast coculture and (2) fibroblast-conditioned media (fCM) on *in vitro* myogenesis. Muscle sheets were prepared by seeding varying ratios of skeletal myoblasts and fibroblasts on a biomimetic substrate and culturing the resulting tissue in either control media or fCM. Muscle sheets were prepared from two cell subpopulations, (1) C2C12 and NOR-10 and (2) primary neonatal rat skeletal muscle cells (nSKM). In C2C12/Nor-10 muscle sheets fCM conferred a myogenic advantage early in culture; at D1 a statistically significant 3.12 ± 0.8-fold increase in myofiber density was observed with fCM. A high purity satellite cell population was collected from an initially mixed population of nSKMs via cell sorting for positive α7-integrin expression. On D6, tissue sheets with low fibroblast concentrations (0 & 10%) cultured in fCM had increased average myofiber density (4.8 ± 0.2 myofibers/field) compared to tissue sheets with high fibroblast concentrations (50%) cultured in control media (1.0 ± 0.1 myofibers/field). Additionally, fCM promoted longer, thicker myofibers with a mature phenotype.

1. Introduction

Pathological abnormalities of the facial musculature can occur as a consequence of congenital defects, traumatic injuries, or surgical ablations [1–4]. Skeletal muscle possesses an innate capacity to self-regenerate following minor injury by recruiting quiescent muscle precursor cells to the site of injury where they then fuse and form mature myofibers [5]. Of the resident stem cell populations found in native muscle, satellite cells are believed to be the principle progenitors during the repair of small tissue defects [6, 7]. Unfortunately, the ability of satellite cells to repair large defects is minimal and exogenous reconstruction may be necessary to restore functionality. Skeletal muscle tissue engineering is a promising therapeutic option for large-scale skeletal muscle myopathies. The ultimate goal of the tissue engineering approach is to develop skeletal muscle with appropriate tissue morphology and functionality that can be implanted *in vivo* to enhance the architecture and contractility of defective muscle [1, 8–10].

Satellite cells have been proposed as a favorable autologous stem cell source for tissue-engineered constructs because of their role in myoblast differentiation [11]. Mononuclear satellite cells, which can be found on the external surface of native muscle fibers, remain predominantly in a quiescent state until the cells become mitotically active in response to transcription factors released during periods of muscle fiber injury [1–4]. α7-Integrin has been shown to be a primary cell marker that signals the linearization of replicating satellite cells and is known for upregulating myoblast fusion in adult muscle cells in the event of injury [12]. Isolation techniques based on α7-integrin immunotagging can be used to collect cells from a muscle biopsy. Utilizing fluorescence-activated cell sorting (FACS), a sample of mixed skeletal muscle can be sorted by the cells expressing α7 for myoblast enrichment [12, 13]. Current techniques for the expansion and differentiation of satellite cells *in vitro* have been hindered by the expanded cells' lack of ability to fuse into myotubes and express normal force generation. The pretransplant muscle tissues have underperformed in

excitability and contractility testing [14]. The scope of this study was to develop a tissue culture that will improve the myogenic process for bioengineered isotropic skeletal muscle sheets based on the role of fibroblasts and the effect of paracrine factors they release for myotube formation.

The physiological relevance of muscle fibroblasts is associated with the regulation of myogenesis. The normal function of fibroblasts is to synthesize the ECM, specifically collagen fibers and laminin for mechanical function [7, 15]. The presence of laminin is important for structural integrity and various cellular responses. Laminin transduces signals within the ECM for secondary myofiber formation by causing allosteric changes to $\alpha 7$. The integrin guides motile primary myoblasts to laminin-rich sites along the basement membrane promoting isotropic orientation [13, 16]. In response to tissue damage, fibroblasts become more transcriptionally active for the release of cytokines and growth factors, a highly ordered regenerative process [17]. After injury, satellite cells and fibroblasts develop in close proximity, each regulating the other. Fibroblasts adjust the expansion of satellite cells by repressing their terminal differentiation into primary myoblasts. During early myogenesis, satellite cells reciprocate fibroblast regulation for connective tissue development. However, satellite cells begin to monitor fibroblast expansion through a negative feedback loop in late myogenesis to prevent excess fibrosis. Excessive ECM can impede structural integrity and regeneration of new tissue [15]. The cellular response to skeletal muscle injury also includes an increase in intracellular paracrine signaling within the tissue. The paracrine proteins expressed are a variety of fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs), predominantly FGF-1, FGF-2, and IGF-1. FGF-1 and -2 are functionally relevant to the repression of terminal differentiation in primary myoblasts [18]. The signaling of IGF-1 has been shown to activate numerous biochemical pathways associated with skeletal muscle hypertrophy induction [19, 20].

2. Materials and Methods

2.1. Isolation of Primary Satellite Cells and Cell Culture. Primary rat neonatal satellite cells were isolated as previously described [8] using a modification of the Bischoff's protocol [21, 22]. Briefly, muscle tissue from the hind limbs of 2-3-day-old rats was excised, minced, separated from connective tissue, incubated with 1.25% protease solution (9.5 g Krebs buffer, 10 mL of 2 M HEPES, 0.5 mL phenol red, 1.25 mg/mL protease, in 1 L filtered H_2O gassed for 20 mins with CO_2) for 90 min while rocking, twice centrifuged, resuspended in SK culture medium (DMEM, 25% fetal bovine serum, 1% antibiotics) with 600 U DNase enzyme, and then preplated on 150 mm culture dish for 120 min. The unattached cells were harvested and cultured in SK medium for 2 days and expanded in growth medium (DMEM, 20% fetal bovine serum, 1% antibiotics) before cell sorting.

As a secondary cell source, Murine C2C12 myoblasts (ATCC), a cell line that originated from normal adult C3H mouse leg muscle, were cultured in growth medium for less than 4 passages. Murine Nor-10 skeletal muscle fibroblasts (ATCC), a cell line that originated from normal C57BL/10 mouse muscle, were cultured in growth medium.

2.2. Satellite Cell Purification Utilizing FACS. Cultures of cells isolated from rat skeletal muscle were sorted using fluorescence-activated cell sorting. Selection was based on the expression of an extracellular epitope of the muscle-specific $\alpha 7$ integrin protein (9 MBL International).

2.3. Preparation of Fibroblast-Conditioned Medium. Nor-10 and alpha-7 negative muscle fibroblasts were cultured separately in growth medium until ~80% confluence was achieved and then switched to differentiation medium (DMEM, 10% horse serum, 1% antibiotics) for 3 days. The conditioned medium (CM) was collected, filtered, and stored at -4° until needed for tissue culture at which point it was thawed, sterilized, and combined 1 : 1 with fresh differentiation medium. Nor-10-CM and alpha-7-negative-CM were exclusively used on tissue sheets composed of C2C12 myoblasts and alpha-7 positive satellite cells, respectively.

2.4. Assembly of Muscle Tissue Sheets. Tissue culture dishes (12 well) were treated with (1 mg/mL) laminin for 1 hour prior to cell seeding. Isotropic muscle sheets were prepared by simultaneously seeding myoblasts and fibroblasts (total of 23×10^3 cells/mL) in the following combinations: (1) 100% C2C12, (2) 90% C2C12 and 10% Nor-10, (3) 50% C2C12 and 50% Nor-10, (4) 100% $\alpha 7$+ satellite cells, (5) 90% $\alpha 7$+ satellite cells and 10% $\alpha 7$-muscle fibroblasts, and (6) 50% $\alpha 7$+ satellite cells and 50% $\alpha 7$-muscle fibroblasts. The tissue sheets were cultured in growth medium for 3 days and then switched to either fibroblast-conditioned medium or control medium (DMEM, 10% Horse serum, 1% antibiotics). C2C12/Nor-10 tissue sheets were cultured for 1 day in differentiation medium and then analyzed for early culture myogenesis while $\alpha 7+/\alpha 7-$ tissue sheets were cultured for 6 days in differentiation medium and then analyzed for late culture myogenesis.

2.5. Quantification of Myofiber Density. Live culture images (4 fields/well) were captured with a phase contract microscope (Nikon Instruments). Total myofibers/images were counted and averaged for each well at time points D1, for C2C12/Nor-10 tissue sheets, and D6, for $\alpha 7+/\alpha 7-$ tissue sheets.

2.6. Immunostaining. Muscle tissue sheets were fixed with 4% formaldehyde at room temperature, permeabilized with 0.1% Triton X, blocked with BSA, and incubated with Desmin primary antibodies (BD Biosciences) for 45 mins and incubated in secondary antibodies (BD Biosciences) together with a Hoechst nuclear dye (Invitrogen) for 1 hr. Images were acquired using a fluorescence microscope (Nikon Instruments).

2.7. Statistics. Data are expressed as mean ± SE. Statistical significance was determined by two-way ANOVA with post

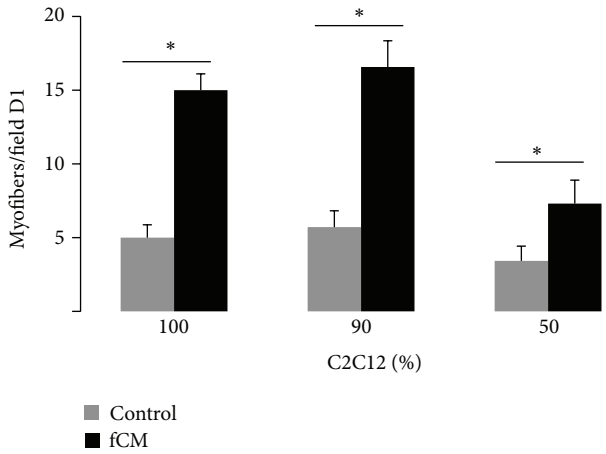


Figure 1: Myofiber density in C2C12/Nor-10 tissue sheets with varied initial cell concentration; 100% C2C12 with 0% Nor-10, to 90% C2C12 with 10% Nor-10, to from 50% C2C12 with 50% Nor-10. Bars show $n = 8$ tissue sheets per group. "$*$" statistically significant between the indicated pairings, $P < 0.05$.

hoc Tukey's test. Differences were considered to be significant when $P < 0.05$.

3. Results and Discussion

It is well established that fibroblasts play a critical role in skeletal muscle formation and function, while also being instrumental in myopathogenesis [7, 15, 17]. Our methods were used to analyze the effect of fibroblasts on myogenesis of tissue skeletal muscle sheets both directly, with addition of fibroblast to tissue culture, and indirectly, with the application of fibroblast-conditioned media (fCM). Our aim for this study was to harness the myogenic capabilities of satellite cells *in vitro* by providing them with a biomimetic environment complete with fibroblast paracrine factors that they are likely to receive *in vivo*.

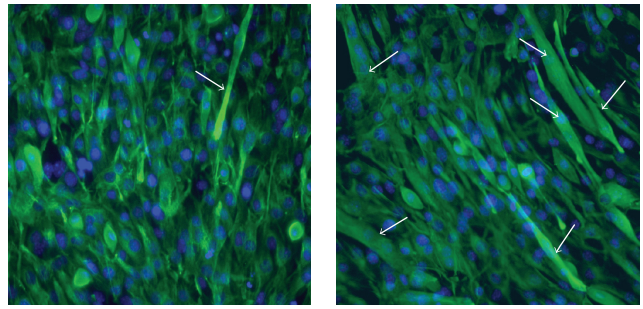
Muscle tissue sheets were successfully prepared from a coculture of C2C12 myoblasts and Nor-10 fibroblasts and analyzed at D1 for early culture myogenesis. All tissue sheets cultured in control medium had comparable myofiber density, an average of 5.0 ± 0.58 myofibers/field (Figure 1). Alternatively, the tissue sheets cultured in fCM were largely impacted by the concentration of Nor-10 fibroblasts. In fCM, the highest myofiber densities, 15.1 ± 1.1 & 16.9 ± 1.8 myofibers/field, were observed in tissue sheets with the lowest concentration of Nor-10 cells, 0% & 10%, respectively, while the myofiber density of tissue sheets with 50% Nor-10 was significantly reduced, 7.8 ± 1.6 myofibers/field (Figure 1). Notably, for each coculture ratio a statistically significant increase in myofiber formation was seen in tissue sheets cultured in fibroblast-conditioned medium (fCM). As compared to control medium, fCM conferred a 4.2 ± 1.5-, 3.3 ± 0.5-, & 4.8 ± 2.1-fold increases in myofiber density were seen in the tissue sheets with 100%, 90%, & 50% C2C12 cells, respectively (Figure 1).

Immunoflourescence staining of representative images from the 90% C2C12 coculture condition (Figure 2) further reveled that tissue sheets cultured in fCM had improved myofiber formation as well as enhanced desmin expression, a marker of early myogenesis [23]. Additionally, multinucleated myofibers were more abundant and had a larger fiber diameter in the tissue sheets cultured in fCM (Figure 2(b)) as compared to control medium indicative of a more mature and functional phenotype.

As a step toward translation, the effect of fCM was explored further with primary isolated neonatal rat skeletal muscle cells (nSKM). Our aim was to selectively sort muscle satellite cells, which are widely considered to be a more clinically relevant cell source as compared to immortalized cell lines [11]. The initial nSKM isolation yielded a mixed population of cells with varying phenotypes, some spindle shaped and some flattened with many pseudopod extensions. Fluorescence-activated cell sorting (FACS) resulted in two populations of cells, (1) $\alpha 7$ positive cells ($\alpha 7+$) and (2) $\alpha 7$ negative cells ($\alpha 7-$) (Figure 3). Satellite cells, known to be $\alpha 7+$, accounted for 36.4 ± 4% of the initial cell population [12, 13]. After sorting, the collected $\alpha 7+$ cells had a purity of 91%. The $\alpha 7-$ cells were passaged several times to ensure high fibroblast purity. Ultimately, autologous muscle biopsies will be a desirable source for satellite cells and muscle fibroblasts to be used in tissue-engineered constructs for therapeutic muscle augmentation, and these methods demonstrate a step toward clinical application.

Muscle tissue sheets were successfully prepared from a coculture of primary satellite cell ($\alpha 7+$) and muscle fibroblasts ($\alpha 7-$) (Figure 4). In culture, tissue sheets with fCM were more active and showed spontaneous contractility, indicating mature and well-assembled contractile machinery, while those cultured in control media did not have spontaneous contractile activity. The resulting tissues were then analyzed at D6 for late culture myogenesis. Similar to C2C12 muscle sheets, the primary cell muscle sheets cultured in control media had minimal dependence on fibroblast concentration with an average of 1.6 ± 0.2 myofibers/field (Figure 5). Alternatively, the sheets cultured in fCM had a greater dependence on fibroblast concentration and those muscle sheets with the lowest concentration of fibroblasts, 100% & 90% $\alpha 7+$, tended to have the greatest myofiber density, 4.1 ± 0.9 & 4.0 ± 0.7 myofibers/field, respectively, compared to 50% $\alpha 7+$ sheets, 2.2 ± 0.6 myofibers/field (Figure 5). Again, for each coculture ratio a significant increase in myofiber formation was seen in tissue sheets cultured in fCM as compared to control medium. 2.5 ± 0.3-, 2.7 ± 0.3-, and 1.4 ± 0.3-fold increases in myofiber density were seen in the tissue sheets with 100%, 90% and 50% $\alpha 7+$ cells, respectively (Figure 5). Finally, the muscle sheets cultured in fCM were composed of longer myofibers with distinctly larger diameter (Figure 4(c)).

Direct addition of fibroblast in culture had a less significant impact on myofiber density compared to conditioned media. The impact of initial fibroblast concentration was amplified in the presence of fCM. This amplification is likely the result of increased cell proliferation induced by the growth factors found in conditioned media. High fibroblast



(a) (b)

Figure 2: Multinucleated myofibers in representative muscle tissue sheets (90% C2C12 and 10% Nor-10) cultured in (a) control medium or (b) fCM. Arrows indicate myofibers that stained positive for muscle-specific Desmin (green) and nuclei (blue). Both tissue sheets were fixed at D1 of differentiation.

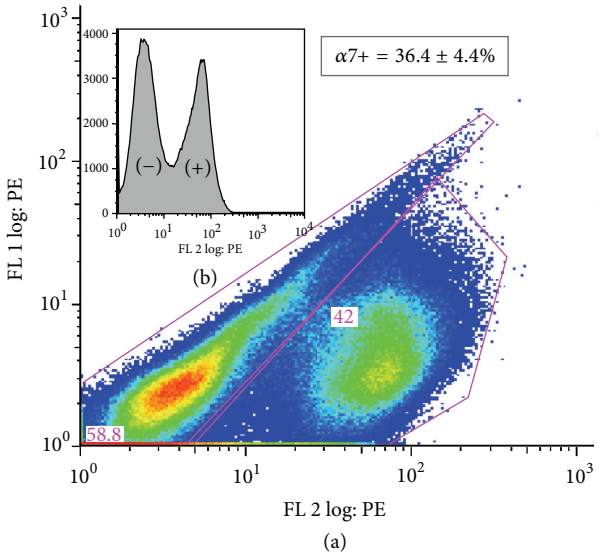


Figure 3: Expression of $\alpha 7$ in a mixed cell population. (a) $\alpha 7$ PE (b) expression histogram.

proliferation could lead to a condition that mimics fibrosis and would inhibit myofiber formation.

Interestingly, the primary satellite cells had a lower overall capacity to form myofibers as compared to C2C12 cells, which readily assemble confluent sheets by D2. Satellite cells may be more dependent on exogenous stimulus to form complete muscle sheets and therefore fCM may represent an important addition to a successful muscle bioreactor. Fibroblasts can be easily isolated from tissue biopsy such that fibroblast-conditioned media would be available along with an autologous cell isolate.

While the present study explores the effect of fibroblast-derived paracrine factors on myogenesis, it is also known that proinflammatory cytokines secreted by monocytes and macrophages may play an important role during *in vivo* satellite cell recruitment and differentiation [24]. In a muscle tissue engineering setting the role of proinflammatory mediators is less clear. It may be desirable to further examine the effect proinflammatory cytokines on tissue-engineered skeletal muscle.

The results of this study demonstrate the effectiveness of fCM in promoting myofiber formation. In the future we will apply this new tissue culture condition to 3D-engineered muscle construct with biomimetic tissue organization, for which we have previously developed a methodology [8], in an attempt to improve the muscle tissue formation and functionality.

4. Conclusions

Engineered skeletal muscle sheets were successfully prepared from a two distinct subpopulations of myoblasts. Initial trials of early tissue culture with myoblast and muscle fibroblast cell lines showed a statistically significant increase in myofiber density when the engineered tissue was cultured in fibroblast-conditioned media (fCM). As a step toward translation, we then explored the impact of fCM on freshly isolated satellite cells. At D6, fCM treatment had a qualitative and quantitative impact on myofiber formation for tissue sheets prepared with primary nSKMs. From these results, we conclude that conditioned media can be used to potentiate myogenesis *in vitro* through improved differentiation of skeletal muscle precursor cells. Paracrine factors released by fibroblasts may represent an important target for potentiating myogenesis in engineered skeletal muscle constructs.

