Journal of Diabetes Research

# The Underlying Mechanisms of Diabetic Myopathy

Lead Guest Editor: Erick O. Hernández-Ochoa
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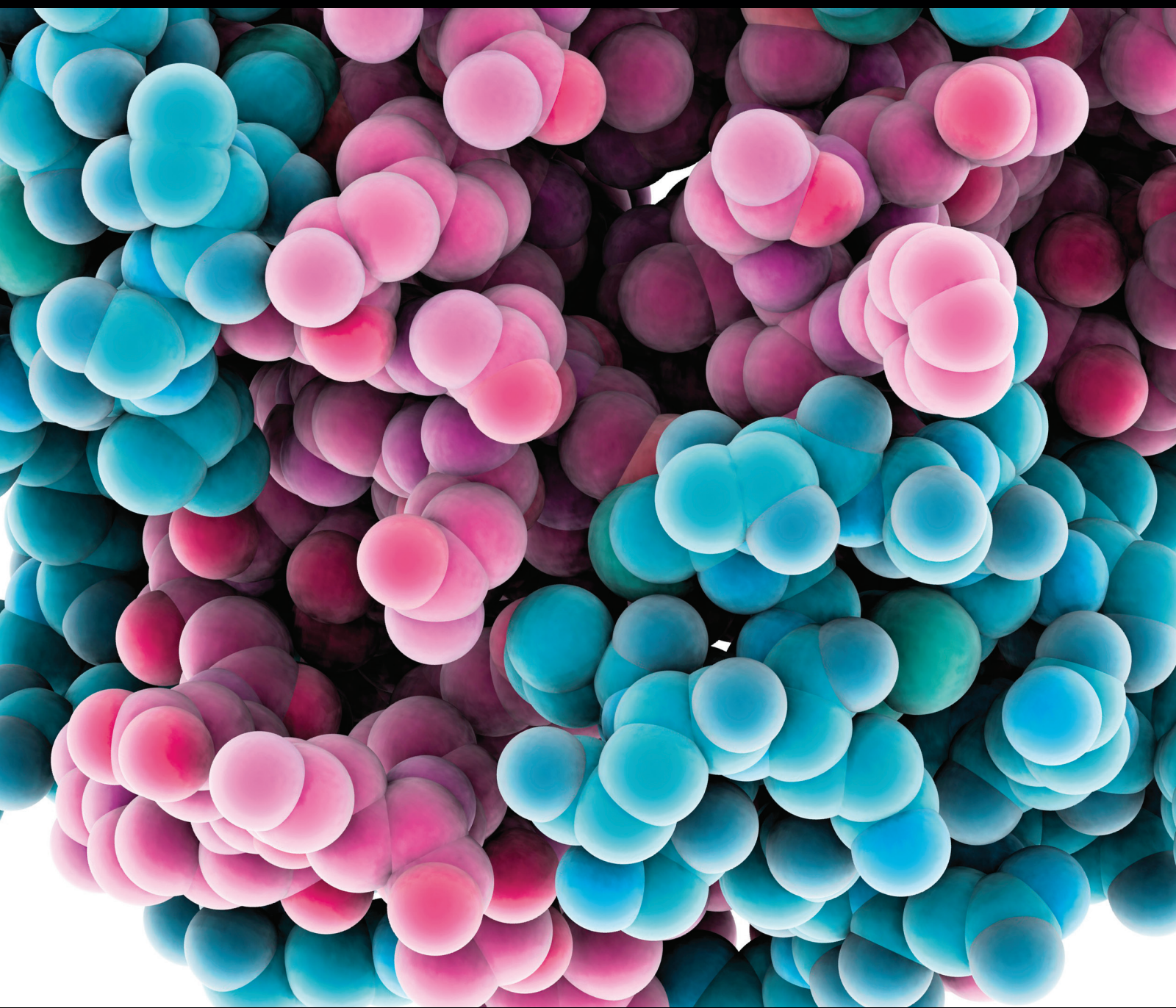

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# Editorial Board

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*Editorial*

# The Underlying Mechanisms of Diabetic Myopathy

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## 1. Editorial

Late complications of diabetes affect both the quality and quantity of life, resulting in major health costs [1]. A common complication of both type 1 diabetes (T1D) and type 2 diabetes (T2D) is the failure to preserve muscle mass and function [2, 3], here referred to as diabetic myopathy [4–6]. Although often overlooked, this complication is believed to contribute to the progression of other diabetic complications and comorbidities (e.g., uncontrolled hyperglycemia, sedentarism, and obesity) based on the key role skeletal muscle plays in glucose homeostasis and locomotion [7–9]. Numerous studies have investigated the link between diabetic myopathy and diverse cellular processes [10]; however, despite the wealth of information on muscle weakness and muscle wasting [11], the specific triggering events of diabetic myopathy in patients with diabetes remain unknown. Further knowledge of the pathophysiological and molecular mechanisms involved in the onset and progression of diabetic myopathy is needed for the development of new pharmacological tools to ameliorate diabetic myopathy. The importance of this area of diabetes research was the motivation for us to develop this special issue.

This special issue includes three review articles and two original research papers, from leading and emerging scientists who study diabetic myopathy in different muscle tissues (cardiac, smooth, and skeletal) and with diverse expertise and interests, aiming to stimulate the continuing effort to understand the impact of diabetes on muscle function. These reviews and research papers represent the joint effort of 23 experts in the field, in which they examine the topic from several angles and levels ranging from translational aspects to whole tissue and single cell.

G. Barrientos and collaborators present a review about membrane cholesterol in skeletal muscle and its role in excitation-contraction coupling and glucose transport. They focus on the muscle plasma membrane network characterized by surface invaginations, also known as the transverse tubular (t-tubular) system [12]. This t-tubular system is rich in cholesterol [13], and it is critical for excitability and bidirectional transport of solutes, ions, nutrients, and metabolic waste. The authors discuss recent findings by other groups [14] and the work that they have done, regarding t-tubular cholesterol dynamics and its effects on excitation-contraction coupling and GLUT4 trafficking in normal and obese animal models [15, 16]. In obesity, the cholesterol content in the t-tubular network is further increased [16]. Interestingly, as suggested by the authors, restoring cholesterol levels and increasing GLUT4 trafficking in the t-tubular system could represent a new therapeutic avenue to ameliorate insulin resistance.

P. E. Morales et al. review the topic of muscle lipid metabolism and the role of lipid droplets and perilipins in rodents and humans. The skeletal muscle is not only important for carbohydrate metabolism, it is also crucial for the metabolism of lipids. Obesity is characterized by aberrant fat storage and increased levels of circulating lipids and fatty acids [17]. Lipotoxicity is characterized by the uncontrolled intracellular accumulation of lipids, oxidative stress, organelle damage, and autophagy [18]. Lipid droplets are intracellular depots,

limited by phospholipid monolayer, and represent an important an immediate source of energy substrates [19]. Lipid droplets are localized mainly in the subsarcolemmal region or in between myofibrils abutted to mitochondria [20]. Here, the authors present insights into the mechanisms underlying lipid trafficking and its metabolism in skeletal muscle, especially focusing on the function of lipid droplets, the PLIN family of proteins and how these entities are modified during mechanical contraction, obesity, and insulin resistance.

The review by D. T. Au et al. focuses on the LDL receptor-related protein 1 (LRP1), a signaling receptor member of the low-density lipoprotein (LDL) receptor family involved in the clearance of chylomicrons from the circulation [21]. LRP1 is widely expressed in different tissues, with high expression in hepatocytes, adipocytes, fibroblasts, macrophages, and vascular smooth muscle cells, where it plays a critical role during angiogenesis and as an atheroprotective factor (i.e., protects against the formation of atherosclerosis) [22]. D. T. Au et al. discuss recent results using liver-specific LRP1 knockout mice [23]. Hepatic LRP1 inactivation resulted in defective insulin signaling, which included impaired phosphorylation of insulin receptor. These authors also summarize recent advances in LRP1 function in adipocytes, including studies using adipocyte LPR1 knockout mice that displayed delayed postprandial lipid clearance and improved glucose tolerance and resistance to high-fat diet-induced obesity [24]. The authors also highlight recent evidence in support of a link between LPR1 from epicardial adipocytes and glucose metabolism in individuals with T2D [25]. Due to the close proximity to muscle cells, intra- and intermuscular adipocytes may communicate with myofibers from skeletal, smooth, and cardiac tissues. Future work on LRP1 signaling could further our understanding of the cross talk between intramuscular adipocytes and the muscle.

E. O. Hernández-Ochoa and colleagues' contribution to this special issue concerns altered action potential-induced $Ca^{2+}$ transients in cultured skeletal muscle fibers challenged with elevated extracellular glucose. As mentioned, patients with T1D and T2D exhibit increased muscle weakness and loss of muscle mass [2, 3, 11]. Yet, the mechanisms underlying diabetic myopathy remain unknown. Previous studies in skeletal muscle reported alterations in the excitation-contraction coupling (ECC)—a coordinated chain of cellular events that links the membrane action potential with intracellular $Ca^{2+}$ release and activation of the contractile machinery [26, 27]. This study shows that muscle fibers cultured in elevated glucose for 48 hrs exhibit predominantly biphasic action potential-induced $Ca^{2+}$ transients in response to single field stimulation. Thus, glucose-induced alterations in $Ca^{2+}$ transients could play a role in the progression of muscle weakness and observed in diabetic myopathy.

The paper by M. L. Mizgier et al. investigated the effect of human myotube-derived media on glucose-stimulated insulin secretion in mice pancreatic islets and rat-isolated beta cells. The glucose homeostasis and insulin secretion are tightly regulated in our body [7–9]. The skeletal muscle accounts for 70–80% of the insulin-dependent glucose disposal in the postprandial state in humans [7]. The skeletal muscle is supposedly also involved in the regulation of insulin secretion from the pancreas via muscle humoral factors, also known as myokines [28, 29]. In this study, they monitored five myokines: IL6, IL8/CXCL8, MCP1/CCL2, fractalkine/CX3CL1, and RANTES/CCL5. They hypothesized that insulin influences the secretion of myokines, which in turn increases the glucose-stimulated insulin secretion (GSIS). Pancreatic islets incubated with conditioned media from human myotubes exposed to glucose exhibited higher GSIS than islets exposed to conditioned media from human myotubes in the absence of glucose stimulation. Furthermore, conditioned media from insulin-treated myotubes did not influence GSIS. They conclude that myokines present in conditioned media from untreated human myotubes regulate insulin secretion in mice pancreatic islets. These results support their hypothesis that myokines can influence insulin secretion.

We hope that more of our colleagues become interested in the study of diabetic myopathy and contribute to further our knowledge of the mechanisms of this understudied pathological process.

## Acknowledgments

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*Research Article*

# Acute Elevated Glucose Promotes Abnormal Action Potential-Induced $Ca^{2+}$ Transients in Cultured Skeletal Muscle Fibers

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A common comorbidity of diabetes is skeletal muscle dysfunction, which leads to compromised physical function. Previous studies of diabetes in skeletal muscle have shown alterations in excitation-contraction coupling (ECC)—the sequential link between action potentials (AP), intracellular $Ca^{2+}$ release, and the contractile machinery. Yet, little is known about the impact of acute elevated glucose on the temporal properties of AP-induced $Ca^{2+}$ transients and ionic underlying mechanisms that lead to muscle dysfunction. Here, we used high-speed confocal $Ca^{2+}$ imaging to investigate the temporal properties of AP-induced $Ca^{2+}$ transients, an intermediate step of ECC, using an acute in cellulo model of uncontrolled hyperglycemia (25 mM, 48 h.). Control and elevated glucose-exposed muscle fibers cultured for five days displayed four distinct patterns of AP-induced $Ca^{2+}$ transients (phasic, biphasic, phasic-delayed, and phasic-slow decay); most control muscle fibers show phasic AP-induced $Ca^{2+}$ transients, while most fibers exposed to elevated D-glucose displayed biphasic $Ca^{2+}$ transients upon single field stimulation. We hypothesize that these changes in the temporal profile of the AP-induced $Ca^{2+}$ transients are due to changes in the intrinsic excitable properties of the muscle fibers. We propose that these changes accompany early stages of diabetic myopathy.

## 1. Introduction

Diabetes mellitus (DM), a complex metabolic syndrome, is due to the inability of the pancreas to produce and/or secrete insulin, referred as insulin deficiency or improper insulin signal transduction by tissues like hepatic, fat, and skeletal muscle, known as insulin resistance. In either insulin deficiency or resistance, the cells are unable to adequately metabolize the glucose, leading to hyperglycemia, the hallmark of the disease. Late complications of diabetes affect both the quality and quantity of life, resulting in major health costs [1]. The disease progression of both type 1 (T1D) and type 2 (T2D) diabetes are different, yet the clinical manifestations and complications are often similar [1]. During episodes of hyperglycemia, glucose levels reach abnormal elevated values ranging from 120 to 1200 mg/dL [2–4]. In addition to the change in glucose concentration, hyperglycemia is accompanied by significant changes in plasma osmolarity [2, 4, 5]. Individuals affected by long-term T2D repeatedly present modest but significant changes in glucose concentration and osmolarity, while patients with acute uncontrolled hyperglycemia (i.e., T1D) can exhibit even larger changes in osmolarity [2–5]. Consequently, it is anticipated that harmful effects of hyperglycemia and/or hyperglycemic-induced osmotic stress contributes to the progression of diabetic complications and comorbidities.

A common comorbidity of both T1D and T2D is sarcopenia and dynapenia—the loss of muscle mass and strength, respectively, and is termed diabetic myopathy [6, 7]. The adequate function of skeletal muscle is fundamental for body movement and glucose metabolism [8–10], and the development of diabetic myopathy, an understudied and commonly overlooked condition, is believed to worsen the metabolic status of the individual already affected with concurrent diabetic complications. Comprehensive studies involving large numbers of patients with chronic T2D have shown increased

sarcopenia and dynapenia when contrasted to healthy individuals [11, 12]. Fatigue and weakness are also common findings in patients with acute episodes of hyperglycemia, particularly in patients with T2D [13]. There are numerous studies related to fatigue, sarcopenia, and dynapenia [14–18]; nevertheless, the precise cellular events linked with these muscular conditions in individuals afflicted by diabetes remain unidentified.

While previous studies have investigated the link between changes in skeletal muscle function and muscle mass [1, 7, 10–12, 14, 17–23], $Ca^{2+}$ homeostasis and signaling in different models of long-term diabetes mellitus [24–27], few have examined the impact of relatively acute elevated glucose on action potential- (AP-) induced $Ca^{2+}$ transients. Direct acute effects of hyperglycemia could have implications for the skeletal muscle myopathy seen in diabetes, especially in patients with poor glycemic control. In particular, studies of experimental diabetes in skeletal muscle have shown alterations in the excitation-contraction coupling (ECC)—a coordinated chain of cellular events that link membrane AP, intracellular $Ca^{2+}$ release, and contractile machinery [28, 29]. We previously reported that muscle fibers exposed to elevated glucose display increased AP-evoked $Ca^{2+}$ signals produced by single brief electric stimulation [25]. Yet, little is known about the consequences of acute elevated glucose on the temporal properties of AP-induced $Ca^{2+}$ transients and the underlying ionic mechanisms that lead to muscle dysfunction. Here, we used ultra-high-speed confocal $Ca^{2+}$ imaging to investigate the temporal properties of AP-induced $Ca^{2+}$ transients, an intermediate step of ECC, using a cellular model of acute hyperglycemia. Our results reveal that elevated glucose-exposed fibers predominantly display abnormal AP-induced $Ca^{2+}$ transients.

## 2. Methods

*2.1. FDB Skeletal Muscle Fibers Culture.* Studies were performed on skeletal muscle fibers enzymatically isolated from the *flexor digitorum brevis* (FDB) muscles of 4- to 5-week-old C57BL/6J mice as previously described [30–33]. Mice were euthanized by $CO_2$ exposure followed by cervical dislocation using protocols approved by the University of Maryland Institutional Animal Care and Use Committee. FDB skeletal muscle fibers were isolated, dissociated, and cultured in a humidified incubator at 37°C (5% $CO_2$) as previously described [30–33]. FDB muscles were dissected and maintained in minimum essential medium (MEM, Life Technologies, Carlsbad, CA, catalog number 11095080) and 2 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, catalog number C-0130) for 3 h. at 37 °C. Muscle fibers were plated on glass-bottomed culture dishes (Matek Inc., Ashland, MA, catalog number P35G-1.0-14-C) coated with laminin (Life Technologies, Carlsbad, CA, catalog number 23017015). After plating, cultures were incubated in MEM, containing 5.56 mM D-glucose, supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, catalog number 10100139) and 50 $\mu g \cdot ml^{-1}$ gentamicin (Life Technologies, Carlsbad, CA, catalog number 15710064). This medium was used as a control isotonic condition (288 mOsm/kg). Two hours after plating, cultures were treated with cytosine $\beta$-D-arabinofuranoside (ara-C; Sigma-Aldrich, St. Louis, MO, catalog number C-1768; 10 $\mu$M for 24 h.) to reduce proliferating cells and to minimize fiber dedifferentiation [30, 33]. For fibers challenged with elevated extracellular glucose media, either D- or L-glucose (25 mM; 48 h.) was added to the control isotonic medium. Over an isotonic reference of 288 mOsm/kg, the addition of 25 mM D-glucose increased the osmolality to 313 mOsm/kg. Osmolarity of the culture media was measured in a Vapro-5520 Osmometer (Wescor Inc., Logan, UT). Here, muscle fiber cultures were 5 days old when used for acute experiments.

*2.2. $Ca^{2+}$ Imaging.* Fluo-4 measurements were carried out on a high-speed confocal system (LSM 5 Live, Carl Zeiss, Jena, DE) as previously described [34, 35]. Muscle fibers were loaded with 1 $\mu$M fluo-4 AM (Life Technologies, Carlsbad, CA, catalog number F14201) in L-15 medium (Life Technologies, Carlsbad, CA, catalog number 21083027). The ionic composition of L-15 in mM is 137 NaCl, 5.7 KCl, 1.26 CaCl2, and 1.8 MgCl2, pH 7.4) supplemented with 0.25% *w*/*v* bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, catalog number A-7906) for 1 h. at room temperature. The dishes were rinsed once with L-15 medium for 5 min to remove residual fluo-4 AM. Individual muscle fibers were imaged with a 60×/1.3 NA water-immersion objective lens. Excitation for fluo-4 was provided by the 488 nm line of a 100 mW diode laser, and emitted light was collected at >505 nm. Action potential- (AP-) induced $Ca^{2+}$ transients were triggered using a brief electrical field stimulus. External field stimulation and inclusion and exclusion criteria of the characteristics of muscle fibers used in this study were performed as previously described [25, 36]. Supramaximal field stimulation (1 ms square pulse, 30 V/cm) was produced by a custom pulse generator and applied via two platinum wires positioned perpendicular to the bottom of the dish, ~5 mm apart, to elicit action potentials. Muscle fibers were centrally positioned relative to the electrodes and to the field of view, at less than about a ±45° angle relative to an imaginary line between the tips of the electrodes, and only fibers exhibiting all or no activation and reproducible responses to field stimulation of alternating polarity were used for the analysis. A variable range of the cultured muscle fibers (3–7%) from the control group or from other groups challenged with elevated glucose did not respond to electrical stimulation of both polarities and were excluded from the analysis. Electrical field stimulation was synchronized relative to the beginning of acquisition. The field stimulus was applied 100 ms after the beginning of the scan sequence, providing control images before stimulation. Confocal line scanning was performed at the ends of the fibers and perpendicular to the long axis of the fibers. These line-scan confocal images were used to calculate the resting steady-state fluorescence level ($F_0$). The average intensity of fluorescence within selected regions of interest (ROIs; dashed rectangles shown in line-scan images in Figures 1 and 2) within a myofiber was measured with Zeiss LSM Image Examiner (Carl Zeiss, Jena, Germany). The ROIs were located in areas spanning the edge and center

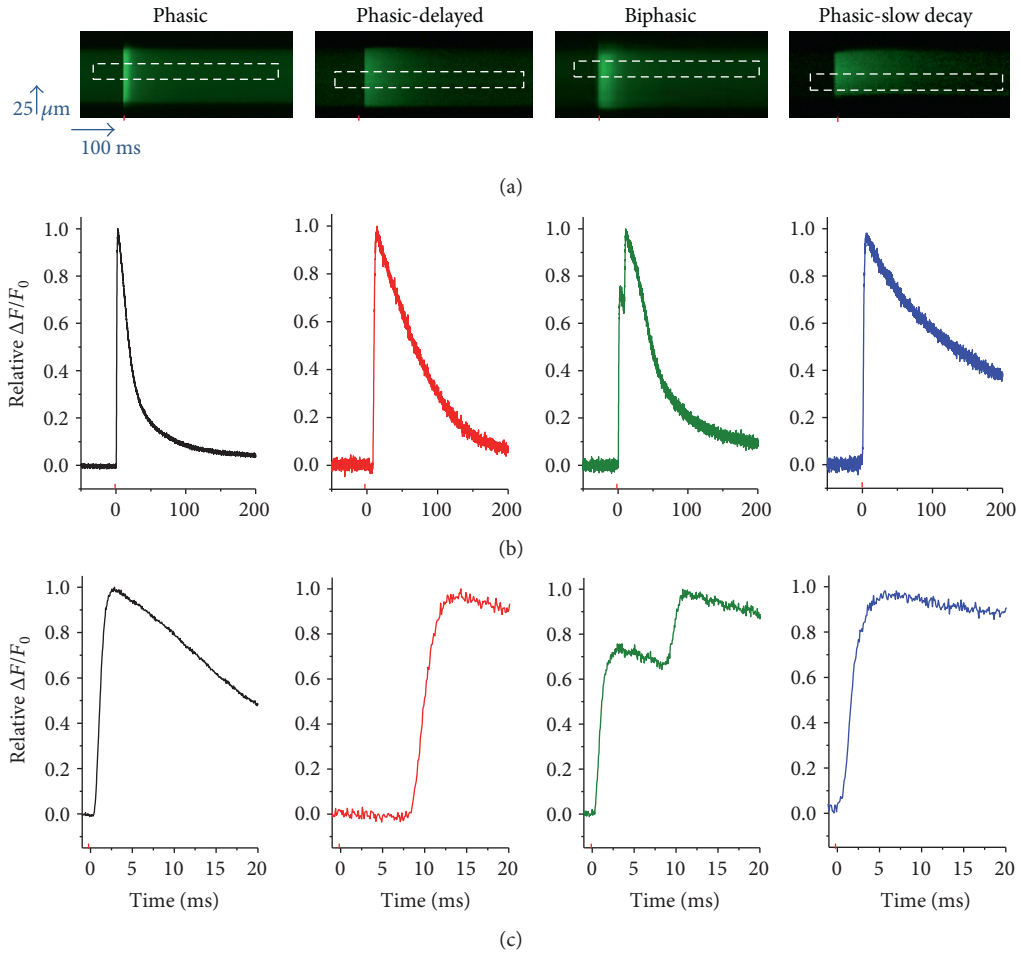


FIGURE 1: Muscle fibers cultured for 5 days exhibit multiple patterns of action potential (AP)-induced $Ca^{2+}$ transients: control conditions. (a) Representative confocal line-scan images of AP-induced $Ca^{2+}$ transients in 5-day-old cultured muscle fibers maintained in control medium. Note that four different patterns were identified: phasic, phasic-delayed, biphasic, and phasic-slow-decay. The red mark indicates the time when field electrical stimulus was applied, and the dashed rectangle illustrates the region of interest used to measure the time course of the $Ca^{2+}$ transient. (b) Time course of the AP-induced $Ca^{2+}$ transients shown in (a). (c) Zoomed-in versions of AP-induced $Ca^{2+}$ transients shown in (b).

of the muscle fiber to monitor $Ca^{2+}$ signals derived from subsarcolemmal and core regions of the fiber, or in regions covering the edges of the fiber to monitor the responses across the fiber width. Images in line-scan ($x$-$t$) mode (frame size: $512 \times 10{,}000$ pixels; scan speed: 100 $\mu$s/line for 1 s acquisition) were background corrected by subtracting an average value recorded outside the cell. The average $F_0$ value in each ROI before electrical stimulation was used to scale $Ca^{2+}$ signals in the same ROI as $\Delta F/F_0$.

