CELL MODELS FOR STUDYING MUSCLE INSULIN RESISTANCE

ČELIJSKI MODELI ZA PROUČAVANJE MIŠIĆNE INSULINSKE REZISTENCIJE

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Abstract

Type 2 diabetes is one of the most prevalent chronic diseases in the world today. Insulin resistance – a reduced responsiveness of tissues to insulin - is a hallmark of type 2 diabetes pathology. Skeletal muscle plays a pivotal role in glucose homeostasis - it is responsible for the majority of insulin-mediated glucose disposal and thus is one of the tissues most affected by insulin resistance.

To study the molecular mechanisms of a disease, researchers often turn to cell models – they are inexpensive, easy to use, and exist in a controlled environment with few unknown variables. Cell models for exploring muscle insulin resistance are constructed using primary cell cultures or immortalised cell lines and treating them with fatty acids, high insulin or high glucose concentrations. The choice of cell culture, concentration and duration of the treatment and the methods for measuring insulin sensitivity, in order to confirm the model, are rarely discussed. Choosing an appropriate and physiologically relevant model for a particular topic of interest is required in order for the results to be reproducible, relevant, comparable and translatable to more complex biological systems. Cell models enable research that would otherwise be inaccessible but, especially when studying human disease, they do not serve a purpose if they are not in line with the biological reality.

This review aims to summarise and critically evaluate the most commonly used cell models of muscle insulin resistance: the rationale for choosing these exact treatments and conditions, the protocols for constructing the models and the measurable outcomes used for confirming insulin resistance in the cells.

Keywords:
insulin resistance, cell model, muscle, cell line, treatment, concentration

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Diabetes is one of the most prevalent chronic diseases in the world, with 422 million adults suffering from it, the majority of who are affected by type 2 diabetes (1). The hallmark of type 2 diabetes pathology is insulin resistance – a reduced responsiveness of tissues to insulin. Insulin is one of the hormones that regulate glucose homeostasis - in the fed state it stimulates glucose uptake, catabolism and storage, which leads to lowering of blood glucose concentration. Symptoms of hyperglycaemia caused by dysregulation of insulin secretion and signalling are increased urine production and increased water and food intake. Liver, muscle and adipose tissue are most affected by insulin resistance. Skeletal muscle has a pivotal role in glucose homeostasis - it composes a majority of body mass, is very metabolically active, and is responsible for up to 80% of insulin-mediated glucose disposal (2).

Signal transduction can be interrupted on numerous levels – starting from the insulin receptor on the cell membrane, down to the main executor enzymes. To study the molecular mechanisms of a disease, researchers often turn to cell models – they are inexpensive, easy to use, and exist in a controlled environment with few unknown variables. Cell lines are clonal, which provides very little biological variability, as opposed to animal models and especially human subjects. This gives results that are highly reproducible, but limits their translatability to more complex systems.

Insulin resistance can be modelled in cells by various treatments: high glucose, high insulin, fatty acids, dexamethasone, pro-inflammatory cytokines, hypoxia. Each of these aims to mimic one of the proposed mechanisms of insulin resistance pathogenesis. This review aims to describe and compare cell models that are commonly used for studying skeletal muscle insulin resistance.

Cell models for exploring muscle insulin resistance are constructed using both primary cell cultures and immortalised cell lines. The adult skeletal muscle is composed of terminally differentiated multinuclear muscle fibers. Fully formed fibers can be isolated from rodent muscle and kept in culture, but only for several days (3). This is why most researchers use satellite cells for making muscle primary cell cultures; these mononuclear myogenic progenitors are easy to obtain (from isolated rodent muscle or human muscle biopsies), maintain in culture and differentiate into myotubes and fibers by mitogen withdrawal (serum deprivation) (3–6).