Acknowledgments

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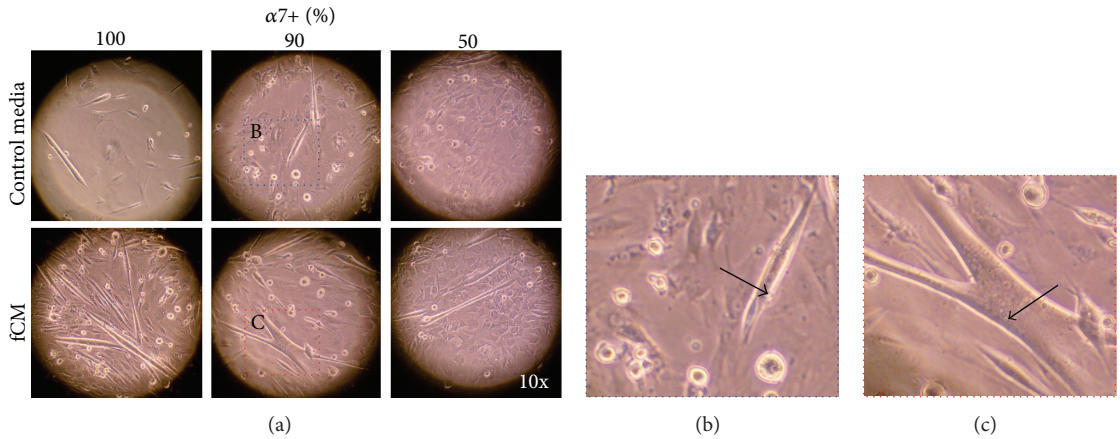

Figure 4: Morphology of muscle tissue sheets. (a) Live culture images from tissue sheets with varying $\alpha 7+$ concentration (100, 50, 90%) in either control media (top panel) or fCM (middle panel). Enlarged representative sections from 90% $\alpha 7+$ tissue sheets cultured in (b) control media and (c) fCM demonstrate that the myofibers in fCM are longer and thicker.

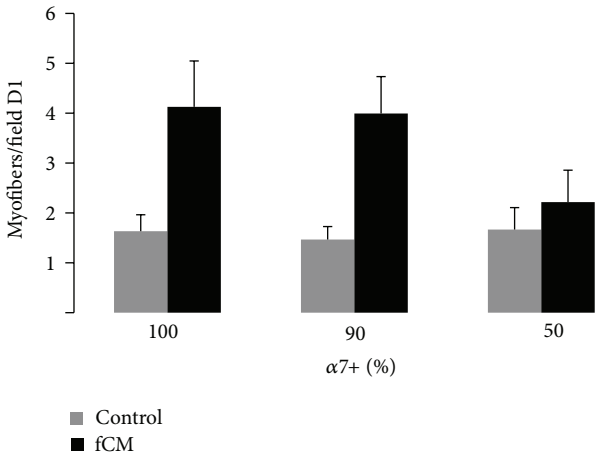

Figure 5: Myofiber density in $\alpha 7+/\alpha 7-$ tissue sheets with varied initial cell concentration; 100% $\alpha 7+$ with 0% $\alpha 7-$, 90% $\alpha 7+$ with 10% $\alpha 7-$, to from 50% $\alpha 7+$ with 50% $\alpha 7-$. Bars show $n = 3$ tissue sheets per group. "$*$" statistically significant between the indicated pairings, $P < 0.05$.

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Clinical Study

Cytokine Response of Cultured Skeletal Muscle Cells Stimulated with Proinflammatory Factors Depends on Differentiation Stage

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Myoblast proliferation and myotube formation are critical early events in skeletal muscle regeneration. The attending inflammation and cytokine signaling are involved in regulation of skeletal muscle cell proliferation and differentiation. Secretion of muscle-derived cytokines upon exposure to inflammatory factors may depend on the differentiation stage of regenerating muscle cells. Cultured human myoblasts and myotubes were exposed to 24-hour treatment with tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS). Secretion of interleukin 6 (IL-6), a major muscle-derived cytokine, and interleukin 1 (IL-1), an important regulator of inflammatory response, was measured 24 hours after termination of TNF-α or LPS treatment. Myoblasts pretreated with TNF-α or LPS displayed robustly increased IL-6 secretion during the 24-hour period after removal of treatments, while IL-1 secretion remained unaltered. IL-6 secretion was also increased in myotubes, but the response was less pronounced compared with myoblasts. In contrast to myoblasts, IL-1 secretion was markedly stimulated in LPS-pretreated myotubes. We demonstrate that preceding exposure to inflammatory factors stimulates a prolonged upregulation of muscle-derived IL-6 and/or IL-1 in cultured skeletal muscle cells. Our findings also indicate that cytokine response to inflammatory factors in regenerating skeletal muscle partially depends on the differentiation stage of myogenic cells.

1. Introduction

Skeletal muscle normally represents 40% of body weight and has a vital role in locomotion and whole body metabolism. Disorders associated with loss of skeletal muscle, including cancer cachexia and age-related sarcopenia, are associated with increased morbidity and mortality [1–4]. Muscle regeneration is vital for maintenance of skeletal muscle mass and function [5]. Different stimuli, including overloading, denervation, direct injury to muscle fibers, or different clinical conditions, trigger regeneration process by activating muscle satellite cells, which transform into proliferating myoblasts. Subsequently, myoblasts fuse with existing muscle fibers or fuse with each other to form multinucleated myotubes [5, 6]. As well as reconstituting muscle tissue following major injury, regeneration process constantly removes smaller lesions due to daily wear-and-tear [7]. Furthermore, satellite cell activation and myoblast proliferation are involved in load-induced muscle hypertrophy [8]. Indeed, myoblasts may contribute new nuclei to existing muscle fibers and thereby support the hypertrophic muscle growth [8, 9]. Additionally other stem cell populations have been identified recently and can participate in muscle regeneration and growth [10]. This indicates that exercise-induced or pharmacological stimulation of muscle regeneration could represent a strategy to treat muscle atrophy associated with different diseases.

Skeletal muscle is an important source of interleukin-6 (IL-6) and other muscle-derived cytokines (myokines), which modulate immune responses and regulate energy metabolism [11]. In skeletal muscle IL-6 is lowly expressed under resting conditions, but is markedly induced during exercise [11, 12]. IL-6 expression is also up-regulated in

regenerating skeletal muscle [13, 14]. Moreover, IL-6 is constitutively secreted in myogenic precursor cells, including myoblasts and myotubes [15, 16]. IL-6 promotes myoblast proliferation [8, 17] and/or myotube formation [18, 19], suggesting a role for IL-6 in muscle regeneration. Additionally, by stimulating myoblast proliferation IL-6 may contribute to load-induced muscle hypertrophy [8]. Regulation of IL-6 secretion during muscle regeneration has not been fully characterized, but may involve inflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1).

Although chronic exposure to TNF-α and IL-1 leads to insulin resistance and proteolysis in skeletal muscle [20–22], both salient characteristics of cachectic states [2], TNF-α and IL-1 could directly or indirectly promote early stages of skeletal muscle regeneration. First of all, TNF-α and IL-1 as well as their receptors are up-regulated in regenerating skeletal muscle [23]. Furthermore, during the initial stages of muscle regeneration macrophages accumulate in injured muscle and represent an important source of TNF-α and IL-1 [5, 24]. As well as removing necrotic debris, macrophages are thought to regulate muscle regeneration directly by stimulating proliferation and/or differentiation of myogenic precursor cells [5, 25]. Also, TNF-α and IL-1 are well-characterized upstream regulators of IL-6 secretion under septic conditions [26, 27] and in cultured skeletal muscle cells [15, 28], suggesting they may indirectly stimulate muscle regeneration by enhancing IL-6 mediated signaling. Finally, TNF-α was shown to promote and/or sustain proliferation [29, 30]. However, the understanding of the role of IL-1 and TNF-α in orchestrating muscle regeneration is incomplete.

During regeneration skeletal muscle cells proceed through an extensive developmental program, which transforms proliferating mononuclear myoblasts into terminally differentiated multinucleated muscle fibers [6]. Considering fundamental phenotypic differences between myoblasts and mature muscle fibers, responsiveness to inflammatory cytokines may depend on the differentiation stage of skeletal muscle cells. The aim of this study was to determine whether endogenous or exogenous inflammatory factors differentially regulate secretion of muscle-derived cytokines in different developmental stages of cultured skeletal muscle.

2. Materials and Methods

2.1. Reagents. ELISA kits for human IL-6 and IL-1 were from Pierce/Thermo Scientific (Waltham, MA, USA). Cell culture flasks and plates were obtained from BD Falcon (Franklin Lakes, NJ, USA). Advanced Minimal Essential Medium (MEM), fetal bovine serum (FBS), Earle's Balanced Salt Solution, trypsin, gentamycin, and Fungizone were obtained from Invitrogen (Paisley, UK). Hoechst was from Molecular Probes (Invitrogen) and Cytotoxicity Detection kit (LDH) from Roche Applied Science (Mannheim, Germany). All other reagents, including lipopolysaccharide (LPS) and TNF-α, were of analytical grade and were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Human Skeletal Muscle Cell Culture. This study was approved by the Ethical Commission at the Ministry of Health of the Republic of Slovenia (no. 63/01/99) and (no. 71/05/12). Human skeletal muscle cultures were prepared from muscle tissue samples obtained during routine orthopaedic surgery from donors without neuromuscular disease. All donors gave their written informed consent. Preparation of primary culture of human skeletal muscle cells was performed as previously described [31–34]. Briefly, muscle samples were cleaned of connective and adipose tissue, cut into small pieces, and then trypsinised for 30 minutes in Earle's Balanced Salt Solution supplemented with trypsin-EDTA at 37°C. The released skeletal muscle cells were grown on Petri dishes in growth medium (Advanced MEM supplemented with 10% (v/v) FBS, 0.3% (v/v) Fungizone and 0.15% (v/v) gentamycin) at 37°C in 5% CO_2/humidified air. After 2-3 weeks and before fusion into myotubes myoblast colonies were selectively trypsinised and transferred to 75 cm^2 cell culture flasks. Growth medium was changed 2-3 times per week and myoblasts were always subcultured before reaching confluence. They were propagated for 2-3 passages before being used for experiments. All experiments were performed on skeletal muscle cell cultures from 4–6 donors.

2.3. TNF-α and LPS Treatments in Cultured Myoblasts and Myotubes. Before the experiment, myoblasts were seeded in six-well plates (BD Falcon), where they were grown on glass cover slips and coated with a 1 : 2 mixture of 1.5% gelatin (Sigma, St. Louis, MI, USA) and human plasma. Experiments on myoblasts were performed on subconfluent cultures before the start of fusion into myotubes. To induce myogenic differentiation and fusion into myotubes, subconfluent myoblast cultures were switched from growth medium to differentiation medium (Advanced MEM supplemented with 2% FBS, 0.3% (v/v) Fungizone and 0.15% (v/v) gentamycin) for 2-3 days. When myotubes started to form, differentiation medium was exchanged for growth medium. Experiments on myotubes were carried out after 3 weeks of differentiation. Cultured myoblasts or myotubes were treated with 100 ng/mL TNF-α (Sigma) or 100 ng/mL LPS (Sigma) or vehicle in growth medium for 24 hours. On the second day of experiment media containing treatments were replaced with growth medium (washout). Experiment was terminated 24 hours later, when media were collected for determination of secreted cytokines.

2.4. Determination of IL-6 and IL-1 Secretion from Cultured Human Myoblasts and Myotubes. Concentrations of IL-6 and IL-1 in cell culture media were measured with human IL-6 and IL-1 ELISA kits (Pierce/Endogen Thermo Scientific) according to the manufacturer's instructions. Growth medium was used as a diluent for the standards as well as the samples to avoid analytical interference. Data were normalized to the number of nuclei per well to allow comparison of cytokine secretion between mononuclear myoblasts and polynuclear myotubes, as described [15]. Briefly, cell cultures on cover slips were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 15 min. They were subsequently exposed to 1 mM Hoechst 33258 (Molecular Probes-Life Technologies, Willow creek, OR, USA), prepared

in phosphate-buffered saline, for 5 min. Number of nuclei per well was estimated by counting Hoechst-stained nuclei in 10 random fields of view per well at 200x magnification. Cytokine secretion data are expressed as the mass of secreted IL-1 or IL-6 in ng/24 h/100,000 nuclei.

2.5. Assessment of Cytotoxicity and Apoptosis. Cytotoxicity was assessed by measuring the activity of lactate dehydrogenase (LDH) in cell culture medium using Cytotoxicity Detection kit (LDH) (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. TNF-α and LPS cause apoptosis in some cell types we tested our cultures for this effect. Apoptosis was assessed by the DNA fragmentation and nuclear chromatin examination, as described [15]. For DNA fragmentation we harvest cells treated with TNF-α and LPS; total DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Electrophoresis was performed on ethidium-bromide stained 1.8% agarose gel and visualized with transilluminator. To obtain a positive control of the DNA ladder, cells were treated with 10% DMSO for three days. For nuclear chromatin examinations, cells, grown on sterile collagen-coated coverslips, or eventually floating dead cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, stained with 1 mM Hoechst 33258 in PBS and examined under fluorescence microscope. Cells were scored as apoptotic if they exhibited unequivocal nuclear chromatin condensation and/or fragmentation.

2.6. Statistics. The data are presented as means ± SEM. Univariate two-way or three-way analysis of variance (ANOVA) was used to analyze the differences between the myoblasts and myotubes for their IL-6 and IL-1 secretion under the different experimental conditions. Bonferroni post hoc test was used. Statistical significance was established at $P < 0.05$. The data were analyzed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft Office Excel 2003).

3. Results

3.1. IL-6 Secretion in Cultured Myoblasts and Myotubes Is Increased after Removal of TNF-α and LPS. Initial stages of skeletal muscle regeneration are characterized by the presence of proinflammatory macrophages, which secrete TNF-α and IL-1 [5]. Later macrophages downregulate TNF-α and IL-1 expression and acquire anti-inflammatory properties, which is thought to underlie resolution of the inflammation [5, 25]. We have previously demonstrated that myoblast and myotubes increase IL-6 secretion in response to treatment with TNF-α [15]. To explore whether preceding exposure to TNF-α affects cytokine secretion in cultured skeletal muscle cells, myoblasts and myotubes were treated with 100 ng/mL TNF-α or vehicle for 24 hours. Treatment media were subsequently removed and fresh growth medium was added. Cytokine secretion was assessed during the 24 hours after removal of TNF-α (Figure 1). Basal IL-6 secretion was similar between myoblasts and myotubes. Increased IL-6 secretion was detected in myoblasts ($P < 0.05$) and myotubes ($P < 0.05$) pretreated with TNF-α. Secretion of IL-6 tended to be lower in myotubes, but the difference did not reach the level of statistical significance. Under *in vitro* conditions, macrophages can be induced to acquire pro-inflammatory phenotype by bacterial lipopolysaccharide (LPS) [25]. LPS also directly stimulates IL-6 secretion from cultured myoblasts and myotubes [15]. We therefore determined whether 24-hour pretreatment with 100 ng/mL LPS affects IL-6 secretion in cultured myoblasts and myotubes (Figure 1). Pretreatment with LPS resulted in a robustly increased IL-6 secretion from myoblasts ($P < 0.05$) and myotubes ($P < 0.05$) during 24 hours after removal of LPS. Consistent with TNF-α treatment, myoblasts were more responsive to stimulation with LPS compared to myotubes ($P < 0.05$). These results indicate that preceding treatment with TNF-α and LPS has a prolonged effect on IL-6 secretion in cultured skeletal muscle.

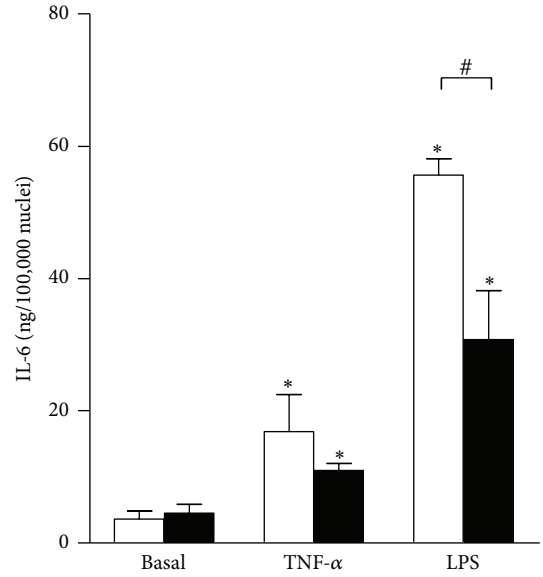

Figure 1: IL-6 secretion from cultured skeletal muscle cells is increased after removal of TNF-α and LPS treatment. Human myoblasts (white bars) and myotubes (black bars) were exposed to TNF-α (100 ng/mL) or LPS (100 ng/mL) or vehicle (Basal) for 24 hours. Medium containing TNF-α or LPS was removed and replaced with growth medium. IL-6 secretion was estimated by ELISA 24 hours after compound removal. Data are means ± SEM ($n = 4$–6). $^{*}P < 0.05$ versus respective Basal. $^{\#}P < 0.05$ myoblasts versus myotubes (95% confidence interval (CI) of difference: −39.96 to −9.74).

3.2. IL-1 Secretion in Cultured Myoblasts and Myotubes Is Increased after Removal of TNF-α and LPS. Next we investigated whether TNF-α and LPS affect secretion of muscle-derived IL-1 in cultured myoblasts and myotubes. IL-1 has a well-characterized role in LPS-triggered cytokine cascade under septic conditions [27], but IL-1 is usually not recognized as a member of myokine family and its function during skeletal muscle regeneration is poorly understood. Cultured myoblasts and myotubes robustly secreted IL-1 in amounts

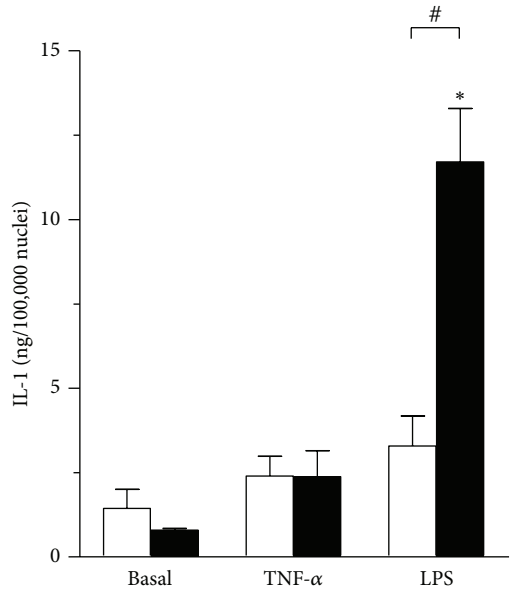

Figure 2: IL-1 secretion from cultured skeletal muscle cells is increased after removal of TNF-α and LPS treatment. Human myoblasts (white bars) and myotubes (black bars) were exposed to TNF-α (100 ng/mL) or LPS (100 ng/mL) or vehicle (Basal) for 24 hours. Medium containing TNF-α or LPS was removed and replaced with growth medium. IL-1 secretion was estimated by ELISA 24 hours after compound removal. Data are means ± SEM ($n = 4$–6). $^{*}P < 0.05$ versus respective Basal. $^{\#}P < 0.05$ myoblasts versus myotubes (95% CI of difference: 5.28 to 11.56).