It is important to note that the temporal resolution of the $Ca^{2+}$ transient is exclusively determined not only by the sampling rate but also by the kinetic properties of the dyes. Here, we used fluo-4, a high-affinity dye, instead of low-affinity dyes merely because in our imaging system this dye provides brighter responses than the low-affinity indicators. Based on our calibration data, fluo-4 was at most 40% saturated with $Ca^{2+}$. The length of the fibers used was 400–600 $\mu$m and the width was 25–80 $\mu$m. No attempts were made to distinguish muscle fiber types or to estimate the actual cytosolic $Ca^{2+}$ concentration. $Ca^{2+}$ imaging experiments were carried out at room temperature, 21–23°C.

*2.3. Toxins and Channel Blockers.* To assess the contribution of different ion channels to the development of the biphasic action potential $Ca^{2+}$ transient, 5-day-old cultured fibers were exposed to either gadolinium (Axxora, San Diego, CA, catalog number 400-023-M500), apamin (Sigma-Aldrich, St. Louis, MO, catalog number A-1289), or Jingzhaotoxin-III (JZTX-III; Alomone Labs, Jerusalem, IL, catalog number STJ-200), blockers of mechanosensitivity, SK channels, and $Na_v1.5$ channels, respectively. Ion channel blockade treatment was carried out using semilocal perfusion. The working concentration of the blockers used here was based on maximal blocking effects described in previous reports [37–39]. Fibers with biphasic action potential-induced $Ca^{2+}$ transient were first identified, then the time course of the $Ca^{2+}$ profile was assessed before and 10 minutes after blocker application.

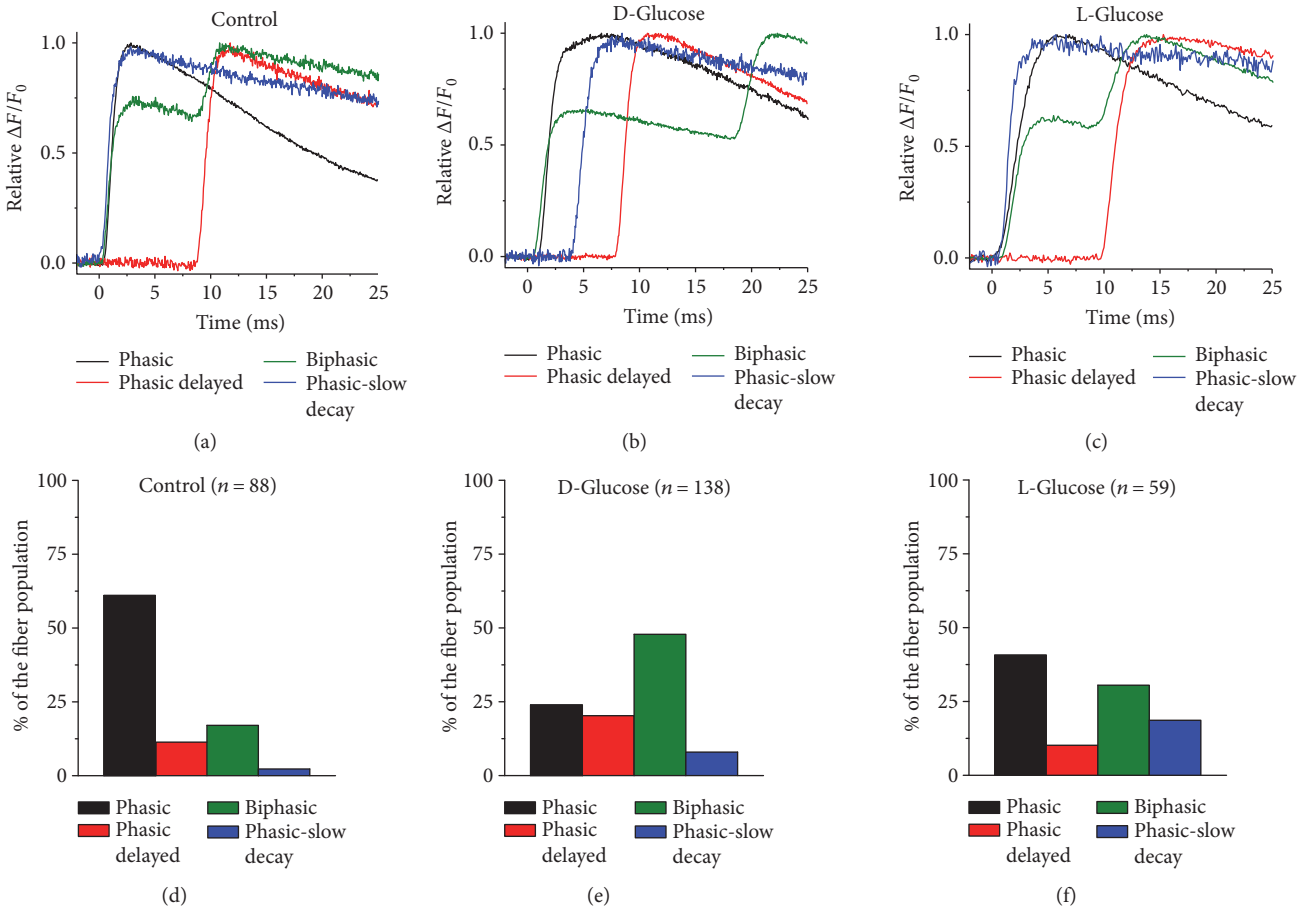


Figure 2: Sustained elevation of extracellular D-glucose modifies the distribution of AP-induced $Ca^{2+}$ transients observed in 5-day-old cultured fibers. Zoomed-in and overlapped version of AP-induced $Ca^{2+}$ transients for control (a), D-glucose (b), and L-glucose (c) challenged fibers. (d–f) Summary of distribution of AP-induced $Ca^{2+}$ transients for fibers exposed to control isotonic medium (d), D-glucose (e), and L-glucose (f). Fibers exposed to D-glucose displayed a significantly larger proportion of biphasic action potential-induced $Ca^{2+}$ transients when compared to control counterparts ($X^2$, $n = 286$, $p$ value $< 0.05$).

*2.4. Data Analysis.* Images of arbitrarily selected muscle fibers were collected and evaluated blindly, using the same settings and enhancing parameters so that all images could be directly compared. Line-scan images were analyzed using LSM examiner (Carl Zeiss, Jena, DE). AP-induced $Ca^{2+}$ signals and statistical analysis were conducted using Origin Pro 8 (OriginLab Corporation, Northampton, MA, USA) and SPSS for Windows ver. 24.0 (SPSS Inc., Chicago, IL, USA). Summary data were reported as mean ± SD. Normal distribution of data was assessed using the Kolmogorov-Smirnov test. Unpaired two-sample Student's $t$-test was used to test for differences between the means of the interspike interval of AP-induced $Ca^{2+}$ transients from two different samples. For experiments that involved the measurements of AP-induced $Ca^{2+}$ transients before or after the addition of ion channel blockers, the interspike interval was compared before and after the addition of blocker in the same muscle fiber. The statistical significance of interspike interval was analyzed using a paired Student's $t$-test on raw data. Cross-classifications and crosstabs were used to examine the relationship between two categorical variables. To test for significant differences, we compared the proportion in each variable, with condition (control and elevated glucose) as the independent variable and AP-induced $Ca^{2+}$ transient pattern (phasic, biphasic, delayed, and slow decay) as the dependent variable, using Pearson chi-square ($X^2$). Differences were considered significant when $p$ value $< 0.05$.

## 3. Results

*3.1. Action Potential-Induced $Ca^{2+}$ Transients in 5-Day-Old Cultured Control Fibers.* Freshly dissociated or 1- to 3-day-old cultured FDB muscle fibers retain many properties of in situ fibers, including muscle contractions and $Ca^{2+}$ transients that correlate with the number and frequency of field stimulation used. In response to a single 1 ms field stimulus, they respond with one single action potential, a single $Ca^{2+}$ transient, and a single twitch) [30, 33], even when challenged with acute (1 h.) elevated glucose (see Supplementary Figure 1 available online at https://doi.org/10.1155/2017/1509048). In a previous study, we analyzed $Ca^{2+}$ handling and action potential- (AP-) evoked $Ca^{2+}$ transients in 5-day-old cultured control and high-glucose-exposed FDB fibers using a ratiometric $Ca^{2+}$ dye and low temporal resolution [25]. To gain

better time resolution of the $Ca^{2+}$ transients, we next monitored fluo-4 transients during stimulation of FDB fibers using an ultra-high-speed (100 $\mu$s/line) confocal microscope in line-scan mode. Figure 1 illustrates representative *x-t* confocal line-scan images (Figure 1(a)) and corresponding fluo-4 $Ca^{2+}$ transients (Figure 1(b)) of four different muscle fibers in control conditions and in response to a supramaximal single field stimulus. Confocal line scanning was performed at one end of the fiber and perpendicular to the long axis of the fiber. Successive vertical lines in each line-scan image reveal the time course of the fluorescence signal before and during repetitive stimulation at 100 $\mu$s resolution. At first glance, the line-scan images appear to display a single transient in the different fibers shown in Figure 1(a). However, fluo-4 $Ca^{2+}$ profiles (Figure 1(b)) show the time course of the $Ca^{2+}$ signals in more detail. In order to evaluate and compare the temporal profile of the $Ca^{2+}$ signals elicited by action potentials, $Ca^{2+}$ transients were normalized relative to peak maximum fluorescence. Using this approach, we identified four distinct and predominant AP-evoked $Ca^{2+}$ transient profiles in 5-day-old cultured control fibers: phasic, phasic-delayed, biphasic, and phasic-slow decay. To further appreciate the temporal properties of these different $Ca^{2+}$ signals elicited by field stimulation, the fluo-4 $Ca^{2+}$ transients are shown in a time-expanded version in Figure 1(c). These distinct patterns of $Ca^{2+}$ signals and their distributions in percentages were phasic (67%), biphasic (18%), phasic-delayed (12%), and phasic-slow decay (3%) (see Figure 2(d)). The rising phase of the $Ca^{2+}$ transient following single stimulation occurred within ~1-2 ms of the applied field stimulus in muscle fibers with phasic responses (Figure 1). In contrast, muscle fibers with a rising phase starting >3 ms after the start of the stimulation were classified as delayed; the duration of this delay was variable (3–15 ms). Another group of fibers exhibited two summated $Ca^{2+}$ transients in response to a single field stimulus, with the first response within 1-2 ms and the second response delayed as in the phasic-delayed fibers, and were classified as biphasic. Finally, another group of fibers exhibited a slow half-time of decay of >100 ms (not shown on the time scale of Figure 1), and were classified as phasic slow decay. These results indicate that 5-day-old cultured FDB fibers exhibit a heterogeneous fiber population that responds to single field stimulation different to freshly dissociated or 1-day-old cultured fibers [31, 35].

*3.2. Action Potential-Induced $Ca^{2+}$ Transients in 5-Day-Old Cultured Muscle Fibers Challenged with Elevated D-Glucose or L-Glucose.* In another series of experiments, muscle fibers were challenged with elevated glucose (25 mM). We assessed the properties of AP-induced $Ca^{2+}$ transients in fibers challenged with either D-glucose or L-glucose (Figure 2) using the same approach applied to 5-day-old cultured control fibers. Fibers exposed to elevated glucose displayed the same patterns of AP-induced $Ca^{2+}$observed in control fibers; however, the distribution of the patterns was different (Figure 2). In D-glucose exposed fibers (Figures 2(b) and 2(e)) the distribution was: biphasic (48%), phasic (24%), phasic-delayed (21%), and phasic-slow decay (7%). In L-glucose exposed fibers (Figures 2(c) and 2(f)) the distribution was: phasic (41%), biphasic (30%), phasic-slow decay (19%), and phasic-delayed (10%), whereas in control the distribution was phasic (67%), biphasic (18%), phasic-delayed (12%), and phasic-slow decay (3%) (Figures 2(a) and 2(d)). We tested whether elevated glucose-exposed fibers exhibit different distribution of patterns of AP-induced $Ca^{2+}$ transients when compared to control counterparts. The two-sided asymptotic significance of the chi-square statistic was less than 0.05; $X^2$ (6, $n = 286$) 43.08 $p = 1.12E - 7$, implying that elevated glucose-exposed fibers exhibit a different distribution of patterns of AP induced-$Ca^{2+}$ transients when compared to control counterparts. Note that the biphasic pattern was more commonly observed in D-glucose challenged fibers, while in L-glucose exposed fibers the phasic pattern was predominant, as seen in control fibers (Figure 2). Also, the interspike interval in biphasic D-glucose challenged fibers was significantly longer ($17.9 \pm 4.2$ ms in D-glucose versus $8.3 \pm 3.4$ ms in control or $9.1 \pm 3.8$ ms in L-glucose, in $n = 12$ fibers, 3 mice per group; $p = 0.09$, two-sample unpaired Student's *t*-test). The effects of D-glucose on the distribution of the AP-evoked $Ca^{2+}$ transients were distinct to those observed in muscle fibers exposed with the same concentration of metabolically inactive L-glucose (Figure 2), suggesting a metabolic rather than an osmoadaptive effect in D-glucose challenged fibers. These observations suggest that elevated D-glucose facilitates excitable mechanism(s) that lead to the development of biphasic action potential-induced $Ca^{2+}$ transients in 5-day-old cultured FDB fibers. Note that while control fibers and those challenged with elevated glucose displayed phasic-slow decay AP-induced $Ca^{2+}$ transients, these fibers represented a variable but small fraction of the overall muscle fiber population and were not studied in detail in the present work.

*3.3. Effect of Electrical Field Stimulation of Alternating Polarity in Fibers with Phasic or Delayed AP-Induced $Ca^{2+}$ Transients.* To examine the propagation time for AP-induced $Ca^{2+}$ signals, fibers were subjected to suprathreshold field stimulation of alternate polarity. The $Ca^{2+}$ transients in fibers with phasic responses occur with a similar short latency (<2 ms) in response to external stimulation of either polarity (Figure 3(a)), indicating AP-induced $Ca^{2+}$ transients. The time course of the $Ca^{2+}$ transients evaluated at two subsarcolemmal regions on opposite sides of the fiber (ROIs 1 and 2 in Figure 3(a)) reveals that suprathreshold pulses elicited synchronous AP-induced $Ca^{2+}$ transients across the fiber width.

In fibers with phasic-delayed $Ca^{2+}$ transients, the response can occur after a delay (3–15 ms) and could, in principle, result from alterations in AP propagation which translate into AP-induced $Ca^{2+}$ propagation deficits (i.e., slow propagation). We wanted to evaluate whether the delay was caused by the absence of a direct response to stimulation at the site of imaging combined with a slow propagation of an AP from the fiber end away from the recording site and towards the recording site. If this were the case, then inverting the polarity of the stimulus applied to a phasic delayed fiber would trigger an AP-induced $Ca^{2+}$ transient with a considerably shorter delay at the site of recording since propagation would no longer be required. Figure 3(b) shows AP-induced $Ca^{2+}$ transients in a fiber with delayed responses

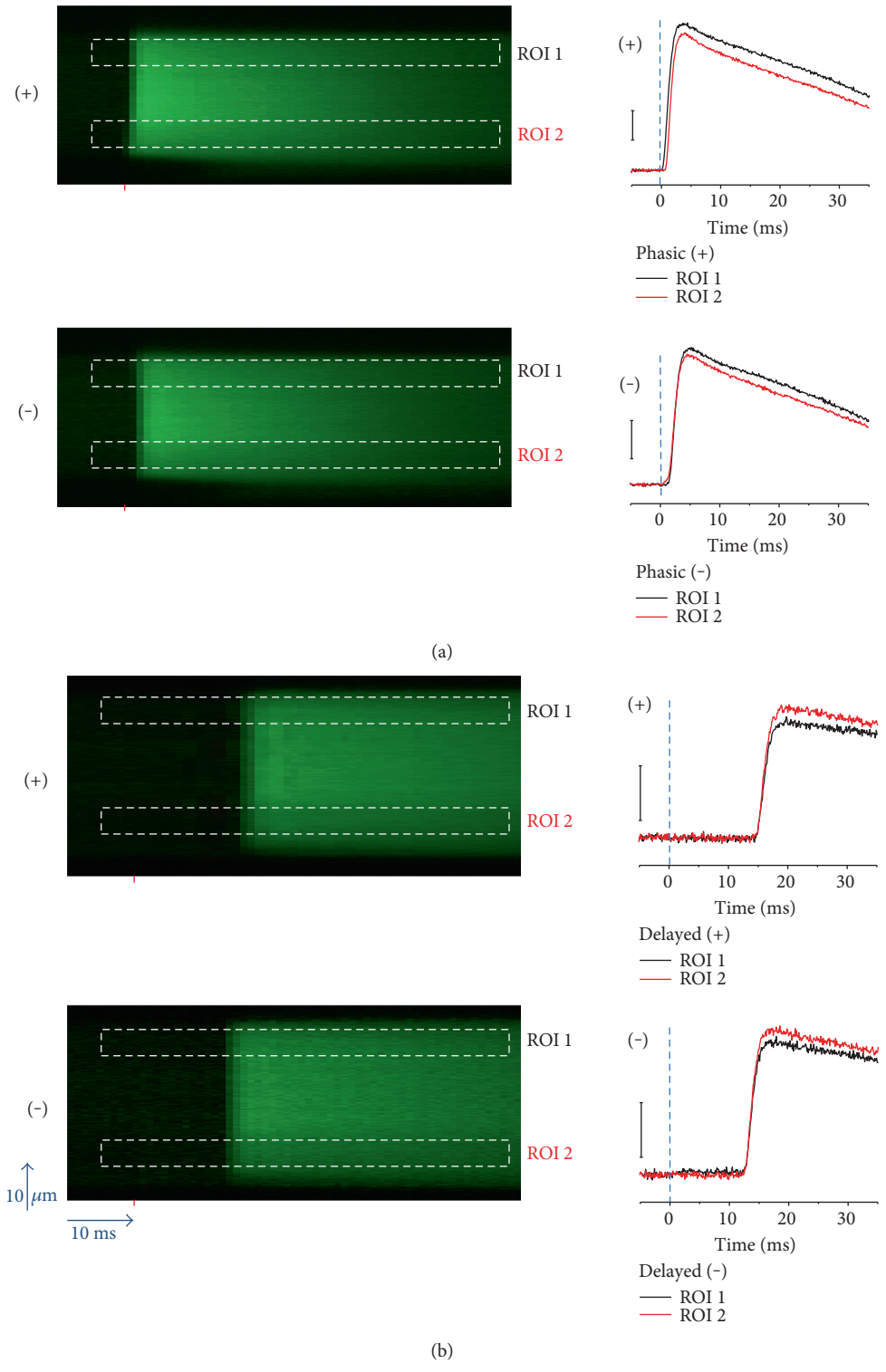


Figure 3: Effect of electrical field stimulation of alternating polarity in fibers with phasic and delayed AP-induced $Ca^{2+}$ transients. (a) *Left*, line-scan images of *phasic* AP-induced $Ca^{2+}$ transients elicited by field stimulation of alternate polarity and measured in two different regions of interest (ROIs); *right*, time course of the phasic AP-induced $Ca^{2+}$ transients measured at two ROIs (ROI 1 and ROI 2) and elicited by the stimulus of positive (*upper traces*) or negative polarity (*lower traces*) at the fiber end where the recordings were made. (b) *Left*, line-scan images of *phasic-delayed* AP-induced $Ca^{2+}$ transients elicited by field stimulation of alternate polarity and monitored in two ROIs; *right*, time course of the delayed AP-induced $Ca^{2+}$ transients measured at two ROIs and elicited by the stimulus of positive (*upper traces*) or negative polarity (*lower traces*) at the fiber end where the recordings were made. The red mark in line-scan images indicates the time when the field stimulus was applied; the dashed rectangles illustrates the location of the ROIs.

using pulses of opposite polarity. As in the phasic fiber, suprathreshold pulses elicited synchronous AP-induced $Ca^{2+}$ transients across the fiber width. The comparison of the time course of AP-induced $Ca^{2+}$ transients measured at the same fiber end but with field stimuli of opposite polarity shows similar delayed time courses and only a modest shift (<3 ms) in their latency upon polarity change. This finding suggests that the $Ca^{2+}$ transients are not delayed because of the time for propagation of the action potential or another signal from one end to the other end of the fiber. In that case, the signal recorded at the end of the fiber where the action potential is initiated would not exhibit the delay. However, this was *not* observed. Since the delay was similar for both polarities of stimulation (i.e., with the AP initiated either at the recording site or at the other end of the fiber), we conclude that the delay could be due to either a delay in the activation of the action potential (prior to its propagation along the fiber) or a delay in the activation of the $Ca^{2+}$ transient after the action potential propagation along the fiber when the action potential is activated at the opposite end of the fiber where the recording is taking place. These possibilities are considered further in the discussion.

*3.4. Blockers of Mechanosensitive Ion Channels, $Ca^{2+}$-Dependent $K^+$ Channels, and $Na_v1.5$ Channels Do Not Affect Biphasic Action Potential-Induced $Ca^{2+}$ Transients Elicited by a Single Field Stimulus.* Next, we investigated whether modifications of the excitable properties of the muscle fiber could account for the occurrence of the biphasic phenotype. We used blockers of ion channels known to modulate the membrane potential and AP properties of the skeletal muscle.

The mechanosensitive ion channels (MsC) in the skeletal muscle are activated by membrane stretch and strong membrane depolarization and are permeable to $Na^+$ and divalent cations [40]. Increased activity of MsC could cause elevated resting $Ca^{2+}$ levels and/or membrane depolarization [37]. We hypothesized that MsC-induced depolarization would eventually trigger an ectopic AP-induced $Ca^{2+}$ transient. To test whether the occurrence of the biphasic action potential-induced $Ca^{2+}$ transient depended on MsC, the time course of fluo-4 $Ca^{2+}$ elicited by field stimulation and a priori identified as a biphasic signal was measured in control external solution, followed by the addition of gadolinium ($Gd^{3+}$, 100 $\mu$M; Figure 4). Ten minutes after the application of $Gd^{3+}$, the $Ca^{2+}$ signal was reassessed. The addition of $Gd^{3+}$ did not affect the time course of the biphasic response. $Gd^{3+}$ did not alter the interspike interval significantly ($n = 8$ fibers; $p > 0.05$, two-sample paired Student's $t$-test); although the amplitude of the $Ca^{2+}$ signal was reduced after the $Gd^{3+}$ addition, this effect was not further evaluated. After washout of $Gd^{3+}$, the amplitude of the $Ca^{2+}$ transient remained reduced (data not shown). The above results suggest that MsC do not contribute to the occurrence of the biphasic action potential-induced $Ca^{2+}$ transients in 5-day-old cultured muscle fibers.

The calcium-sensitive potassium channel with small potassium conductance, $K_{Ca}2.3$, is normally expressed at low level; however, its expression is markedly increased in denervated and myotonic dystrophy muscle [41]. $K_{Ca}2.3$ channel activity in the T-tubules of denervated skeletal muscle causes a local increase in potassium ion concentration that leads to hyperexcitability [38]. Because of their involvement in hyperexcitability, we next considered the possibility that $K_{Ca}2.3$ could be involved in the development of the biphasic action potential-induced $Ca^{2+}$ transient. To test whether the occurrence of the biphasic $Ca^{2+}$ transient involved $K_{Ca}2.3$ channels, the time course of fluo-4 $Ca^{2+}$ elicited by field stimulation was measured in a control external solution, followed by the addition of apamin (1 $\mu$M) (Figure 4). $K_{Ca}2.3$ channels can be blocked by apamin [42]. Ten minutes after the application of apamin, the $Ca^{2+}$ signal was measured again. As in the case of MsC, the addition of apamin did not alter the interspike interval significantly ($n = 8$ fibers; $p > 0.05$, two-sample paired Student's $t$-test), although a reduction in the amplitude of the $Ca^{2+}$ signal was also observed (Figure 4). These results suggest that $K_{Ca}2.3$ channels do not contribute to the occurrence of the biphasic action potential-induced $Ca^{2+}$ transients.