To skip the difficult and time-consuming process of isolating and culturing primary cells, most researchers use commercially available cell lines. They are able to proliferate in culture for a long period of time, compared to primary cultures which lose their proliferative capacity after a few rounds of mitosis. Commonly used muscle cell lines are C2C12 mouse myoblasts and L6 rat myoblasts. C2C12 is a fast-fusing subclone of C2 cells – a cell line derived from a leg muscle of an adult C3H mouse (4). L6 are derived from a neonatal rat hind limb preparation (4). Both cell lines are easily maintained in culture as proliferating myoblasts, and are readily differentiated by reducing the serum concentration in the medium from 10% foetal bovine serum to 2% horse serum (4).

When studying a cell disease model, one should consider the characteristics of a cell line. For example, C2C12 have a good signalling response to insulin stimulation, but a very mild response in terms of glucose uptake and glycogen synthesis (7). On the other hand, L6 cells are stated to be better for studying insulin signalling than C2C12, as their signalling response is more preserved and better...
representing of the wild type muscle – observations like these are rare in the published scientific literature, but can be found on on-line scientific forums (8). Immortalised cell lines have their own disadvantages – they can deviate from normal cells in cell metabolism and signalling, and have some neoplastic potential (3).

Rationale for insulin resistance models

Insulin resistance cell models can be constructed using various treatments that aim to mimic mechanisms of insulin resistance pathogenesis. Three of the models that are commonly found in the literature are fatty acid-induced, insulin-induced and high glucose-induced cell models.

In the first stages of diabetes pathogenesis, insulin resistance causes the pancreas to compensate by secreting more insulin, which leads to hyperinsulinemia. As is the case with most hormones, insulin downregulates its own signalling response in a negative feedback loop, so hyperinsulinemia leads to further worsening of insulin resistance, as shown by transgenic mice overexpressing insulin (9) and patients with primary insulinoma who develop an impaired response to insulin (10).

Elevated blood levels of lipids observed in patients with diabetes, obesity and metabolic syndrome are presumed to contribute or even be the leading cause of insulin resistance. High levels of plasma triglycerides correlate with lower insulin sensitivity in healthy subjects (11). Experimentally increasing plasma free fatty acids (FFA) leads to impaired insulin-mediated glucose disposal and insulin signalling in healthy human subjects (12, 13) and animals (14–16). Imbalance of lipid availability and utilisation results in ectopic fat deposition, like intramyocellular fat, which has a stronger correlation with lowered insulin sensitivity than plasma FFA level (17).

It has historically been considered that hyperglycaemia, as well as being the consequence of diabetes, also leads to insulin resistance and inhibition of insulin secretion. There are only few studies on human subjects (18, 19) and animal models (20, 21) that explore this, possibly because of the difficulty of causing isolated hyperglycaemia in vivo, and consideration of the subjects’ safety, but numerous in vitro studies have proven that high glucose concentrations lead to decreased insulin responsiveness of isolated tissues and cultured cells.

Modelling insulin resistance

Most studies that aim to model and study muscle insulin resistance in vitro use cells treated with fatty acids or high concentrations of insulin. To test the responsiveness of these cells to insulin, they are additionally subjected to a short insulin treatment – usually 15 to 30 minutes long – which is called an insulin challenge. This way, researchers can compare insulin sensitivity of control (untreated) and treated cells, both in the basal state, and after acute insulin stimulation.

The reference range of fasting plasma concentration of insulin is 12-150 pmol/L (2 - 25 µIU/mL) for healthy adult human subjects (22). Insulin concentration can reach 1200 pmol/L (200 µU/mL) during a glucose tolerance test (22). Treatment concentrations used for establishing insulin resistance cell models are almost 100-fold higher – most studies reviewed in this paper use a chronic (24 - 72 h) 100 nmol/L insulin treatment.

Fatty acid-induced insulin resistance is another approach to modelling insulin resistance in cells, which is commonly used for in vitro studies. Palmitic acid is the most common treatment used for constructing these models; concentrations range from 0.1 to 0.8 mmol/L, and treatment durations from 2 to 24 h, the most common being 16 and 24 h. The reference range of fasting plasma free fatty acids for healthy individuals is 0.3 - 1.1 mmol/L (8 - 31 mg/dL) (22), which means that the treatment concentrations used for cell models are in the range of the concentrations measured in vivo. Palmitic acid consists about 25% of plasma FFA (23).