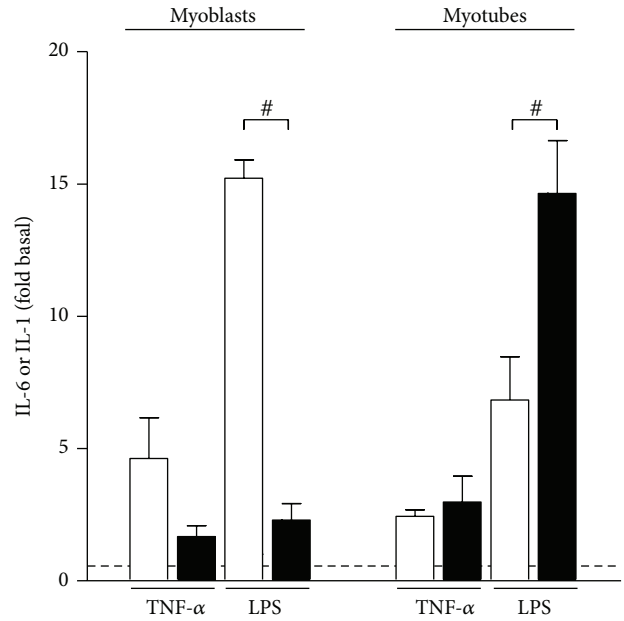

Figure 3: Cultured skeletal muscle cells are characterized by differential responsiveness to pro-inflammatory factors. Human myoblasts and myotubes were exposed to TNF-α (100 ng/mL) or LPS (100 ng/mL) for 24 hours. Medium containing TNF-α or LPS was removed and replaced with growth medium. Fold increase in IL-6 (white bars) and IL-1 (black bars) secretion following exposure to TNF-α or LPS was calculated to assess responsiveness of myoblasts and myotubes to stimulation with pro-inflammatory factors TNF-α or LPS. The dashed line symbolically represents the level of IL-6 or IL-1 secretion under basal conditions. Data are means ± SEM ($n = 4$–6). $^{\#}P < 0.05$ versus IL-6 secretion in LPS-treated myoblasts (95% CI of difference: −18.34 to −7.53) and myotubes (95% CI of difference: 2.42 to 13.23).

comparable to IL-6 (Figure 2). Pre-treatment with TNF-α tended to increase secretion of IL-1 from myoblasts and myotubes, but the increase did not reach the level of statistical significance (Figure 2). In sharp contrast, LPS pre-treated myotubes displayed a robust increase in IL-1 secretion ($P < 0.05$) during 24 hours after removal of LPS. Myotubes were markedly more responsive to LPS pretreatment compared with myoblasts ($P < 0.05$). These data suggest IL-1 secretion in regenerating myoblasts is not increased upon removal of pro-inflammatory factors.

3.3. Cytokine Response Upon Exposure to Proinflammatory Factors Depends on Differentiation Stage of Cultured Skeletal Muscle Cells. Proliferating myoblasts and differentiated myotubes are two distinct developmental stages, characterized by fundamental phenotypic differences. IL-6 promotes myoblast proliferation [8, 17], whereas IL-1 has a prominent role in immune response and may act as a negative regulator of myogenic differentiation [35]. To assess the potential differences in cytokine response in myoblasts and myotubes display different, we analyzed IL-1 and IL-6 secretion pattern following exposure to TNF-α and LPS (Figure 3). Myoblasts and myotubes were responsive to both TNF-α and LPS, but their response pattern was markedly different. TNF-α and LPS pretreatment in myoblasts resulted in robustly increased IL-6 secretion, whereas IL-1 was not appreciably increased. By contrast, in LPS-treated myotubes IL-1 was more strongly induced than IL-6 ($P < 0.05$). IL-6 and IL-1 secretion in myotubes was higher following LPS pretreatment compared with TNF-α ($P < 0.05$). Taken together our data suggest that pro-inflammatory factors induce prolonged up-regulation of IL-6 in myoblasts, whereas myotubes display a robust increase in IL-1 secretion concomitant with less pronounced stimulation IL-6 production.

3.4. TNF-α and LPS Treatment Did Not Induce Cell Death. LPS and TNF-α treatment can lead to cytotoxicity and cell death [36]. Cytotoxicity of TNF-α and LPS treatment was assessed by measuring the activity of lactate dehydrogenase in cell culture media as describes in Materials and Methods. There was no difference in lactate dehydrogenase activity in myoblasts or myotubes under basal condition compared to TNF-α and LPS treatment (data not shown). Apoptosis was not induced, as assessed by evaluation of DNA fragmentation and chromatin condensation. Also, the number of cells per well was similar between different treatments.

4. Discussion

Skeletal muscle regeneration is an essential process for maintenance of muscle mass and function [5, 6]. Although

exposure to pro-inflammatory cytokines like TNF-α and IL-1 may has deleterious effects on skeletal muscle [2, 20, 21], cytokine signaling plays an important role in skeletal muscle regeneration and/or hypertrophy [5, 6, 8, 25]. TNF-α and IL-1 are produced by immature skeletal muscle cells [15, 16, 37] and/or by macrophages, which infiltrate injured skeletal muscle [5, 25]. Moreover, evidence suggests TNF-α stimulates and/or prolongs myoblast proliferation [29, 30], indicating that TNF-α may promote muscle regeneration. Molecular mechanisms underlying divergent roles of pro-inflammatory cytokines in skeletal muscle remain to be established, but differential responsiveness to external stimuli could depend on the developmental stage of skeletal muscle cells during regeneration and on dosage and time of exposure. Here we show that proliferating myoblasts respond to pro-inflammatory factors TNF-α and LPS with prolonged increase in IL-6 secretion, while IL-1 secretion remained unaltered. By contrast myotubes displayed markedly increased IL-1 secretion, but exhibited less pronounced IL-6 up-regulation.

Skeletal muscle is an important source of cytokines, which regulate many aspects of muscle function and participate in regulation of immune responses as well as whole body metabolism [11, 38]. We observed robust IL-6 secretion under basal conditions, which is consistent with earlier studies in cultured skeletal muscle cells [15, 16, 39]. Several factors are known to modulate IL-6 secretion from skeletal muscle cells, including LPS, TNF-α, glucocorticoids, and hypoxia [15, 16, 33], but regulatory mechanisms underlying basal IL-6 secretion are incompletely understood. Interestingly, myoblasts and myotubes constitutively secreted IL-1 in amounts comparable to IL-6, whereas substantially lower level of IL-1 secretion was previously observed in cultured myoblasts [16]. However, we did not detect increased IL-1 production in myoblasts exposed to TNF-α or LPS although IL-6 secretion was markedly stimulated. This suggests that putative autocrine stimulation by IL-1 does not play a major role in inflammatory factor-induced up-regulation of IL-6 in cultured skeletal muscle cells. Of note, endogenous TNF-α expression was observed in C2C12 cells under basal conditions [37]. Similarly, we detected modest secretion of TNF-α in cultured human skeletal muscle cells (data not shown). Taken together this indicates that endogenous TNF-α production could represent an additional stimulus for basal IL-6 secretion in myoblasts and myotubes [15].

Early stages of muscle regeneration are characterized by increased expression of TNF-α and IL-1 as well as LPS-binding protein [23]. This is coincident with influx of neutrophils and macrophages into the injured skeletal muscle. Initially, resident macrophages display inflammatory phenotype and secrete pro-inflammatory cytokines like TNF-α and IL-1 [5]. Subsequently, macrophages suppress TNF-α and IL-1 expression and acquire an anti-inflammatory phenotype, which is thought to be particularly important for the outcome of regeneration process [5, 25]. We previously demonstrated that pro-inflammatory milieu, mimicked by TNF-α or LPS treatment strongly induces IL-6 secretion in cultured myoblasts and myotubes [15]. In the work presented here we determined whether preceding exposure to pro-inflammatory environment has a prolonged effect on IL-6 up-regulation. Indeed, stimulation of cultured myoblasts by TNF-α or LPS led to prolonged increase in IL-6 secretion even as pro-inflammatory treatment was removed. We also found that cultured myotubes robustly increase IL-1 production in response to LPS, whereas IL-1 secretion remained unaltered in myoblasts. As previously reported, TNF-α exposure did not increase IL-1 secretion from cultured myoblasts [16]. These data demonstrate that cytokine response in cultured human skeletal muscle cells persists even after removal of pro-inflammatory factors, indicating withdrawal of inflammatory stimuli may not be sufficient for immediate resolution of inflammation associated with skeletal muscle injury and regeneration. These findings are also compatible with the notion that switches to pro-inflammatory macrophage phenotype, which involves secretion of anti-inflammatory cytokines, actively promotes termination of acute inflammation [5, 25].

Our data show that, in contrast to constitutive level of cytokine secretion, the production of cytokines following exposure to inflammatory factors TNF-α and LPS is related to the developmental stage of myogenic cells. This is in agreement with our previous observations that induction of IL-6 secretion in human myoblasts during TNF-α or LPS treatment is more pronounced compared with response in myotubes [15]. The biological basis of these differences is not fully understood but could reflect phenotypic differences between myoblasts and myotubes. Mononuclear myoblasts are actively proliferating cells, which markedly differ from the differentiated multinucleated myotubes as regards protein expression and responses to various extrinsic factors [40]. Moreover, while myoblasts proliferate intensively in order to reach the critical number of cells that are necessary for successful fusion, myotubes are destined for further differentiation, innervation and maturation to adult muscle fibers [5, 6, 41]. The distinctive biological difference in the role played by myoblasts and myotubes during muscle regeneration is reflected in different expression patterns of TNF-α receptors [42] and Toll-like receptors [43] in myoblasts and myotubes, with consecutive activation of different intracellular signaling pathways during muscle ontogenesis and regeneration [7, 44].

Inflammation during initial stages of regeneration has an important role in restoration of muscle function after injury [5, 24]. However, chronic inflammation and/or exposure to pro-inflammatory cytokines has deleterious effect on skeletal muscle [2, 20, 21, 45]. Timely suppression of inflammation by anti-inflammatory macrophages is therefore considered crucial for a favorable outcome of regeneration [5, 25]. Here we demonstrate increased IL-6 and IL-1 secretion even after removal of TNF-α and LPS treatment. Considering that IL-6 increases myoblast proliferation [8, 17], this indicates pro-inflammatory factors could promote early stages of muscle regeneration by indirectly increasing the number of myogenic cells. Additionally, TNF-α may directly increase myoblast proliferation [30]. However some studies demonstrated that TNF-α may have also inhibitory effect upon myoblast differentiation [46–48]. In contrast to IL-6, increased IL-1 secretion was detected only in myotubes. IL-1 is an important mediator of the immune response

and plays a role in the local response to infection and tissue injury as well as regulation of cell proliferation, differentiation and apoptosis [49], but its role in muscle regeneration is not well-characterized. Thus, increased IL-1 production upon exposure to inflammatory factors may concomitantly reduce myotube formation and promote IL-6-stimulated myoblast proliferation. Transient stimulation with pro-inflammatory factors may therefore promote myogenesis by increasing the number of myoblasts, whereas diminished differentiation of myogenic cells would tend to impair the outcome of regeneration during chronic exposure to pro-inflammatory factors. Altogether, our findings are compatible with the observations that pro-inflammatory (TNF-α and IL-1 expressing) macrophages stimulate myoblast proliferation even as they suppress myoblast differentiation and fusion into myotubes [5, 25].

Collectively, our findings demonstrate that skeletal muscle cells display different cytokine response patterns upon stimulation with endogenous or exogenous pro-inflammatory factors TNF-α or LPS, respectively. Moreover, we show that TNF-α or LPS treatment has a prolonged effect on increased cytokine secretion in cultured myoblasts and myotubes even as pro-inflammatory treatment is terminated. Although myoblasts and myotubes display similar basal IL-1 and IL-6 secretion, myoblasts tend to respond to pro-inflammatory stimuli with increased IL-6 secretion, whereas IL-1 production was particularly strongly augmented in myotubes. Regulation of cytokine production therefore depends on the differentiation stage and is probably cytokine-type specific. These findings shed further light on the complex interactions between the cytokines and skeletal muscle cells during regeneration.

Conflict of Interests

The authors declare no competing interests.

Authors' Contribution

M. Podbregar and T. Mars carried out the experiments and wrote the paper. M. Lainscak and O. Prelovsek participated in study design, results analysis, and paper preparation. All authors read and approved the final paper.

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Review Article

Exercise-Induced Rhabdomyolysis and Stress-Induced Malignant Hyperthermia Events, Association with Malignant Hyperthermia Susceptibility, and *RYR1* Gene Sequence Variations

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Exertional rhabdomyolysis (ER) and stress-induced malignant hyperthermia (MH) events are syndromes that primarily afflict military recruits in basic training and athletes. Events similar to those occurring in ER and in stress-induced MH events are triggered after exposure to anesthetic agents in MH-susceptible (MHS) patients. MH is an autosomal dominant hypermetabolic condition that occurs in genetically predisposed subjects during general anesthesia, induced by commonly used volatile anesthetics and/or the neuromuscular blocking agent succinylcholine. Triggering agents cause an altered intracellular calcium regulation. Mutations in *RYR1* gene have been found in about 70% of MH families. The *RYR1* gene encodes the skeletal muscle calcium release channel of the sarcoplasmic reticulum, commonly known as ryanodine receptor type 1 (RYR1). The present work reviews the documented cases of ER or of stress-induced MH events in which *RYR1* sequence variations, associated or possibly associated to MHS status, have been identified.

1. Introduction

Rhabdomyolysis is an acute syndrome determined by a direct or indirect muscle injury. It results from skeletal muscle breakdown and massive release of the intracellular content into blood circulation, which can lead to potentially fatal events, such as acute renal failure, hyperkalemia, and other metabolic complications [1, 2]. The etiology of rhabdomyolysis is broad and includes inherited diseases, drugs, toxins, muscle compression, overexertion, and infections. Regardless of the mechanism, these muscle injuries ultimately lead to a leakage of Ca^{2+} ions into the intracellular space, and the excess of Ca^{2+} ions gives rise to a persistent muscle contraction that ends in energy depletion and cell death (Figure 1) [1]. Rhabdomyolysis syndrome may also occur as a result of a strenuous or not strenuous physical exercise (exertional rhabdomyolysis or ER) often in hot and humid climates. Although anyone may develop ER under extreme physical and environmental conditions, some individuals seem to be more predisposed than others, suggesting a genetic link. The most commonly identified predisposing conditions of ER are deficiencies of carnitine palmitoyltransferase II (*CPT2* gene, OMIM *600650), myophosphorylase (McArdle disease, *PYGM* gene, OMIM *608455), and myoadenylate deaminase (*AMPD1* gene, OMIM +102770). Events similar to those occurring in ER are triggered after exposure to anesthetic agents in malignant hyperthermia susceptible (MHS) patients. Therefore, an association between ER and malignant hyperthermia (MH) has been investigated and reported [3–10]. However, two studies on the effect of exercise on thermoregulatory and metabolic responses in MHS subjects gave controversial results [11, 12]. Moreover, cases of MH-like events in the absence of anesthetic agents, and caused by high environmental or core body temperature, or even by emotional stress, have been reported [13–16].

Malignant hyperthermia (OMIM #145600) is an autosomal dominant hypermetabolic condition that occurs in genetically predisposed subjects during general anesthesia, induced by commonly used volatile anesthetics and/or the neuromuscular blocking agent succinylcholine. Triggering agents cause an altered intracellular calcium regulation. An MH attack, unless immediately recognized and treated, is

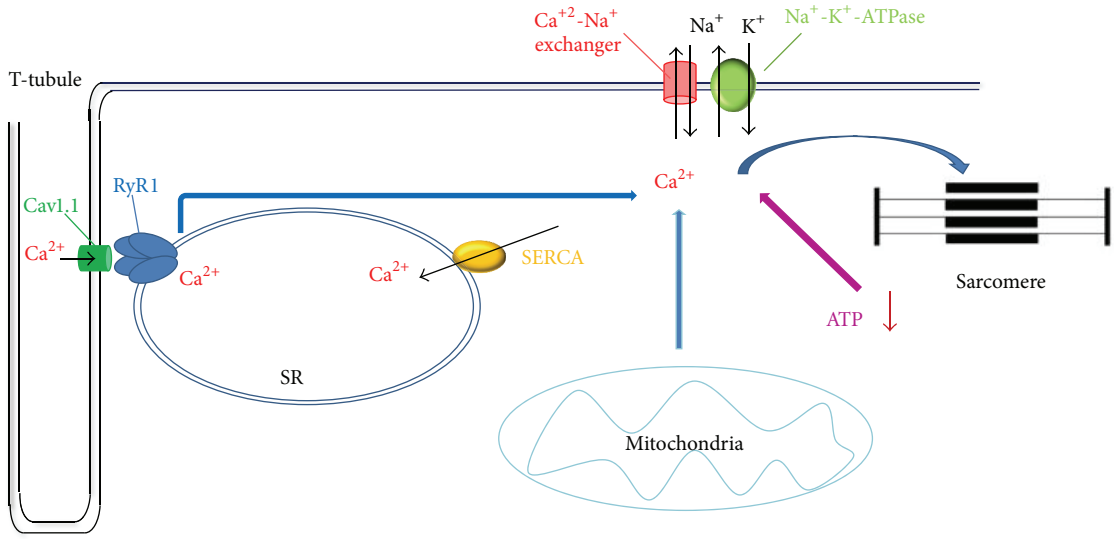

Figure 1: Schematic representation of a skeletal muscle cell and of Ca^{2+} and Na^{+} ion fluxes across the sarcolemma and sarcoplasmic reticulum (SR). Activation of Cav1.1 by membrane depolarization causes the RyR1 channel to open and to release Ca^{2+} from SR, thus triggering muscle contraction. Ca^{2+} concentration is regulated by the Ca^{2+}-ATPase membrane pump (SERCA) that sequesters Ca^{2+} in the SR and by the Na^{+}-K^{+}-ATPase membrane pump and the Ca^{2+}-Na^{+} antiport that exchange Ca^{2+} for Na^{+} across the sarcolemma. Regulation of calcium flux may be disrupted at any of these sites. ATP depletion, by consumption during muscle contraction, or reduced ATP production, results in intracellular Ca^{2+} increasing, muscle contraction, and continued energy consumption, leading to rhabdomyolysis.

often fatal. Clinical symptoms of a classic MH attack are accelerated muscle metabolism, muscle contractions, metabolic acidosis, tachycardia, and hyperthermia. These symptoms are correlated with some altered biochemical parameters, such as metabolic acidosis with increased pCO_2 and lactate production and release of potassium and muscle proteins, as creatine kinase and myoglobin, into the blood. Frequent late events are damage of kidney function due to massive myoglobin release and/or a diffuse intravascular coagulation, which is often the main cause of death [17]. The prevalence of MH episodes is estimated to range from 1 : 10,000 to 1 : 220,000 [17]. Malignant hyperthermia susceptibility can be diagnosed by an *in vitro* test, based on the differential contractile response of normal (MHN) and MHS muscles to caffeine and halothane. Protocols for MH contracture testing of human skeletal muscle have been developed by the European [18] and North American [19] MH Groups, namely, *in vitro* contracture test (IVCT) and caffeine halothane contracture test (CHCT), respectively. A considerable genetic heterogeneity has been reported for MH. Six genetic *loci* (MHS1, OMIM #180901; MHS2, OMIM #154275; MHS3, OMIM #154276; MHS4, OMIM #600467; MHS5, OMIM #601887; MHS6, OMIM #601888-6), associated with MH, have been identified. About 70% of affected families are linked to the MHS1 *locus*, where the *RYR1* gene encoding the skeletal muscle calcium release channel of the sarcoplasmic reticulum, commonly known as ryanodine receptor type 1 (RyR1), maps. Dantrolene is an RyR1 antagonist that blocks calcium release from the sarcoplasmic reticulum stores and is the only specific agent available for the treatment of an MH attack. Less than 1% of MHS cases can be attributed to mutations in the *CACNA1S* gene (*locus* MHS5) encoding the α1S subunit of the voltage-dependent L-type calcium channel of the skeletal muscle, Cav1.1. Only three MH-causing mutations identified in the *CACNA1S* gene were hitherto functionally characterized [20–22]. RyR1 and Cav1.1 are the two major proteins involved in the excitation-contraction coupling in skeletal muscle.