The expression of $Na_v1.5$ channels is low in 1- to 2-day-old cultured muscle fibers. However, $Na_v1.5$ expression increases in fibers cultured for over 3 days [43–45]. This $Na_v1.5$ increased expression could explain the occurrence of abnormal excitability and $Ca^{2+}$ signals. To test whether increased $Na_v1.5$ function is involved in the altered action potential-induced $Ca^{2+}$ signals seen in 5-day-old cultured fibers, we exposed the fibers to JZTX-III, a $Na_v1.5$ channel blocker [39, 46]. The addition of JZTX-III (1 $\mu$M; 10 min) to the external solution did not affect the time course of the biphasic response (Figure 4). JZTX-III caused a nonsignificant reduction in the interspike interval ($17.3 \pm 3.8$ ms in D-glucose versus $15.8 \pm 3.3$ ms in D-glucose treated with JZTX-III, $n = 6$ fibers, 2 mice per group; $p = 0.509$, two-sample paired Student's $t$-test). Contrary to $Gd^{3+}$ or apamin, the addition of JZTX-III to the recording solution did not reduce the amplitude of the AP-induced $Ca^{2+}$ signals (Figure 4). These results suggest that $Na_v1.5$ channels do not contribute to the occurrence of the biphasic action potential-induced $Ca^{2+}$ transients.

## 4. Discussion

Numerous studies have investigated how changes in skeletal muscle excitability, $Ca^{2+}$ signaling, and contractility occur in acute and long-term hyperglycemia [12, 13, 20, 21, 47, 48]; however, few studies have examined the impact of diabetes mellitus on the excitability [22], contractility [49], and $Ca^{2+}$ signaling [24] of the skeletal muscle at the cellular level. In particular, little is known about the temporal properties of AP-evoked $Ca^{2+}$ signals during acute hyperglycemia. Using an in cellulo model and high-speed confocal $Ca^{2+}$ imaging, we assessed the impact of acute elevated extracellular glucose (48 h.) on the temporal properties of AP-evoked $Ca^{2+}$ signals. The present study shows that muscle fibers cultured in control medium (5 mM D-glucose) for 5 days display 4 distinct temporal waveforms of AP-induced $Ca^{2+}$ transients: phasic, biphasic, phasic delayed, and phasic-slow decay, in order of predominance. Our study also shows that fibers challenged with elevated extracellular D-glucose (25 mM for 48 h; a condition

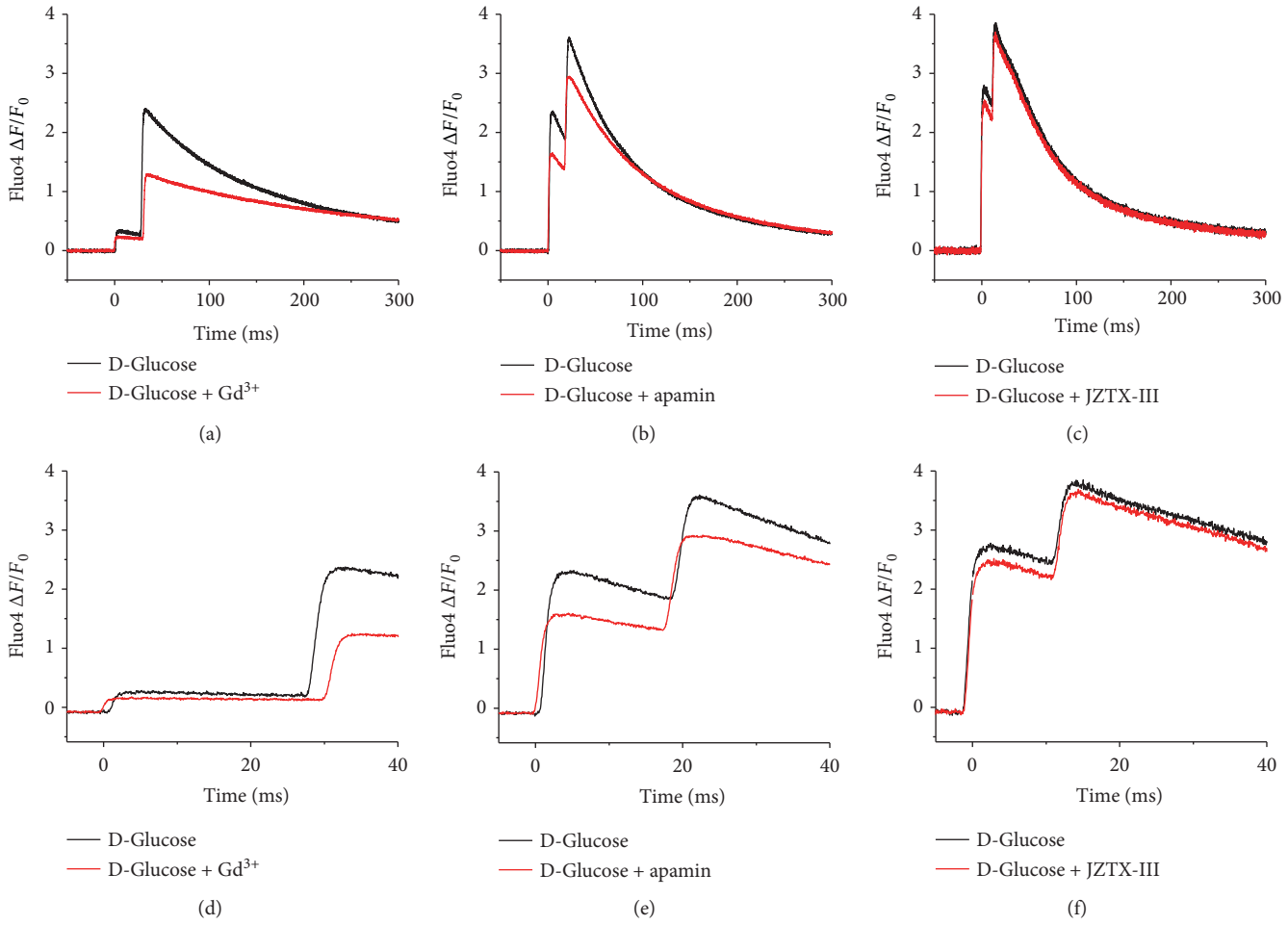


FIGURE 4: Inhibition of ion channels known to modulate the excitable properties of skeletal muscle: impact on biphasic AP-transients on 5-day old cultured fibers challenged with elevated glucose. Representative time course of a biphasic AP-induced $Ca^{2+}$ transient (left panels) measured in fibers challenged with elevated D-glucose (25 mM; 48 h.) before (black traces) and 10 minutes after (red traces) the treatment with gadolinium (100 $\mu$M) (a), apamin (1 $\mu$M) (b), and JZTX-III (1 $\mu$M) (c). Panels (d–f) are zoomed-in versions of the records shown in (a–c) to better appreciate biphasic responses before and after channel blockers addition. No significant changes in time course of the $Ca^{2+}$ transient (i.e., interspike interval) were found in fibers challenged with 25 mM D-glucose-exposed fibers and treated with gadolinium ($n = 8$ fibers; $p > 0.05$, two-sample paired Student's $t$-test), apamin ($n = 8$ fibers; $p > 0.05$, two-sample paired Student's $t$-test), or JZTX-III ($n = 6$ fibers; $p > 0.05$, two-sample paired Student's $t$-test), when compared to fibers challenged with D-glucose.

that could mimic severe uncontrolled hyperglycemia) also exhibit these 4 distinct patterns. However, under these conditions, the biphasic pattern is the predominant waveform, suggesting that elevated glucose promotes the biphasic responses. To our knowledge, this study is the first report of these abnormal AP-induced $Ca^{2+}$ signals in relation to elevated glucose in fibers of normal morphology.

How was a short (1 ms) field stimulus capable of generating such delayed (>3 ms) or biphasic AP-induced $Ca^{2+}$ transients? We have previously shown that the electrode array used in our study allows for the application of field pulses resulting in the depolarization of the end of the fiber close to the cathode, and hyperpolarization of the opposite end of the fiber (near the anode) [36]. In *phasic* fibers, pulses of alternating polarity elicited propagated AP-induced $Ca^{2+}$ transients at the end of the fiber facing the cathode, near the recording site, and its longitudinal propagation along the fiber [36] (see Figure 3). The time to $Ca^{2+}$ transient peak following single AP stimulation occurs in ~1-2 ms in phasic muscle fibers. The delayed responses (time to $Ca^{2+}$ transient peak > 3 ms) and the second phase of biphasic responses were variable from fiber to fiber. We do not currently know the nature of this variation. We found that fibers with delayed AP-induced $Ca^{2+}$ transient pulses of opposite polarity resulted in subtle latency changes of the $Ca^{2+}$ transient (see Figure 3). This implies that the delay is *not* due to slow AP propagation through the T-tubule system [50, 51], which would cause a major delay for $Ca^{2+}$ transients initiated at the opposite end from which the recordings are made, but not in responses initiated at the same end where recording occurs.

In the case of *phasic-delayed* fibers, we hypothesize that increased transient outward currents, like $K_v1.4$ and $K_v3.4$ type-A $K^+$ channels, channels expressed in skeletal muscle that oppose membrane depolarization [52, 53], will activate at the cathode near the recording site and will

cause membrane potential to reach AP threshold with a delay longer than the stimulus. This delay in AP initiation will cause the observed delay of the AP-induced $Ca^{2+}$ transients in the depolarized end of the fiber near the recording site. The fiber end undergoing hyperpolarization will only be depolarized after the delayed AP is initiated at the other end, followed by rapid propagation of the AP along the fiber. Thus, the (relatively long) delay will be similar at both ends of the fiber for a given polarity stimulation or at the same end of the fiber with alternating polarity stimulation (Figure 2(b)), as observed. Alternatively, AP initiation could have no delay at both ends of the fiber (and/or for both polarities of stimulation), but the $Ca^{2+}$ release response could be delayed due to some as yet undetermined mechanism. In fibers with *biphasic* responses, the negative electrode induces an AP, which triggers the first AP-induced $Ca^{2+}$ transient that propagates towards the positive electrode. The other end, subjected to hyperpolarization, could display voltage sags that counteract hyperpolarization and contribute to rebound membrane potential, triggering the second AP that will propagate toward the other end near to the recording site. Increased inward currents activated by hyperpolarization, such as Kir2.1 channels [54], could explain the membrane potential rebound. Thus, both fiber ends exhibit both a phasic and a delayed response. The $Ca^{2+}$ transients seeing in *phasic-slow decay* fibers could arise from differences in $Ca^{2+}$ binding and transport [55]. These possibilities, and others, require further experimental investigation.

Our results show that the inhibition of MsC with $Gd^{3+}$ did not affect the time course of biphasic AP-induced $Ca^{2+}$ transients during acute hyperglycemia. Note that $Gd^{3+}$ is a nonspecific channel blocker; in addition to inhibiting MsC, it also blocks other ion channels such as voltage-gated $K^+$, $Na^+$, and L-type $Ca^{2+}$ channels [56]. Fiber treatment with apamin, a $K_{Ca}$ channel blocker [42], did not reverse the effects of elevated glucose on biphasic AP-induced $Ca^{2+}$ transients. Similarly, JZTX-III, a $Na_v1.5$ channel blocker [39, 46], did not alter the occurrence of the biphasic responses in fibers challenged with elevated glucose. These findings suggest that neither MsCs, $K_{Ca}$, nor $Na_v1.5$ plays a role in the origin of the biphasic AP-induced $Ca^{2+}$ transients. It is yet to be determined whether other ion channels play a role in the abnormal AP-induced $Ca^{2+}$ transients in long-term cultures and/or exposure to elevated glucose.

$Ca^{2+}$ signals are essential in numerous aspects of muscle function [57, 58]. A previous study reported the occurrence of local $Ca^{2+}$ signals by acute (≤1 h.) hyperosmotic stress in the cell periphery of cultured muscle fibers [59]. Whether local $Ca^{2+}$ signals are present in muscle fibers challenged with hyperosmotic stress induced by elevated glucose for more prolonged periods (>24 h.) remains to be determined.

Do these defects on AP-induced $Ca^{2+}$ signals seen in long-term cultured fibers and experimental hyperglycemia occur in patients with diabetes? Most adults with diabetes have at least one coexisting condition, either acute or chronic [60]. Muscle weakness and fatigue are common complaints of diabetic patients during periods of acute [13] and long-term hyperglycemia [61] and are also common in muscle disuse atrophy [62, 63]. Because $Ca^{2+}$ signals and excitable properties are critical for skeletal muscle function [52, 57], we hypothesize that if the changes in excitability and abnormal $Ca^{2+}$ signals observed in five-day-old cultured fibers occur in vivo, these could contribute to the development of muscle weakness, fatigue, and diabetic myopathy. Further work exploring the underlying mechanisms and relationship between diabetes and skeletal muscle disuse (denervation/physical inactivity, etc.) would be of pathophysiological interest.

In this study, we used an in cellulo model of hyperglycemia using 25 mM glucose for 1-2 days. This paradigm is an extreme model of hyperglycemia, and it is restricted to a short spectrum of metabolic abnormalities and hormonal changes seen in diabetes (i.e., severe uncontrolled diabetes). The abnormalities in AP-induced $Ca^{2+}$ signals that we observed may be influenced or caused by fiber disuse and/or denervation which may occur in long-term cultured muscle fibers [25, 64] and in some extent by in vitro dedifferentiation [33]. Both muscle disuse/denervation and dedifferentiation are characterized by abnormal excitability [33, 45]. Because ara-C treatments minimize the dedifferentiation process [25, 33] (see also Supplementary Figure 2), we believe that dedifferentiation could play a minor role in our observations. Nevertheless, the cultured muscle fibers used here represent a cellular model of muscle disuse/denervation [25, 65] and is a valuable alternative to animal studies to explore severe and acute effects of hyperglycemia on the function of skeletal muscle fibers.

## Conflicts of Interest

The authors Erick O. Hernández-Ochoa, Quinton Banks, and Martin F. Schneider declare that they have no competing interests.

## Authors' Contributions

Erick O. Hernández-Ochoa designed and performed the research and data analysis. Quinton Banks performed the research. Erick O. Hernández-Ochoa, Quinton Banks, and Martin F. Schneider edited and approved the manuscript. Erick O. Hernández-Ochoa wrote the paper.

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*Review Article*

# Muscle Lipid Metabolism: Role of Lipid Droplets and Perilipins

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Skeletal muscle is one of the main regulators of carbohydrate and lipid metabolism in our organism, and therefore, it is highly susceptible to changes in glucose and fatty acid (FA) availability. Skeletal muscle is an extremely complex tissue: its metabolic capacity depends on the type of fibers it is made up of and the level of stimulation it undergoes, such as acute or chronic contraction. Obesity is often associated with increased FA levels, which leads to the accumulation of toxic lipid intermediates, oxidative stress, and autophagy in skeletal fibers. This lipotoxicity is one of the most common causes of insulin resistance (IR). In this scenario, the "isolation" of certain lipids in specific cell compartments, through the action of the specific lipid droplet, perilipin (PLIN) family of proteins, is conceived as a lifeguard compensatory strategy. In this review, we summarize the cellular mechanism underlying lipid mobilization and metabolism inside skeletal muscle, focusing on the function of lipid droplets, the PLIN family of proteins, and how these entities are modified in exercise, obesity, and IR conditions.

## 1. Introduction

Obesity and type 2 diabetes mellitus (T2DM) have become hallmark pandemic events of our century, currently affecting 600 million [1] and 380 million adult people [2], respectively. These pathologies have a longstanding stablished relation, as obese patients are often diabetic as well [3, 4]. Increased free fatty acid (FFA) plasma levels are present in both pathologies [5, 6], and therefore, they have been conceived as a major link between obesity and T2DM.

T2DM is a disorder of variable etiology characterized by sustained hyperglycemia, with alterations of carbohydrate, fat, and protein metabolism [7]. In the case of T2DM, this overt hyperglycemia results from the reduced action of insulin on its target tissues, such as skeletal muscle, liver, and adipose tissue, at least on initial stages [4, 8, 9]. In this regard, the proper function of skeletal muscle is of paramount importance, given that it is involved in the clearance of 25% of plasmatic glucose in a basal, fasting state [8] and up to nearly 70–85% of plasmatic glucose in postprandial state [10, 11]. This substantial increment in skeletal muscle glucose uptake is the result of increased presence of the facilitative glucose transporter 4 (GLUT4) in the sarcolemma and T-tubule in response to insulin action. This transporter is basally located in intracellular vesicles and moves to and fuses with the plasma membrane as a result of insulin-mediated signaling [12, 13]. The particularities of glucose transport, kinetics, and mechanisms are beyond the scope of this review and can be read elsewhere [14, 15].

As mentioned before, alteration of FA metabolism is also an important feature of T2DM patients, as their plasmatic levels are often increased [5, 16]. However, different lines of evidence have indicated that accumulation of different lipidic entities inside muscle cells leads to insulin resistance. Increased ceramide [17], intramyocellular lipids (IMCLs) [18], diacylglycerol (DAG) [19, 20], and long-chain fatty acyl-CoA [21] levels have been negatively correlated with insulin action, depicting the importance of understanding the link between obesity and the lack of insulin response in skeletal muscle. The mechanisms involved in intracellular lipid accumulation and how these phenomena are involved in IR is relevant to understand the extent of obesity-

induced damage in skeletal muscle. In this review, we begin with a comprehensive view of lipid metabolism in healthy skeletal muscle, covering uptake, metabolization, and storage. We then focus on the function of lipid droplets (LDs), an organelle responsible for both intracellular storage and trafficking of FAs between different cellular compartments, and provide information on how LDs contribute to insulin resistance in the obese state, with special interest on specific LD proteins, the PLIN protein family.

## 2. Overview of Lipid Metabolism in Healthy Skeletal Muscle

Skeletal muscle is responsible for the body's energy expenditure, participating in thermogenic functions, glucose and lipid uptake, and other metabolic processes. The fuel supply is obtained from metabolic machinery involving enzymatic pathways in charge of obtaining energy from glucose and FAs, through glycolysis and $\beta$-oxidation, respectively. These processes are dependent on substrate availability [22, 23].

Lipolysis is the process in which triacylglycerides (TAGs) are broken down to produce FFAs. Increased FA turnover is triggered by various stimuli, including $\beta$-adrenergic agonists and exercise [24, 25]. FA uptake in the muscle is dependent on metabolic demands and lipid availability. Once inside the cell, FAs enter the oxidative process, TAG synthesis, or if uptake exceeds metabolization, they undergo accumulation in confined compartments, often LD. Acute lipid oversupply produces inhibition of glucose oxidation, and mitochondria preferentially switch from carbohydrate to FA utilization, depicting the high degree of metabolic flexibility in skeletal muscle [26, 27]. In fact, the sole alteration of FA entrance machinery levels is able to modulate FA oxidation rate [28] indicating a high level of metabolic interregulation. Figure 1 depicts the key points regarding FA flux inside skeletal muscle cells, as will be discussed in the following sections.

*2.1. Lipid Uptake.* FAs move from plasma into skeletal fibers using different proteins, such as FA binding proteins [29] and FA transport proteins (FATPs) [30]. All of these proteins are upregulated by classic stimuli that are often associated to skeletal muscle: insulin and contraction [31–33]. For instance, FA translocase (also known as cluster of differentiation 36, CD36), which translocates from cytoplasm to plasma membrane in specialized vesicles, is able to relocate in response to muscle contractions [34]. Furthermore, CD36 mRNA and protein levels are upregulated by high-lipid diet feeding, increasing FA uptake in skeletal muscle [35]. As for FATPs, these proteins are expressed in a tissue-specific manner, with FATP1, FATP4, and FATP6 variants being the predominant forms in skeletal muscle [36]. Its function is still debated, as they have been also shown to possess enzymatic activity (as acyl-CoA synthetases), besides its transport function [37]. FATP1 is present in T-tubules, and its overexpression has been shown to increase FA oxidation in skeletal muscle [38, 39]. After contraction, FATP4 and CD36 are both increased in the sarcolemma, while insulin stimulation induces the translocation of FATP1 and FATP4 to the T-tubules, increasing FA uptake [38].

Once FAs enter the skeletal fiber, they have different fates depending on the metabolic status of the cells. In resting condition, plasma FAs are driven into TAG synthesis as the first destination instead of being moved to the mitochondria for oxidation [40].

*2.2. Lipid Storage.* Increased lipid deposition in skeletal muscle develops when skeletal fiber FA uptake outpaces FA oxidation. An excessive lipid flux into the skeletal muscle is a factor that influences the accumulation of lipid intermediates, which in turn produces lipotoxic stress [41, 42]. Lipid excess generates fatty infiltrations, also called intermuscular adipose tissue, and IMCLs. Evidence suggests that intermuscular adipose tissue is related to the aging process, loss of muscle strength, and decrease in muscle insulin sensitivity [43]. IMCLs are stored in LDs localized between the sarcomeres and adjacent to mitochondria [44], providing an energy pool used in acute and chronic exercise, as will be discussed in the next sections.

IMCLs are composed of triacylglycerol, diacylglycerol (DAG), long-chain acyl-CoA, and ceramides and both DAGs and ceramides are implicated in muscle lipotoxic effect [45]. As mentioned earlier, IMCL accumulation is associated with alterations in insulin signaling [18]. It is worth noting, however, that IMCLs are not always related to obesity. In healthy subjects, women can accumulate 57% more lipids than men in skeletal tissue, without being obese [46]. Hoeg et al. suggested that there is not a clear association between TAG content and impairment of insulin-stimulated muscle glucose uptake [46]. Indeed, accumulation of LDs is often present in endurance athletes [47], who have functional insulin signaling. This phenomenon is known as the "athlete's paradox," and its mechanism remains unclear [48].

Several lines of evidence show that elevated lipid intermediates such as DAGs and ceramides are associated with impaired insulin signaling in skeletal muscle [19, 20, 49–51]. Curiously, DAGs are also higher in trained athletes, which were associated with improved insulin sensitivity [52], suggesting that DAG content is not always indicative of insulin signaling derangement.

*2.3. Lipolysis in Skeletal Muscle.* There are three lipases expressed in skeletal muscle that are responsible for the breakdown of TAGs: monoacylglycerol lipase, adipose triglyceride lipase (ATGL), and hormone-sensitive lipase (HSL) [53]. ATGL catalyzes the first step of TAG lipolysis in skeletal muscle from humans and mice, resulting in the release of one fatty acid molecule. Monoacylglycerol lipase is responsible for the hydrolysis of monoacylglycerol, releasing glycerol and FAs. Overexpression of ATGL in skeletal muscle cells generates an increase in lipolysis and in the expression of the transcription factor PPAR$\delta$, suggesting a role of ATGL in mitochondrial biogenesis [54]. This in turn is associated with increased oxidative capacity in skeletal muscle [54, 55]. Endurance training leads to increased ATGL levels, enhancing intramuscular lipolysis, mainly in type I oxidative fibers [56]. On the contrary, a decrease in ATGL expression characterizes aged muscle, which is accompanied by defects in the antioxidant response and sarcopenia [57].

