The Role of 2-Hexadecenal in the Development of Metabolic Disturbances in Human Skeletal Cells

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July 10, 2019

Abstract

Type II diabetes (T2D) is the most common type of diabetes mellitus. It can lead to further metabolic diseases such as kidney and cardiovacular diseases, which lead to premature death. The main cause of T2D is impaired insulin sensitivity which leads to β-cell failure followed by insulin deficiency. The skeletal muscle plays a major role in postpandrial glucose uptake. Furthermore, the prevention of insulin resistance in such tissue can prevent or ameliorate the development of T2D. Previous studies have shown that lipid aldehydes can lead to impaired insulin sensitivity.

The effect of 2-hexadecenal (2-HE) on skeletal muscle metabolism was studied by examining the efficiency of the fatty acid oxidation of skeletal muscle cells. The transformation of glucose into glycogen was examined. But also the expression of the gene interleaukin 6 (IL6). The results indicated that 2-HE lead to an increase in both the fatty acid oxidation and glycogen synthesis efficiency. Additionally, it lead to a decrease in the expression of IL-6.

Acknowledgements

First, I would like to extend gratitude to my mentor Laura Sardon Puig for her guidance and patience during this project. Her major effort and time has helped me tremendously and I am very thankful for having her as my mentor. Secondly, I would like to thank my co-worker Lina Cao Zhang for helping and supporting me during this project, as well as medical student Erik Hermelin for sharing his knowledge on the subject. Lastly, special recognition to the research academy Rays for Excellence, Svenska Tekniska Vetenskapsakademin i Finland as well as Kjell and Märta Bejers Stiftelse for giving me this opportunity.

Contents

List of Abbreviations

2-HE 2-hexadecenal.

BCA Bicinchoninic acid assay.

DMEM Dulbecco's Modified Eagle Medium.

DMSO Dimethyl sulfoxide.

FPG Fasting Plasma Glucose.

IL6 Interleukin-6.

PBS Phosphate-buffered saline.

T2D Type II Diabetes.

1 Introduction

Diabetes mellitus is a major public health problem, affecting ∼5% people worldwide. There are three different types of diabetes, where $90 - 95\%$ of all diabetic patients have type II diabetes (T2D) [1, 2]. The criteria for the diagnosis of diabetes is a plasma glucose concentration of $\geq 7.0\,\mathrm{mmol\,L^{-1}}$ after eight hours of fasting [3]. It is expected that without intervention, there will be approximately 300 million people with T2D by the year 2025, which is double the current number in 2019 [4]. Patients with T2D can develop blindness, as well as cardiovascular and kidney diseases [5, 6].

The main cause of T2D is deficient insulin sensitivity or responsiveness to the metabolic actions of insulin. That occur in the tissues and lead to increased glucose levels in the blood. If not corrected, the two defects can lead to β-cell failure followed by insulin deficiency [7]. The predominant site of postpandrial, insulin-related glucose uptake is located in the skeletal muscle. Therefore, studies have examined skeletal muscle cells in order to understand insulin sensitivity, and they have showed that muscle lipid accumulation can be a factor in the development of T2D in skeletal muscle cells [8].

1.1 Background

There are approximately 10^{16} cells in the human body, all with different functions [9]. Some of these cells are skeletal muscle cells, which form in the muscle tissue [10]. Like all cells, skeletal muscle cells perform gene expression and cellular respiration. Another purpose is extracting energy (ATP) using glucose as well as fatty acids, which occurs in the mitochondrion.

1.1.1 Gene Regulation

In the nucleus of the cell, DNA is transcribed to mRNA by the enzyme RNA-polymerase (Figure 1). The RNA then exits the nucleus and is transported to the ribosome. There, it is translated into a protein with the help of free amino acids. Occasionally, a gene can

be turned on or off due to environmental conditions. An example of that is an increase in concentration of a substance in the body. The part of DNA that codes for the gene gets transcripted to RNA and is later translated to protein. This way, the production of that protein increases [10]. Some proteins function as enzymes that catalyze chemical reactions, while others are receptors that receive signals to the cell and regulate what substances enter the cell. Subsequently, specific substances have a noticeable effect on the body because they result in the decrease in the production of essential proteins. [10, 11]

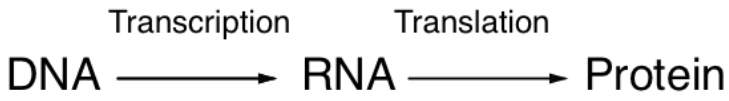

Figure 1: DNA is trancribed to RNA, which is translated into protein. Protein has various essential functions in the body.

1.1.2 Glycogen Synthesis

There are many receptors in the body, including the insulin receptors which receive signals from the hormone insulin. The insulin binds to the receptors on the cell membrane, activates a complex cascade by phosphorylation reactions and activates a subunit. This allows glucose to enter the cell (Figure 2). The glucose can then either be converted into ATP through the glycolysis pathway, krebs cycle and electron transport chain or into glycogen through glycogen synthesis. Insulin is therefore a prerequisite for glucose to enter into the cell in order for energy to be extracted [7].