Some in vitro studies use a combination of high glucose (25 mmol/L) and other treatments (24). The reference range for fasting plasma glucose is 4.1 - 5.6 mmol/L (74 - 100 mg/dL) (22). C2C12 cells (and other muscle cell lines) are commonly grown in high glucose DMEM, as recommended by the American Type Culture Collection (ATCC) (25), which contains 25 mmol/L glucose - a concentration analogue to severe hyperglycaemia. Negative control experiments are often done in high glucose medium as well, and this practice should be questioned, as this does not make a perfect control for insulin signalling studies. If studying the effects of high glucose on insulin resistance, researchers should have in mind that control (untreated) cells should be grown and studied in low glucose (4.5 mmol/L), or glucose free medium. Studies using insulin or glucose (or both) are far less numerous than studies using FFA.

When comparing concentrations of substances for in vivo and in vitro experiments, it is important to consider the pharmacokinetic properties of the substance – how it is absorbed, metabolised and excreted from the body. Considering that the substances discussed here are biological compounds, and are found in blood in the same form as in the cell medium (FFA are albumin-bound, glucose and insulin are free), we think that concentrations in cell medium should be as close as possible to the blood concentration. It would be ideal to test a wide range of concentrations, and choose the lowest one that causes the expected biological response to establish a cell model (in the case of insulin resistance models that would be lower insulin signalling pathway activity - phosphorylation of Akt, or lower glucose uptake, compared to control). Treatments used in in vitro studies are often chosen in order to induce a (more or less immediate) desired effect, without considering if the treatment concentration is comparable to that found in vivo. For example, insulin concentrations used for establishing cell models are much higher than plasma insulin concentrations. There is no
data on testing significantly lower insulin concentrations in vitro. It is possible that since the exposure of cells to elevated insulin in patients is prolonged, the same effects could not be achieved in several days in vitro. On the other hand, when testing the insulin response, researchers use a short (15 - 30 min) insulin treatment. It would be logical if this insulin treatment matched the postprandial plasma concentration of insulin. If anything, the concentration of insulin reaching muscle cells is expected to be lower than the plasma concentration, and the cells in culture are directly subjected to all the insulin in a small volume of cell medium. Paradoxically, the insulin concentration used for the insulin challenge is almost 100-fold higher than measured in vivo.

Reference ranges for healthy human subjects and treatment concentration and duration used in in vitro studies are compared in table 1.

Table 1. Plasma reference ranges for healthy human subjects and treatment concentrations and durations for cell models.

<table>
<thead>
<tr>
<th>glucose (mmol/L)</th>
<th>insulin (μmol/L)</th>
<th>fatty acids (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference range in vivo</td>
<td>Treatment concentration</td>
<td>Treatment duration (h)</td>
</tr>
<tr>
<td>4.1 - 5.6</td>
<td>12x10^{-9} - 150x10^{-9}</td>
<td>0.3 - 1.1</td>
</tr>
<tr>
<td>25</td>
<td>100x10^{-4}</td>
<td>0.1 - 0.8</td>
</tr>
<tr>
<td>24 - 72</td>
<td>2 - 24</td>
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Measuring insulin sensitivity

Insulin sensitivity can be measured by different methods – measuring the effect of insulin on cell metabolism, like glucose uptake and glycogen synthesis, or measuring the activity of the insulin signalling pathway. A decrease in these responses is interpreted as a sign of reduced insulin sensitivity.