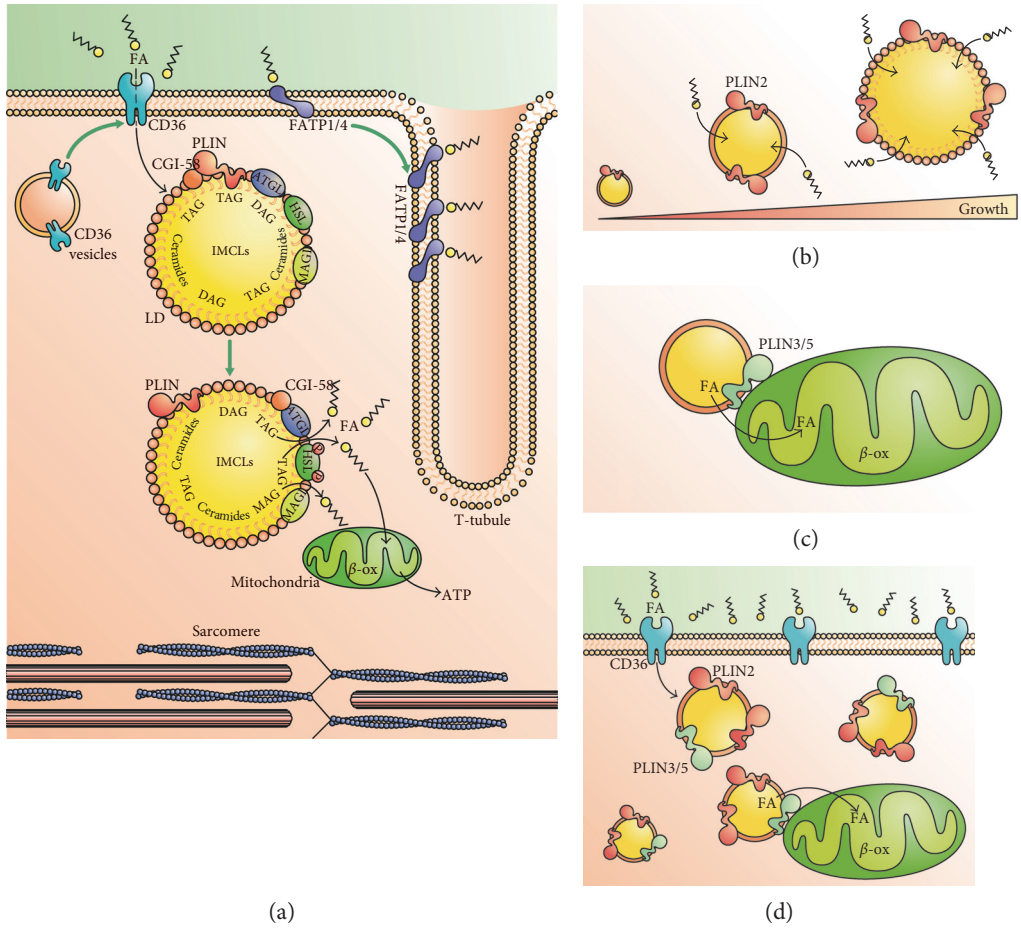


FIGURE 1: FA flux in skeletal muscle and role of perilipins. (a) Schematic representation of FA uptake and deposition in lipid droplets (LD). FA uptake is mediated by FAT/CD36, located in the plasmalemma. FATP1/4 are located in the plasmatic membrane and cooperate with FA intake and metabolism. Movement of CD36 to the plasmatic membrane and an increment in FATP1/4 can be triggered by insulin or contraction (green arrow, see text for details). Once inside the cell, FAs can be accumulated in LDs as acyl-glycerides (TAG, DAG). Perilipins (PLINs) coat the LD membrane, along with the lipases ATGL and HSL and the coactivator CGI-48. In response to contraction (green arrow), ATGL activity increases, as a result of dissociation from PLINs, while HSL is activated by PKA-dependent phosphorylation. This leads to increased FA flux to cytosol and mitochondria, undergoing further $\beta$-oxidation ($\beta$-ox) and ATP synthesis. (b) Main function of PLIN2 in skeletal muscle. PLIN2 coats LD and promotes FA intake, leading to increased size of LD. (c) PLIN3 is part of LD mitochondria contact sites, promoting efficient transfer of FA from LD to mitochondria for oxidation. (d) In cases of excess plasmatic FAs, such as obesity and T2DM, FAs uptake into skeletal muscle is increased. Higher levels of PLIN2 promote an increase in both size and number of LDs. Furthermore, increased levels of PLIN5 foster FA metabolization inside mitochondria.

ATGL is activated by comparative gene identification-58 (CGI-58), a protein member of $\alpha/\beta$-hydrolase fold enzyme family [58]. These proteins are located on the surface of LDs and mitochondria [59], CGI-58 being preferentially expressed in oxidative muscle, as cardiac and soleus muscle [60]. The increase in lipolytic activity results from direct interaction between CGI-58 and ATGL [58]. CGI-58 knockdown reduces lipolysis and incorporation of FAs into TAGs, along with reducing mitochondrial membrane potential [60]. Likewise, muscle-specific inactivation of CGI-58 in mice induces skeletal steatosis but only in oxidative muscle [61]. It is not surprising then that CGI-58 mutations produce lipid storage diseases, such as the Chanarin-Dorfman syndrome, which is characterized by neutral lipid accumulation in skeletal muscle and ichthyosis [62]. Interestingly, Xie et al. found that diminished CGI-58 levels improve glucose tolerance and insulin sensitivity in mice fed with high-fat diet (HFD). The authors explain these findings by suggesting that storing fat in glycolytic muscle is detrimental, whereas it is healthy in oxidative muscle [61].

Similar to ATGL, HSL catalyzes TAG hydrolysis to release FAs into the cytoplasm. Like CGI-58, HSL is highly expressed in type I oxidative fibers of skeletal muscle, and it is activated by adrenergic stimulation and contraction [63, 64]. Both stimuli are capable of regulating HSL activity through PKA- and AMPK-dependent phosphorylation, thus modulating the breakdown of TAGs from IMTG [65].

## 3. Role of Lipid Droplets and Perilipins

As shown in the previous sections, FA metabolism in skeletal muscle requires a tight balance between the uptake and usage

processes, to avoid accumulation of detrimental lipid intermediaries. In this regard, LDs play a pivotal role in maintaining intracellular lipid homeostasis.

LDs are intracellular vesicle-like organelles composed mainly of neutral lipids, including TAGs and sterol esters (Figure 1(a)), and are present in different cellular types, with adipose tissue and skeletal muscle being the most studied [66]. Its formation is a consequence of different metabolic processes, such as lipid storage, lipid exchange between organelles, and cell signaling. LDs are limited by a phospholipid monolayer, which includes phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol [67]. This composition is similar to the endoplasmic reticulum (ER) membrane, supporting the idea that LDs are a specialized domain of the ER [68].

LDs are covered with different members of lipid droplet-associating protein family, referred as the PAT family [69]. This family is composed of perilipins (PLINs), S3-12, and TIP47, among other proteins, which vary on tissue-specific expression and their constitutive or differentiated permanence on LD surface [70, 71]. The most characterized members of the PAT family are PLINs, which are discussed in detail below.

Perilipin family is composed by 5 members (PLIN1–5) that play a major role in the control of TAG hydrolysis and lipolysis in adipose tissue, with a less clear role in skeletal muscle. PLIN1 is expressed in adipocytes and steroidogenic cells but not in skeletal muscle [72, 73], and therefore, its function is not further discussed.

On the other hand, PLIN2 is highly expressed in adipose tissue and skeletal muscle in both rodents and humans [74]. The levels of this protein correlate positively with LD content in skeletal muscle [74], where it can interact with ATGL [75] and HSL [76]. PLIN2 is necessary for both differentiation and regulation of lipolysis in adipose tissue, but its role in skeletal muscle remains unclear. Overexpression of PLIN2 results in increased intracellular TAG storage and larger and more numerous LDs in tibialis anterior muscle [77]. In agreement, downregulation of PLIN2 in cultured myotubes prevented oleate-induced lipid droplet storage, suggesting its involvement in LD stabilization [77]. Evidence suggests that PLIN2 participates in IMCL synthesis and LD growth (Figure 1(b)), but it seems that it is not implicated in skeletal muscle lipolysis, as PLIN2 is not phosphorylated under adrenergic or contractile stimulation [76]. Interestingly, PLIN2-coated LDs increase with age and are associated with lack of muscle strength [78]. This is interesting, as aging is associated with insulin resistance [79].

Unlike PLIN2, the role of PLIN3 in skeletal muscle is not clear. Pharmacological and genetic activation of AMP-activated protein kinase leads to increased gene expression of PLIN3 in skeletal muscle, resulting in higher IMCL content [80]. Conversely, PLIN3 levels in muscle biopsies from healthy patients are positively correlated with whole-body oxidative capacity, while PLIN3 knockdown results in decreased FA oxidation [81]. Subcellular localization of PLIN3 may play a role in its function, as it has been observed that endurance training, but not electrically induced contraction, induces PLIN3 expression and its association with mitochondria in rats (Figure 1(c)) [82]. Whether species-specific function or intracellular localization is responsible for this discrepancy is still unknown.

Little is known about PLIN4 in skeletal muscle. This perilipin is expressed in skeletal muscle, heart, and adipose tissue, and it is preferentially located in LDs containing cholesterol esters [83]. Of all perilipins, *Plin4* mRNA is the most expressed in vastus lateralis biopsies from healthy individuals, and its levels are higher in slow- than fast-twitch muscle [84]. Unlike PLIN3, PLIN4 expression is reduced in response to prolonged endurance training [84].

PLIN5 is found both on the surface of LDs and in the cytoplasm, and it is transcriptionally regulated by PPAR$\delta$ in skeletal muscle. PLIN5 is highly expressed in oxidative tissue such as cardiac and skeletal muscle [85]. Laurens et al. conclude that PLIN5 has an important role in lipolysis, facilitating FA oxidation in response to contraction and increased metabolic demand [86]. PLIN5 is involved in the communication between the LDs and the mitochondria, presumably to facilitate the direct transfer of FFAs released during lipolysis (Figure 1(c)). In fact, there is close structural proximity between PLIN5-decorated LDs and mitochondria [87] and PLIN5 overexpression leads to increased transcription of mitochondrial biogenesis, electron transport chain complexes, and FA oxidation genes [88]. PLIN5 overexpression increases both expression and serum concentration of fibroblast growth factor 21, a major insulin- and exercise-responsive myokine [89]. Furthermore, its overexpression can induce gene expression of factors involved in the ER stress response in order to preserve mitochondrial function [89].

## 4. Lipid Droplet Remodeling in Skeletal Muscle after Contraction

Skeletal muscle is a plastic tissue susceptible to different stimuli but mainly adrenergic stimulation and contraction. Studies have been designed to evaluate PLIN distribution after contraction-induced muscle lipolysis in skeletal muscle, in order to understand the physiological role of PLINs in metabolism. The mechanisms that regulate exercise-induced lipolysis in skeletal muscle are poorly understood, and reports indicate that they may be more complex than lipolysis in adipose tissue. Electrical stimulation is used to induce acute contractile stimulation in isolated fibers [90]. Interestingly, LD and PAT associations are differentially modified in adipose tissue and skeletal muscle when chronic stimulation is applied, such as endurance training [91].

LDs, PLIN2, and PLIN5 are located in the subsarcolemmal region during rest. PLIN2 and PLIN5 are found in LDs and in the cytosol. Neither PLIN2 nor PLIN5 localization changes after contraction in soleus muscle, even when LD content is decreased after electrical stimulation [92]. As mentioned above, ATGL and CGI-58 are necessary for the activation of HSL in the regulation of lipolysis. In this context, both PLIN2 and HSL translocate to LDs after electrically stimulated acute contraction [93]. In fact, exercise rapidly triggers protein kinase A-dependent HSL activation in humans, promoting FA release [65, 94]. ATGL-CGI-58 protein interaction increases and ATGL-PLIN2 decreases after electrical

stimulation [75]. The same acute stimulation is capable of increasing mitochondrial PLIN5 content in rats [76]. However, chronic exercise does not change PLIN5 level in muscle biopsies obtained from patients subjected to training intervention [84]. PLIN4 mRNA, in turn, is decreased after exercise programs consisting of combined strength and endurance training for 12 weeks [84].

## 5. Findings on Insulin Resistance in Skeletal Muscle

*5.1. Obesity as a Main Cause of Insulin Resistance.* Despite the great knowledge that has been generated in the recent years by studying the molecular mechanisms involved in the generation of IR as a result of obesity, several questions remain unresolved. Obesity is characterized by enhanced accumulation of FAs in adipose tissue, liver, and skeletal muscle, and HFD feeding is an accepted model for obesity and IR. Long-term HFD feeding produces an increase in LD content and PLIN5 expression in skeletal muscle [95]. As discussed above, one of the mechanisms involved in HFD-dependent IR is the presence of toxic lipid intermediates as a result of lipid management derangements.

*5.2. Oxidative Stress.* An intracellular pro-oxidative environment has been reported in skeletal muscle from obese and insulin-resistant individuals. We have shown that insulin-resistant mice show increased insulin-stimulated $H_2O_2$ release, and a decreased reduced-to-oxidized glutathione ratio [96]. Some reports suggest that PLIN5 plays a protective role against oxidative burden in the heart, suppressing excess ROS production by sequestering FAs in TAGs [97], but there is no direct evidence in skeletal muscle showing that ROS are involved in the gene expression of PLINs.

*5.3. Lipotoxicity.* Lipotoxicity leads to the damage of organelles that are necessary for intracellular metabolic control, due to an excessive accumulation of lipid intermediates such as lipid-derived DAGs and ceramides [41]. As described before, it has been proposed that the generation and accumulation of these lipid intermediates alters insulin-stimulated glucose uptake [19, 49–51]. FA intermediates activate serine/threonine kinases that impair the ability of the insulin receptor to activate downstream targets, as IRS-1. This leads to decreased translocation of GLUT4 and therefore reduced glucose uptake into skeletal muscle cells [98–100]. Skeletal muscle uses LDs as a protective mechanism against the accumulation of these lipid intermediates. As for the role of PLIN2 in lipotoxicity, it has been reported that PLIN2 knockdown prevents intramyocellular TAG storage, while PLIN2 overexpression augments myocellular fat storage and neutral TAG accumulation in LDs [77]. DAG levels are not increased in this model. Accordingly, PLIN2 overexpression reverts palmitate-induced impairments in insulin signaling [77]. Increased PLIN2 expression inhibits GLUT-mediated glucose uptake into skeletal cells apparently via the retention of SNARE fusion machinery proteins for vesicular fusion at the plasma membrane. This suggests that PLIN2 may play an important role in regulating skeletal insulin response [101]. In fact, PLIN2 is increased in skeletal muscle from rats with genetic-induced diabetes, which might be considered as a compensatory mechanism to deal with excessive lipid load (Figure 1(d)) [74]. Interestingly, skeletal muscle from patients with T2DM had lower PLIN2 gene expression compared to the skeletal muscle of obese control subjects, although a trend to increased protein levels was observed [102].

Studies from two separate groups report that overexpression of PLIN5 in skeletal muscle results in increased LD size and richness in TAGs but does not impair insulin sensitivity [88, 89]. In fact, T2DM patients' muscle biopsies do not show significant differences in PLIN5 levels compared to matched control patients [74]. These findings may be explained by the reports that PLIN5 overexpression in skeletal muscle is capable of protecting the cells against lipotoxicity by increasing the amount of esterified lipid chains into LDs [103]. DAG accumulation is reduced in PLIN5 overexpressing myotubes treated with palmitate, which elevates ceramides and DAG content [103]. On the other hand, fasting is a physiological IR model, in which PLIN5 has been proposed to decrease lipotoxicity by promoting interaction of LDs with mitochondria. Fasting produces an increase in insulin resistance and mitochondrial dysfunction associated with higher presence of PLIN5 in LDs (Figure 1(d)). The authors hypothesized that this effect could be explained by an expanded capacity for inert lipid storage [104].

*5.4. Autophagy.* Autophagy is a cellular process that generates the degradation of damaged cytoplasmic organelles and proteins [105]. LDs contribute to the initiation of autophagy, promoting autophagosome biogenesis through phosphatidylcholine generation from TAG hydrolysis [106].

Mitochondrial function has been shown to be impaired in IR-related diseases. Maintaining autophagy flux is necessary to prevent accumulation of dysfunctional mitochondria and conserve the skeletal muscle mass [105]. Recently, it has been proposed that LDs are linked to the dynamic mitochondrial process. In skeletal muscle, the presence of damaged mitochondria generates oxidative stress and apoptosis, both of which can produce atrophy and muscular weakness [107]. Skeletal muscle from IR individuals has a higher degree of oxidative stress and toxic lipid intermediates accumulation, both conditions associated to mitochondrial damage [108]. Dynamin-related protein-1 (Drp-1) participates in mitochondrial fragmentation, which may result in mitochondrial dysfunction and IR [109]. Specifically, it was described that CGI-58 promotes mitochondrial fission through upregulation of Drp-1 expression producing mitochondrial fragmentation. Reports also showed that CGI-58 overexpression leads to significantly higher basal levels of both autophagy and mitophagy in the C2C12 myotube cell line [110]. Intracellular lipidic stores can be broken down through the process of autophagy, a catabolic pathway that ultimately delivers specific cargo to lysosomal degradation. It has been suggested that PAT- and LD-interacting proteins are part of the autophagy machinery, driving the process of LD recycling [111, 112]. Elegant genetic and imaging approaches have shown that LDs are engulfed by autophagosomes and

then associated with lysosomes for degradation [113]. Furthermore, this process is accompanied by degradation of PLIN2 and PLIN3 in the LDs, allowing for effective recruitment of the autophagic machinery and metabolism of the TAG contained within [114]. The LDs' role in autophagy in skeletal muscle is an open field of research.

*5.5. Insulin Resistance Independent of Impaired Insulin Signaling.* A great proportion of specialized literature suggest that IR in skeletal muscle is a result of impaired insulin signaling. Considering that GLUT4 appears to be responsible for most of both contraction- and insulin-stimulated glucose transport, any defect produced in GLUT4 subcellular trafficking could be responsible of glucose uptake impairment, which is the ultimate step in insulin action.

Upon contraction or insulin stimulation, GLUT4-containing vesicles translocate, dock, and fuse with the plasma membrane through the action of N-ethylmaleimide-sensitive factor attachment protein receptors, known as SNARE fusion machinery proteins [115, 116]. In this regard, PLIN2 overexpression inhibits GLUT4-mediated glucose uptake, with the apparent mechanism being retaining SNARE proteins for vesicular fusion at the plasma membrane, suggesting that PLIN2 may play an important role in regulating glucose uptake [101]. Furthermore, SNARE proteins have been found in LDs and mitochondria in skeletal muscle [117], which suggests that other PLIN isoforms, as PLIN3 or PLIN5, might also be involved. GLUT4-vesicule fusion to plasma membrane is also dependent on $Ca^{2+}$ signaling [118, 119], and therefore, disturbances in $Ca^{2+}$ homeostasis could be a factor involved in insulin resistance, independent of impaired insulin signaling.

## 6. Conclusions

Until now, questions regarding the specific functions of different PLINs in skeletal muscle remain unanswered. The fact that two completely different physiological conditions, as IR and exercise, results in the accumulation of LDs in skeletal muscle continues to be a topic of debate. Could it be that different patterns of PATs in LDs causes this difference? Another possibility is that the intracellular distribution of the LDs differs in these two conditions, as their interactions with organelles such as mitochondria and lysosomes is becoming an apparent regulatory mechanism in LD dynamics. Despite the current uncertainties, it may be conceived that PLIN protein function in skeletal muscle is similar to that observed in adipose tissue: regulators and promoters of FA intake into LDs, and physical mediators that facilitate its further metabolization in mitochondria.

Throughout this review, we have depict the different and multiple processes involved in lipid metabolism in skeletal muscle. The myriad of stimuli that act on skeletal muscle (such as insulin, contraction, and adrenergic stimulation) and the tone of activation (whether it is acute or chronic); all of them can impinge on lipid metabolic pathways. In consequence, a detailed study on all of these variables is necessary in order to more precisely conclude the role of LDs in skeletal muscle in both healthy and disease states.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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*Review Article*

# The LDL Receptor-Related Protein 1: At the Crossroads of Lipoprotein Metabolism and Insulin Signaling

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The metabolic syndrome is an escalating worldwide public health concern. Defined by a combination of physiological, metabolic, and biochemical factors, the metabolic syndrome is used as a clinical guideline to identify individuals with a higher risk for type 2 diabetes and cardiovascular disease. Although risk factors for type 2 diabetes and cardiovascular disease have been known for decades, the molecular mechanisms involved in the pathophysiology of these diseases and their interrelationship remain unclear. The LDL receptor-related protein 1 (LRP1) is a large endocytic and signaling receptor that is widely expressed in several tissues. As a member of the LDL receptor family, LRP1 is involved in the clearance of chylomicron remnants from the circulation and has been demonstrated to be atheroprotective. Recently, studies have shown that LRP1 is involved in insulin receptor trafficking and regulation and glucose metabolism. This review summarizes the role of tissue-specific LRP1 in insulin signaling and its potential role as a link between lipoprotein and glucose metabolism in diabetes.

## 1. Introduction

The metabolic syndrome is comprised of interrelated physiological, metabolic, and biochemical risk factors for type 2 diabetes and cardiovascular disease (CVD). It is well established that the metabolic syndrome correlates with a sedentary lifestyle and obesity, and the syndrome is escalating as a worldwide public health concern due to its prevalence and burden. Furthermore, individuals with the metabolic syndrome have a five-fold increased risk for type 2 diabetes and are twice as likely to develop CVD over the next five to ten years [1]. The metabolic syndrome is currently diagnosed in individuals who present three of the five following risk factors: elevated waist circumference, as defined by population- and country-specific criteria, elevated triglycerides (TGs), reduced high-density lipoprotein cholesterol (HDL-C), elevated blood pressure, and elevated fasting glucose [1]. Insulin resistance has been widely discussed as a potential linking factor for the metabolic syndrome; however, the pathogenesis is unclear and further research is needed to establish a linkage among risk factors.

Type 2 diabetes, a clinical outcome of the metabolic syndrome and the most common form of diabetes, is a complex group of heterogeneous metabolic disorders that includes insulin resistance. The etiology of diabetes is multifactorial and can consist of both genetic and environmental factors, such as low physical activity levels, poor diet, and excess body weight [2]. A metabolic disorder that commonly accompanies diabetes is dyslipidemia, which consists of postprandial lipemia, increased plasma very low-density lipoprotein (VLDL), increased small dense low-density lipoprotein (LDL), and reduced high-density lipoprotein (HDL) [3]. It is speculated from the results of several studies (reviewed in [3]) that diabetic dyslipidemia is caused by several factors, including the effects of insulin on liver apoprotein production, peripheral effects of insulin on adipose and muscle, lipoprotein lipase (LPL) regulation, and effects of cholesteryl ester transfer protein (CETP). This abnormal lipid metabolism is likely a large contributor to the

increased risk of atherosclerosis and CVD in diabetics. In addition to CVD, diabetes is also associated with microvascular and macrovascular pathologies, including retinopathy, nephropathy, and neuropathy [4].

The low-density lipoprotein receptor-related protein 1 (LRP1) is a member of the low-density lipoprotein receptor (LDLR) family and is highly expressed in hepatocytes, adipocytes, neurons, vascular smooth muscle cells, fibroblasts, and macrophages. LRP1 is essential for embryonic development [5, 6] and plays a role in the recruitment and maintenance of mural cells during angiogenesis [6]. LRP1 was originally identified as an endocytic receptor for $\alpha_2$-macroglobulin- ($\alpha_2$M-) proteinase complexes [7, 8] and apolipoprotein E (apoE) [9], and studies now reveal that LRP1 can bind numerous, unrelated ligands with high affinity (summarized in [10, 11]). In addition to its endocytic function, LRP1 also functions in signal transduction pathways and can interact with other cellular receptors. Several studies have demonstrated that LRP1 is atheroprotective and can regulate processes involved in vascular integrity (reviewed in [12]). More recently, research has shown that LRP1 is involved in insulin signaling and glucose homeostasis in several different tissues. This review briefly summarizes the growing body of literature on LRP1, its role in lipoprotein and glucose metabolism, and its potential influence on the metabolic syndrome and diabetes.

## 2. LRP1 Structure and Mode of Ligand Binding

LRP1 is a large receptor consisting of a 515 kDa heavy chain ($\alpha$ subunit) and a noncovalently associated 85 kDa light chain ($\beta$ subunit). As a member of the LDLR family, LRP1 contains modular structures that are shared by all family members and includes cysteine-rich complement-type repeats (CR), epidermal growth factor- (EGF-) like repeats, $\beta$-propeller (YWTD repeat) domains, a transmembrane domain, and an intracellular domain (ICD) (reviewed in [10, 13]). CR, commonly referred to as ligand-binding repeats, occur in four clusters (termed I–IV) on the LRP1 heavy chain. Interactions between LRP1 and many of its known ligands have been mapped to these clusters. The LRP1 EGF repeats and $\beta$-propeller domains are predicted to be involved in ligand uncoupling and are located between CR clusters. The LRP1 light chain contains a short extracellular segment, a single-pass transmembrane domain, and an ICD, which contains two dileucine (LL) motifs and two NPxY motifs.