The aim of this paper is to review the documented cases of ER or of stress-induced MH events in which sequence variations (SVs) of the *RYR1* gene, associated or possibly associated to MHS, have been identified.

2. Methods

The PubMed and Web of Science databases were consulted to search for studies on documented cases of ER or of stress-induced MH events in which *RYR1* SVs, associated or possibly associated to MHS, have been identified. Search terms included "*RYR1*," "mutation," "malignant hyperthermia," "exercise," "heat stress," "stress-induced malignant hyperthermia," and "nonanesthetic malignant hyperthermia." Single-nucleotide polymorphism (SNP) databases (http://www.ncbi.nlm.nih.gov/snp, http://www.dmd.nl/nmdb2/variants.php?select_db=RYR1) were also searched. Three different programs, namely, PMut (http://mmb.pcb.ub.es/PMut/), SIFT (http://sift.jcvi.org/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), were used to predict the pathological character of *RYR1* SVs which have not been functionally characterized. PMut is based on the use of neural networks trained with a very large database of human disease-associated mutations and neutral SVs [23] and combines sequence alignment/position-specific scoring matrix with structural factors; score >0.5 predicts a pathological effect. SIFT is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences [24]. The SIFT scores range from 0 to 1; the amino

acid substitution is predicted as damaging if the score is ≤0.05 and as tolerated if the score is >0.05. PolyPhen-2 predicts the effects of an amino acid substitution using both structure and sequence information [25] and classifies variants as "probably damaging," "possibly damaging," or "benign," based on pairs of false positive rate thresholds.

3. Results

3.1. RYR1 Gene Sequence Variations (SVs) in ER and Stress-Induced MH Patients. Thus far, more than 300 missense SVs have been identified in the *RYR1* gene (http://www.ncbi.nlm.nih.gov/snp, http://www.dmd.nl/nmdb2/variants.php?select_db=RYR). Some *RYR1* SVs have been characterized by *in vitro* functional studies. The demonstration that a SV alters the kinetic properties of the RyR1 channel allows to define its role in the pathogenesis of MHS. Various methods have been developed to characterize the function of RyR1 variants: analysis of calcium release in human primary myotubes [26–28] and in immortalized B lymphocytes from patients or after expression by transfection in various cell types [29–31], determination of the channel openings in a ryanodine binding assay [32], and a metabolic test *in vitro* based on the measurements of proton release rate in immortalized B lymphocytes from patients [33]. MHS-associated *RYR1* mutations cause the channels to become hypersensitive to activation by electrical and pharmacolog-i-cal (caffeine, halothane, 4-chloro-m-cresol) stimuli. Identification of causative *RYR1* mutations is an aid to the diagnosis of MHS. In fact, although the IVCT/CHCT are the gold standard to establish the risk of MHS, an individual harboring an MH causative mutation can be considered MHS even without an IVCT/CHCT result (http://www.emhg.org). Furthermore, genetic analysis is crucial to identify and evaluate the few cases of discordance between genotype, characterized by the presence of a causative mutation, and MHN-typed phenotype [34, 35]. A retrospective study reported these discordant cases in approximately 2.6% of *RYR1* mutation-positive families [35]. Such discordant subjects are regarded as MHS for clinical purposes on the basis of genetic data alone, since they bear a causative mutation [34, 35].

Table 1 shows a list of *RYR1* gene missense SVs and the corresponding amino acid substitutions, identified in patients who experienced ER or stress-induced MH events [10, 13–16, 36–38]. Four *RYR1* SVs, corresponding to the amino acid substitutions p.R163C, p.G341R, p.G2434R, and p.T4826I, have already been demonstrated to be causative of MHS (http://www.emhg.org). The p.R3983C substitution was identified in two unrelated children who had fatal, nonanesthetic awake episodes associated with febrile illness and heat stress [15]. One of the children also had the variant p.D4505H. Interestingly, the child who only had the p.R3983 variant also had an MH attack during general anesthesia with halothane. These two SVs were functionally characterized by evaluating the caffeine sensitivity of Ca^{2+} release in transfected myotubes. Both p.R3983C and p.D4505H RyR1 channel variants exhibit an increase in the sensitivity to activation by caffeine, although the effect of the p.R3983C substitution alone is quite modest [15]. The SVs p.R401C, p.A933T, p.G2160S, p.R2336H, p.T4288_A4290dup, p.T4294 M, p.L4320_R4322dup, and p.R4645Q were reported to be absent in at least 100 control chromosomes. Instead, the p.S1342G and the p.S1352G variants are present among the African American population with a frequency of 4% and 2.7%, respectively [39], indicating that they are neutral polymorphic changes in RyR1. The p.R2336H, p.T4288_A4290dup, p.L4320_R4322dup, and p.R4645Q SVs have already been reported in MHS families [40–42].

3.2. In Silico Analysis of RYR1 Variants Reported in Patients Who Experienced ER and Stress-Induced MH Events. To predict the pathological character of p.E209 K, p.R401C, p.A933T, p.G2160S, p.R2336H, p.T4294 M, and p.R4645Q SVs, I tested them with 3 different prediction programs, namely, PMut (http://mmb.pcb.ub.es/PMut/) [23], SIFT (http://sift.jcvi.org/) [24], and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) [25]. Table 2 shows the results obtained by this analysis. The p.R401C, p.A933T, and p.R2336H variants were predicted to have a pathological character, while the predictions generated for p.E209 K, p.G2160S, p.T4294 M, and p.R4645Q variants were divergent. The p.E209 K variant, that has been predicted to be neutral by two programs and only possibly damaging by PolyPhen-2, has been found in association with p.R2336H in one patients who experienced stress-induced MH events and was typed MHS by CHCT (see Table 1) [36]. All the programs tested predict a pathological effect for the p.R2336H variant, that could be the molecular basis of both phenotypes. However, functional studies are needed to conclusively define the exact pathogenic effects of this amino acid substitution and to assess if it is the cause of stress-induced MH events in the patient.

Wappler et al. [10] found causative mutations (p.R163C, p.G341R, and p.G2434R) in only three out of ten MHS patients who experienced ER. They screened only eight *RYR1* exons located in the hotspot region; therefore, this limited analysis can explain the low mutation detection rate. Moreover, Sambuughin et al. [39], by sequencing the *RYR1* cDNA, found putative causative SVs (p.A933T and p.T4294 M) in only two out of six ER/MHS patients studied. In the remaining cases, the ER/MHS phenotype could be caused by *RYR1* SVs which may escape the *RYR1* cDNA screening because they determine unbalanced allelic expression [43–46] or, alternatively, could be caused by mutations in other candidate MHS *loci* genes.

4. Conclusions and Perspectives

ER and stress-induced MH events are syndromes with diverse etiologies that afflict particularly military recruits in basic training and athletes. This paper reports an overview of the literature on cases associated with MHS and with *RYR1* causative mutations or putative causative SVs. The possible disease-causing role of SVs, identified in patients who experienced ER and stress-induced MH events and that have not been functionally characterized, was investigated by computational analysis by using three different approaches, to increase the predictive power. Although only the molecular

Table 1: *RYR1* sequence variants reported in patients who experienced ER and stress-induced MH events.

Nucleotide change	Exons	Aminoacid change	MH-causative mutation (http://www.emhg.org)	Unrelated patients (*n*)	Regions of the *RYR1* gene investigated	dbSNP	MH status	References
c.487C>T	6	R163C	Yes	1 1	gDNA hot spot	rs118192161	MHS n.d	[10] [13]
c.625G>A c.7007G>A	7 43	E209K/ R2336H		1	cDNA complete	— rs112563513	MHS	[36]
c.1021G>A	11	G341R	Yes	1	gDNA hot spot	rs121918592	MHS	[10]
c.1201C>T	12	R401C		2	cDNA hot spot	—	MHS	[16]
c.2797G>A c.4024A>G c.4055C>G	23 28 28	A933T/ S1342G/ A1352G		1	cDNA complete	rs148623597 rs34694816 rs112105381	MHS	[39]
c.4024A>G	28	S1342G		3	cDNA complete	rs34694816	MHS	[37, 39]
c.4024A>G c.4055C>G c.12861_12869dup c.12881C>T	28 28 91 91	S1342G/ A1352G/ T4288_A4290dup/ T4294M		1	cDNA complete	rs34694816 rs112105381 — —	MHS	[39]
c.2797G>A c.6478G>A	28 39	S1342G/ G2160S		1	cDNA complete	rs34694816 rs143398211	MHS	[39]
c.7300G>A	45	G2434R	Yes	1	gDNA hot spot	rs121918593	MHS	[10]
c.11947C>T	87	R3983C	Yes	1*	gDNA (106 exons)	—	n.d.	[15]
c.11947C>T c.13513G>C	87 92	R3983C/ D4505H	Yes Yes	1*	gDNA (106 exons)	—	MHS	[15]
c.12959_12967dup c.13934G>A	91 95	L4320_R4322dup/ R4645Q		1*	gDNA (106 exons)	—	n.d.	[14]
c.14473C>T	100	T4826I	Yes	1*	cDNA complete	rs121918595	n.d.	[38]

*patients who experienced stress-induced MH events; n.d.: not determined. Nucleotide substitutions were numbered on the cDNA sequence (GenBank NM_000540.2); gDNA: genomic DNA.

Table 2: In silico analysis of *RYR1* sequence variants reported in patients who experienced ER and stress-induced MH events.

Sequence variant	PMut	SIFT	Polyphen-2
p.E209K	**0.6598**	0.29	Possibly damaging
p.R401C	**0.8400**	**0.04**	**Probably damaging**
p.A933T	**0.5969**	**0.01**	**Probably damaging**
p.G2160S	0.2159	0.49	Possibly damaging
p.R2336H	**0.8377**	**0.00**	**Probably damaging**
p.T4294M	**0.8994**	0.11	Benign
p.R4645Q	**0.8261**	**0.00**	Benign

Scores predicting pathological effect are in bold: PMut, > 0.5; SIFT ≤ 0.05. Polyphen-2 classifies the sequence variants as probably damaging, possibly damaging, or benign.

characterization of RyR1 channel variants can define the functional impact of a given SV, in silico predictions, which are fast and relatively inexpensive methods, may filter out SVs that are unlikely to affect protein function and allow phenotype prediction based on the biochemical severity of the amino acid substitution and on the protein sequence and structural information. Overall, the data presented in this paper emphasize the concept that some *RYR1* SVs are associated with both phenotypes and underline the importance of performing contracture testing and *RYR1* variant screening in these patients.

A mouse model of heat- and anesthetic-induced MHS has been created by introducing the p.Y522S mutation in the *RYR1* gene [47]. Only mice which are heterozygous for the p.Y522S mutation ($RyR1^{Y522S/wt}$) are viable and exhibit whole body contractions and elevated core temperatures in response to anesthetic exposure or heat stress [47]. Elevated environmental temperatures induce muscle contractures, rhabdomyolysis, and death in these mice. The Ca^{2+} leaking caused by the p.Y522S mutation, combined with temperature, generates increases in reactive nitrogen species and S-nitrosylation of the mutant channel that enhances RyR1 channel activity. Ultimately, the exposure to elevated temperatures produces abnormal muscle contractures in the $RyR1^{Y522S/wt}$ mice [48]. Recently, it has been reported that AICAR, an activator of the AMP-activated protein kinase (AMPK), prevents Ca^{2+} leaking, generation of reactive oxygen and nitrogen species, and heat-induced sudden death in $RyR1^{Y522S/wt}$ mice [49]. The effect of AICAR is not due to an increase in AMPK

activity but to the inhibition of RyR1 channel activity. On the basis of these results, Lanner et al. [49] proposed "the potential use of AICAR for prophylactic treatment in humans with enhanced susceptibility to exercise and/or heat-induced sudden death associated with RyR1 disease mutations." Moreover, studies on the effects of prior eccentric exercise on isolated mouse RyR1$^{Y522S/wt}$ muscle indicated that high-force eccentric contractions, run under nonthermally stressful conditions, may attenuate the thermal stress-induced loss of function [50]. This finding can have important implications because it suggests that the exercise-induced muscle injury may mitigate the severity of stress-induced MH episodes, possibly in humans as well.

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Review Article

Exercise-Induced Muscle Damage and Running Economy in Humans

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Running economy (RE), defined as the energy demand for a given velocity of submaximal running, has been identified as a critical factor of overall distance running performance. Plyometric and resistance trainings, performed during a relatively short period of time (~15–30 days), have been successfully used to improve RE in trained athletes. However, these exercise types, particularly when they are unaccustomed activities for the individuals, may cause delayed onset muscle soreness, swelling, and reduced muscle strength. Some studies have demonstrated that exercise-induced muscle damage has a negative impact on endurance running performance. Specifically, the muscular damage induced by an acute bout of downhill running has been shown to reduce RE during subsequent moderate and high-intensity exercise (>65% VO_2max). However, strength exercise (i.e., jumps, isoinertial and isokinetic eccentric exercises) seems to impair RE only for subsequent high-intensity exercise (~90% VO_2max). Finally, a single session of resistance exercise or downhill running (i.e., repeated bout effect) attenuates changes in indirect markers of muscle damage and blunts changes in RE.

1. Introduction

Running economy (RE), defined as the energy demand for a given velocity of submaximal running, is an important predictor of aerobic running performance, particularly in elite runners who have a similar aerobic power (i.e., maximal oxygen uptake, VO_2max) [1]. Runners with high RE demonstrate lower energetic cost at submaximal velocity and consequently tend to run faster at given distance or longer at a constant velocity.

A number of biomechanical (e.g., gait patterns, kinematics, and the kinetics of running) and physiological factors (e.g., oxidative muscle capacity) seem to influence RE in trained athletes [2, 3]. Moreover, some interventions (plyometric, resistance and altitude training) performed during relatively short periods of time (~15–30 days) have been successfully used to improve RE [4–6]. Plyometric and resistance trainings lead to neuromuscular adaptations such as increased neural drive to the muscles and changes in muscle stiffness and muscle fiber composition, which might reduce the energetic cost during submaximal exercise. However, plyometric and resistance trainings, especially when they are unaccustomed activities, may cause delayed onset muscle soreness (DOMS), swelling, and reduced muscle strength. The negative effect of muscle-damaging exercises on endurance running performance has been experimentally demonstrated in both animal [7, 8] and human experiments [9]. However, studies that have investigated the effect of exercise-induced muscle damage (EIMD) on RE have produced equivocal results [9–12]. This review discusses the effects of EIMD induced by different exercise types (strength, long-distance running, and downhill running) on RE. Different recovery strategies aiming to enhance the RE after EIMD are also addressed.

2. Running Economy

Aerobic fitness, as well as running performance, can be measured by different variables, for example, maximal oxygen uptake (VO_2max), lactate threshold (LT), onset of blood

lactate accumulation (OBLA), movement economy (ME), or running economy (RE). The VO_2max, which reflects an individual's maximal rate of aerobic energy expenditure, has been considered the gold standard for measuring aerobic power [13]. Indeed, the VO_2max has a positive association with aerobic running performance obtained during middle- and long-distance events (1,500 m–42,195 m) [14–16]. However, some studies [17–20] have shown that subjects with similar VO_2max values may attain different aerobic running performance or VO_2 values during exercise of similar duration and intensity. These differences are most likely due to variations in ME among subjects.

The ME is defined as the amount of energy necessary ($Kcal \cdot min^{-1}$) to perform a given task [21]. However, due to the difficulty to determine the external work performed during running, RE, expressed as the volume of oxygen uptake ($mL \cdot Kg^{-1} \cdot min^{-1}$) during a specific submaximal running intensity ($Km \cdot h^{-1}$), has been adopted. There is a strong association between RE and aerobic running performance, with RE being a better predictor of performance than VO_2max, particularly in athletes who have similar VO_2max [19, 22].

Several factors have been proposed to influence RE in trained subjects. These include oxidative muscle capacity and muscle stiffness. Muscle stiffness corresponds to the ability of the muscles to store and release elastic energy. Moreover, some interventions such as training, environment, and muscle damage can modify the oxygen cost over a range of running speeds [6, 11, 23–25].

Improved oxidative muscle capacity can be associated with reduced oxygen consumption per mitochondrial respiratory chain during submaximal exercise. Trained subjects are known to have better RE than untrained individuals, and long-distance runners are more economical than middle-distance runners [26]. Additionally, a high weekly volume of training has also been associated with better RE. However, a short period (4–6 weeks) of high-intensity aerobic training (near or above VO_2max) can also lead to improvement in the RE of trained runners [27].

In addition to aerobic characteristics and adaptations, neuromuscular profile factors have also been considered important aspects of RE. Type II muscle fibres seem to be positively correlated with submaximal energy consumption, especially at lower speeds [28]. Furthermore, both muscular stiffness and the ability to rapidly develop muscular force (i.e., rate of force development (RFD)) have demonstrated significant correlations with RE [29, 30]. Stiffer muscle-tendon complexes may increase elastic energy storage by reducing the ground contact time, thus decreasing the running oxygen cost. Similar to stiffness, a higher RFD is associated with a shorter time to generate a contraction. This effect could also diminish the ground contact time and running oxygen cost. Heavy weight and plyometric training associated with endurance training have improved RE in well-trained runners [5, 31]. Basically, these types of strength training induce neuromuscular adaptations such as increased neural drive to the muscles, altered muscle-tendon complex stiffness, and changed muscle fibre composition (i.e., I $\rightarrow$ IIA $\leftarrow$ IIX).