Glucose uptake and metabolism

Glucose uptake measurement is considered a gold standard for measuring insulin sensitivity in insulin-dependent tissues like muscle. The most commonly used and the most sensitive method is measuring the uptake of radiolabeled \( ^2 \text{H} \) 2-deoxyglucose (2-DG), which is transported into the cell in the same manner as glucose, but cannot be further metabolised, so it accumulates inside the cell (26). The amount of 2-DG in cell lysates is measured by scintillation counting. An alternative approach, which is safer for the researcher but less sensitive, is measuring the uptake of the fluorescent 2-DG analogue, 2-NBDG (2-deoxy-2-\( ^-[(7\text{-nitro-2,1,3-benzoxadiazol-4-yl} \text{-amino}) \text{-D-glucose}] \) with a fluorescence plate reader or by flow cytometry (27). Glycogen synthesis measurement is done by incubating the cells with radiolabeled D-(U-C\(^3\)) glucose and measuring the rate of its incorporation into glycogen (28). It is not used as much as glucose uptake as a measure of insulin sensitivity, but it can be useful for studying insulin-stimulated glucose metabolism, especially in cells like hepatocytes, where glucose uptake is not insulin-dependent.

Studies showed that acute insulin stimulation (insulin challenge) increased glucose uptake for 20% in C2C12 (29), but in L6 cells this effect was more pronounced – the glucose uptake doubled (24, 30). Chronic insulin treatment (72 h) completely abolished the insulin-stimulated increase in glucose uptake in C2C12 cells (29). L6 cells treated with insulin, with or without high glucose, for 24 h showed an increase in basal glucose uptake, but the acute insulin-stimulated increase was significantly reduced (24, 30).

Similarly, palmitate decreased insulin-stimulated glucose uptake in a time- (31) and dose-dependent manner (32). In some of the studies, this decrease in glucose uptake was observed very early, after only 2 h of treatment (33). Insulin-stimulated glycogen synthesis in C2C12 myotubes was modest (2-fold, compared to an 8-fold increase in 3T3-L1 adipocytes), but was nonetheless completely abolished by palmitate treatment (7). This effect was also observed in primary culture of rat skeletal muscle, using a fairly low palmitate concentration (100 μmol/L) (34). Insulin signalling pathway activity

The other approach is to measure protein levels or phosphorylation states of insulin signalling pathway proteins by immunoblot. The insulin signalling pathway consists of two main branches – the PI3K/Akt pathway and the MAP kinase pathway. The PI3K/Akt pathway acts by changing the activity of enzymes by phosphorylation and in this way regulating glucose metabolism. These outcomes are easy to measure by immunoblot, and are thus studied extensively. A commonly used parameter of insulin signalling pathway activation is phosphorylation of protein kinase B (PKB, Akt) - one of the key proteins in the insulin signalling pathway. Akt has two major phosphorylation sites – Thr308 and Ser473, and phosphorylation of both is necessary for full activation (35, 36). It is not clear why most researchers choose to measure Ser473 phosphorylation, made by mammalian target of rapamycin complex 2 (mTORC2) (37), and not the Thr308 which is made by phosphoinositide-dependent kinase (PDK) (38), and is thus a more direct link in the signalling pathway.

Chronic insulin treatment reduced insulin-stimulated phosphorylation and expression of insulin receptor (24, 29, 39), and reduced insulin-stimulated insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation (24, 29). Total IRS-1 protein level was unaltered (29) or decreased (24) by insulin treatment. Further downstream in the signalling pathway, insulin together with high glucose
decreased phosphoinositide 3-kinase (PI3K) activity (24, 29) and insulin-stimulated Akt phosphorylation on both sites (24, 39). Similarly, palmitate decreased the insulin-stimulated phosphorylation of Akt, using various treatment concentrations and durations (7, 32–34, 40–42). Palmitate treatment also decreased insulin-stimulated phosphorylation of glycogen synthase kinase 3β (GSK3β) (7), a direct substrate of Akt.