In a canonical mode of ligand binding, ligand recognition occurs when a lysine $\varepsilon$-amino group on the ligand forms salt bridges with aspartic acid carboxylates on LRP1 CR. The aspartic acid residues are coordinated and stabilized by a calcium ion and form an acidic pocket. Ligand binding is further strengthened by van der Waals interactions between CR aromatic residues and the aliphatic portion of the ligand lysine residue docked within the acidic pocket. Weak electrostatic interactions can also occur between other ligand lysine residues and additional acidic residues on the CR. The ICD of LRP1 contains two NPxY motifs which can be phosphorylated by activated protein-tyrosine kinases. Both the unphosphorylated and phosphorylated states of the NPxY motifs can serve as a binding site for other proteins [14].

Due to its ability to bind to numerous ligands, LRP1 associates with a chaperone termed the receptor-associated protein (RAP) which prevents premature ligand binding during receptor trafficking from the endoplasmic reticulum (ER) to the Golgi. RAP is a 39 kDa ER-associated chaperone that binds tightly to LRP1 and enables delivery of the receptor to the plasma membrane. Because it binds to LRP1 with high affinity, RAP is frequently used as a competitive inhibitor of LRP1 ligand binding and can inhibit LRP1 receptor function [15, 16].

## 3. LRP1 Single-Nucleotide Polymorphisms (SNPs) Are Associated with Several Diseases

Advances in genome-wide association studies (GWAS) have revealed that LRP1 SNPs are associated with several diseases, including coronary heart disease [17], abdominal aortic aneurysm [18, 19], and migraines [20, 21]. In a recent study by Delgado-Lista et al. [22], top SNPs affecting carbohydrate metabolism were identified in subjects with the metabolic syndrome (European Union LIPGENE project cohort). An LRP1 SNP, rs4759277, was found to be highly linked to several phenotypic features of carbohydrate metabolism, including fasting insulin, C-peptide, HOMA-IR, and QUICKI. Many of the LRP1 SNPs are located within introns, and the effect of these polymorphisms on LRP1 expression and function is currently unknown.

## 4. Glucose Transporters

In most mammalian cells, glucose transport occurs by the process of ATP-independent facilitative diffusion and is mediated by members of the GLUT (SLC2A) family of membrane transport proteins. Fourteen GLUT proteins are encoded by the human genome and include transporters that bind nonglucose substrates, such as fructose, myoinositol, and urate [23]. Although GLUT1–4 are well studied, primary substrates are unknown for at least half of the GLUT family. GLUT proteins contain approximately 500 amino acids with twelve transmembrane $\alpha$-helices and a single N-linked oligosaccharide [24]. GLUT family members are divided into three subclasses based on sequence similarity [25]. GLUT1–4, which are abundantly found in the central nervous system (CNS), pancreatic $\beta$-cells, hepatocytes, neurons, adipocytes, and skeletal muscles, are briefly discussed below.

*4.1. GLUT1.* GLUT1 is the predominant GLUT isoform expressed in human pancreatic islets [26–28], and glucose sensing in pancreatic $\beta$-cells are dependent on glucokinase activity (reviewed in [29, 30]). GLUT1 is also the most abundant glucose transporter in the CNS with widespread distribution in the brain [31]. As the most metabolically active organ, the brain depends on capillary bed transport of glucose from the blood to the brain. In contrast to peripheral tissue, glucose transport across the blood-brain barrier (BBB) into the CNS is not dependent on insulin. This process

is mediated by GLUT1 [32], which is located in endothelial cells at the BBB. In addition to its function at the BBB, GLUT1 also functions in glial-mediated uptake of glucose from brain interstitial fluid.

*4.2. GLUT2.* GLUT2 is highly expressed in the liver, intestine, and kidney and is expressed at lower levels in the CNS, neurons, astrocytes, and tanycytes [33]. Although GLUT2 is highly expressed in rodent pancreatic islets and is the primary glucose sensor and transporter, GLUT2 is expressed at low levels in human islets [26, 27]. GLUT2 has a low affinity for glucose ($K_m$ ~17 mM), but its high expression level in select tissues ensures rapid glucose equilibration between the extracellular space and cell cytosol. In pancreatic $\beta$-cells, GLUT2 is involved in glucose-stimulated insulin secretion (GSIS), and impaired GSIS in diabetic rats was shown to be associated with decreased GLUT2 expression [34, 35]. In the liver, GLUT2 functions to uptake glucose in the fed state and release glucose in the fasting state. Hepatic glucose uptake inhibits glycogenolysis and stimulates glycogen synthesis. Interestingly, GLUT2 in the brain regulates several cellular and physiological functions, including feeding initiation and termination, glucagon secretion, thermoregulation, and the melanocortin pathway (reviewed in [33]). GLUT2 is also involved in autonomic nervous activity and taste preference.

*4.3. GLUT3.* In addition to GLUT1, GLUT3 is the other predominant GLUT isoform expressed in human pancreatic islets [26, 27]. GLUT3, often referred to as the neuron-specific glucose transporter, is also expressed in the brain, testis, spermatozoa, and lymphocytes in humans [23, 31]. Levels of GLUT3 expression in different regions of the brain directly correlate with regional cerebral glucose utilization. GLUT3 has a high affinity for glucose ($K_m$ ~1.5 mM) and high turnover rate, resulting in efficient glucose uptake in neurons.

*4.4. GLUT4.* GLUT4 is highly expressed in adipose tissue and skeletal muscle and is critical to whole-body glucose homeostasis. The expression and translocation of GLUT4 are highly regulated by insulin, and disruption of this regulatory process results in insulin resistance and an increased risk for developing diabetes (reviewed in [36]). Interestingly, transgenic mice expressing high levels of GLUT4 in adipose tissue or skeletal muscle are highly glucose tolerant and insulin sensitive. Currently, the structure of GLUT4, the cellular components involved in its trafficking, and the insulin-mediated signaling pathways regulating its trafficking are unclear. Recent studies have identified several components of GLUT4 containing vesicles (discussed below), but additional studies are needed to elucidate how each of these components affects GLUT4 function.

## 5. Insulin Enhances Postprandial Lipoprotein Clearance via LRP1 Translocation to the Cell Surface in Hepatocytes

LRP1 is highly expressed in hepatocytes where it functions to mediate the endocytosis and degradation of chylomicron remnants as well as VLDL, $\alpha_2$M-proteinase complexes, serpin-enzyme complexes, and blood coagulation factor VIII (reviewed in [10]). The binding of chylomicron remnants to LRP1 is mediated by apoE and further enhanced by LPL [37], which remains attached to triglyceride-rich lipoproteins (TRLs) following lipolysis and facilitates hepatic clearance from the circulation [38]. It was recently shown that apolipoprotein C-III (apoC-III), which is highly correlative to plasma TG levels, inhibits the hepatic clearance of TRLs by both LRP1 and LDLR [39].

Early studies found that LRP1 is also abundant in adipocytes [40] and its endocytic function increases 2-3-fold following exposure of the cells to insulin as indicated by increased uptake of $^{125}$I-labeled activated $\alpha_2$M ($\alpha_2$M$^*$) [41, 42]. These findings raised the possibility that insulin may also stimulate LRP1-specific uptake of chylomicron remnants in the liver. To test this hypothesis, Laatsch et al. [43] investigated the effect of insulin on hepatic LRP1-mediated uptake of postprandial chylomicron remnants. Their studies revealed that like adipocytes, insulin stimulated LRP1 translocation to the cell surface in human hepatic HuH7 tumor cells, a rat hepatoma cell line (FAO cells), as well as primary hepatocytes isolated from wild-type (WT) and low-density lipoprotein receptor knockout (LDLR$^{-/-}$) mice.

These results were replicated in vivo by demonstrating that glucose-injected mice showed a significant increase in the hepatic uptake of $\alpha_2$M$^*$ compared to NaCl-injected control mice [43]. Enhanced uptake of $\alpha_2$M$^*$ upon insulin stimulation was not observed in leptin-deficient obese mice (ob/ob), which exhibit glucose intolerance and insulin resistance. To confirm the role of LRP1 in the insulin-stimulated hepatic uptake of postprandial chylomicrons, hepatic LRP1 expression was ablated by injecting LRP1$^{flox}$ mice with an adenovirus expressing Cre recombinase. Following intraperitoneal glucose injection, chylomicron remnant uptake was significantly reduced in hepatic LRP1 knockdown animals compared to that in control animals injected with an adenovirus expressing EGFP. Together, these studies demonstrate that insulin stimulates hepatic LRP1 translocation to the cell surface resulting in enhanced chylomicron remnant uptake, thus connecting insulin-mediated signaling events to postprandial lipoprotein catabolism.

## 6. Hepatic Inactivation of LRP1 Impairs Insulin Signaling and Suppresses GLUT2 Translocation to the Plasma Membrane

To further investigate the role of hepatic LRP1 in insulin resistance, mice were generated in which LRP1 was specifically deleted in the liver (h-LRP1$^{-/-}$) [44]. When placed on a high-fat diet (HFD), h-LRP1$^{-/-}$ mice exhibited an accelerated body weight gain attributed to an increase in fat mass, glucose intolerance, pyruvate intolerance, insulin resistance, and dyslipidemia. Furthermore, the mice developed nonalcoholic steatohepatitis. To define potential mechanisms by which h-LRP1$^{-/-}$ mice develop insulin resistance, components of the insulin signaling pathway were investigated. These studies revealed that hepatic LRP1 inactivation

resulted in defective insulin signaling, which included impaired phosphorylation of insulin receptor (IR), AKT, and GSK3$\beta$ and incomplete suppression of gluconeogenic genes. Interestingly, h-LRP1$^{-/-}$ hepatocytes had significantly lower levels of IR expression at the cell surface; however, the extent of insulin-stimulated IR internalization was similar between h-LRP1$^{-/-}$ and h-LRP1$^{+/+}$ hepatocytes. These results suggest that efficient IR expression at the cell surface is LRP1 dependent.

Similar to the studies conducted by Laatsch et al. [43], insulin treatment of primary hepatocytes isolated from WT mice stimulated LRP1 translocation to the cell surface. Interestingly, LRP1 translocation was inhibited by the saturated fatty acid palmitate. In contrast, oleic and linoleic acids, unsaturated fatty acid components of the HFD, did not inhibit insulin-stimulated LRP1 translocation. Insulin also increased GLUT2 translocation to the plasma membrane in WT hepatocytes, and in the presence of palmitate, GLUT2 levels in the plasma membrane were further increased. Insulin failed to mediate GLUT2 translocation to the plasma membrane in h-LRP1$^{-/-}$ hepatocytes, suggesting a critical role for LRP1 in insulin-mediated GLUT2 translocation.

These studies provide further evidence that hepatic LRP1 is important for maintaining insulin sensitivity and preventing diet-induced steatosis by regulating insulin signaling and modulating GLUT2 translocation to the plasma membrane in response to insulin. It is not presently clear how LRP1 regulates the trafficking of GLUT2 containing vesicles; however, substantial work has revealed insights into how LRP1 regulates the trafficking of another glucose transporter, GLUT4 (see below).

## 7. LRP1 Regulates Glucose Homeostasis in Adipocytes

The postprandial function of LRP1 has been largely focused on studies in the liver where LRP1 clears apoE-enriched chylomicron remnants and VLDL from the circulation. An early study by Lossow et al. [45] showed that glucose-fed rats incorporated twice the amount of injected chylomicron [$^{14}$C]cholesterol into adipose tissue as fasted rats. This result suggests that insulin may influence chylomicron uptake in adipose tissue. As mentioned earlier, studies by Descamps et al. [42] revealed that LRP1 is abundantly expressed on adipocytes, and receptor activity is significantly increased when exposed to physiological concentrations of insulin. Upon insulin stimulation, primary adipocytes isolated from rat epididymal fat pads exhibited increased uptake of $^{125}$I-$\alpha_2$M$^*$ and apoE-enriched $\beta$-VLDL; however, uptake of $^{125}$I-$\alpha_2$M$^*$ and apoE-enriched $\beta$-VLDL could be inhibited by the GST-39 kDa fusion protein (i.e., GST-RAP) or anti-LRP1 IgG. Insulin also rapidly increased the number of GST-RAP binding sites on the cell surface, suggesting that LRP1 is not synthesized de novo in response to insulin but rather is rapidly translocated to the cell surface from intracellular stores. In vivo rat studies showed that chylomicron uptake was significantly enhanced in animals that were fasted and subsequently given glucose/insulin compared to animals that were only fasted. Chylomicron uptake was further increased in fed rats given glucose/insulin compared to fasted rats given glucose/insulin. The authors proposed that the significant increase in chylomicron uptake in fed rats may be due to a synergistic effect of insulin-induced LRP1 expression on the cell surface and increased LPL activity due to a fed state. This seminal work by Descamps et al. demonstrates that insulin stimulates LRP1 translocation to the cell surface and increases the uptake of chylomicron remnants in adipose tissue.

Further insight into the role of LRP1 in adipocytes was derived from mice in which LRP1 was specifically inactivated in adipose tissue (ad-LRP1$^{-/-}$) [46]. Adipocyte LRP1 knockout mice displayed delayed postprandial lipid clearance compared to ad-LRP1$^{+/+}$ littermates, and delayed lipid clearance was not attributed to defective LPL expression in ad-LRP1$^{-/-}$ mice. Adipose-specific LRP1 inactivation also produced mice with significantly lower body weights due to a reduction in fat mass. Further histological analysis revealed a reduction in both the size and the number of lipid droplets in white and brown adipocytes from ad-LRP1$^{-/-}$ mice. Despite the reduced body weight, ad-LRP1$^{-/-}$ mice consumed more food and were more susceptible to body fat loss under fasting conditions. Observations revealed that ad-LRP1$^{-/-}$ mice displayed difficulty in maintaining body temperature and had a significant increase in energy expenditure compared to ad-LRP1$^{+/+}$ littermates. The enhanced energy expenditure was correlated to an increase in muscle thermogenesis and is likely a compensatory mechanism for the reduction in brown adipose tissue and body temperature in ad-LRP1$^{-/-}$ mice. Interestingly, ad-LRP1$^{-/-}$ mice had improved glucose tolerance and were resistant to HFD-induced obesity and glucose intolerance. This protective effect in ad-LRP1$^{-/-}$ mice is likely due to a shift in energy metabolism consisting of defective lipid uptake and storage and muscle thermogenesis. Together, these studies show that LRP1 regulates adipocyte energy homeostasis and can influence glucose metabolism and insulin sensitivity.

These early cell-based and animal studies revealed a link between adipose LRP1 and glucose metabolism. Nasarre et al. [47] analyzed LRP1 expression in epicardial and subcutaneous fat from type 2 diabetic and nondiabetic patients. Epicardial fat is a metabolically active fat deposit and has been associated with several components of the metabolic syndrome (reviewed in [48]). LRP1 mRNA and protein expression in epicardial fat were significantly higher in diabetic patients compared to nondiabetic patients; however, LRP1 mRNA and protein expression in subcutaneous fat were similar in both patient populations. Moreover, LRP1 mRNA expression in epicardial fat positively correlated with plasma TG ($R^2 = 0.50$; $P = 0.01$) and plasma glucose ($R^2 = 0.33$; $P = 0.03$) levels. The authors noted, however, that diabetic patients had a higher, but nonsignificant, incidence of atherosclerosis which may be a confounding factor in the upregulation of LRP1. Results from this patient study suggest that overexpression of epicardial LRP1 may play an important role in the alterations of lipid metabolism associated with T2D.

Collectively, these studies illustrate the complex interplay between insulin signaling and adipose LRP1. Additional

Table 1: $t_{1/2}$ values calculated from rate constants for LRP1 recycling in cells.

| Ligand | Cell | Condition | $t_{1/2}$ endocytosis (min) | Fold | $t_{1/2}$ exocytosis (min) | Fold | [a]PM | Fold | Ref |
|---|---|---|---|---|---|---|---|---|---|
| AF647-$\alpha_2$M | 3T3-L1 cells | Basal | 1.69 | 1.0 | 6.93 | 1.0 | 0.22 | 1.0 | [54] |
| AF647-$\alpha_2$M | 3T3-L1 cells | Insulin | 1.69 | 1.0 | 6.30 | 0.9 | 0.24 | 1.1 | [54] |
| AF647-$\alpha_2$M | 3T3-L1 differentiated | Basal | 2.48 | 1.5 | 33.00 | 4.8 | 0.07 | 0.3 | [54] |
| AF647-$\alpha_2$M | 3T3-L1 differentiated | Insulin | 2.04 | 1.2 | 16.12 | 2.3 | 0.11 | 0.5 | [54] |
| $^{125}$I-8G1 | WI-38 fibroblasts | Basal | 4.81 | 2.8 | ND | ND | ND | ND | [71] |
| $^{125}$I-$\alpha_2$M | Human SMCs | Basal | 1.08 | 0.6 | ND | ND | ND | ND | [b] |
| $^{125}$I-$\alpha_2$M | CHO LRP1 null transfected with chicken LRP1 | Basal | 2.68 | 1.6 | ND | ND | ND | ND | [72] |

[a]Fraction of total expressed in plasma membrane [54]. [b]M. Migliorini, S. C. Muratoglu, D. T. Au, and D. K. Strickland, unpublished data. ND: not determined.

studies are needed to determine if the LRP1-mediated uptake mechanism is altered in insulin-resistant individuals and whether altered LRP1 function further influences glucose metabolism and insulin sensitivity in patients with T2D.

## 8. LRP1 Is a Major Component of GLUT4 Containing Vesicles

The mechanism by which LRP1 is translocated to the cell surface following insulin exposure was not fully understood until LRP1 was identified as one of the most abundant proteins in GLUT4 containing vesicles by proteomic analysis [49] (see below). GLUT4 cell surface levels are regulated by insulin, and upon insulin exposure, glucose uptake is substantially enhanced due to the rapid translocation of GLUT4 containing vesicles to the plasma membrane. In adipocytes cultured under basal conditions, 99% of the total GLUT4 is located within the cell [50]. In contrast, approximately 40% of the total GLUT4 is present at the cell surface in insulin-stimulated cells [50].

To identify the proteome of GLUT4 containing vesicles, Jedrychowski et al. [49] developed a unique purification protocol involving differential immunoadsorption of vesicles isolated from light microsomal fractions of primary rat adipocytes. The purified GLUT4 containing vesicles were demonstrated to be sensitive to insulin-mediated translocation and were subjected to semiquantitative and quantitative proteomic analysis. Using this approach, Jedrychowski et al. identified LRP1 and several other membrane proteins as components of these vesicles and confirmed that like GLUT4, LRP1 is translocated to the cell surface in rat epididymal adipocytes, which is consistent with earlier studies [41, 42].

## 9. LRP1 Forms a Complex with Sortilin, IRAP, and GLUT4 and Regulates GLUT4 Trafficking

To identify interacting proteins, the light microsomal fraction isolated from rat epididymal adipocytes was subjected to crosslinking studies [49]. Immunoprecipitation of fraction extracts with anti-GLUT4 IgG identified not only GLUT4 but also LRP1, insulin-regulated aminopeptidase (IRAP), and sortilin, suggesting that these proteins associate as a complex. To determine the impact of LRP1 expression on GLUT4 vesicle formation, LRP1 was silenced in 3T3-L1 adipocytes. Expression of IRAP, sortilin, and GLUT4 decreased in LRP1-silenced adipocytes; furthermore, the LRP1-depleted 3T3-L1 adipocytes demonstrated an approximately 50% decrease in insulin-stimulated glucose uptake. The in vivo impact of LRP1 expression on GLUT4 levels was confirmed by immunoblotting epididymal fat tissue from adipose-specific LRP1 knockout mice, which revealed a decrease in both sortilin and GLUT4 levels. Interestingly, coprecipitation experiments using the LRP1 cytoplasmic domain, prepared as a fusion protein with GST, demonstrated an association with Tbc1D4 (also known as Akt substrate of 160 kDa or AS160). Tbc1D4/AS160 contains two phosphotyrosine binding domains (PTB) and regulates GLUT4 trafficking by catalyzing the hydrolysis of Rab-bound guanosine triphosphate. The two PTB domains in Tbc1D4/AS160 are phosphotyrosine-independent, indicating that they recognize the NPxY motif that is not tyrosine phosphorylated [51]. LRP1 contains two NPxY motifs within its cytoplasmic domain, and tyrosine 4507 is phosphorylated by Src family kinases [52]. Interestingly, Bilodeau et al. [53] discovered that LRP1 is tyrosine phosphorylated in mouse livers following insulin injection, and how this impacts the association of LRP1 cytoplasmic domain with Tbc1D4/AS160 remains to be determined.

## 10. Differentiation of 3T3-L1 Cells into Adipocytes Reduces LRP1 Recycling Rate

By employing quantitative flow cytometric assays, Brewer et al. [54] determined the endocytic rate constant, exocytic rate constant, and fraction of molecules located in the plasma membrane for GLUT4, transferrin receptor, and LRP1. These experiments were performed in 3T3-L1 cells, a murine fibroblast-like cell line that can be differentiated into an adipocyte-like cell line. The results for LRP1 are summarized in Table 1 as $t_{1/2}$ values along with additional data from the literature. Under basal conditions, the LRP1 endocytic rate in 3T3-L1 cells is greater than the recycling rate. The net effect is that only 22% of total functional LRP1 is located on the cell surface. Furthermore, the results revealed that insulin has little impact on LRP1 endocytosis and recycling in 3T3-L1 cells. In contrast, upon differentiation into adipocyte-like cells, the LRP1 endocytic rate is slowed by 1.5-fold while the recycling rate is slowed by 4.8-fold, with the combined effect resulting in a 68% reduction of LRP1 surface levels. Interestingly, treatment with insulin increased both the

endocytic rate and the recycling rate, resulting in increased levels of LRP1 on the cell surface. Interestingly, knockdown of Tbc1D4/AS160 accelerated LRP1 exocytosis under basal (2.5-fold) and insulin-stimulated (1.6-fold) conditions [54], confirming the functional importance of the interaction of Tbc1D4/AS160 with LRP1.

## 11. A Link between Alzheimer's Disease and Glucose Transport across the Blood-Brain Barrier

Alzheimer's disease (AD) is a neurodegenerative disorder resulting in dementia that affects a large number of the elderly and is characterized by amyloid plaque deposits made up of aggregates of misfolded amyloid $\beta$ (A$\beta$) oligomers and neurofibrillary tangles in the brain [55, 56]. Since numerous studies have concluded that patients with diabetes have an increased risk of developing Alzheimer's disease (AD) compared to healthy individuals [57–59], the interaction between insulin signaling and the CNS has received significant attention in the last decade. In the CNS, insulin has a well-established role as a growth factor, including effects on synaptogenesis and nerve growth. Furthermore, insulin signaling is crucial for synaptic plasticity, learning, and memory (reviewed in [60]), and thus, neuronal insulin receptor dysfunction could lead to cognitive decline [61, 62].

To examine the impact of glucose transport across the BBB on the progression of AD, elegant experiments were performed by Winkler et al. [32] who employed a mouse model of AD and demonstrated that GLUT1 deficiency intensifies A$\beta$ peptide accumulation in the brain of these mice. While blood glucose levels were unchanged in GLUT1$^{-/-}$ mice, cerebrospinal fluid (CSF) glucose levels were dramatically reduced. Interestingly, a significant reduction in capillary length in the somatosensory cortex and hippocampus were noted in GLUT1$^{-/-}$ mice resulting in reduced blood flow. The study also demonstrated that lower levels of GLUT1 lead to diminished levels of LRP1 expression, further aggravating A$\beta$ accumulation as LRP1 plays a major role in clearing this peptide. Upon rescue of GLUT1 expression, LRP1 protein levels normalized in the brain capillaries of the AD mouse model, demonstrating a connection between GLUT1 and LRP1 expression levels. This effect of GLUT1 on LRP1 expression was connected to increased levels of the sterol regulatory element binding protein 2 (SREBP2), which is a known transcriptional suppressor of LRP1 [63].