Environmental variables can also be used to reduce the energetic cost of running. RE can be improved (2-3%) after relative short periods (~15–20 days) of altitude exposure (~2.000–4.500 m). Altitude exposure during daily activities, sleeping, or training can enhance RE at sea level altitude through haematological and muscle changes in favour of oxygen transport [32–34]. Moreover, heat exposure during training sessions can also improve RE by enhancing the thermoregulatory process, thus reducing the cardiovascular and muscle work for a given exercise intensity [23, 35, 36].

More recently, EIMD has also proposed to generate important modifications in RE. Muscular damage induced by an acute bout of downhill running has shown to reduce RE in the days following the intervention (24–120 hours) [9, 11]. Specific aspects of this intervention are addressed hereafter.

3. Muscle Damage and the Repeated Bout Effect

Skeletal muscle damage has been considered an important factor contributing to DOMS and strength loss after eccentric exercise [37]. Basically, the exercise conditions at which muscle damage can be induced are unaccustomed exercises and exercises with higher intensity or longer duration than those to which the subject is adapted [37, 38]. The resulting metabolic overload and mechanical strain have been suggested the main factors generating muscle damage [38]. Warren et al. [39] have suggested that measures of muscle function such as strength and power are effective indicators of both the magnitude and time course of muscle damage. Depending on the magnitude of muscle damage, muscle force at isometric, and dynamic testing conditions may be impaired for 1–7 days after the exercise [40–43]. Other important symptoms of muscle damage are disruption of the sarcolemma and extracellular matrix [44, 45], increased blood levels of creatine kinase (CK) and myoglobin (MB), stiffness, and swelling [46–48].

In general, muscle damage can be induced by both static (isometric) and dynamic (concentric and eccentric) muscle contractions. However, there is substantial evidence that eccentric muscle actions result in greater muscle damage than isometric or concentric actions [49–52]. The magnitude of strength loss after EIMD may vary between 5–10% and ~60% [43, 52], depending on the characteristics of the protocol and the type of muscle actions (i.e., isometric, concentric or eccentric) used during the posttest. The different effects of eccentric versus isometric or concentric actions have also been verified in the context of whole body exercises (i.e., running, cycling, and cross-country skiing) [43, 53]. For example, muscle damage and strength loss are higher during running (~20–30%), which involves concentric and eccentric actions, when compared with cycling (~10–15%), which involves mainly concentric actions [53]. In accordance with Millet and Lepers [53], although concentric and eccentric actions are present during cross-country skiing, muscular damage is considerably lesser during this exercise than in running because shock waves are present only during running. The main factors attributed to the greater effect of eccentric contractions on muscle damage are the higher peak torque

values [54] and reduced motor unit activation for a given force [54–56], both of which induce a higher mechanical stress on the muscles [54]. Other important aspects of the greater muscle damage induced by eccentric muscle actions are that no energy (ATP) is necessary to detach the cross-bridges formed during muscle contraction [57] and that the longer length of the muscles during the contraction generates greater muscle damage.

In addition to the main mechanical factors (i.e., the force level produced and the change in muscle length) [37, 58, 59], some metabolic factors such as substrate depletion, calcium influx, and reactive oxygen species have also been proposed to influence muscle damage [38, 60]. The effects of the different mechanical and metabolic factors that would contribute to muscle damage do not occur at the same time. The time course of the events involves damage in components of excitation-contraction system and sarcomeres [59] and degeneration and regeneration of muscle fibres, during which DOMS, stiffness, and swelling occur [37]. Additionally, there is an inflammatory response generating a transfer of fluid and cells to remove damaged contractile proteins and cellular debris from the damaged muscles [61]. Thereafter, the muscle regeneration process is initiated [61]. Although some of these effects may appear only some hours after the exercise, muscle strength may be impaired during and immediately after the exercise. Thus, mechanisms other than muscle damage can also explain the muscle fatigue (i.e., strength loss).

It has been suggested that the magnitude of muscle damage and the loss of muscle function might be attenuated after one bout of eccentric exercise [62–64]. This concept is known as the repeated bout effect (RBE). The RBE has been demonstrated after both eccentric muscle actions [65] and downhill running [66]. In general, this protective effect is confirmed by the reduced decrements and faster recovery of muscle strength, less swelling and DOMS, and attenuated changes in CK and MB in the blood [62, 67, 68]. In addition, alterations in muscle circumference or echo intensity (inflammation) are also smaller after the first eccentric exercise bout [68]. This protective effect has been demonstrated after a few days of the eccentric exercise [66] and may last up to 6 months (circumference, DOMS, and inflammation) or 9 months (maximal isometric force, CK), depending on the marker of muscle damage [65].

It has been hypothesized that the RBE is mediated by neural, cellular, and mechanical mechanisms [63, 64]. The neural changes proposed to contribute to the RBE are increased slow-twitch fibre recruitment and synchronisation of motor unit firing, better distribution of the workload among muscle fibres, higher participation of synergist muscles to torque production, and increased motor unit activity relative to torque produced [69–71]. Neural mechanisms have been suggested based on the reduced median frequency [69], which reflects some central aspects related to motor unit recruitment. Howatson et al. [69] have demonstrated a 10% decrease in median frequency 14 days after a bout consisting of either 10 or 45 maximal eccentric actions. RBE has also been observed in the untrained contralateral limb, referred to as the contralateral RBE [72]. These studies [69, 72] confirm that, in addition to intramuscular adaptations, central aspects regarding motor unit recruitment are also involved in RBE.

The main mechanical adaptations associated with RBE are increased muscle stiffness and intramuscular connective tissue and changes in the intermediate filament system (maintenance of structural integrity of sarcomeres) [63]. Cellular adaptations are associated with higher number of sarcomeres in myofibrils [59, 73], which might decrease myofibrillar disruption in the next exercise bout, strengthened plasma membranes, increased protein synthesis, removal of stress-susceptible fibres [59, 74, 75], and remodelling of the cytoskeleton, including effects on proteins such as titin and desmin, talin and vinculin [76], which might improve the strength and the stability of sarcomeres and protect muscle fibers against injuries. Other adaptation that has been hypothesized to explain the RBE is the lesser inflammatory response. Since the mechanical disruption is decreased after the first eccentric exercise bout, the stimulus for the inflammatory response is also reduced after the exercise [73, 74]. Some of these alterations have been associated with reduced muscle damage (strengthened extracellular matrix) and a change in the optimal angle for torque production toward a longer muscle length (increases in number of sarcomeres).

The magnitude of muscle damage induced by eccentric exercise is greater at longer muscle lengths [65, 77]. When the muscles are elongated, the sarcomere length is also greater. Because the severity of muscle damage is influenced by the muscle strain generated [73, 78], it has been suggested that the RBE would be greater under conditions of longer muscle lengths. Nosaka et al. [73] have investigated the effect of the range of motion of the exercise used to induce muscle damage on the RBE. The protocol used to induce muscle damage involved 24 maximal eccentric contractions of the elbow joint, using amplitudes of 50–100° or 130–180°. Although the changes (maximal isometric strength, range of motion, upper arm circumference, muscle soreness, and CK) induced by the first bout were significantly greater using the higher amplitude, both exercise conditions induced RBE. However, the effect generated by the short range of motion was lesser than that promoted by the higher amplitude.

Other factor that can modify this protective effect (i.e., RBE) of eccentric exercise is the magnitude of muscle damage [65, 66], which is influenced by the exercise intensity of the first bout. Chen et al. [66] showed that 30 eccentric contractions performed at 40% of maximal isometric strength generated a smaller attenuation of the changes in indirect markers of muscle damage (20–60%) than maximal eccentric exercise (65–100%).

It has been also demonstrated that both the muscle damage level (i.e., CK) and strength impairment and recovery (i.e., isometric torque) are progressively greater with increases in the number of bouts (1–4). However, Chen et al. [68] have demonstrated that repetitive submaximal eccentric exercise bouts (40% MVC) performed every two weeks promote a protective effect similar to that induced by one maximal eccentric exercise bout. In this study, the main indirect markers of muscle damage were less affected by the second to the fourth bouts of submaximal eccentric exercise than the first; that is, the protective effect is promoted under

Table 1: Comparison of the effects of the resistance exercise on running economy.

Study	Subjects	EIMD	Muscle damage	VO_2max (%)	RE (%)
Paschalis et al. [10]	10 healthy males	120 eccentric actions	↑ CK, ↑ DOMS, and ↓ ROM, and ↓ strength	55 and 75	√
Burt et al. [12]	9 healthy men	100 squats at 80% body mass	√ CK, ↑ DOMS, and ↓ strength	90	↓ 4-5
Vassilis et al. [87]	24 young healthy men	120 eccentric actions	↑ CK, ↑ DOMS, ↓ strength	70	√
Scott et al. [88]	8 active men and 8 active women	3-4 × 10 repetitions of squat, lunges, step up and step down, and stiff-legged deadlift	↑ DOMS	70	√

EIMD: exercise-induced muscle damage; %VO_2max: exercise intensity at which running economy was measured; RE: running economy; CK: creatine kinase; DOMS: delayed onset muscle soreness; ROM: range of motion; ↓ indicates decrease; √ indicates no change; ↑ indicates increase.

conditions of reduced levels of induced muscle damage. Even after repeated submaximal bouts the magnitude of muscle damage was still smaller than that induced by one maximal bout. The authors suggested that the effect of exercise intensity on the protective effect of the first bout does not apply when some bouts of low-intensity exercise are performed. Therefore, the magnitude of muscle damage does not necessarily affect the protective effect of eccentric exercise. Moreover, Howatson et al. [69] have compared two protocols of maximal eccentric contractions to induce muscle damage with 45 or 10 contractions. After 14 days, subjects performed the same protocol with 45 contractions. Although the effect of the higher volume of the first bout on damage markers (CK, DOMS, and isometric torque) was greater, the protective effects of both protocols were similar. Therefore, the intensity of the first bout seems to be the main aspect of the magnitude of muscle damage and RBE.

Because one exercise bout is enough to generate the RBE, some studies have also investigated whether resistance training could also reduce the effects of eccentric exercise on muscle damage markers [67, 79]. Specifically, Newton et al. [67] found that resistance-trained subjects demonstrated smaller RBE when compared with untrained subjects. Moreover, Falvo et al. [79] did not find changes in indirect markers of muscle damage (maximal isometric torque and CK) in resistance-trained men. The authors attributed the absence of RBE to a lack of neural adaptation. Thus, it is likely that strength training induces to adaptations that reduce the RBE.

The majority of studies that have analysed the RBE used relatively short time periods after the eccentric exercise (i.e., from approximately 7–14 days to 6–9 weeks). However, some studies [80, 81] have reported that the RBE induced by 24 maximal eccentric actions of the elbow flexors may last up to 6 months. Nosaka et al. [80] aimed to investigate the responses of the main indirect markers of muscle damage (CK, maximal isometric torque, DOMS, and swelling) five days after the eccentric exercise bout, with sessions six, nine, and twelve months apart. The main finding of this study was that the RBE for strength, swelling, DOMS, and CK lasted up to six months.

4. Strength Exercise, Muscle Damage, and Running Economy

A variety of studies have investigated the influence of EIMD and DOMS on neuromuscular performance indicators (i.e., strength and rate of force development) [82–84]. These studies verified that the isomeric peak torque is compromised immediately after the damaging exercise that causes DOMS, with a gradual recovery in subsequent days. The magnitude and the recovery rate from strength loss seem to be related to the training history of the muscle group. For instance, when performing maximal eccentric contractions, upper limb muscles (less active) demonstrate greater loss of strength (50–70%) and slower recovery (60–90 days) when compared to lower limb muscles (locomotory muscles) (20–30% and 10–30 days, resp.) [37, 85]. However, only a few studies have investigated the effects of EIMD and DOMS on aerobic performance indexes (e.g., VO_2max, lactate response to exercise, VO_2 kinetics, and movement economy) [86]. These studies analysed the effects of EIMD on RE [10, 12] and VO_2 kinetics during submaximal cycling exercise [86]. In this context, studies that investigated the effects of strength exercises (i.e., jumps, isoinertial and isokinetic eccentric exercises) on RE will be addressed (Table 1).

Paschalis et al. [10] analysed the effects of eccentric exercises on indirect muscle damage markers (CK, DOMS, ROM, and isometric force) and RE in active individuals who were not engaged in strength training programs. The eccentric exercise protocol consisted of 120 (12 × 10) maximal voluntary contractions (MVC) at an angular velocity of 1.05 $rad \cdot s^{-1}$. Although indirect muscle damage markers were significantly altered in the subsequent days (24–72 h), the RE (assessed at 55 and 75% VO_2max) was not modified. Similar data were obtained by Vassilis et al. [87], who analysed

Table 2: Comparison of the effects of the downhill running on running economy.

Study	Subjects	EIMD	Muscle damage	VO_2max (%)	RE (%)
Chen et al. [11]	50 male students	30 min DHR at −15%	↑ CK, ↑ DOMS, ↓ strength, and ↑ LDH	70, 80, and 90	↓ 5
Hamill et al. [92]	10 recreational female runners	30 min DHR at −15%	↑ CK, ↑ DOMS	80	√
Braun and Dutto [93]	9 endurance trained men	30 min DHR at −10%	↑ DOMS	65, 75, and 85	↓ 3
Chen et al. [94]	10 soccer trained men	30 min DHR at −15%	↑ CK, ↑ DOMS, ↓ strength, and ↑ MB	65, 75, and 85	↓ 4–7

EIMD: exercise-induced muscle damage; DHR: downhill running; %VO_2max: exercise intensity at which running economy was measured; RE: running economy; CK: creatine kinase; DOMS: delayed onset muscle soreness; MB: myoglobin; LDH: lactate dehydrogenase; ↓ indicates decrease; √ indicates no change; ↑ indicates increase.

the effects of eccentric exercise (120 MVC at a $60^{\circ}\cdot s^{-1}$) on RE in recreational athletes with no previous experience in resistance training. The RE (assessed at 70% VO_2max) was not changed 48 hours after the damaging bout. Therefore, EIMD induced by isokinetic eccentric contractions do not seem to interfere on RE measured at moderate intensities (55–75% VO_2max).

Using closed kinetic-chain exercises, Scott et al. [88] have also analysed the effects of EIMD on RE. The volunteers performed a series of lower extremity resistance exercises designed to induce DOMS. RE was analysed at 70% VO_2max, 24–30 hours after the EIMD. Although the subjects demonstrated a higher rate of perceived exertion values, RE was maintained unaltered throughout the days after EIMD. In another study, Marcora and Bosio [9] did not find any alteration in RE (70% VO_2max) after 100 drop jumps, although DOMS, CK, and knee extensors strength were significantly affected by EIMD. Therefore, the evidence suggests that muscle damage induced by both open and closed kinetic-chain exercises dose not alter RE at moderate intensities (55–75% VO_2max).

However, in a recent study, Burt et al. [12] presented conflicting data regarding the effect of EIMD on RE. In this study, indirect markers of muscle damage and RE were measured, 24–48 h after EIMD (10 sets of 10 squats at 80% body mass). Significant increases in all indirect markers of muscle damage, kinematic parameters (stride length and stride frequency), and oxygen uptake during submaximal running (~90% VO_2max) were observed at 24–48 h following the initial bout of EIMD. Some authors [82, 89] have suggested that the changes in RE are associated with decrements in neuromuscular function (i.e., MVC) after EIMD. However, both the magnitude and the time course of the changes in muscular function (MVC) and RE can be different. Therefore, changes during submaximal exercise (i.e., RE) may not be strictly associated with neuromuscular function.

As a whole, these data suggest that the effects of muscle damage induced by strength exercise (i.e., jumps, isoinertial and isokinetic eccentric exercises) on RE are intensityd-dependent. During moderate exercises (55–75% VO_2max), RE is not altered by EIMD. However, during high-intensity exercise (~90% VO_2max), RE is impaired. During high intensity exercise, additional type II fibres, which are the most affected by EIMD, are recruited. Moreover, at these intensities, the VO_2 either attains a delayed steady state (heavy domain) or continues to increase slowly (i.e., VO_2 slow component (VO_2SC)) reaching its maximal values at the end of exercise (severe domain) [90]. Although the physiological determinants of VO_2SC remain poorly understood, some authors have proposed that an increased ATP and/or O_2 cost of power production in fatigued fibres, rather than the additional recruitment of poorly efficient muscle fibres, is responsible for the VO_2SC [91]. Therefore, the effects of strength exercise-induced muscle damage on RE seem to depend on the fibre recruitment pattern and/or on the mechanisms determining the VO_2SC.

5. Downhill and Long-Distance Running, Muscle Damage, and Running Economy

Adopting a more specific approach, some studies have investigated the influence of muscle damage induced by strenuous exercise (e.g., long-distance running) or downhill running on neuromuscular parameters, and RE. This aspect and the effect of some interventions on RE during the recovery period after EIMD are also addressed in this topic (Table 2).

As mentioned previously, muscle damage is usually induced by maximal and submaximal eccentric contractions, but it can also be observed when a high volume of eccentric/concentric contractions are performed, due to the eccentric contractions per se [95] or because of metabolite accumulation that may lead to stress and impairment of the muscle fibres [96]. Because a high number of concentric and, particularly, eccentric contractions are performed during long-distance running, the symptoms of muscle damage

are usually observed immediately and a few days after the running bout.

In a study conducted by Millet et al. [97], changes in muscle function and muscle damage markers from 22 experienced marathon runners were collected and analysed after they had run an international extreme mountain ultramarathon. The race consisted of a 166 km marathon through mountainous terrain with the final destination set at 9500 m below the starting point. This predominately downhill configuration required a high number of eccentric contractions particularly for the knee extensors. Indirect muscle damage markers (strength, CK, LDH, and MB) were analysed before, immediately after, and 2, 5, 7, 9, and 16 days after the marathon. The authors found higher decreases in force production immediately after the ultramarathon, most likely because of the fatigue experienced during the race. However, some of the strength markers remained altered until 5 days after exercise, as usually occurs after muscle damaging activities. Blood markers also demonstrated the highest value immediately after the race, returning to baseline values 5 days later. The authors found that even though this type of activity can induce extreme muscle damage, after 16 days, all the alterations induced by muscle damage and/or fatigue had returned to normal. Considering that force production is intimately related to RE, these findings may indicate that an extremely damaging activity may induce high levels of force loss and decreases in RE. Force production is usually fully recovered 5 days after the damaging activity. However, RE may recover at a faster rate than force.