**Figure 1.** Insulin and palmitate affect the activity of the insulin signalling pathway and other signalling proteins that regulate this pathway. IRS-1 – insulin receptor substrate 1; PI3K – phosphoinositide-3 kinase; PDK – phosphoinositide-dependent kinase; Akt – protein kinase B; GSK3β – glycogen synthase kinase 3β; mTORC1 – mammalian target of rapamycin complex 1; AMPK – adenosine monophosphate-activated kinase; p70S6K – S6 kinase; JNK - c-Jun N-terminal kinase; NF-κB - nuclear factor κB; PTP1B – protein tyrosine phosphatase 1B.

**Regulation of the insulin signalling pathway**

Apart from the insulin signalling pathway, several other pathways are being studied in the context of insulin resistance, for their potential role in the regulation of insulin sensitivity. Both chronic insulin and palmitate treatment increased Ser307 and Ser636/639 inhibitory phosphorylations of IRS-1 (30, 40). Insulin signalling is mainly regulated by Ser/Thr kinases or tyrosine phosphatases that inhibit IRS-1 - many of these proteins are also affected by palmitate and insulin treatment (figure 1).

Apart from being downstream signalling components of the PI3K-Akt signalling pathway, mTORC1 and its substrate S6 kinase (p70S6K) can negatively regulate insulin signalling by phosphorylating IRS-1 on serin and threonin residues (43–45). Palmitate inhibited AMP-activated protein kinase (AMPK), which lead to activation of mTORC1 and p70S6K. High insulin increased phosphorylation of mTOR and p70S6K, but did not change phosphorylation of AMPK (Thr172) (30).

 Intramyocellular lipid accumulation has been shown to induce inflammation and stress signalling in the muscle, which may negatively regulate insulin signalling. Palmitate increased the basal activation of the stress kinases JNK and p38 (39), increased levels of phosphorylated nuclear factor κB (NF-κB) and decreased total inhibitor of κB (IκB) (42). Palmitate treatment induced NF-κB activation and interleukine 6 (IL-6) expression and secretion in C2C12 cells (40). Also, L6 myotubes treated with high insulin and glucose had increased p38 MAPK phosphorylation (activation) and total protein content. On the other hand, JNK phosphorylation was not changed by the treatment (24). Protein tyrosine phosphatase 1B (PTP1B) is well-known for its impact on insulin sensitivity - by dephosphorylating tyrosine residues of the insulin receptor and IRS, it inhibits downstream signal transduction. Palmitate increased PTP1B mRNA levels, promotor activity and protein level (33, 46). This is caused by accumulation of ceramides and subsequent activation of the JNK-NFκB pathway (47).
Lipid Accumulation and Metabolism

A widely accepted mechanism by which palmitate induces insulin resistance is by leading to accumulation of lipid products, such as diacylglycerol (DAG) and ceramides (7) - fatty acids are precursors for the synthesis of DAG and ceramides, so they upregulate their synthesis by increasing substrate availability. Fatty acids may play an additional role by regulating the activity and transcription of the enzymes involved in lipid metabolism. Both DAG and ceramides inhibit insulin signalling by various mechanisms. For example, DAG activates novel protein kinases C (PKCs) (like PKCθ), which phosphorylate and inhibit TAG synthesis; and Pgc1α, that regulates fatty acid oxidation (40). Some authors report fairly low concentrations of palmitate in muscle cells; higher concentrations lead to cell death (33-35). In addition, DAG and ceramides inhibit insulin signalling by various mechanisms. For example, DAG activates novel protein kinases C (PKCs) (like PKCθ), which phosphorylate and inhibit TAG synthesis; and Pgc1α, that regulates fatty acid oxidation (40).

Impaired fatty acid oxidation or storage as triacylglycerols (TAG) could lead to the build-up of DAG. Palmitate decreased the expression of diacylglycerol O-acyltransferase (Dgat2), that catalyses the last step of TAG synthesis; and Pgc1α, that regulates fatty acid oxidation in muscle cells by co-activating peroxisome proliferator-activated receptors (PPARs) (40). In contrast, unsaturated fatty acids other than palmitate caused accumulation of DAG, but not all caused an inhibition of insulin signalling which means that DAG accumulation is not sufficient to cause insulin resistance (7).