## 12. Neuronal LRP1 Regulates Glucose Metabolism

To determine a role of LRP1 on neuronal glucose metabolism, Liu et al. [64] generated a forebrain neuron-specific LRP1 knockout (n-LRP1$^{-/-}$) mouse and observed that the insulin receptor $\beta$ (IR$\beta$) and phosphorylated Akt levels were significantly decreased in n-LRP1$^{-/-}$ mice compared to those in control mice. These results were consistent with cell-based studies in which LRP1 expression was knocked down. Coimmunoprecipitation experiments confirmed that LRP1 interacts (directly or indirectly) with the IR$\beta$. Importantly, cellular glucose uptake levels were demonstrated to be impaired in LRP1-deficient cells. The levels of GLUT3 and GLUT4 were reduced in primary neurons in which LRP1 was silenced, as were the levels of these two glucose transporters in the cortex of n-LRP1$^{-/-}$ mice. Finally, the study demonstrated that LRP1 deficiency in neurons leads to glucose intolerance in the brain. Together, these studies revealed that LRP1 modulates insulin signaling in neurons similar to its role in hepatocytes.

Prior studies have shown that insulin treatment alters intracellular trafficking of LRP1, stimulating recycling of LRP1 to the plasma membrane in both adipocytes [41] and hepatocytes [43]. To determine if this occurs in neurons, SH-SY5Y human neuronal cells were treated with insulin, and the results confirmed enhanced surface expression of LRP1 upon insulin treatment [64]. Furthermore, the study demonstrated that hyperglycemia suppresses LRP1 expression in the brain. Since LRP1 plays an important role in the clearance of the A$\beta$ peptide in the brain, lower levels of LRP1 could exacerbate AD pathology.

## 13. Circulating Lipoprotein Delivery to the Brain Regulates Insulin Signaling in *Drosophila*

Contrary to the earlier dogma that the CNS was not considered to be an insulin-sensitive tissue, the CNS is now regarded as an insulin-sensitive organ. The IR is widely expressed in the brain [65], and IR-mediated signaling promotes neuronal development, glucoregulation, feeding behavior, body weight, cognitive processes, executive functioning, learning, and memory formation [66]. Local insulin production in the CNS in mammals has been under intense debate. While a body of work supports the expression of insulin in the CNS (reviewed in [67]), there is some debate regarding the existence of definitive evidence [68]. Interestingly, in *Drosophila*, three of the seven circulating insulin-like peptides are secreted from the brain [69] and act locally on feeding behavior and systemically to regulate metabolism.

Since insulin and its signaling pathway have been well conserved over the course of evolution, Brankatschk et al. [70] used a *Drosophila melanogaster* model for investigating insulin signaling in neurons and discovered that the BBB is the main sensor to report the nutritional status in these organisms. Surprisingly, it is not the total calories absorbed, but rather the lipid composition of consumed food that is sensed by special neurons which in turn regulate insulin signaling.

In *Drosophila*, the fat body is an organ analogous to the liver and produces lipophorin, the major hemolymph (vertebrate blood analog) lipid carrier. In addition, the fat body also produces lipid transfer particle (LTP) which is responsible for transferring lipids onto lipophorin. Brankatschk et al. [70] discovered that LTP can cross the BBB in a process mediated by LRP1 and LRP2 (megalin). Furthermore, they found that fat molecules derived from yeast-based food (as opposed to those derived from plant-based food) promoted LTP accumulation in the brain which enhanced insulin

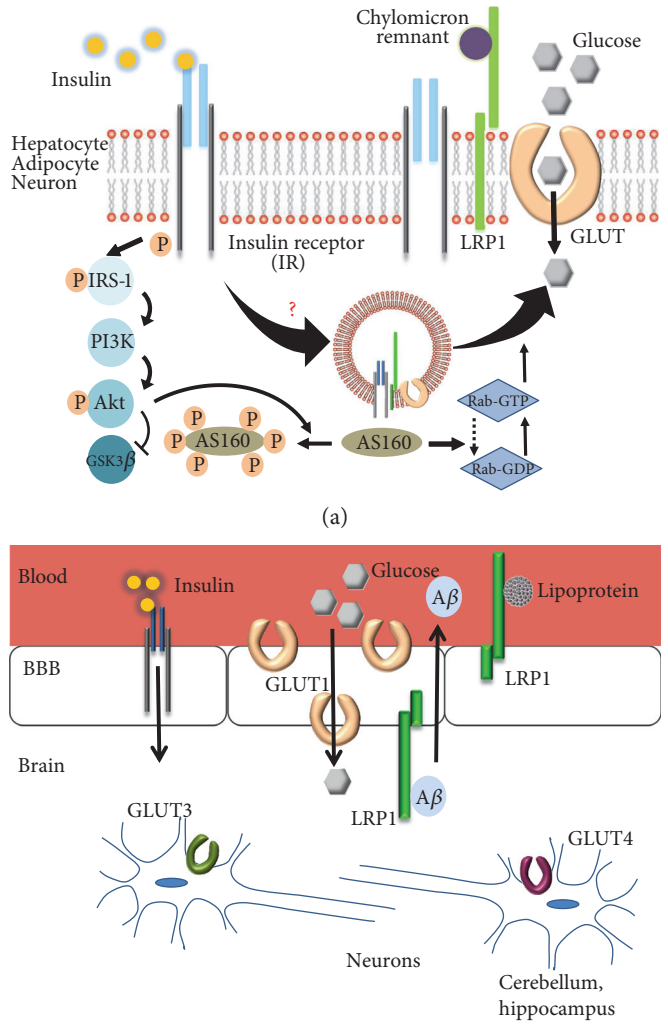


Figure 1: (a) LRP1-dependent insulin signaling in peripheral tissues. Insulin stimulates LRP1 trafficking to the plasma membrane of hepatocytes, adipocytes, and neurons. GLUT2 in hepatocytes, GLUT4 in adipocytes, and GLUT3 and GLUT4 in neurons are translocated to the plasma membrane in an LRP1-dependent manner upon insulin stimulation. Upon binding to its receptor, insulin initiates signaling pathways that mediate GLUT4 translocation to the plasma membrane in a process regulated by LRP1 expressed in GLUT4 containing vesicles. Akt activation by insulin is crucial for this process as it causes AS160 phosphorylation at multiple sites and inactivates its GAP activity. AS160 is known to associate with the LRP1 cytoplasmic domain. Rab GTPase activation in turn stimulates GLUT4 translocation. (b) Glucose and insulin metabolism is modulated by LRP1 in the brain. Insulin production by cells in the brain is somewhat controversial, although insulin is delivered from the blood to the brain by receptor-mediated transcytosis. The insulin receptor rarely induces glucose uptake by brain cells. Instead, it has effects on feeding that are largely opposite to those produced by insulin in the periphery. CNS insulin also impacts cognition. In the brain, the most metabolically active organ, glucose acquisition is independent of insulin, and glucose transporter proteins (GLUTs) mediate glucose delivery from the blood to the brain through the blood-brain barrier (BBB). GLUT1 is detected exclusively in the endothelial cells of the BBB as well as all other neural cells (in a distinct molecular form different than BBB). GLUT3 is specifically expressed in neurons. GLUT4 is an insulin-sensitive transporter and is only expressed at lower levels in specialized neurons of the hippocampus and the cerebellum. The function of the BBB-localized LRP1 in actively removing A$\beta$ from the brain is regulated by insulin levels. Through the endocytic function of LRP1, the BBB may act as the main sensor to report the nutritional status, especially the lipid composition of consumed food to special neurons which in turn regulate insulin signaling, as it was reported to be the case in *Drosophila*.

signaling and promoted fly larvae growth. These studies revealed that in *Drosophila*, fat-containing molecules carry specific nutrient information to sensory cells in the brain. This finding opens the intriguing question of whether a generalized mechanism in mammals exists in which lipoproteins carry information from the periphery to the brain to regulate glucose homeostasis.

## 14. Conclusions

The initial observations that LRP1 cell surface levels and functional activity increased in response to insulin treatment led to the proposal that insulin signaling may modulate lipoprotein catabolism [42]. This proposal was proven in subsequent studies where intraperitoneal injection of glucose

enhanced LRP1 levels on the hepatic surface and increased the uptake of chylomicron remnant lipoprotein particles [43]. Additional work revealed that LRP1 directly regulates the insulin signaling pathway (Figure 1(a)) [44, 64], although the molecular mechanism of how this occurs is not yet clear and requires further investigation. Studies have also identified LRP1 as a major component of GLUT4 containing vesicles and have further shown that cellular trafficking of these vesicles depends on LRP1 expression [49]. Again, the molecular mechanism is largely unknown and will require additional studies. Whether LRP1 is contained in GLUT2 containing vesicles in the liver and can modulate vesicle trafficking remains to be established. Finally, studies in the brain employing mouse models suggest a connection between GLUT1 expression levels and LRP1 expression levels [32], but it is presently unknown how these two molecules are linked. Interestingly, studies performed in *Drosophila* [70] revealed that fat-containing molecules absorbed from the diet carry specific nutrient information to sensory cells in the brain. This finding raises the intriguing question of whether a generalized mechanism in mammals exists in which lipoproteins carry information from the periphery to the brain to regulate glucose homeostasis. As the current debate continues on whether AD represents a form of diabetes (type 3 diabetes) that selectively afflicts the brain, LRP1's protective role against neurodegenerative disease is now highlighted as a molecule converging the roles of removing excess A$\beta$ peptide from the brain, lipoprotein metabolism, and mediating insulin signaling (Figure 1(b)).

## Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Membrane Cholesterol in Skeletal Muscle: A Novel Player in Excitation-Contraction Coupling and Insulin Resistance

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Membrane cholesterol is critical for signaling processes in a variety of tissues. We will address here current evidence supporting an emerging role of cholesterol on excitation-contraction coupling and glucose transport in skeletal muscle. We have centered our review on the transverse tubule system, a complex network of narrow plasma membrane invaginations that propagate membrane depolarization into the fiber interior and allow nutrient delivery into the fibers. We will discuss current evidence showing that transverse tubule membranes have remarkably high cholesterol levels and we will address how modifications of cholesterol content influence excitation-contraction coupling. In addition, we will discuss how membrane cholesterol levels affect glucose transport by modulating the insertion into the membrane of the main insulin-sensitive glucose transporter GLUT4. Finally, we will address how the increased membrane cholesterol levels displayed by obese animals, which also present insulin resistance, affect these two particular skeletal muscle functions.

## 1. Introduction

The physiological relevance of plasma membrane cholesterol levels has attracted increased attention in recent years. Cholesterol is an essential component of eukaryotic membranes, which display molar ratios of cholesterol to phospholipids in the range of 7–55 mol% [1–4]. Physiological levels of cholesterol in the cellular membranes are critical to preserve membrane fluidity and thickness and to structure the lipid domains that are involved in signal transduction processes [2, 5].

Contraction of skeletal muscle takes place via the excitation-contraction (EC) coupling process [6]. Action potential propagation into the fiber interior through the transverse tubule (T-tubule) system initiates EC coupling, which results in the cytoplasmic $Ca^{2+}$ increase that triggers muscle contraction [7]. In addition to contraction, insulin-sensitive glucose transport and glucose homeostasis represent additional key functions of skeletal muscle which occur predominantly at the level of the T-tubule system [8]. Accordingly, T-tubule composition and structure are likely to regulate both the EC process and insulin-sensitive glucose transport.

The mammalian T-tubule membranes are highly enriched in sphingomyelin and cholesterol compared to the surface sarcolemma [9]. This feature endows these membranes with a highly ordered lipid environment [10]. We have reported recently that single fibers isolated from adult skeletal muscle display a 26% decrease in cholesterol content following incubation with the cholesterol removing agent methyl-$\beta$-cyclodextrin (M$\beta$CD). This agent also alters the distribution pattern of the voltage-dependent calcium channel Cav1.1 in T-tubules and suppresses electrically evoked $Ca^{2+}$ transients [11].

Skeletal muscle is the largest body organ in nonobese subjects and represents the major site of insulin-stimulated glucose disposal [12]. Insulin increases glucose uptake into skeletal muscle and adipose tissue by redistributing type 4 glucose transporters (GLUT4) from their intracellular

location to the plasma membrane [13]. In skeletal muscle, insulin resistance (IR) is associated with disturbed insulin signaling, leading to defective GLUT4 traffic to the T-tubules and the surface membrane [8].

Animals fed a high-fat diet (HFD) become insulin-resistant and exhibit elevated levels of membrane cholesterol compared with normal chow-fed animals [14]. Likewise, mice fed a HFD become obese, develop IR, and display increased levels of cholesterol in isolated skeletal muscle T-tubule membranes and adult muscle fibers [15]. Conversely, new insights into GLUT4 trafficking reveal that compounds that partially reduce membrane cholesterol content increase insulin-independent GLUT4 translocation and glucose uptake, both in adipocytes [16] and in muscle cell lines [14]. Therefore, altering the physiological levels of membrane cholesterol may lead to cellular malfunction and thus may contribute to the pathological processes triggered in humans by obesity or by cholesterol depletion caused by pharmacological agents. Here, we will review the critical evidence that supports a role of membrane cholesterol as a new player in physiological muscle function and in the IR condition.

## 2. Role of Cholesterol in Plasma Membrane Properties

Cholesterol is the single most abundant lipid molecule of plasma membranes, representing up to 55 mol% of the total lipid composition [1–4]. The cholesterol molecule is essential for membrane biogenesis [4] and influences the structure and physical properties of biological membranes, including membrane thickness [17] and fluidity [18]. Membrane cholesterol participates in a wide range of physiological functions including limiting ion leakage through membranes [19], modulation of signal transduction pathways [5], and traffic of membrane proteins [20].

Cholesterol is a polycyclic amphipathic molecule derived from a sterane backbone (Figure 1). It has a polar head formed by a single hydroxyl group which in membranes can form hydrogen bonds with polar groups of proteins or lipids [21]. The nonpolar section of cholesterol has two faces, a planar face called the $\alpha$-surface and a rough face called the $\beta$-surface. These sections allow specific cholesterol interactions with protein $\alpha$-helical regions and $\beta$-surfaces [21, 22].

Cholesterol modifies the organization of lipids in artificial bilayers. Phosphatidylcholine molecules with unsaturated hydrocarbon chains can adopt a liquid-disordered fluid phase in bilayers; however, cholesterol addition induces a change to the liquid-ordered phase, decreasing the fluidity of the membrane [23–25]. The spatial distribution of cholesterol responds according to the umbrella model, whereby the polar head groups of phospholipid function as an umbrella, shielding the hydrophobic moiety of cholesterol molecules from water [26]. Cholesterol has higher affinity for sphingolipids, leading to a highly regular distribution in membranes which minimizes cholesterol-cholesterol contact [26].

Lipid rafts are small-scale (10–200 nm) domains found in live cell membranes enriched in cholesterol and sphingolipids. In lipids rafts, the interaction of cholesterol with sphingolipids is more stable; cholesterol presents its $\alpha$-surface to these lipids leaving its $\beta$-surface to interact with transmembrane domains of integral proteins [21]. Lipid rafts play an essential role in membrane-protein sorting and in the formation of signaling complexes [27–29]. In these lipids domains, cholesterol increases the order of lipid-acyl chains, increasing the local membrane thickness and limiting the type of integral membrane proteins located on this hydrophobic environment. These changes around membrane proteins can modulate the local lipid environment and modify the internal protein conformation states and their function [30].

A previous report [31] described a Cholesterol Recognition/Interaction Amino Acid Consensus sequence (CRAC domain), which is a short linear amino acidic motif with a specific vectorial direction (from N- to C-terminal). The CRAC domain starts at the N-terminus with a Leu (L) or Val (V) residue, followed by a segment comprising 1 to 5 residues. The segment continues with a mandatory Tyr (Y) residue, a segment comprising 1 to 5 residues, and ends with a basic Lys (K) or Arg (R) residue, (L/V)-$X_{1-5}$-(Y)-$X_{1-5}$-(K/R) [32]. CRAC domains interact with cholesterol in the cytoplasmic leaflet of the membrane. These motifs belong to some transmembrane protein domains and have a favorable fit for cholesterol binding [21].

Another newly recognized cholesterol-binding sequence, known as CARC domain, has recently been described; it has almost the same sequence as CRAC but runs in the opposite direction (from C- to N-terminal) and has a central aromatic amino acid, which can be either Tyr or Phe, (K/R)-$X_{1-5}$-(Y/F)-$X_{1-5}$-(L/V) [33]. In both cases, the van der Waals forces and H-bonds between the Y residue and the OH group of the cholesterol molecule participate in the interaction between cholesterol and the CRAC/CARC-containing protein [21].

Both CRAC and CARC motifs represent oriented amino acid sequences, with an apolar amino acid residue at one terminal and a highly polar, positively charged basic residue at the other end [32]. In some cases, these amino acidic sequences are located in the same transmembrane segment but CARC is associated with the outer leaflet and CRAC is located at the inner leaflet of the plasma membrane. There are several examples of membrane receptors that have such a dual interaction with CRAC and CARC. These receptors include neuropeptide FF receptor, metabotropic glutamate receptor 5, GABA type B receptor subunit 2, CB1 receptor, 5-HT7 receptor, adenosine receptor A1, VIP receptor 1, prolactin-releasing peptide receptor, oxytocin receptor, TRVP1 receptor, and corticotrophin-releasing factor receptor 1 [34].

The alignment of the dipolar components of phospholipids at the water interface generates a membrane-internal potential known as the dipole potential [35], with an estimated magnitude around 280 mV [36]. It has been suggested that the dipole potential modulates the translocation rates of ions across lipid membranes [37]. The activities of $Na^+/K^+$-ATPase and phospholipase A2 increase with increasing dipole potential [38, 39]. Cholesterol intercalation strongly affects the dipole potential; cholesterol removal generates a reduction close to 50 mV [40]. Restoration of cholesterol levels reverses this reduction in natural membranes [41]. However, cholesterol levels have negligible effects on the dipole

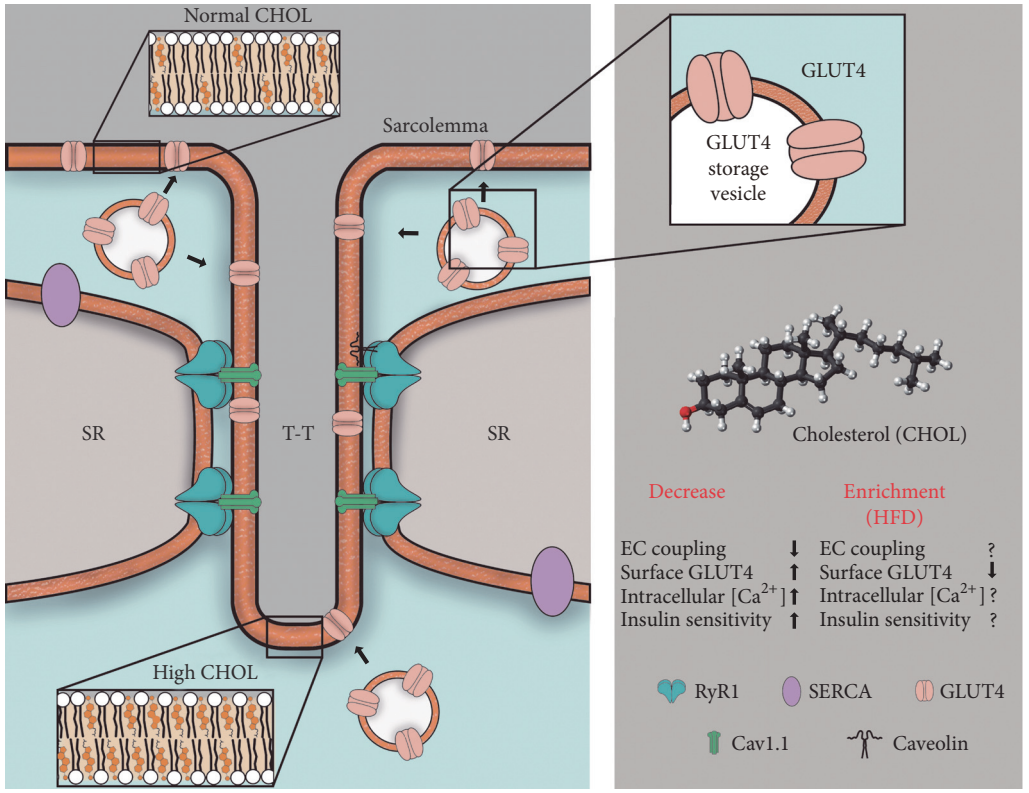


Figure 1: Schematic figure showing the T-tubule (T-T) system and its high cholesterol content compared with the surface membrane (sarcolemma, see inserts). Note the main proteins of the EC coupling complex and the sites of GLUT4 translocation. In response to insulin, the GLUT4 transporters translocate mainly to the T-tubule system, where EC coupling takes place, and also to the surface membrane region. Modifications of cholesterol content affect both EC coupling and GLUT4 mediated glucose transport in skeletal muscle. ↑: increment; ↓: decrease; ?: unknown effect.

potential exhibited by polyunsaturated membranes [42]. Currently, there is no information to our knowledge regarding the effects of obesity, metabolic syndrome, or cholesterol lowering treatments on dipole potential.

The manipulation of membrane cholesterol content is useful to study its effects on cellular physiology. The most common and simple approach has been to treat membranes with cyclodextrins, a family of cyclic compounds, which have a central hydrophobic pocket that extracts cholesterol from the cell membranes [43]. To date, however, only few studies, some of which are presented below, have provided information on how cholesterol modulation affects skeletal muscle protein functionality.

## 3. T-Tubule Structure and Composition

The T-tubule system of skeletal muscle is an intricate network composed of narrow tubules of around 40–85 nm in diameter which originate from deep invaginations of the surface plasma membrane [44]. This membrane system represents around 80% of the total plasma membrane surface of skeletal muscle [45]. Early studies using differential centrifugation reported that mammalian T-tubule membranes have a high proportion of cholesterol and sphingolipids [9] which resemble the composition of cholesterol-enriched lipid rafts domains [11]. Moreover, electron paramagnetic resonance assays indicate that at physiological temperature the lipid phase of T-tubule membranes is remarkably less fluid than that of other mammalian plasma membranes [10], resembling the low fluidity of thermophilic bacterial membranes. The T-tubules contain many proteins involved in EC coupling and other signaling processes; membrane cholesterol levels modulate the function of several of these proteins, including Cav1.1 [46], caveolin-3 [47], and $Na^+/K^+$- ATPase [48].

## 4. T-Tubule Cholesterol Levels Influence the EC Coupling Process

During muscle contraction, the action potential elicited at the neuromuscular junction propagates through the surface membrane into the T-tubule network, which is a key element in the EC coupling process [7]. The T-tubule membrane is flanked by two junctional sarcoplasmic reticulum (SR) membranes, forming structures known as triads which allow the direct interaction of the T-tubule residing protein Cav1.1 with Ryanodine receptor type 1 (RyR1), an integral SR membrane protein [7]. During EC coupling, Cav1.1 works as voltage sensor and commands transient RyR1 opening in response to membrane depolarization; the subsequent $Ca^{2+}$ release from the SR produces muscle contraction [6].

It has been proposed that membrane cholesterol can modulate Cav1.1 activity [46]. In mechanically skinned fibers, in which the surface membrane is removed leaving the T-system intact, cholesterol depletion with M$\beta$CD induces T-tubule system depolarization without changes in its integrity [49]. Moreover, cholesterol depletion from intact fetal skeletal muscle using M$\beta$CD decreases Cav1.1 $Ca^{2+}$ currents and shifts their voltage dependence to more positive values; it is important to remark that M$\beta$CD saturated with cholesterol does not affect Cav1.1 function [46].

We have shown that partial cholesterol removal from dissociated adult fibers inhibits EC coupling and depolarizes the fibers [11]. Cholesterol removal with M$\beta$CD also increases the resting $Ca^{2+}$ level, apparently by stimulating $Ca^{2+}$ release from internal stores [11], suggesting that plasma membrane cholesterol might modulate functional interactions of the T-tubule membranes with the intracellular $Ca^{2+}$ stores.

Caveolin-3 is a cholesterol-binding protein [47], which directly interacts with Cav1.1 [50] and modulates its $Ca^{2+}$ channel function [51]. A recent report showed that cholesterol also modulates cardiac EC coupling and contraction [52]. Collectively, these results raise the possibility that cholesterol modulates striated muscle EC coupling by direct interaction with the protein complex engaged in this process and indirectly by modulating the lipid environment and accessory proteins such as caveolin-3.