To investigate factors that could influence RE, Kyrolainen et al. [89] subjected 7 experienced runners to a protocol simulating a marathon. RE and kinematic variables (stride length and frequency, mean contact time, external mechanical work and power, and angular displacements and velocities of the hip, knee, and ankle joints) were collected before, during (at the 1st, 13th, 26th, and 42nd kilometres), two hours after, and in the days (2, 4, and 6) after the marathon. Muscle damage markers (CK and SOR) were also collected after the exercise. The impairment of RE (i.e., higher oxygen consumption) was observed only at the end of the marathon (42nd kilometre and two hours afterward). CK and SOR were significantly increased immediately after the marathon and returned to baseline values only at the 6-day postexercise time point. These data may indicate that alterations in RE after marathon running may not be exclusively in the result of muscle damage but may be affected by other factors such as thermal stress. To better understand the time course of recovery of the various parameters of muscle function following marathon running, it is important to investigate other indirect EIMD markers, such as force and inflammatory response.

Muscle damage induced by downhill running has also been widely studied in the last decades. This type of exercise has been proven to induce muscle damage even when performed for relatively short periods (e.g., 30 minutes) due to higher mechanical stress applied to the lower limb muscles during the contact with the ground phase [95]. Some studies have shown that downhill running can lead to muscle damage of the same magnitude as plyometric or maximal eccentric exercises [92, 98]. Considering that downhill running induces muscle damage, a series of studies have investigated its influence on neuromuscular and metabolic markers in animals [99] and humans [11, 93, 94, 98]. In animals, downhill running has been utilised to induce overtraining [100] as well as a training method to increase the number of sarcomeres [101]. In humans, this exercise model has been recently studied in attempt to understand its influence on specific running and aerobic parameters, such as RE [93, 98] and running kinematics.

To the best of our knowledge, Hamill et al. [92] performed the first study investigating the influence of downhill running on RE. In this study, 10 recreational female runners underwent a 30 min downhill running bout (DRB) with −15% slope at 73.5% of maximal heart rate. Indirect markers of muscle damage (SOR and CK), RE (80% VO_2max), and kinematic parameters were measured before and 2 and 5 days after the DRB. SOR and CK levels increased 2 days after the DRB, returning to baseline values 5 days after the exercise. Although kinematic parameters were modified, the DRB did not alter RE. The authors proposed that changes in kinematics might be due to increases in SOR, which compromises the range of motion, and thus alters the movement patterns.

In another model to investigate the influence of downhill running-induced muscle damage on RE, Braun and Dutto [93] conducted a study in which 9 endurance-trained subjects underwent a DRB (30 minutes at 70% VO_{2peak} with a −10% slope). Assessments of SOR, RE (65%, 75% and 85% VO_2max), and stride length were performed before and 48 hours after the DRB. SOR was increased and RE was impaired 48 hours after the DRB, suggesting that muscle damage might have increased the energy cost of running. The authors stated that the muscle damage decreased the range of motion and strength, thus compromising running kinematics, which is known to be related to RE.

To better describe the time course of changes in RE, Chen et al. [94] subjected 10 soccer-trained volunteers to a downhill running protocol similar to that proposed by Braun and Dutto [93]. Muscle damage (MVC, SOR, CK and MB) and RE (65%, 75%,d and 85% VO_2max) were assessed before and 1 hour and 1–5 days after the DRB. Alterations in muscle damage markers were consistent with those found in the literature, including increases in CK, MB, and SOR, with peak values attained 48 hours after the DRB. Strength loss was also maximal immediately after the DRB. All muscle damage markers returned to baseline values 5 days after the DRB. The magnitude of change was smaller, and the time course recovery was faster for RE (4–7% and 4 days, resp.) than for the indirect markers (i.e., isometric peak torque (IPT)) of muscle damage (7–21% and 4 days, resp.). The authors suggested that the alterations in running kinematics, the need to recruit more muscle fibres, the impairment in the stretch-shortening cycle, and the reduced levels of muscle glycogen might impair RE following a DRB.

Because alterations in the muscular tissue due to EIMD have been shown to affect RE because of differences in muscle fibre recruitment and other neuromuscular properties, Chen et al. [11] assessed RE at 3 different intensities (70, 80, and 90%VO_{2peak}) after a DRB. Muscle damage markers showed the expected alterations, peaking 2 days after DRB. The

alteration in RE measured at 90% VO_{2peak} was significantly higher than at 80% VO_{2peak}. No significant change in RE was found at 70% VO_{2peak}. Previous studies have indicated that fast-twitch motor units are progressively recruited with increased levels of exercise intensity [102]. Because several investigations have reported selective damage to type II muscle fibres after eccentric muscle actions in humans [40, 103], there appears to be a relationship between the motor unit recruitment pattern and impairment in RE.

Therefore, the effects of strength exercises and downhill running on RE seem to be different. While strength exercises seem to affect RE only during high intensity submaximal exercises (~90% VO_2max), downhill running also increases the energetic cost during moderate exercises (>65% VO_2max). It is important to note that during running exercise, the VO_2SC is attenuated (heavy intensity) and/or nonexistent (moderate). Thus, the effect of strength exercises on RE seems to occur only at running intensities at which the VO_2SC is present. Greater muscle mass and/or the magnitude or specificity of muscle damage induced by downhill running may partially explain these results.

A variety of interventions have been proposed to enhance recovery from EIMD, that is, to reduce the severity and duration of injury and SOR. It is a common belief that low-intensity training (i.e., active recovery) enhances the recovery process by accelerating the return to homeostasis after EIMD. To investigate whether submaximal running would influence the recovery from DRB, Chen et al. [98] analysed the effect of 30-minute daily running exercises performed at different intensities (40%, 50%, 60%, and 70% VO_{2peak}) by different groups on the recovery of muscle damage and RE. Muscle damage was induced by a DRB (30 minutes at 70% VO_{2peak} with −10% slope). The authors found that the time-course recovery of muscle damage markers and RE was similar for all groups, regardless of whether submaximal running was performed. Thus, low-to-moderate-intensity running seems not to improve the recovery from muscle damage and/or RE impairment.

Performing a similar subsequent bout of eccentric exercise results in significantly less change in the markers of muscle damage. This phenomenon is known as the RBE [104]. In fact, Byrnes et al. [105] and Chen et al. [66] demonstrated that when a DRB was repeated 1–6 weeks after the first bout, the indirect markers of muscle damage (isometric peak torque, CK, SOR, and range of motion) were significantly reduced. Moreover, Chen et al. [106] verified that the RBE was also observed in RE and running kinematics parameters. In this study, 12 male subjects underwent the same downhill running protocol adopted by Chen et al. [11] except the interval allowed between bouts that was twice as long, to allow full recovery from the first bout. The authors found significant changes in all markers of muscle damage after both protocols. However, the RE, kinematics parameters, and SOR were less affected after the second DRB. Therefore, one bout of DRB might induce a protective effect, leading to reduced levels of SOR and blunted changes in RE and biomechanical parameters.

In another study, Burt et al. [12] subjected 9 subjects to repeated bouts of 100 squats and measured muscle damage markers (isometric peak torque, vertical jump height, CK, and SOR) and RE before, immediately after the bouts, and 1-2 days after the bouts. The bouts were separated by enough time to recover from the EIMD symptoms. All muscle damage and RE markers were significantly affected by the first bout. However, no alterations in some muscle damage markers and RE were observed after the second bout. Thus, a previous damaging activity leads to blunted or nonexistent alterations in muscle damage markers and RE.

6. Supplementation and Muscle Damage Recovery

A series of recent studies has investigated the influence of different types of supplementation on recovery from and prevention of muscle damage. The main supplements that seem to protect against muscle damage are the flavonoids, which are known for their efficient anti-inflammatory and antioxidant properties. Studies investigating supplementation with flavonoid rich substances and their influence on muscle damage will be discussed.

Howatson et al. [107] conducted a study in which muscle damage, inflammatory response, and oxidative stress were measured before, immediately after, and 24 and 48 hours after a marathon. The purpose of this study was to investigate whether a tart cherry juice supplement would affect recovery from muscle damage after marathon running in 20 recreational marathon runners, using a double-blind placebo intervention. Muscle damage markers determined in this study were CK, LDH, DOMS, and IPT. Other parameters (total antioxidant status, thiobarbituric acid reactive species,d and protein carbonyls) were measured to identify inflammatory response, and oxidative stress. Both groups (control versus supplemented) demonstrated similar decreases in IPT. However, IPT was higher for the supplementation group at all time points, showing faster recovery. Moreover, the authors found that the supplementation enhanced the anti-inflammatory response as well as reduced the oxidative stress.

Kuehl et al. [108] investigated the effects of tart cherry juice supplementation on SOR immediately after a long-distance running (~26 km) bout. The study was performed in a randomised, double-blind placebo fashion in 54 experienced runners. The subjects were separated in two groups: placebo and tart cherry supplement. Both groups started ingesting their supplements 7 days prior to the running bout. The increase in SOR was significantly greater for the placebo group when compared to the tart cherry group. These findings are similar to those of Howatson et al. [107] and indicate that the anti-inflammatory and antioxidant properties of the tart cherry supplement might reduce SOR after EIMD.

Supplements containing flavonoid compounds have been shown to confer a protective effect against muscle damage either by attenuating a vast number of markers or by accelerating their recovery after the EIMD. This type of protection has been hypothesised to be due to the anti-inflammatory and antioxidant properties present in these types of compounds [107]. A number of studies have shown a direct relationship between flavonoid supplementation and protection against

muscle damage. Because muscle damage may affect RE, it would be interesting to analyse if this type of supplementation can protect against RE impairment after EIMD.

7. Conclusion

Despite the systematic implications of RE on aerobic running performance, only a few experiments have specifically studied the response of this index after EIMD. Recent studies have analysed RE after strength exercises (i.e., jumps, isoinertial and isokinetic eccentric exercises) and downhill running. These studies have found that the magnitude of reduction in muscle function (MVC) after EIMD is greater than the RE and kinematic parameters. Moreover, the time course for the changes in muscle function, RE, and kinematic parameters are not similar. As a whole, these data suggest that the putative mechanisms underlying muscle function and RE during the recovery from EIMD are not completely shared. The effects of muscle damage on RE seem to depend of the interaction between the type of eccentric exercise and the intensity at which the RE is measured. Strength exercises seem to modify RE preferentially during high-intensity exercise (~ 90% VO_2max). However, the effects of downhill running can also be observed at moderate intensities (>65% VO_2max). Finally, a single session of strength exercise or downhill running attenuates changes in indirect markers of muscle damage and blunts changes in RE.

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Review Article

Mitochondria as a Potential Regulator of Myogenesis

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Recent studies have shown that mitochondria play a role in the regulation of myogenesis. Indeed, the abundance, morphology, and functional properties of mitochondria become altered when the myoblasts differentiate into myotubes. For example, mitochondrial mass/volume, mtDNA copy number, and mitochondrial respiration are markedly increased after the onset of myogenic differentiation. Besides, mitochondrial enzyme activity is also increased, suggesting that the metabolic shift from glycolysis to oxidative phosphorylation as the major energy source occurs during myogenic differentiation. Several lines of evidence suggest that impairment of mitochondrial function and activity blocks myogenic differentiation. However, yet little is known about the molecular mechanisms underlying the regulation of myogenesis by mitochondria. Understanding how mitochondria are involved in myogenesis will provide a valuable insight into the underlying mechanisms that regulate the maintenance of cellular homeostasis. Here, we will summarize the current knowledge regarding the role of mitochondria as a potential regulator of myogenesis.

1. Introduction

Mitochondria generate most of the energy necessary for cellular function via oxidative phosphorylation (OXPHOS) as well as contribute to metabolism, Ca^{2+} signaling, and apoptosis. Besides, several lines of evidence suggest that mitochondrial function and activity are linked to cell differentiation, as have been shown in a wide variety of cell types including myoblasts [1–9]. When the myoblasts differentiate into myotubes, mitochondrial enzyme activity is drastically increased [10–12]. Likewise, muscle regeneration is also accompanied by an increased mitochondrial enzyme activity [13–15]. These findings suggest that the metabolic shift from glycolysis to OXPHOS as the major energy source occurs during myogenesis. The metabolic shift has been reported in embryonic stem cells (ESCs) [16, 17] and induced pluripotent stem cells (iPSCs) [18]. For example, iPSCs have low mitochondrial activity, relying predominantly on glycolysis for ATP generation and maintaining a state of dedifferentiation, while differentiation is accompanied by an increased mitochondrial activity [18]. Therefore, the metabolic shift may be a key event initiating cell differentiation. This shift requires an activation of mitochondrial biogenesis through coordinated expression of nuclear and mitochondrial genomes. Mitochondrial biogenesis is tightly controlled by transcriptional coactivators, transcription factors, and nuclear receptors [19–23]. Their expression is coordinately induced during myogenic differentiation [11, 12] and muscle regeneration [13, 15]. To further elucidate the relationship between mitochondria and cell differentiation, the effects of impairment of mitochondrial function and activity on myogenic cells have been investigated using antimycin [24], azide [24–27], chloramphenicol [4, 6–9], carbonyl cyanide m-chlorophenylhydrazone carbonyl (CCCP) [24], cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) [6], ethidium bromide (EtBr) [1–3, 24], myxothiazol [7], rhodamine 6G [28], rifampicin [1], rotenone [7], oligomycin [6, 7, 26], tetracycline [5], and valinomycin [24]. Overall, these antibiotics and chemicals can exert a negative influence on myogenesis. For example, respiration-deficient myoblasts

devoid of mitochondrial DNA (rho° cells) by EtBr, an inhibitor of mtDNA replication and transcription, fail to differentiate into myotubes [1–3]. Rifampicin, which inhibits mitochondrial RNA synthesis, shows reversible inhibition of myotube formation [1]. Tetracycline, an inhibitor of mitochondrial protein synthesis, blocks myoblasts fusion [5]. Chloramphenicol, an inhibitor of mitochondrial protein synthesis, restricts myogenic differentiation [4, 6–9] and interferes with muscle regeneration [15]. Despite the data being accumulating, little is known about the molecular mechanisms underlying the regulation of myogenesis by mitochondria. In this paper, we will summarize the current knowledge regarding the role of mitochondria as a potential regulator of myogenesis.

2. Mitochondrial Biogenesis

Mitochondrial biogenesis (also referred to as mitochondriogenesis) is characterized as a vital process in the synthesis and degradation of the organelle [29, 30]. Therefore, this fundamental process comprehends (1) the synthesis import and incorporation of lipids and proteins to the existing mitochondrial reticulum; (2) the stoichiometric assembly of multisubunit protein complexes into a functional respiratory chain; (3) replication of the mitochondrial DNA (mtDNA); (4) selective degradation of mitochondria by autophagy (mitophagy) [22, 31, 32]. When it is not indicated, in this paper, mitochondrial biogenesis simply considers an increase in mitochondrial volume and changes in organelle composition per tissue or cell [31]. Mitochondrial biogenesis requires a coordination of expression of nuclear and mitochondrial genomes [20].

3. Transcriptional Regulation of Mitochondrial Biogenesis

Recent technological and scientific advances have allowed it to systematically identify the complement of over 1,000 different proteins that comprise the mammalian mitochondrial proteome [33]. The majority of the mitochondrial proteins are encoded by the nuclear genome and synthesized on cytoplasmic ribosomes [20, 34], whereas the minority are encoded and synthesized within the mitochondria. The mitochondrial genome contains 37 genes encoding 13 enzymes involved in OXPHOS, 22 types of transfer RNAs, and 2 types of ribosomal RNAs [35]. To maintain mitochondrial functionality, it is necessary for two genomes to be coordinately regulated [20]. It has been widely accepted that peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) plays a central role in a regulatory network governing the transcriptional control of mitochondrial biogenesis [20–23]. PGC-1α works in concert with a wide variety of interacting partners, which are transcription factors and nuclear receptors [21, 22]. PGC-1α was discovered in a yeast two-hybrid screen for brown adipose-specific factors that interact with the nuclear receptor PPARγ and are dramatically induced by exposure to cold in brown fat and skeletal muscle [36]. Subsequently, two additional PGC-1 family members were identified, PGC-1-related coactivator (PRC) [37] and PGC-1β [38]. The three coactivators regulate expression of a broad set of mitochondrial genes and promote mitochondrial biogenesis [39]. Since these coactivators lack DNA-binding activity [40, 41], PGC-1 family coactivators exert their effects through interactions with transcription factors and nuclear receptors bound to specific DNA elements in the promoter region of genes. For example, nuclear respiratory factor-1 (NRF-1) and NRF-2 (GA-binding protein; GABP) were the first regulatory factors implicated in the global expression of multiple mitochondrial functions in vertebrates [23]. Both NRF-1 and NRF-2 are involved in the transcriptional control of nuclear and mitochondrial genes involved in OXPHOS, electron transport (complex I-V), mtDNA transcription/replication, heme biosynthesis, protein import/assembly, ion channels, shuttles, and translation [19]. For more complete details, excellent review articles are already available on this subject [19–23].

4. Mitochondrial Enzyme Activity and Function during Muscle Regeneration and Myogenesis *In Vitro*

Muscle regeneration, which partially recapitulates embryonic myogenesis [42], would stimulate mitochondrial biogenesis [13]. Muscle injury was induced by intramuscular injection of either bupivacaine (which induces Ca^{2+} release from the sarcoplasmic reticulum (SR) and simultaneously inhibits Ca^{2+} reuptake into the SR, resulting in persistently increased [Ca^{2+}] levels and leads to myofiber death), notexin (which involves Ca^{2+} overload and activation of Ca^{2+}-dependent proteases, resulting in tissue necrosis), or freezing (which causes uniform and complete necrosis of myofibers). Such acute muscle injury shows a rapid loss of the activities of citrate synthase [13–15], a mitochondrial matrix enzyme participating in the Krebs cycle, which is often used as marker for the mitochondria content of a tissue, after 2-3 days, a time when degenerative myofibers still persist and proliferating myoblasts reside [13, 15]. The activity of citrate synthase then is increased drastically between days 5 and 10, a time when myoblasts differentiate into myotubes [13, 15]. Similarly, the rate of state-3 respiration (respiratory rate during active phosphorylation of ADP) is recovered [13]. The rate of state-4 respiration (respiratory rate after exhaustion of ADP) is comparable between control and injured muscles [13]. Accordingly, the pattern of changes in the respiratory control ratio (RCR), a measure of the "tightness of coupling" between electron transport and oxidative phosphorylation, is calculated as the ratio of state-3/state-4 respiration [43], closely resembling the pattern obtained with citrate synthase activity [13]. Consistent with the *in vivo* findings, the activity of mitochondrial enzymes including citrate synthase, isocitrate dehydrogenase, 3-hydroxyacyl CoA dehydrogenase, cytochrome *c* reductase, succinate dehydrogenase, and cytochrome oxidase is drastically increased during myogenic differentiation [10, 12]. The content of respiratory chain complexes is higher in myotubes than in myoblasts [11,

44]. Similarly, the rate of state-3 respiration exhibits the same trend observed in respiratory chain complexes [44]. Leary et al. examined the changes in metabolic rate during myogenic differentiation [25]. In proliferating myoblasts, approximately 30% of the ATP used by the cells is provided by OXPHOS, whereas terminally differentiated myotubes rely on mitochondrial respiration as their major source of metabolic energy (approximately 60%) [25]. Intriguingly, the total metabolic rate remains constant throughout the culture period, but there is a steady shift toward a greater reliance on mitochondrial pathways [25]. Taken together, these findings suggest that the metabolic shift from glycolysis to oxidative phosphorylation as the major energy source occurs during myogenesis.