Changes in muscle composition (32). Except for these, most studies that report a decrease in cell viability use higher concentrations of palmitate (> 0.6 mmol/L). Primary rat skeletal muscle cells seem to be more sensitive to palmitate treatment than cell lines – cytotoxicity was observed for 0.2 mmol/L for palmitic, oleic, and linoleic acids; 0.4 mmol/L for stearic and docosahexaenoic acids; and 0.8 mmol/L for eicosapentaenoic acid (34). Palmitate is more cytotoxic than other fatty acids, especially unsaturated (50).

A number of studies further explore the type of cell death that occurs upon FFA treatment. 0.75 mmol/L palmitate treatment for only 2 h was found to decrease cell viability, increase the number of TUNEL positive cells and increase caspase-3 activity in C2C12 myotubes, which indicates that palmitate induces apoptosis in these cells (33). Palmitate treatment (0.8 mmol/L for 24 h) induced a positive propidium iodide (PI) staining, cytochrome-c release, and caspase-3 cleavage and activation – all indicating apoptosis (41).

Cell viability is rarely studied when high insulin or high glucose are used for inducing insulin resistance in cells. Due to its mitogenic effect, insulin increases cell proliferation, but as most of the insulin resistance studies are done using differentiated non-proliferating cells, these effects are not observed. One study showed that low concentration of insulin (10 nmol/L) increased the proliferation of C2C12 myoblasts, but higher concentrations (50 and 100 nmol/L) decreased the viability of both myoblasts and differentiated C2C12 myotubes (51). Glucotoxicity is studied in the context of diabetes on different cell types, like endothelial cells (52) and cardiomyocytes (53), but there is almost no data on the effects of high glucose on skeletal muscle viability.

Conclusion

This review aims to summarise and critically evaluate the most commonly used cell models of muscle insulin resistance: the rationale for choosing these exact treatments and conditions, the protocols used for constructing the models and the measurable outcomes used for confirming insulin resistance in the cells.

Having all this in mind, the following conclusions and recommendations are made for consideration:

1. -FFA-induced models are constructed using concentrations comparable to those in vivo, but insulin-induced models use concentrations much higher than this - we should consider using the lowest concentration that produces the desired effects. When using high glucose concentrations, there should be a low glucose negative control.

2. When measuring insulin signalling activity, Ser473 phosphorylation of Akt is measured more frequently than Thr308, which is a more direct consequence of insulin receptor phosphorylation. Measuring both...
phosphorylations and other downstream substrates of Akt would give a more complete and accurate picture of the signalling pathway activity. Ideally, other metabolic outcomes should be measured in combination with this, like glucose uptake and glycogen synthesis.

3. It should be explored if the treatment used for inducing insulin resistance causes changes in cell viability - it is shown that FFA treatment can cause cell death, but this is rarely done in studies using insulin and high glucose. In this case, it should be measured in combination with this, like glucose uptake and glycogen synthesis. Evaluating the effects of different treatments and comparing them to each other and to data from animal models or human subjects can get us closer to finding the cell models that are the most relevant for studying insulin resistance. The limitations of the specific cell types, treatments and methods used for evaluating our results should also be considered. Nevertheless, cell models are a first step in preclinical studies, and should be treated as such – a good vessel for studying the basic molecular mechanisms, and building a knowledge basis to test hypothesis on more complex models and finally, human subjects.

We should be aware that none of the treatments can accomplish all the changes in cell physiology that happen in vivo. Evaluating the effects of different treatments and comparing them to each other and to data from animal models or human subjects can get us closer to finding the cell models that are the most relevant for studying insulin resistance. The limitations of the specific cell types, treatments and methods used for evaluating our results should also be considered. Nevertheless, cell models are a first step in preclinical studies, and should be treated as such – a good vessel for studying the basic molecular mechanisms, and building a knowledge basis to test hypothesis on more complex models and finally, human subjects.

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