## 5. Cholesterol: A Novel Regulator in GLUT4 Translocation

Skeletal muscle is a major contributor to whole-body metabolism; it is the largest insulin-sensitive tissue in the body, which makes it a key locus for insulin-stimulated glucose uptake [53]. In humans under euglycemic, hyperinsulinemic conditions, around 80% of body glucose uptake occurs in skeletal muscle, which represents a central component of glucose homeostasis [12]. In addition, this tissue is also an important consumer of fatty acids, which together with glucose constitute the principal energy sources of skeletal muscle [54].

The glucose transporter GLUT4 is one of fourteen members of the glucose transport family and displays high affinity for glucose [55]. Patients with IR and type 2 diabetes mellitus (T2DM) show defects in insulin-stimulated glucose metabolism in skeletal muscle. These alterations have been attributed to a disturbance in glucose transport, resulting mainly from dysregulated GLUT4 trafficking, the predominant insulin-sensitive glucose transporter expressed in skeletal muscle [56–58]. Both insulin and muscle contraction induce GLUT4 translocation to the skeletal muscle plasma membrane [59], presumably by engaging separate signaling pathways. The increase of surface GLUT4 occurs as a result of translocation of GLUT4-containing intracellular vesicles to the plasma membrane [60].

In order to enter into the muscle cell, glucose delivered by blood flow must be transported across the surface membrane and the T-tubule membranes into the cytoplasm, where it is trapped by hexokinase II action [58]. Insulin binding to the insulin receptor (InsR) promotes a conformational change in the receptor which leads to the transphosphorylation in tyrosine residues of its cytoplasmic $\beta$ subunits [61]. The activated InsR phosphorylates, among other proteins, the main InsR substrate (IRS) proteins, including IRS-1 and IRS-2. Tyrosine-phosphorylated IRS-1 and IRS-2 serve as docking sites for SH2 domain-containing proteins, such as class IA (p85/p110-type) phosphatidylinositol 3 kinase (PI3K) [62]. The activation of this lipid kinase increases phosphatidylinositol-3,4,5-trisphosphate (PIP3) levels at the inner face of the plasma membrane and recruits pleckstrin (PH) homology domain-containing proteins, which are essential for insulin-stimulated GLUT4 translocation and the ensuing glucose uptake [8]. The PH domain-containing Akt protein is a cytoplasmic serine-threonine kinase that plays a fundamental role in mediating insulin-stimulated GLUT4 translocation; Akt organizes several downstream molecules that involve successive steps, including AS-160 activation and Rab Family GTPases, which finally position GLUT4 in the plasma membrane and promote GLUT4-mediated glucose transport [13, 63]. The increase in plasma membrane GLUT4 occurs due to a large increase in the rate of GLUT4 exocytosis, coupled with a smaller decrease in the rate of GLUT4 endocytosis [60]. The continuous recycling of GLUT4 offers the flexibility to regulate both its exocytic and endocytic rates. In cultured adipose and muscle cells, insulin rapidly stimulates the rate of exocytosis of GLUT4 transporters [64]. However, there is limited information about the mechanisms that regulate GLUT4 endocytosis. Although insulin reduces GLUT4 endocytosis in adipose cells [65], it does not affect the rate of GLUT4 internalization in rat cardiomyocytes [66] or in skeletal muscle cell lines [67]. In adult skeletal muscle, GLUT4 accumulates in several intracellular compartments, and although it locates preferentially in perinuclear regions, it is also present in peripheral vesicles [68, 69]. Functional studies in adipocytes and skeletal muscle cell lines indicate that insulin-derived cellular signals promote GLUT4 translocation to the plasma membrane from a specialized compartment termed GLUT4 storage vesicles [60].

Various proteins including actin, actin dynamics, and microtubular motors intricately regulate the process of GLUT4 vesicle mobilization, tethering, docking, and fusion in response to insulin. GLUT4 is internalized via clathrin-mediated endocytosis or via cholesterol-dependent but clathrin-independent endocytosis [60]. However, there are few studies addressing the role of membrane cholesterol in GLUT4 traffic.

In adipocyte and skeletal muscle cell under basal conditions, around 5% of GLUT4 is present in the surface membranes; insulin stimulation increases its level to about 50% [70]. Early studies by nuclear magnetic resonance [71, 72] complemented with more recent reports have shown that GLUT4 translocation is defective in T2DM patients [8, 73]. The majority of GLUT4-containing vesicles do not move long distances but are depleted locally in the surface membrane or T-tubule regions [8, 74]. Moreover, analysis of GLUT4 translocation in insulin-resistant muscle showed that GLUT4 recruitment is affected primarily in the T-tubule region [8]. Muscles subjected to osmotic shock to dissociate the T-tubule connection with the surface membrane have their T-tubule

network with no access to insulin and glucose from the extracellular fluid [8, 75]. The dissociation of the T-tubule system reduces basal glucose transport by 50% and completely abolishes the insulin-induced increase in glucose transport [8], highlighting the critical role of the T-tubule system in insulin-mediated glucose transport [75]. Nevertheless, it is not clear why the dissociation of the T-tubule system inhibits insulin-dependent glucose transport through the surface membrane.

The mammalian T-tubule membranes are highly enriched in cholesterol and sphingolipids [9] endowing them with a rigid lipid environment with highly restricted membrane fluidity properties [10]. GLUT4 translocation occurs at cholesterol-rich microdomains [76], suggesting that changes in cholesterol levels modulate insulin-stimulated GLUT4 exocytosis. In fact, current evidence supports the hypothesis that increased plasma membrane cholesterol levels have a key role in the impaired GLUT4 traffic observed in IR and T2DM, since glucose-intolerant animal models and humans accumulate cholesterol in their skeletal muscle membranes [14]. Recently, using HFD-fed animals as a model of IR, we reported that triad-enriched fractions isolated from the skeletal muscle of these obese animals have around 30% higher cholesterol content than triads from lean control animals [15]. In addition, muscle fibers isolated from HFD-fed obese mice show a 40% decrease in insulin-stimulated glucose uptake rates compared to fibers from lean control mice. In HFD-fed mice, four subcutaneous injections of M$\beta$CD improved their defective glucose tolerance test, normalized their high fasting glucose levels, and restored insulin-stimulated glucose uptake in adult skeletal muscle fibers [15]. In addition, preincubation of isolated muscle fibers with relatively low concentrations of M$\beta$CD increases both basal glucose uptake and insulin-induced glucose uptake in fibers from controls or HFD-fed mice. In muscle fibers from HFD-fed mice, M$\beta$CD improves insulin sensitivity and Indinavir, a GLUT4 antagonist, prevents the stimulatory effects of M$\beta$CD on glucose uptake [15]. In addition, M$\beta$CD increases membrane GLUT4 content and elicits intracellular calcium signals that are inhibited by Dantrolene, an agent which blocks the functional interaction of Cav1.1 and RyR1 [77] and reduces M$\beta$CD-mediated glucose uptake [15]. Interestingly, L6 myotubes cultured in a hyperinsulinemic medium resembling in vivo conditions that promote the progression of insulin resistance display an increase in membrane cholesterol [78]. The increased cholesterol levels in the plasma membrane of L6 myotubes cells may impair insulin action through a loss of cortical filamentous actin (F-actin), leading to defective GLUT4 regulation by insulin and an increase in the hexosamine biosynthesis pathway [14].

Treatment with chromium picolinate, a compound that removes membrane cholesterol, activates GLUT4 trafficking and enhances insulin-stimulated glucose transport via a cholesterol-dependent mechanism [79]. In addition, chromium supplementation significantly improves fasting glycemia in T2DM patients [80]. Treatment with M$\beta$CD reversibly decreases the cholesterol content of membranes in a dose-dependent manner, leading to increased GLUT4 incorporation into the plasma membrane of L6 myotubes [14]. More recently, it has been reported that AMP-activated protein kinase (AMPK) enhances insulin-stimulated GLUT4 regulation via lowering membrane cholesterol levels [78]. All together, these reports suggest a novel aspect of GLUT4 regulation by cholesterol in skeletal muscle.

## 6. Concluding Remarks and Perspectives

The cholesterol content of the T-tubule membrane in skeletal muscle is significantly higher than the levels present in the plasma membrane of most cells. A decrease in membrane cholesterol with cholesterol-removing agents alters muscle function, affecting both the excitation-contraction coupling process and glucose transport mediated by GLUT4 translocation to the T-tubule membrane (Figure 1). In conditions such as high-fat diet-induced obesity, the cholesterol content of T-tubule membranes increases even further, making it likely that pathological conditions, such as insulin resistance and type 2 diabetes, entail increased T-tubule cholesterol content. Accordingly, restoring membrane cholesterol levels in the T-tubule system via increasing surface GLUT4 levels in response to insulin may constitute an interesting therapeutic target to ameliorate insulin resistance.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this review article.

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*Research Article*

# Effect of Human Myotubes-Derived Media on Glucose-Stimulated Insulin Secretion

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Fasting to postprandial transition requires a tight adjustment of insulin secretion to its demand, so tissue (e.g., skeletal muscle) glucose supply is assured while hypo-/hyperglycemia are prevented. High muscle glucose disposal after meals is pivotal for adapting to increased glycemia and might drive insulin secretion through muscle-released factors (e.g., myokines). We hypothesized that insulin influences myokine secretion and then increases glucose-stimulated insulin secretion (GSIS). In conditioned media from human myotubes incubated with/without insulin (100 nmol/L) for 24 h, myokines were qualitatively and quantitatively characterized using an antibody-based array and ELISA-based technology, respectively. C57BL6/J mice islets and Wistar rat beta cells were incubated for 24 h with control and conditioned media from noninsulin- and insulin-treated myotubes prior to GSIS determination. Conditioned media from insulin-treated versus nontreated myotubes had higher RANTES but lower IL6, IL8, and MCP1 concentration. Qualitative analyses revealed that conditioned media from noninsulin- and insulin-treated myotubes expressed 32 and 23 out of 80 myokines, respectively. Islets incubated with conditioned media from noninsulin-treated myotubes had higher GSIS versus control islets ($p < 0.05$). Meanwhile, conditioned media from insulin-treated myotubes did not influence GSIS. In beta cells, GSIS was similar across conditions. In conclusion, factors being present in noninsulin-stimulated muscle cell-derived media appear to influence GSIS in mice islets.

## 1. Introduction

Regulation of insulin secretion is critical for understanding glucose homeostasis under (patho)physiological conditions. Such regulation is particularly complex in the transition from fasting to postprandial state on which its secretion must be tightly adjusted to insulin needs, so tissue glucose supply is assured while hypo- and hyperglycaemia are prevented.

Skeletal muscle plays an active role controlling circulating glucose concentration. On the one hand, this tissue is a major site of insulin-stimulated glucose disposal [1], which is crucial in the adaptation to the rapid increase in glucose flux into

circulation after a meal. On the other hand, skeletal muscle might also influence insulin secretion by interacting with pancreas through humoral factors [2–6]. Interestingly, two skeletal muscle-specific genetic mice models characterized by altered glucose metabolism (a knock-out for peroxisome proliferator-activated receptor gamma coactivator 1-alpha [PGC1-alpha$^{-/-}$] [5] and a transgenic for muscle-specific RING-finger 1 protein [Murf1] [6]) present abnormal in vivo insulin secretion. Such findings have been considered to be indicative of a putative endocrine factor mediating a muscle-pancreas crosstalk.

In humans, support for this hypothesis comes indirectly from in vivo studies aimed at assessing the effect of insulin on its secretion [7, 8]. These studies found higher glucose-stimulated insulin secretion (GSIS) following a 4 h isoglycemic-hyperinsulinemic clamp when compared with a 4 h saline infusion [7, 8]. This finding was in line with an in vitro study reporting higher insulin secretion after insulin stimulation [9]. However, most of the in vitro studies have shown that insulin inhibits its own secretion [10–12]. Thus, enhanced GSIS after insulin versus saline infusion may have an alternative explanation. We propose that the drastic increase in skeletal muscle glucose disposal after insulin (versus saline) infusion may trigger the release of a humoral factor having influence on insulin secretion [2]. In this regard, we recently highlighted an inverse, insulin sensitivity-independent association between 24 h whole-body carbohydrate oxidative disposal (an indirect marker of skeletal muscle glucose metabolism) and 24 h insulin secretion in humans [13]. Such finding may suggest that glucose disposal, particularly at the level of its oxidation, may drive insulin secretion [14]. Further support to this hypothesis underlies on the observation that skeletal muscle-specific PGC1-alpha$^{-/-}$ versus wild-type mice had impaired in vivo but normal in vitro (isolated islets) GSIS. This observation suggests that a humoral factor is mediating an in vivo interaction between skeletal muscle and pancreas.

The notion that a muscle-pancreas crosstalk exists has been fairly accepted [15, 16], although the effect of muscle-released factors including myokines on pancreatic insulin secretion has not been proven in in vivo animal or human models. A reasonable, feasible, and first-step approach to test this hypothesis is to evaluate the effect of conditioned media from insulin- and noninsulin-treated human myotubes on GSIS from isolated pancreatic islets or beta cells. This model was used by Bouzakri et al. [3], who observed increased GSIS in rat and human primary beta cells incubated with conditioned media from nonstimulated human muscle cells.

We also included the determination of 5 proteins in the conditioned media as candidate mediators. We selected IL6, IL8/CXCL8, MCP1/CCL2, fractalkine/CX3CL1, and RANTES/CCL5, which are known to be released from muscle cells [3, 17, 18]. Some of them (IL6, fractalkine/CX3CL1, and RANTES/CCL5) have been also shown to influence in vitro insulin secretion [4, 5, 19, 20]. In turn, we recently reported that circulating IL8/CXCL8 concentration directly associates with in vivo insulin secretion in humans [21].

## 2. Methods

*2.1. Experimental Design.* Human primary myotubes were incubated for 24 h with or without 100 nmol/L insulin [22]. Then, conditioned media were collected and stored at −80°C for later analysis, which included (i) characterization of the myokine profile and (ii) incubation of pancreatic mice islet and rat beta cells to evaluate basal insulin secretion and GSIS. In parallel, insulin-induced metabolic changes at the level of glycogen content and synthesis, glucose oxidation, and extracellular lactate concentration were assessed.

*2.2. Myotubes Differentiation.* Skeletal muscle cells were obtained from *vastus lateralis* biopsies of lean healthy volunteers (4 males, 24 ± 1 years, 23 ± 1 kg/m$^2$, and 85 ± 2 mg/dL fasting blood glucose). All volunteers gave written informed consent and the protocol was approved by an institutional ethics committee of the Toulouse Hospital (number 0816302). Studies were performed according to the latest version of the Declaration of Helsinki and the Current International Conference on Harmonization guidelines. The clinical study was registered at Clinicaltrials.gov NCT01083329 and EudraCT 2009-012124-85.

Myoblasts were isolated and grown as previously described [23]. Briefly, myoblasts were isolated by trypsin digestion, preplated on an uncoated Petri dish for one hour to remove fibroblasts, and subsequently transferred to T-25 collagen-coated flasks in Dulbecco's modified Eagle's medium (DMEM) low glucose (5.5 mmol/L) supplemented with 10% foetal bovine serum (FBS) and growth factors (human epidermal growth factor, BSA, dexamethasone, gentamicin, fetuin, and amphotericin B [Fungizone, Invitrogen]). Cells were pooled and grown at 37°C and 5% $CO_2$. Differentiation of myoblasts into myotubes was initiated at approximately 90% confluence by switching to alpha-minimum essential medium (alpha-MEM), Glutamax™ supplement with antibiotics, 2% FBS, and fetuin. The medium was changed every other day, and cells were grown up to 5-6 days.

*2.3. Myotube-Conditioned Media Generation.* Myotubes were incubated for 24 h with/without 100 nmol/L recombinant human insulin (Sigma-Aldrich) in alpha-MEM, Glutamax supplement, without FBS. At the end of the treatment, conditioned media were collected and stored at −80°C until utilization. Once thawed on ice, conditioned media were centrifuged at 14000*g*, 10 min at 4°C to eliminate any cell debris. Cell death was assessed by chemiluminescent quantification of adenylate kinase activity (ToxiLight, Lonza Group Ltd., Basel, Switzerland) in conditioned media from noninsulin- and insulin-treated myotubes.

*2.4. Total Glycogen Assay.* After 24 h with/without 100 nmol/L insulin, total glycogen content was determined using an enzymatic method (amyloglucosidase [Sigma-Aldrich]) and the glucose amount obtained was quantified using a commercial kit (DiaSys "Glucose GOD FS"). All assays were performed in triplicate and were normalized to protein amount (BCA Protein Assay kit, Pierce).

*2.5. Glycogen Synthesis Assay.* Myotubes were preincubated with glucose- and serum-free alpha-MEM for 90 min and then exposed to DMEM supplemented with D[U-$^{14}$C]glucose (1 $\mu$Ci/mL; PerkinElmer) with/without 100 nmol/L insulin for 3 h. After incubation, glycogen synthesis was determined as described previously [22, 23]. All assays were performed in triplicate and normalized to protein amount (BCA Protein Assay kit, Pierce).

*2.6. Glucose Oxidation Assay.* Myotubes were preincubated with glucose- and serum-free alpha-MEM for 90 min. This incubation was followed by a 3-hour incubation with D[U-$^{14}$C]glucose (1 $\mu$Ci/mL) and 5.5 mmol/L of nonlabelled (cold) glucose with/without 100 nmol/L insulin. After incubation, $^{14}CO_2$ was measured as previously described [22, 23]. All assays were performed in triplicate and normalized to protein amount (BCA Protein Assay kit, Pierce).

*2.7. Extracellular Lactate Determination.* Lactate was measured in myotube supernatant after 24 h treatment with/without insulin using a commercial kit (Lactate PAP, Biomérieux, France). All assays were performed in triplicate and normalized to protein amount (BCA Protein Assay kit, Pierce).

*2.8. Myokine Concentration in Conditioned Media.* In conditioned media from myotubes treated for 24 h with/without 100 nmol/L insulin, quantitative determination of 5 a priori selected cytokines/chemokines was performed by using multiplex analysis (Luminex, R&D System for IL6, IL8/CXCL8, RANTES/CCL5, and MCP1/CCL2) or ELISA (for fractalkine/CX3CL1; R&D System).

Further analysis included qualitative determination of 80 proteins through a membrane-based antibody array (C-series AAH-CYT-5, from Raybiotech®). Membranes were revealed using a blot scanner (C-Digit®, LI-COR) and spots densitometry was measured using the Image Studio™ software, following the manufacture instructions.

*2.9. Murine Pancreatic Islets and $\beta$ Cell Isolation.* Male C57BL6/J mice were used for all mouse islets experiments and Wistar rats for all rat beta cells experiments. Animals were housed in a temperature- and light-controlled room and were allowed to consume standard chow and water ad libitum. All mice experiments were carried out in accordance with the National Research Council (NRC) Publication Guide for Care and Use of Laboratory Animals (copyright 1996, National Academy of Science) and approved by the Ethics Committee for Animal Welfare from the School of Medicine of the Pontifical Catholic University of Chile. Rat experiments were carried out according to protocols approved by the State Commissioner on Animal Care. Adult 8-week old mice and 150–200 g rats were anesthetized with a mix of ketamine: xylazine (0.18 mg : 0.012 mg per gram of animal) by intraperitoneal injection. Pancreas was perfused with collagenase (0.21 mg/mL of Liberase TL [Roche] for mice and 0.90 mg/mL of collagenase [Sigma] for rats) through the common bile duct prior to euthanasia (by incision of the chest cavity to produce a bilateral pneumothorax). After verification of death, pancreas was removed out of the animal. Islets were isolated after pancreas digestion (37°C for 14 min), followed by Histopaque® 1077 (Sigma) density gradient separation and handpicked purification. Mice islets were cultured until the next day in RPMI 1640 medium containing 11.2 mmol/L glucose, 10% FBS, 110 $\mu$g/mL sodium pyruvate, and antibiotics (110 U/mL penicillin and 110 $\mu$g/mL streptomycin). Rat islets were trypsinized and beta cells were purified using a fluorescence-activated cell sorter (FACS), by autofluorescence to yield a population of more than 95% beta cells. Sorted rat beta cells were washed in 10 mL sterile DMEM (GIBCO, Invitrogen) containing 11.2 mmol/L glucose, 10% FCS, 110 $\mu$g/mL sodium pyruvate, and antibiotics (110 U/mL penicillin, 110 $\mu$g/mL streptomycin, and 50 $\mu$g/mL gentamycin). Aliquots of $3 \times 10^5$ cells were seeded in nonadherent Petri dishes and incubated overnight at 37°C. The next day, cells were resuspended at a density of $4 \times 10^5$ cells/mL in DMEM. Aliquots (50 $\mu$L) of this suspension were plated as droplets at the center of Petri dishes previously coated with 804G extracellular matrix [24] and incubated at 37°C until the next day.

*2.10. Murine Pancreatic Islets and Beta Cell Incubation with Myotube-Derived Conditioned Media.* Mice islets (5 per condition) and rat beta cells were incubated for 24 h with (i) unconditioned (control) media; (ii) conditioned media from noninsulin-treated myotubes; (iii) conditioned media from insulin-treated myotubes; and (iv) unconditioned (control) media with 100 nmol/L recombinant insulin. All media were supplemented with 10% SBF prior to islet or beta cell incubation.

*2.11. Glucose-Stimulated Insulin Secretion.* After 24 h incubation, mice islets and rat beta cells were washed for one or two hours (resp.) by incubating with Krebs Ringer HEPES buffer (KRH in mmol/L: 137 NaCl, 4.8 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 5 $NaHCO_3$, 16 HEPES, and 0.1% BSA) at 2.8 mmol/L glucose, and supernatant was eliminated. Then, mice islets and rat beta cells were incubated for one hour with KRH 2.8 mmol/L glucose (basal insulin secretion) followed by one-hour incubation at 16.7 mmol/L glucose (i.e., GSIS). All incubations were performed at 37°C and 5% $CO_2$. Supernatants were collected, while islets and beta cells were lysed in HCl-Ethanol. Supernatants and lysates were stored at −20°C for insulin determination. For mice islets, insulin was determined by ELISA (Merck-Millipore) by RIA for rat beta cells. Insulin secretion is expressed as a percentage of the total content. All experiments were run in triplicate.

*2.12. Quantitative Real-Time PCR of Protein Receptors in Mice Pancreatic Islets.* Two independent pools of at least 400 islets were obtained from ~10 mice each. Islets were incubated overnight in complete RPMI 1640 medium as described above and stored at −80°C in lysis buffer with 1% 2-mercaptoethanol (Life Technologies) for later extraction and analysis. Total RNA was isolated using the PureLink™ RNA Mini Kit (Life Technologies), treated with DNase (on column

Table 1: Sequences of forward and reverse primers used for PCR analyses.

| Gene | Forward ($5' \rightarrow 3'$) | Reverse ($5' \rightarrow 3'$) | Product length (bp) |
|---|---|---|---|
| *Cxcr2* | atccaccttgaattctcccatc | gcctcactttcttccagttca | 145 |
| *Il6ra* | cctctgacttccatttctgct | caagaatcctcgtccatgtcc | 118 |
| *Cxcr1* | tcccgtgatatttccaaattctttc | tcccgcacacaaggaac | 120 |
| *Ccr3* | ggtgcccactcatattcatagg | ctactggactcataaaggacttagc | 125 |
| *Ccr5* | gtgctgacataccataatcgatg | tgtcttcatgttagatttgtacagc | 147 |
| *Ccr1* | aggaactggtcaggaataatagc | caaaggcccagaaacaaagtc | 125 |
| *Gpr75* | tcaggatctcagctcacaga | agatagggtcactactgcga | 102 |
| *Ccr2* | actgaggtaacatattattgtcttcca | gagccatacctgtaaatgcca | 148 |
| *Cx3cr1* | cacaatgtcgcccaaataacag | tcccttcccatctgctca | 112 |
| *Cyclophilin* | tggagagcaccaagacagaca | tgccggagtcgacaatgat | 66 |

PureLink DNAse, Life Technologies), and quantified with a Nanodrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized with the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies), using 500 ng total RNA in a 20 $\mu$L reaction volume. Gene amplification was carried out using the Brilliant II SYBR® Green QRT-PCR AffinityScript kit (Agilent Technologies) on a Stratagene MX3000P thermocycler. Primers were purchased from Integrated DNA Technologies, Inc. (Table 1). Relative expression of mRNAs was determined after normalization against cyclophilin as an internal reference and calculated by the $2^{-\Delta\Delta Ct}$ method.