5. Gene Expression Involved in Mitochondrial Biogenesis during Muscle Regeneration and Myogenesis *In Vitro*

An activation of mitochondrial biogenesis occurs through the coordinated expression of PGC-1 transcriptional coactivators, transcription factors, and nuclear receptors. Surprisingly, PGC-1α expression remains unchanged during muscle regeneration, whereas PRC and PGC-1β are upregulated 3 days after injury [15]. This finding may be in line with other studies using mouse myoblasts [37, 45, 46]. PGC-1α fails to detect in either myoblasts or myotubes, whereas PRC and PGC-1β are readily detectable [46]. In contrast, Duguez et al. have reported that PGC-1α is upregulated 10 days after injury [13]. Irrespective of whether PGC-1α is upregulated in injured muscle, these findings lead us to hypothesize that PRC and PGC-1β may contribute to the mitochondrial biogenesis, at least in part, at the early stage of muscle regeneration. It has been shown that PRC coactivates NRF-1 [37, 45], and PGC-1β also interacts with NRF-1 and estrogen-related receptor α (ERRα) [47], suggesting that both PRC and PGC-1β may functionally replace PGC-1α during muscle regeneration. In support of this hypothesis, several studies have revealed the potential roles of PRC and PGC-1β in mitochondrial biogenesis in myogenic cells. PRC-overexpressing myotubes show an elevated fatty acid oxidation and increased expression of mitochondrial genes [48]. Forced expression of PGC-1β in C2C12 cells results in increased mitochondrial biogenesis and oxygen consumption [49]. Skeletal muscle-specific PGC-1β transgenic mice exhibit increased mtDNA amount, mitochondrial content, mitochondrial enzyme activity, upregulation of mitochondrial genes, and enhanced exercise performance [50]. On the other hand, mice lacking PGC-1β show a reduced number of mitochondria, decreased respiration function, and decreased expression of mitochondrial genes [51]. However, the possibility cannot be excluded that PGC-1α may contribute to the mitochondrial biogenesis during muscle regeneration, as has been shown in gain-of-function and loss-of-function studies [52–56]. Accordingly, further studies are required to elucidate the role of PGC-1α in mitochondrial biogenesis during muscle regeneration.

Not only PGC-1 family coactiuators but also NRF-1, NRF-2, and mitochondrial transcription factor A (TFAM) are also upregulated during muscle regeneration [15]. This is in line with the findings that PGC-1 stimulates an induction of NRF-1 and NRF-2 gene expression and can also interact directly with and coactivate NRF-1 on the promoter for TFAM [57]. TFAM plays a key role in mammalian mtDNA transcription/replication [21]. Likewise, when myoblasts differentiate into myotubes, PGC-1α, NRF-1, and TFAM are upregulated, and mtDNA content and copy number are increased 2–4-fold in myotubes relative to myoblasts [11, 12]. Therefore, upregulation of these genes contributes to increase the template availability for transcription and translation of key mitochondrial proteins necessary for myogenesis.

6. Possible Role of Mitochondria in Regulating Muscle Regeneration

Recent studies have extended our knowledge of the potential role of mitochondrial biogenesis in muscle regeneration [15, 58]. It has been reported that muscle regeneration is impaired when mitochondrial protein synthesis is inhibited with chloramphenicol [15]. Chloramphenicol inhibits protein synthesis in mitochondria but not in mammalian cytoplasmic ribosomal systems [59] since mammalian mitochondrial ribosomes are susceptible to peptidyl-transferase inhibition by it [60]. Chloramphenicol reversibly binds to the 50S subunit of the 70S ribosome and blocks prokaryotic protein translation primarily by inhibiting peptidyl-transferase and blocking elongation [61]. Consequently, chloramphenicol inhibits the proper assembly of 4 out of 5 respiratory chain complexes within mitochondria and therefore potentially attenuates mitochondrial biogenesis in mammalian cells. Mice were intramuscularly injected with chloramphenicol at days 3, 5, and 7 after the initial freeze injury, and the muscle specimens were histochemically analyzed at day 10. Impairment of mitochondrial activity induced by chloramphenicol results in poor muscle regeneration with small myofibers and increased connective tissues [15]. Overall, this supports *in vitro* data that show that chloramphenicol blocks myogenic differentiation [4–9]. Therefore, *in vivo* data, when combined with the previous data *in vitro*, suggests a role for mitochondrial biogenesis for sustaining muscle regeneration. However, the molecular mechanisms remain unknown although chloramphenicol downregulates myogenin, which is required for terminal differentiation and myotube formation, in an avian QM7 myoblast [6, 8] and mouse C2C12 myoblast [9].

It has been reported that muscle regeneration is effectively accelerated using a method for complex mediated delivery to intracellular mitochondria [58]. The method is based on the mitochondriotropism of a multisubunit RNA import complex (RIC) [62]. Muscle injury was induced by piercing repeatedly with a 26-gauge hypodermic needle at an angle of ~45° to the longitudinal axis of the fiber, resulting in ~3000 myofibers being damaged at each insertion [58]. When a combination of polycistronic RNAs encoding the guanine-rich heavy-strand (H-strand) of the mitochondrial genome is

administrated to injured muscle, it rejuvenates mitochondrial mRNA levels, organellar translation, respiratory capacity, and intramuscular ATP levels with reduced intracellular reactive oxygen species levels [58]. It increases proliferative potential of satellite cells and differentiation capacity of myoblasts concomitantly with upregulation of myogenic regulatory factors including Myf5, MyoD, myogenin, and MRF4, promoting muscle regeneration with the recovery of muscle contractility [58]. One of the most intriguing aspects of RIC-mediated transfection strategy, MyoD, and Numb-positive cells are detected and attached to old myofibers at the injury site [58]. This may provide new insight into the possible mechanism regulating muscle regeneration through enhancing mitochondrial activity. Numb protein has been generally considered to be a negative regulator of Notch signaling [63], which inhibits myogenic differentiation [63]. Numb segregates asymmetrically in dividing adult mouse muscle satellite cells [64, 65]. Attenuation of Notch signaling by Numb overexpression leads to the commitment of progenitor cells to the myoblast cell fate with increased expression of Myf5 and desmin [64]. Therefore, RIC-induced Numb protein may play a certain role in regulating muscle regeneration by modulating Notch signaling. However, recent evidence suggests that although forced expression of Numb in myogenic progenitors does not abrogate canonical Notch signaling, it can stimulate the self-renewal of myogenic progenitors [66]. Therefore, a role of Numb in regulating muscle regeneration remains to be elucidated. Furthermore, it is unknown how mitochondrial activity modulates Notch signaling at the present time.

7. Do Mitochondria Act as a Potential Regulator of Myogenesis?

Korohoda et al. [4] have reported that chloramphenicol inhibits the fusion of myoblasts isolated from chick embryo skeletal muscle. This is among the first study to show the effect of chloramphenicol on myogenesis. They show that tryptose phosphate broth and nucleosides can restore the cell capacity to proliferate but not to fuse and differentiate in the presence of chloramphenicol [4]. Subsequently, it has been demonstrated that mitochondrial activity is an important regulator of myogenic differentiation in quail myoblasts of the QM7 cell line and mouse myoblasts of the C2C12 cell line using chloramphenicol [6, 8, 9]. Chloramphenicol-treated myoblasts proliferate at a slower rate than control myoblasts without inducing any alteration of cell viability [6]. When chronically exposed to chloramphenicol throughout the culture period, it severely suppresses myogenic differentiation [6, 8, 9]. The possibility can be excluded that intracellular ATP depletion induced by chloramphenicol could be responsible for the inhibition of myoblast differentiation for the following the reasons: (1) glycolysis fully compensates for mitochondrial impairment just before and during terminal differentiation, as shown in a marked accumulation of L-lactate in the culture medium [6], and this has been already reported in C2C12 cells using tetracycline [5]; (2) differentiation of myoblast is repressed especially when exposing to chloramphenicol at the onset of terminal differentiation [6]. These findings indicate that mitochondrial activity regulates myogenic differentiation independently of their implication in ATP synthesis [6].

Chloramphenicol inhibits myogenic differentiation by downregulating myogenin but not MyoD and Myf5 [6, 8]. Intriguingly, this downregulation is commonly observed in FCCP, myxothiazol [7], rotenone [7], and oligomycin [6, 7], which affect mitochondria at different levels. These findings suggest that myogenin could be an important target of mitochondrial activity. Chloramphenicol has no effect on myogenin mRNA stability [6], suggesting that mitochondrial activity could regulate myogenin expression at the transcriptional level [6]. Unexpectedly, overexpression of neither myogenin nor MyoD fails to restore differentiation capacity in chloramphenicol-treated myoblasts [6]. This indicates that mitochondrial activity could regulate myogenic differentiation by decaying ability of myogenic regulatory factors via other negative regulators. Chloramphenicol has no effect on the expression of MEF2C (myocyte enhancer factor 2C) and Id (inhibitor of differentiation) [8]. Seyer et al. have identified c-Myc (cellular myelocytomatosis oncogene) gene, which could be a target gene regulated by mitochondrial activity [8]. c-Myc is a proto-oncogene encoding a transcription factor [67], which plays a role in regulating myogenesis [68–74]. Impairment of mitochondrial activity by chloramphenicol abrogates the downregulation of c-Myc normally occurring at the induction of differentiation in control cells [8]. Overexpression of c-Myc mimics the influence of mitochondrial activity inhibition on myogenic differentiation [8]. A triiodothyronine-dependent mitochondrial transcription factor (p43) overexpression, which stimulates mitochondrial activity, downregulates c-Myc expression [8]. These findings suggest the possibility that c-Myc could be a primary target of mitochondrial activity. Indeed, the endogenous c-Myc is downregulated within the first 24 h after switching to a differentiation medium [70]. Ectopic expression of c-Myc in quail myoblasts fails to form myotubes and downregulates MyoD, myogenin, and Myf5 expression [73]. Cotransfection of c-Myc with MyoD and myogenin in NIH 3T3 cells inhibits myogenic differentiation [71].

While these findings are compelling, a role of c-Myc should be carefully considered. First, irreversible repression of c-Myc is not required for terminal myogenic differentiation, and its expression is insufficient to suppress the differentiated phenotype, since nuclear runoff transcription assay demonstrates that c-Myc and skeletal muscle-specific genes could be simultaneously transcribed in both biochemically differentiated cells (no fusion) and terminally differentiated cells [69]. The c-Myc- transformed C2C12 cells retain the ability to undergo commitment and biochemical differentiation, but they are strikingly unable to fuse into multinucleated myotubes with no change in the expression of MyoD, myogenin, and myosin heavy chain [72]. These findings lead us to rethink how c-Myc modulates myogenic differentiation. Secondly, c-Myc represses $p21^{Cip1/WAF1}$ expression through transcriptional activator, Miz-1- (c-Myc interacting zinc-finger protein 1-) dependent interaction with $p21^{Cip1/WAF1}$ core promoter [75]. In addition, c-Myc interacts with Miz-1

and recruits DNA methyltransferase 3A to $p21^{Cip1/WAF1}$ promoter to silence p21 transcription [76]. The expression of $p21^{Cip1/WAF1}$ is known to be a key event triggering the withdrawal of myoblasts from the cell cycle to G_0, a prerequisite to myogenic differentiation [77]. Indeed, chloramphenicol and overexpression of c-Myc decrease the proportion of myoblasts in the G_0-G_1 phase, whereas overexpression of p43 exerts opposite influence [8]. These findings suggest the possibility that mitochondrial activity could regulate myoblast cell cycle withdrawal by modulating expression of $p21^{Cip1/WAF1}$ through c-Myc/Miz-1 complex. Thirdly, Myc is a member of the Myc/Max (Myc-associated factor X)/Mad (MAX dimerization protein) transcriptional network that comprises a group of widely expressed transcription factors [78]. c-Myc/Max heterodimers transactivate its downstream genes by binding to the E-box sequence 5′-CACGTG-3′ in the target promoter, whereas Mad/Max heterodimers act as transcriptional repressors at the same E-box-related DNA-binding sites [78]. Therefore, c-Myc/Max heterodimers function by competing with Mad/Max heterodimers, resulting in controlling the expression of their target genes. Intriguingly, a switching from c-Myc/Max to Mad/Max heterodimers occurs when leukemia cells differentiate into monocyte/macrophage [79, 80]. These findings lead us to hypothesize that mitochondrial activity may be involved in this switching during myogenic differentiation. It requires additional studies to validate this observation in myogenic cells. Finally, a new mode of Myc regulation has been recently reported in myogenic differentiation [81]. Myc protein is cleaved by a calpain to generate a cytoplasmic form, "Myc-Nick," which retains Myc box regions but lacks nuclear localization sequence and the basic helix-loop-helix/leucine zipper domains essential for heterodimerization with Max and DNA binding activity [81]. During myogenic differentiation, while the full-length Myc decreases, Myc-nick is increased. Ectopic expression of Myc-nick in human primary myoblasts, human rhabdomyosarcoma (RD) cells, and mouse C2C12 myoblasts accelerates their differentiation and increases expression of skeletal muscle-specific markers [81]. Taken together, the mechanisms underlying the regulation of biological function of c-Myc are complicated. Therefore, further studies are needed to elucidate the role of c-Myc in the regulation of myogenesis by mitochondria.

To further understand the molecular mechanisms underlying the regulation of myogenic differentiation by mitochondria, Seyer et al. [9] conducted a comprehensive differential display analysis using total RNA from control and chloramphenicol-treated myoblasts to search for other gene modulating by mitochondrial activity [9]. They identified calcineurin (also referred to as protein phosphatase 2B) as another candidate molecule [9], in which serine/threonine protein phosphatase under the control of a eukaryotic Ca^{2+}- and calmodulin plays a critical role in the coupling of Ca^{2+} signals to cellular responses [82]. It is a heterodimeric enzyme consisting of a 60 kDa catalytic A subunit (calcineurin A) and 19 kDa calcium-binding regulatory B subunit (calcineurin B) [82]. Calcineurin signaling has been implicated in regulating myogenesis [83–90]. Chloramphenicol attenuates the differentiation-induced upregulation of calcineurin A, whereas overexpression of p43 increases calcineurin A expression in proliferating myoblasts [9]. Based on these findings, they suggest that calcineurin could be a novel target regulated by mitochondrial activity. Intriguingly, expression of a constitutively active form of calcineurin upregulates the expression of myogenin [85]. Calcineurin regulates expression of the myogenin gene at the transcriptional level by activating MEF2 and MyoD transcription factors [87]. Taken together, mitochondrial activity may regulate myogenesis through calcineurin-mediated myogenin expression. On the other hand, it has been shown that calcineurin A and its direct downstream transcriptional effector, NFATc (nuclear factor of activated T-cells), are upregulated concomitantly with a modest increase in calcineurin B in mtDNA-depleted cells (only ~20% of the mtDNA content compared with normal untreated cells) [24]. Biswas et al. developed myogenic cell lines with partially depleted mtDNA when chronically exposed to EtBr for many passages to investigate the mechanism of mitochondrial-nuclear crosstalk [24]. The mtDNA-depleted cells have an elevated steady-state cytosolic Ca^{2+} level ($[Ca^{2+}]i$), as shown in other mitochondrial inhibitors including antimycin, azide, CCCP, and valinomycin [24]. Therefore, increased cytosolic Ca^{2+} may stimulate the expression of calcineurin-related molecules in the myoblasts treated with these drugs. It is to be noted that increased expression of calcineurin is observed by mtDNA depletion or acute treatment (30 min) with high amounts of mitochondrial inhibitors. As already described, mtDNA-depleted myoblasts by EtBr fail to differentiate into myotubes [1–3], and NFAT is not an essential downstream target of calcineurin during myogenesis [85]. Therefore, the activation of calcineurin pathway induced by impairment of mitochondrial function and activity could not contribute to myogenesis.

The nuclear factor-κB (NF-κB) functions as a negative regulator of myogenesis [91]. NF-κB is a heterodimeric or homodimeric complex formed from five distinct subunits: RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) [91]. Only RelA, c-Rel, and RelB possess C-terminal transcriptional transactivation domains, whereas NF-kB1 and NF-kB2 lack intrinsic transactivating properties and instead function as homodimeric transcriptional repressors or modulators of transactivating dimer partners [91]. When stimulated by a wide variety of different stimuli, IκB is phosphorylated by IκB kinase (IKK) complex and subsequently degraded by the proteasome, allowing NF-κB to translocate into the nucleus where they regulate target gene expression [91]. Respiration-deficient myoblasts devoid of mitochondrial DNA by EtBr show a decreased expression of RelA, increased expression of IκB and p50, and unchanged expression of RelB and p52 [24]. Intriguingly, other mitochondrial inhibitors also have same effects on their expression [24]. These findings suggest that mitochondrial activity can modulate NF-κB transcriptional activity although it is required for measuring its DNA binding activity, for example, by an electrophoretic mobility shift assay.

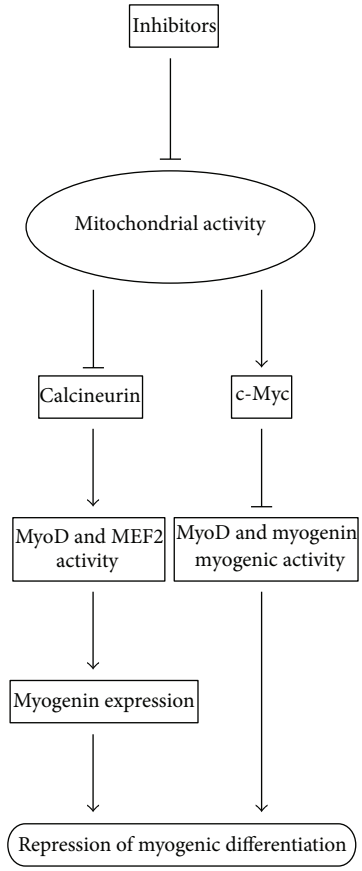

Figure 1: Hypothetic model of mitochondrial activity in myogenic differentiation.

8. Conclusion

This paper provides the current knowledge about the role for mitochondria as a potential regulator of myogenesis. Several studies have highlighted that mitochondria play a role in regulating myogenic differentiation possibly through a number of mechanisms. In particular, myogenin, c-Myc, and calcineurin have been identified as candidate molecules of mitochondrial target [6, 8, 9]. Together with previous data [8, 9, 87], a hypothetical model involving c-Myc and calcineurin in the regulation of myogenic differentiation by mitochondrial activity is presented in Figure 1. In this model, when myoblasts are induced to differentiate in the presence of mitochondrial inhibitors, downregulation of c-Myc could be inhibited, which depresses the activity of MyoD and myogenin, resulting in blocking myogenic differentiation. Decreased calcineurin signaling by inhibiting mitochondrial activity could contribute to myogenin expression through modulating MyoD and MEF2 activity. Understanding how mitochondria are involved in myogenesis will provide a valuable insight into the underlying mechanisms that regulate the maintenance of cellular homeostasis. Recently, it has been reported that the transgenic mice with skeletal muscle-specific expression of PGC-1α preserve mitochondrial function as well as neuromuscular junctions and muscle integrity during ageing [92], and mitochondrial gene therapy may be effective in the treatment of muscle injury [58]. These efforts may facilitate to understand the molecular mechanisms of mitochondrial disorders.