*2.13. Statistical Analyses.* Unless stated otherwise, all data are expressed as mean ± SEM of multiple experiments. All statistical comparisons were done by two-tailed *t* tests or two-way ANOVA with Tukey post hoc test, as appropriate. Values of $p < 0.05$ were considered significant. Statistical analyses were performed using GraphPad Prism 6.0 for Windows (GraphPad Software Inc., San Diego, CA).

## 3. Results

*3.1. Myotubes Glucose Metabolism.* As expected, insulin increased myotubes glycogen content after 24 hours by 1.6 ± 0.3-fold ($p < 0.001$) and its synthesis over 3 hours by 2.3 ± 0.9-fold ($p < 0.002$) (Figures 1(a) and 1(b)). However, glucose oxidation and lactate concentration remained similar when compared with nontreated myotubes (Figures 1(c) and 1(d)).

*3.2. Myokine Content in Conditioned Media from Human Myotubes.* Conditioned media from noninsulin-treated and insulin-treated human myotubes showed less than 10% cell mortality relative to the cell lysate positive control (Figure 2). Four out of 5 selected myokines (IL6, IL8/CXCL8, MCP1/CCL2, and RANTES/CCL5) were found in both conditioned media (Figures 3(a)–3(d)), whereas fractalkine/CX3CL1 was not detected in any media. Conditioned media from insulin- versus noninsulin-treated myotubes had lower IL6 and IL8/CXCL8 concentration ($p < 0.05$; Figures 3(a) and 3(b)) but higher RANTES/CCL5 concentration ($p < 0.05$; Figure 3(d)). In turn, MCP1/CCL2 showed a borderline significant lower concentration in conditioned media from insulin-treated myotubes ($p = 0.06$; Figure 3(c)). Further analysis explored the presence of a larger number of proteins by using a qualitative protein array including 80 chemokines and cytokines. We detected 32 and 23 out of 80 proteins in conditioned media from noninsulin-treated and insulin-treated myotubes, respectively (see Figure S1 in Supplementary Material available online at https://doi.org/10.1155/2017/1328573). Importantly, 10 proteins that were detectable in conditioned media from noninsulin-treated myotubes became undetectable in media from insulin-treated myotubes. In turn, one protein turned detectable in response to insulin. A full list of the detected proteins before and after insulin treatment is shown in Table S1.

*3.3. Insulin Secretion in Murine Pancreatic Islets and Beta Cells Incubated with Myotube-Derived Conditioned Media.* Mice islets incubated with control media (with or without added recombinant insulin) or myotube-conditioned media (from noninsulin- and insulin-treated myotubes) had similar basal insulin secretion (Figure 4(a)). In addition, GSIS increased at similar extent in both control conditions (Figure 4(a)). However, islets incubated with noninsulin-treated myotube-conditioned media showed higher GSIS versus control (2.4 ± 0.4 vs. 1.5 ± 0.3%; $p < 0.05$. Figure 4(a)). In turn, islets incubated with conditioned media from insulin-treated myotubes had similar GSIS versus its respective control ($p = 0.71$) and also when compared with GSIS induced by conditioned media from noninsulin-treated myotubes ($p = 0.43$) (Figure 4(a)). In rat primary beta cells, insulin secretion (both at low and high glucose concentration) was not different across conditions ($p = 0.71$) (Figure 4(b)).

*3.4. Myokine Receptors mRNA Expression in Isolated Mice Islets.* Gene expression of selected myokine receptors was determined in isolated mice islets (receptor's and their ligands are listed in Table 2). Three out of 4 RANTES/CCL5 receptors were found in islets. Among them, GPR75 had the highest expression level followed by CCR1 and CCR5. In turn, IL6 and fractalkine/CX3CL1 receptors (IL6Ra and CX3CR1, resp.) were also expressed. Among

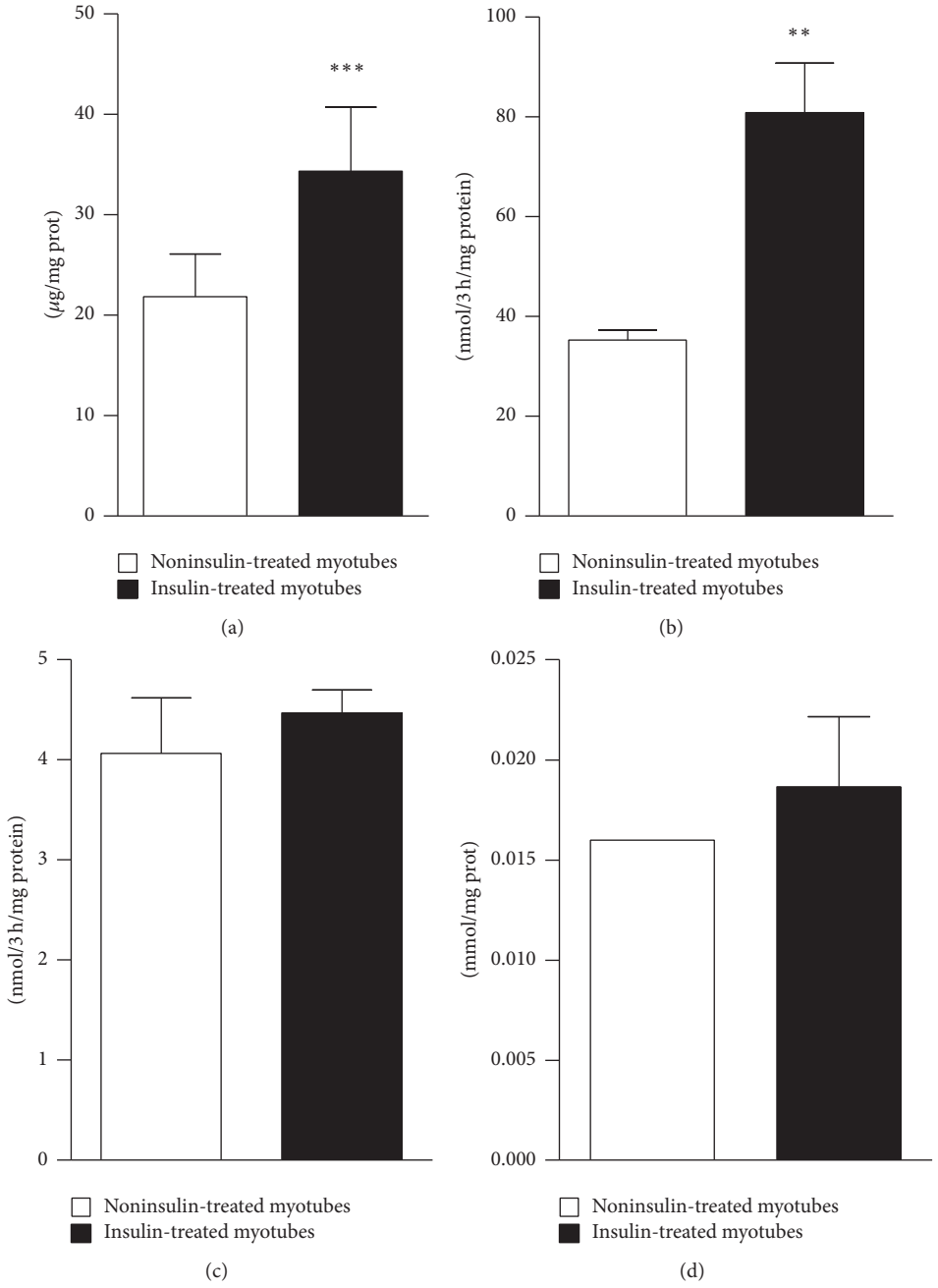


Figure 1: Myotubes metabolic changes in response to insulin. Glycogen content (a) and extracellular lactate content (d) were determined after 24 h with/without 100 nmol/L insulin treatment. Glycogen synthesis (b) and glucose oxidation (c) were determined after 3 h incubation with D[U-$^{14}$C]glucose with/without 100 nmol/L insulin. Mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$, two-tailed $t$ test.

all protein receptors measured, CXC3R1 showed the highest relative expression, whereas the common receptor for IL8/CXCL8, GRO/CXCL1, 2, and 3, NAP2/CXCL7 and ENA78/CXCL5 (CXCR1, CXCR2), MCP1/CCL2 (CCR2), and RANTES/CCL5 (CCR3) receptors did not have detectable expression (Figure 5).

## 4. Discussion

We found that human myotubes-derived media increased GSIS in isolated mice islets, whereas such effect was not observed in rat primary beta cells. In turn, conditioned media of insulin-treated myotubes did not change GSIS, both in isolated pancreatic islets and beta cells, when compared with control condition. Previously, Bouzakri et al. [3] detected increased GSIS in primary human and rat beta cells incubated with conditioned media from human myotubes. Those findings are consistent with our observation in isolated islets; however, we did not confirm that observation using primary rat beta cells. Eventually, different incubation times between studies (24 h in the present study versus 48 h) play a role. Alternatively, a potential interaction between beta and

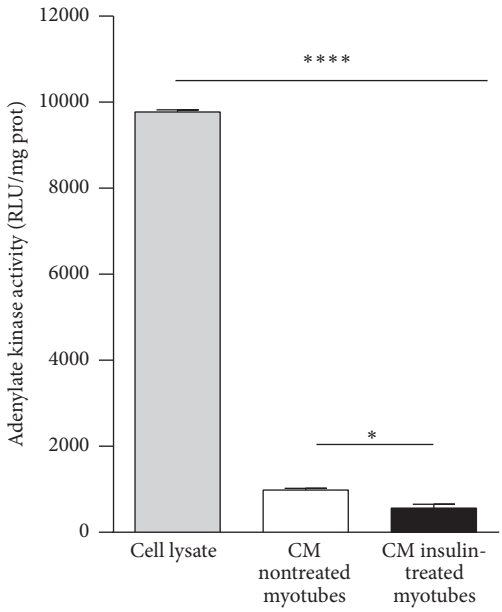


Figure 2: Myotube death in conditioned media from noninsulin- and insulin-treated myotubes. Cell death was assessed by chemiluminescent quantification of adenylate kinase activity normalized to total protein content. RLU, relative light units. Mean ± SEM. $^{*}p < 0.05$ and $^{****}p < 0.0001$, one-way ANOVA with Tukey post hoc test.

nonbeta cells may underlie the contrasting outcome between islets and beta cells. In this regard, Ellingsgaard et al. reported that IL6 enhanced GSIS in islets through increased alpha cell GLP1 secretion [4].

Our hypothesis that skeletal muscle interacts with pancreas regulating insulin secretion is mostly grounded on the classical inverse association between insulin sensitivity and its secretion [2]. Several factors found in conditioned media from muscle cells, including proteins (i.e., myokines), metabolites (e.g., lactate), and also vesicular-like structures (e.g., exosomes) [16], may mediate such putative muscle-pancreas crosstalk. Here, we focused on the presence of myokines in conditioned media.

Human myotubes conditioned media expressed 32 out of 80 detectable proteins. Apparently, none of the most highly expressed myokines (GRO/CXCL1, 2, and 3, MCP1/CCL2, IL8/CXCL8, TIMP1, TIMP2, NAP2/CXCL7, and ENA-78/CXCL5) according to our qualitative approach seemed to play a role in insulin secretion, considering that their receptors in isolated pancreatic islets showed undetectable mRNA expression under cultured conditions (i.e., RPMI 1640 at 11.2 mmol/l glucose). Certainly, alternative myokines as well as nonprotein factors may underlie our finding.

In this regard, a nonprotein factor representing a new cell-to-cell communication mode comes from exosomes. These are microvesicles carrying molecules such as microRNA that can reach distant organs and exert a (patho)physiological effect. Indeed, a recent study showed that skeletal muscle cells-derived exosomes when injected to mice targeted beta cells [15].

Table 2: Cytokines/chemokines receptors name and its ligands.

| Receptor name | Ligand |
|---|---|
| IL6R | IL6 |
| CXCR1 | IL8/CXCL8 GRO/CXCL1,2&3 NAP2/CXCL7 |
| CXCR2 | IL8/CXCL8 GRO/CXCL1,2&3 NAP2/CXCL7ENA78/CXCL5 |
| CCR3 | RANTES/CCL5 |
| CCR5 | RANTES/CCL5 |
| CCR1 | RANTES/CCL5 |
| GPR75 | RANTES/CCL5 |
| CCR2 | MCP1/CCL2 |
| CX3CR1 | Fractalkine/CX3CL1 |

Additional interest was focused on the role of insulin on myokine secretion and then the effect of conditioned media from insulin-treated myotubes on GSIS. As observed for other conditions (e.g., muscle contraction-induced glycogen depletion increases muscle IL6 secretion [25, 26]), the secretion of some myokines might be sensitive to changes in insulin-dependent muscle glucose metabolism. By using a widely accepted in vitro insulin concentration (100 nmol/l) to induce muscle cell glucose metabolism [22, 27–29], we found differences in three of the studied proteins expression in conditioned media from noninsulin- versus insulin-treated myotubes (IL6, IL8/CXCL8, and RANTES/CCL5).

Thus, we observed that an increase in glycogen synthesis/content was accompanied by reduced IL6 concentration in conditioned media of insulin-treated human myotubes. Eventually, IL6 and additional myokines may mediate the well-known inverse association between insulin sensitivity and its secretion [22, 25–28]. However, such distinct myokine pattern apparently did not lead to differential GSIS from isolated pancreatic or beta cells incubated with media from insulin-treated versus control myotubes.

In part, insulin induced only minor changes in myokine secretion and GSIS from islets incubated with nonconditioned media and conditioned media from insulin-treated myotubes was similar. It cannot be ruled out that 24 h insulin exposure (100 nmol/L) may have impaired muscle cell insulin sensitivity and altered any insulin-dependent difference in the pattern of myokine secretion. Thus, some factors might be mostly released during the period of preserved insulin sensitivity, and then their release will be altered as insulin resistance develops. Even so, those factors should still be found in the media independent of the change over time in their secretion pattern. It is also possible that the myokine concentration at which islets were exposed may have been insufficient to influence GSIS. Furthermore, our model based on different species (human myotube-conditioned media and murine islets/beta cells) may add complexity to interpretation. Still, an earlier study found comparable results when rat and human beta cells were treated with conditioned media from human myotubes [3]. Finally, our in vitro model might not successfully emulate

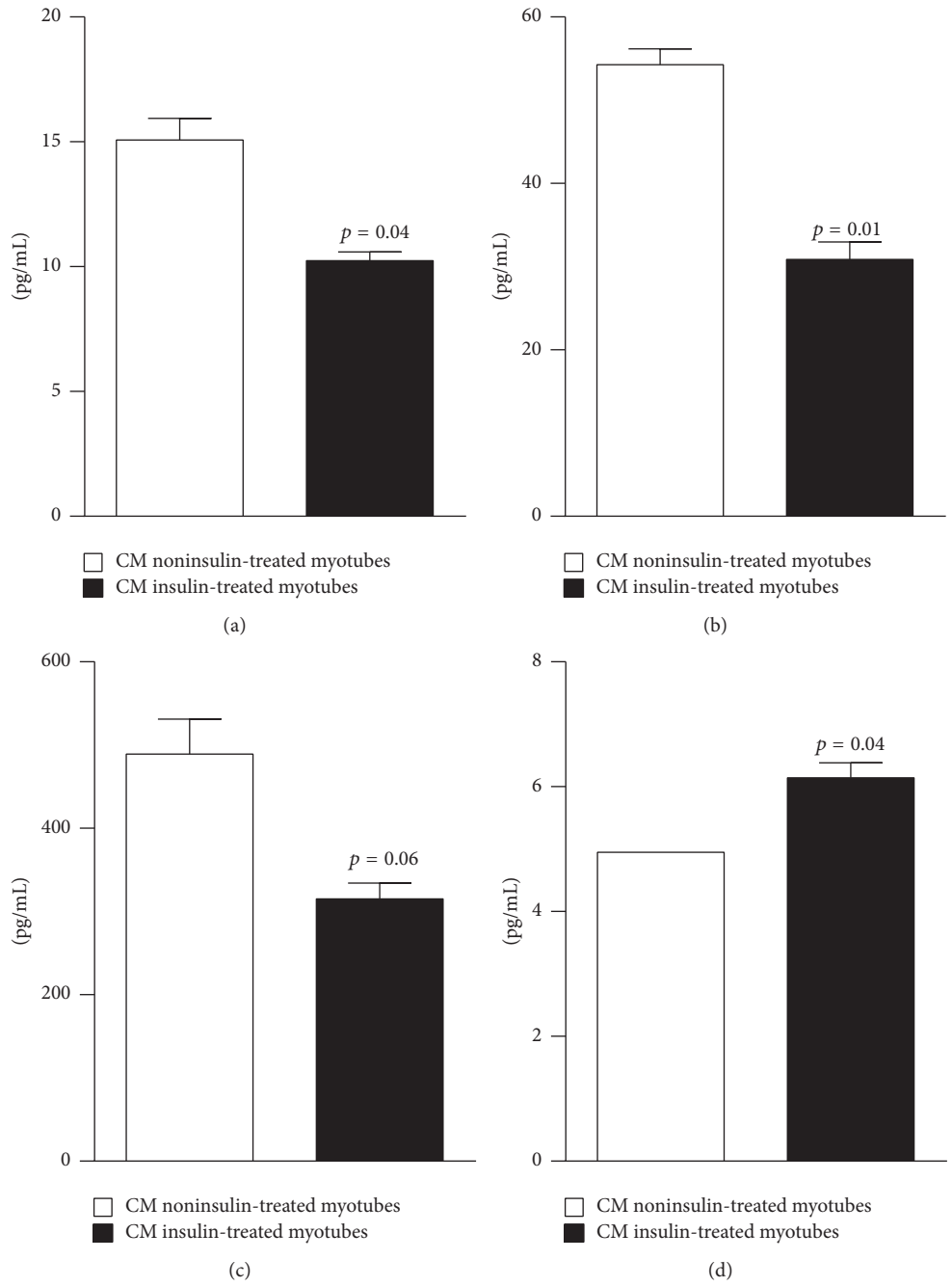


Figure 3: Myokine expression in conditioned media from noninsulin- and insulin-treated myotubes. IL6 (a), IL8/CXCL8 (b), MCP1/CCL2 (c), and RANTES/CCL5 (d) determined by multiplex in myotube-conditioned media from noninsulin- and insulin-treated myotubes. Mean ± SEM. Analysis by two-tailed *t*-student.

in vivo conditions on which insulin (directly or indirectly) appears to stimulate insulin secretion [8, 30]. Although such effect has been considered to be in line with in vitro evidence indicating that insulin directly enhances its own secretion [9], it must be considered that most in vitro studies found that insulin inhibits its secretion [10–12]. Thus, an alternative explanation based on humoral factors coming from insulin-sensitive tissues including skeletal muscle becomes appealing.

Taken together, these findings support the hypothesis that skeletal muscle-released factors can influence insulin secretion, which encourages the quest for identifying the nature of such factor as well as its potential in vivo role on glucose homeostasis. Any eventual physiological relevance of our findings appears independent of insulin and its action in muscle, at least under the context of our study. Thus, a mechanism mediating the interaction between insulin sensitivity and its secretion remains uncovered.

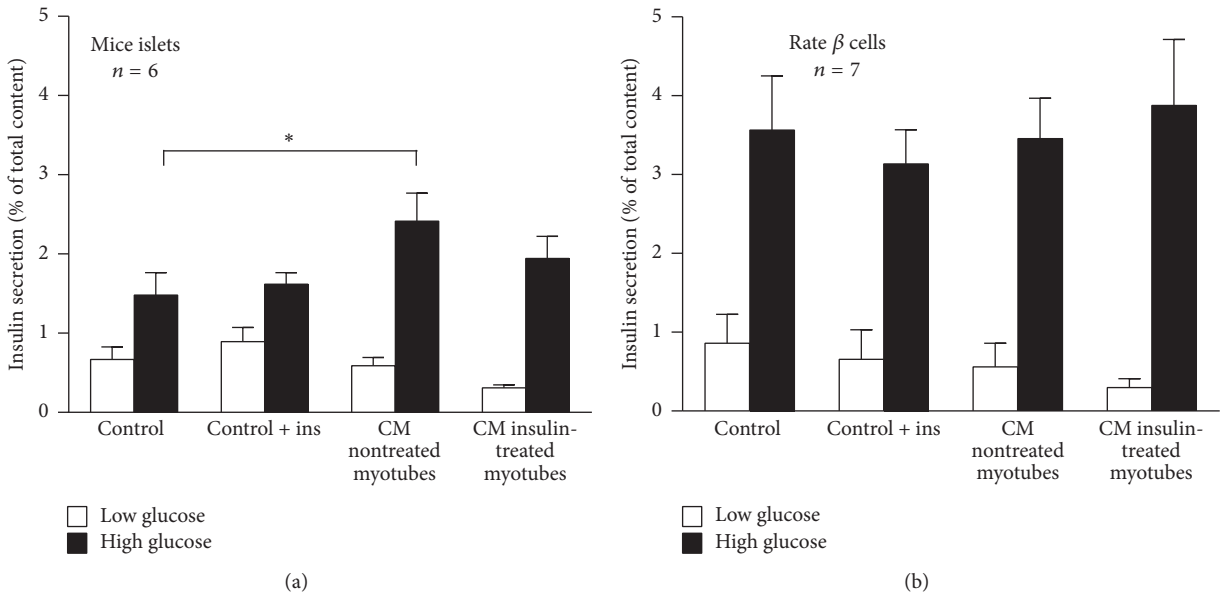


Figure 4: Effect of conditioned media from noninsulin- and insulin-treated myotubes on glucose-stimulated insulin secretion. Insulin secretion in isolated mice islets (a) and primary rat beta cells (b). Islets and beta cells were incubated with conditioned media from nontreated and insulin-treated myotubes for 24 h before hormone secretion assessment. Controls are unconditioned media without/with added insulin (100 nmol/L). Low glucose = 2.8 mmol/L glucose and high glucose = 16.7 mmol/L glucose. Mean ± SEM. $^{*}p < 0.05$, two-way ANOVA with Tukey post hoc test.

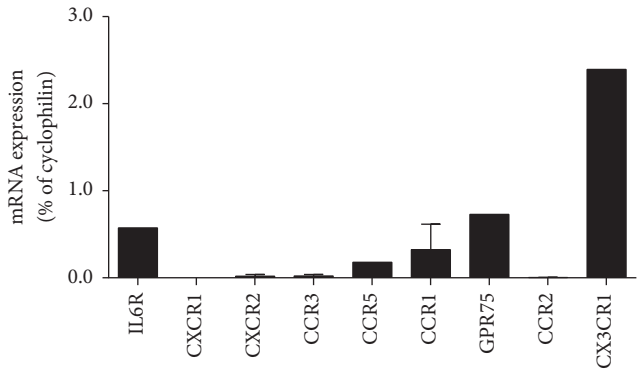


Figure 5: mRNA expression of myokine receptors in mouse islets. Quantification of myokine receptors mRNA in ~400 pooled islets expressed as a percentage of cyclophilin mRNA levels in the same samples. Mean ± SEM. $n = 2$.

## Competing Interests

The authors declare that there is no conflict of interests associated with this manuscript.

## Authors' Contributions

Maria Luisa Mizgier and Jose E. Galgani designed the study, analysed and interpreted the data, and drafted/revised and approved the final version of the manuscript. Luis Rodrigo Cataldo analysed qRT-PCR, interpreted the data, and revised and approved the final version of the manuscript. Juan Gutierrez, José L. Santos, Ariel E. Contreras-Ferrat, Paola Llanos, and Mariana Casas interpreted the data and revised and approved the final version of the manuscript. Cedric Moro collaborated in the generation of myotube-conditioned media, interpreted the data, and revised and approved the final version of the manuscript. Karim Bouzakri collaborated in the primary beta cells experiments, interpreted the data, and revised and approved the final version of the manuscript.

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