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Review Article

Muscle Wasting and Resistance of Muscle Anabolism: The "Anabolic Threshold Concept" for Adapted Nutritional Strategies during Sarcopenia

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Skeletal muscle loss is observed in several physiopathological situations. Strategies to prevent, slow down, or increase recovery of muscle have already been tested. Besides exercise, nutrition, and more particularly protein nutrition based on increased amino acid, leucine or the quality of protein intake has generated positive acute postprandial effect on muscle protein anabolism. However, on the long term, these nutritional strategies have often failed in improving muscle mass even if given for long periods of time in both humans and rodent models. Muscle mass loss situations have been often correlated to a resistance of muscle protein anabolism to food intake which may be explained by an increase of the anabolic threshold toward the stimulatory effect of amino acids. In this paper, we will emphasize how this anabolic resistance may affect the intensity and the duration of the muscle anabolic response at the postprandial state and how it may explain the negative results obtained on the long term in the prevention of muscle mass. Sarcopenia, the muscle mass loss observed during aging, has been chosen to illustrate this concept but it may be kept in mind that it could be extended to any other catabolic states or recovery situations.

The main function of skeletal muscle is to provide power and strength for locomotion and posture, but this tissue is also the major reservoir of body proteins and amino acids. Thus, although the loss of muscle proteins has positive effects in the short term by providing amino acids to other tissues, an uncontrolled and sustained muscle wasting impairs movement, leads to difficulties in performing daily activities, and has detrimental metabolic consequences with reduced ability in mobilizing enough amino acids in case of illness and diseases. The resulting weakness increases the incidence of falls and the length of recovery and when advanced, muscle wasting is correlated to morbidity and increased mortality. Consequently, one of the challenges we have to face is to supply amino acids to the tissues with higher requirements in catabolic states [1] but also to prevent a too important loss in muscle proteins and ultimately improve muscle recovery.

During the day, protein metabolism is modified by food intake. Whole-body proteins are stored during postprandial periods and lost in postabsorptive periods. With a muscle protein mass that remains constant, the loss of muscle proteins is compensated for the same protein gain in the postprandial state. In adult volunteers, oral feeding is associated with an increase in whole-body protein synthesis and a decrease in proteolysis [2–5]. These changes are mediated by feeding-induced increases in plasma concentrations of both nutrients and hormones. Many studies suggest that amino acids and insulin play major roles in promoting postprandial protein anabolism [6]. Thus, in case of muscle wasting, muscle protein loss results from an imbalance

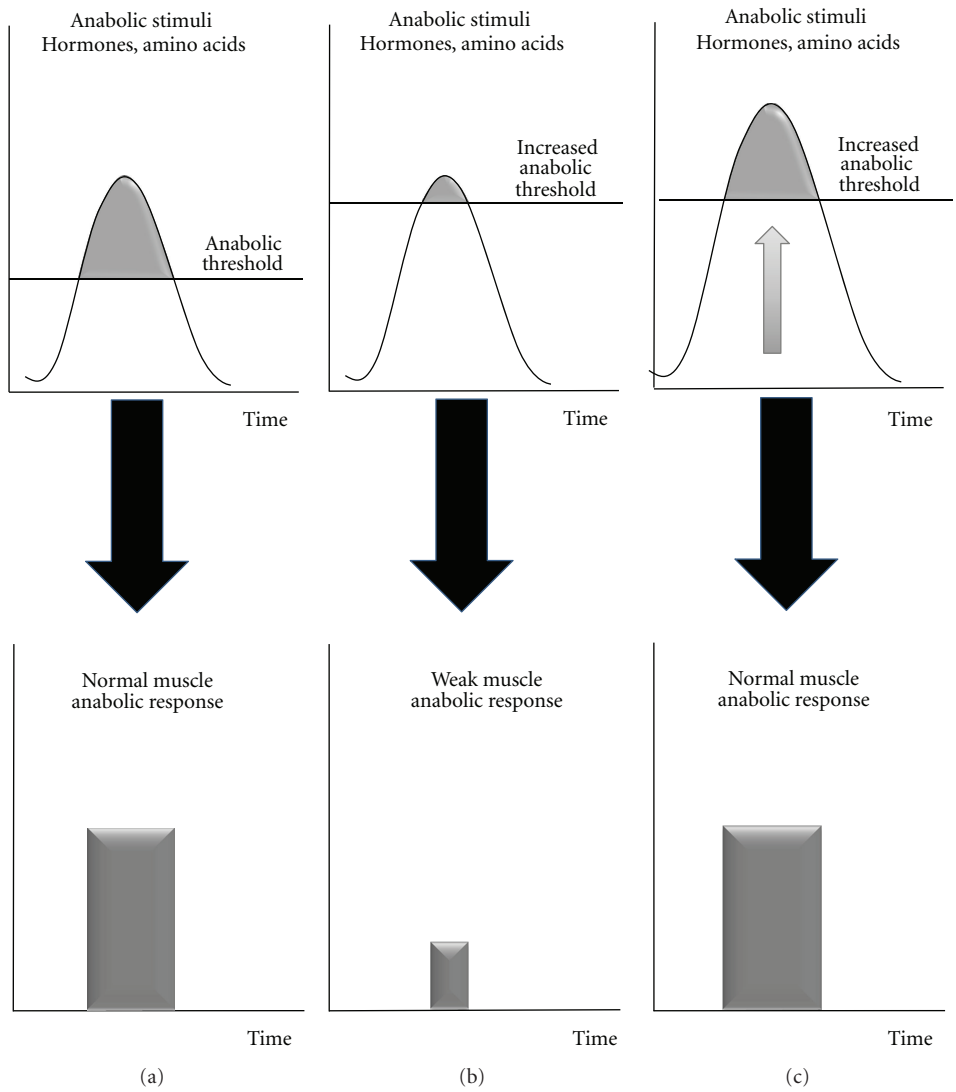

Figure 1: The concept of increased anabolic threshold with associated altered muscle protein anabolism during the postprandial period.

between protein accretion and break-down rates which, in part, comes from a defect in the postprandial anabolism.

Although each muscle wasting situation is characterized by its specific mechanism(s) and pathways leading to muscle loss, an increase of catabolic factors such as glucocorticoids, cytokines, and oxidative stress, often occurs and it is now well established that these factors have potential deleterious effects on the amino acids or insulin signalling pathways involved in the stimulation of muscle anabolism after food intake [7–11].

These signalling alterations lead to an "anabolic resistance" of muscle even if the anabolic factor requirements (amino acids e.g.) are theoretically covered, that is, with a normal nutrient availability fitting the recommended dietary protein allowances in healthy subjects. This anabolic resistance may be in part explained by an increase of the muscle "anabolic threshold" required to promote maximal anabolism and protein retention (Figures 1(a) and 1(b)). Because the muscle "anabolic threshold" is higher, the anabolic stimuli (including aminoacidemia) cannot reach the anabolic threshold anymore and by consequence, muscle anabolism is reduced with the usual nutrient intake (Figure 1(b)). A possible nutritional strategy is then to increase the intake of anabolic factors (especially amino acids) to reach the new "anabolic threshold" (Figure 1(c)). There are several ways to increase amino acid availability to skeletal muscle: increase protein intake, to supplement the diet with one or several free amino acids or to select the protein source on its amino acid composition and physicochemical properties when digested in the digestive tract. These nutritional strategies tested to increase postprandial amino acid levels above the increased anabolic threshold and ultimately to restimulate muscle protein synthesis in situations of anabolic resistance led to conflicting results with no or more or less positive effects of the supplementation on nitrogen retention. This could be explained by variations in amino acid kinetics. The duration of the hyperaminoacidemia postprandially can also be of a variable

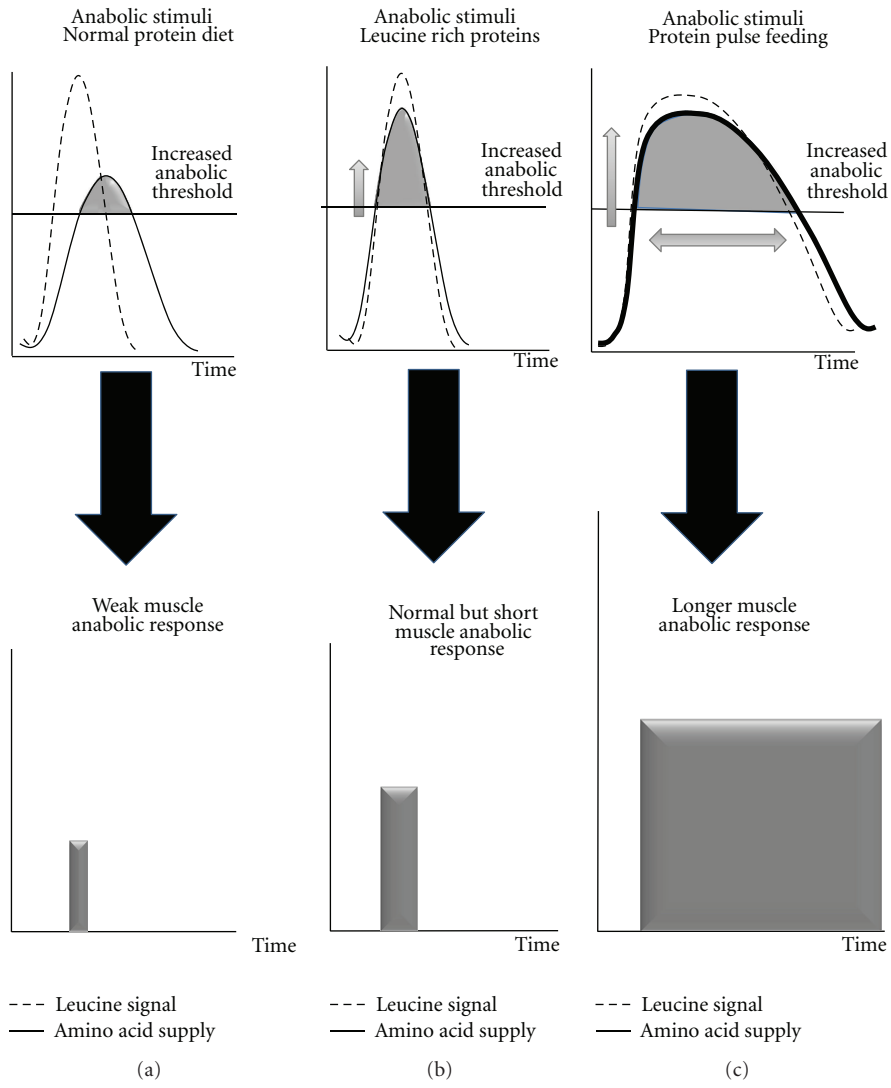

Figure 2: Free leucine, leucine rich proteins, and high protein diet in terms of amino acid kinetic and associated anabolic response in situation of increased muscle anabolic threshold.

magnitude and duration, depending of the form of the protein/amino acid supply in the diet. To illustrate this concept, we will take one example, that is, the loss of muscle mass during aging, while keeping in mind that this could be translated to any situation of muscle wasting.

Sarcopenia, as other catabolic states, has been found to result from a decreased response and/or sensitivity of protein synthesis and degradation to physiologic concentrations of amino acids [12–14]. This is related to a defect of the leucine signal to stimulate the mTOR signalling pathway activity [15]. These data suggest that increasing leucine availability may then represent a nutritional strategy to overcome the "anabolic threshold" increase observed during aging. Studies in both elderly humans and rodents subjected to free leucine supplementation have shown that such supplementations indeed acutely improved muscle protein balance after food intake by increasing muscle protein synthesis and decreasing muscle proteolysis in the postprandial state (reviewed in Balage and Dardevet [16]). However, the few chronic studies conducted with such free leucine supplementations did not succeed in promoting an increase in muscle mass [17–19]. Choosing free leucine as a supplement over a normal protein diet creates a desynchronization between leucine signal and the rise in all amino acids (Figure 2(a)). Indeed the free leucine is absorbed immediately whereas the other amino acids are released later after gastric emptying and proteolytic digestion in the gut. This nonsynchronization between the stimulation of muscle leucine-associated protein metabolism pathways and the delayed availability of amino acids as substrates can explain that protein anabolism was only stimulated for a very short period of time during the postprandial period and then could not translate into a significant muscle protein accretion.

Studies with a synchronized leucine signal and amino acid availability have been performed by using leucine rich proteins that are rapidly digested (whey proteins) [20]. With

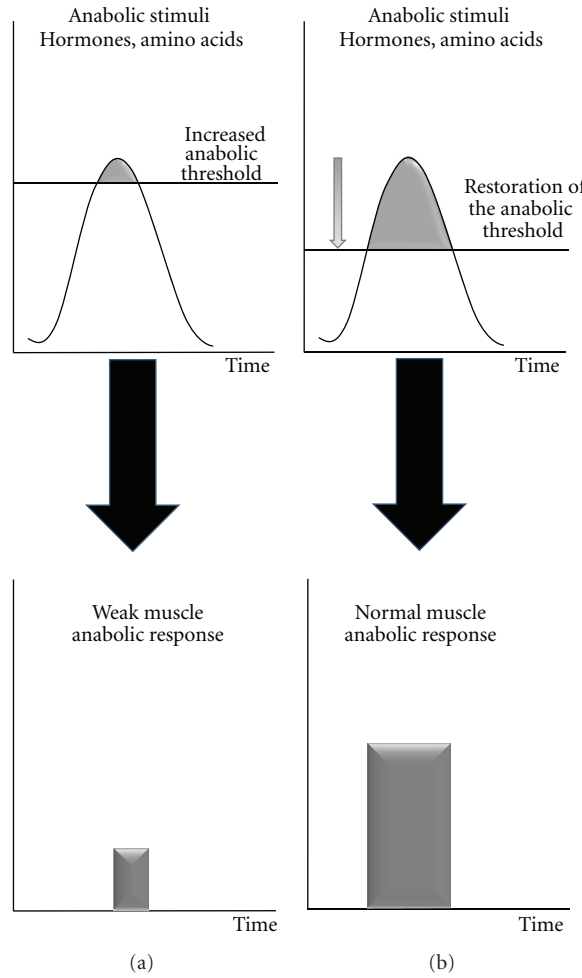

Figure 3: Strategies aiming at partially decreasing the muscle "anabolic threshold" and increasing the efficiency of the postprandial period.

such proteins, leucine availability is increased simultaneously with the other amino acids to reach the increased muscle anabolic threshold (Figure 2(b)). However, as observed for free leucine supplementation, when such dietary proteins where given on the long term in elderly rodents [21], muscle anabolism was acutely improved but muscle mass remained unchanged. However, Magne et al. [22] have shown that in elderly rodents recovering from acute muscle atrophy, leucine rich proteins were nevertheless efficient in improving recovery of muscle mass whereas free leucine supplementation remained ineffective. It may be postulated that, when given on the long term, protein muscle metabolism was adapted by increasing also protein catabolism in parallel with the increase of protein anabolism. However, after a catabolic state with an important muscle mass loss occurring within few days, this adaptation may be delayed and leucine rich proteins remained efficient in improving muscle mass.

According to these data, it can be concluded that besides counteracting the muscle anabolic resistance, the duration during which the anabolic resistance is muzzled also plays a critical role in leading to a significant muscle protein accretion. A prolonged stimulation could not be achieved with fast proteins at normal dietary level (even enriched with leucine) since the concentration of amino acids as substrates declines rapidly after their intake [23]. However, by strongly increasing protein intake, such ideal situations could be nevertheless achieved (Figure 2(c)). The "protein pulse feeding" initially developed by Arnal et al. [24–26] have shown that, by concentrating 80% of the total daily protein intake in one meal, protein retention was improved in elderly women subjected to a such nutritional strategy. Similarly, when very large amount of amino acids (wherein leucine formed the highest percentage of the mixture), positive results have been observed [27–30].

The above nutritional strategies discussed raised the problem that the organism has to cope with large amount of nitrogen to eliminate. This point can be critical with already frail sarcopenic subjects or patients for who the renal function will be oversolicited whereas it may be already altered.

In order to minimize this deleterious side effect of high protein intake, a strategy to reverse the increase in the "anabolic threshold" would restore the anabolic stimulation during the postprandial period with lower intake of dietary proteins or amino acid supplementations (Figures 3(a) and

3(b)). This requires the knowledge of the factors involved and responsible in the "anabolic threshold" elevation. The causes can be multiple and specific for each catabolic state. However, most of these muscle loss situations have in common an increase of the inflammatory status. Regarding aging, levels of inflammatory markers, such as interleukin-6 (IL6) and C reactive protein (CRP), increase slightly, and these higher levels are correlated with disability and mortality in humans [31, 32]. Even if the increase is moderate, higher levels of cytokines and CRP increase the risk of muscle strength loss [33] and are correlated with lower muscle mass in healthy older persons [34]. We have recently shown that the development of a low grade inflammation challenged negatively the anabolic effects of food intake on muscle protein metabolism and that the pharmacologic prevention of this inflammatory state was able to preserve muscle mass in old rodents [7, 8]. A resensitization of muscle protein synthesis to amino acids could be also achieved with other nutrients such as antioxidants [11, 35] but it is not known yet if such supplementations could be effective in preserving muscle mass. Interestingly, Smith et al. [36] have tested n-3 polyunsaturated fatty acids supplementation to increase the sensitivity of muscle protein metabolism to anabolic factors (amino acids and insulin) by increasing the cellular membrane fluidity in elderly volunteers. Although they obtained a resensitization of the mTOR signalling pathway with the n-3 fatty acids, it is not known if the decrease of the "anabolic threshold" has been large enough to translate into sufficient postprandial protein accretion and then preserve muscle mass in the long term if not associated with a concomitant dietary increase in amino acids.

By choosing muscle mass loss during aging as an example of muscle wasting, it becomes obvious that skeletal muscle "anabolic threshold" is increased in such situations and that muscle protein metabolism becomes resistant to dietary anabolic factors even if these factors are supplied at the level they elicit maximal effects in normal physiological situations. It is important to note that this anabolic resistance during aging may be specific to amino acids [37]. Because the muscle "anabolic threshold" is more elevated, the duration of the stimulation by anabolic signals (as leucine) and the overcome of amino acid supply above the threshold is reduced with usual nutrient intake. Two strategies can be used (alone or in combination) to deal with this decreased "efficient" postprandial period: (1) by increasing the anabolic signals, and particular amino acid availability; however, it is necessary to synchronize the anabolic stimuli with the substrates in order to optimize the incorporation of amino acids into muscle proteins; (2) by increasing the efficiency of the postprandial period with strategies aiming at partially restoring (i.e., decreasing) the muscle "anabolic threshold".

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