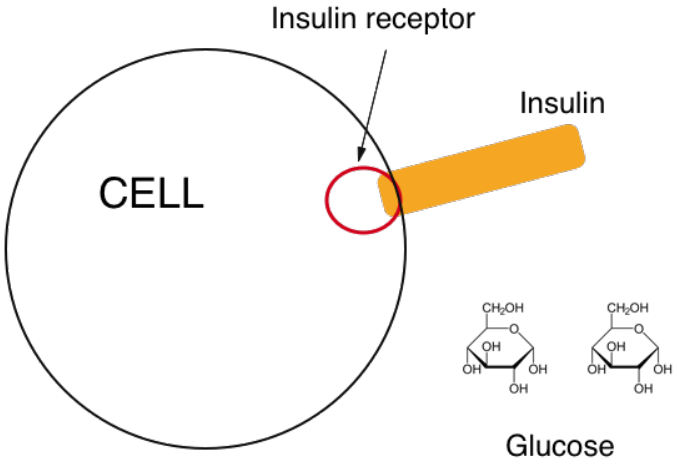

Figure 2: The insulin binds to the receptors on the cell membrane, so that glucose can enter the cell.

There can however occur complications in the process of glucose uptake connected to insulin. This can lead to many medical conditions, one of them being insulin resistance. Insulin resistant diabetic patients are approximated to have a mean blood glucose level of $158 \pm 15\,\mathrm{mg\,dL^{-1}}$ after an insulin-infusion rate of $120\,\mathrm{nmol\,m^{-2}}$ $120\,\mu\mathrm{L/\,min}$ for two hours. That occurs when the number of insulin receptors have decreased or stopped responding to insulin. This results in a decreased amount of glucose entering the cell and an accumulation of glucose to form in the blood. That condition, hyperglycemia, is known to be a characteristic feature of T2D.

When an individual develops insulin resistance, they do not instantly become hyperglycemic and develop diabetes. During the early stages of insulin resistance, the pancreatic β-cells, which are the cells that produce insulin, are able to create higher amounts of insulin, (Figure 3). Because of this, the same amount of glucose can enter the cell. This is called hyperinsulinemia and offsets the hyperglycemia, as well as the insulin resistance. Consequently, T2D is not instantly developed.

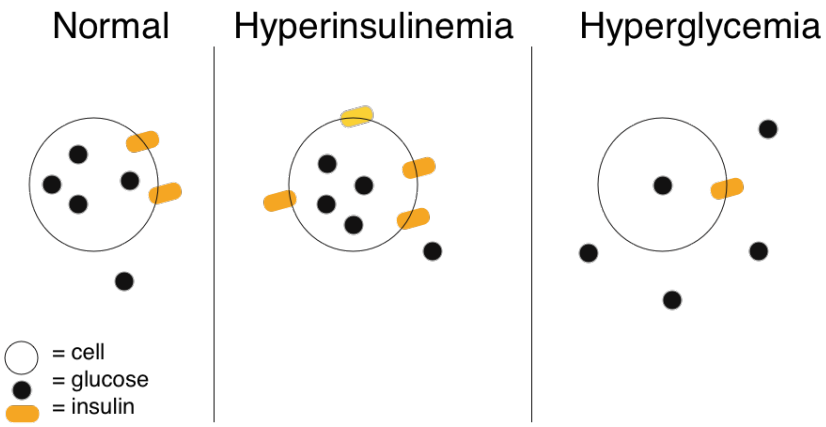

Figure 3: Hyperinsulinimia is when higher amounts of insulin is produced so that the same amount of glucose can enter the cell. Hyperglycemia is when sufficient amounts of insulin is no longer produced, which results in a decreased amount of glucose entering the cell.

When the β-cells cannot proceed producing insulin, the amount of insulin in the body decreases. This results in decreased level of glucose able to enter the cell and later, hyperglycemia, which results in T2D. Subsequently, insulin resistance leads to T2D. However the pathogenesis requires a long time. Therefore, early treatment is crucial. Furthermore, it is also important to understand what substances cause insulin resistance, in order to understand the reasons for the pathogenesis of T2D [7, 12, 13].

1.1.3 Fatty Acid Oxidation

Cells cannot only extract energy from glucose, but also from fatty acids. The fatty acid oxidation is a pathway where fatty acids are utilized in order to produce energy. Fatty acids are oxidized to CO_2 and H_2O [10]. An example of a fatty acid is palmitate ($C_{16}H_{32}O_2$), which is the most common fat found in animals [14]. Palmitate can become acids that are part of a particular group of fatty acids called sphingolipids or ceramides. Sphingolipids are known to interfere with key insulin intermediates, resulting in insulin resistance as well as with the fatty acid oxidation [15, 16].

1.2 2-Hexadecenal

2-hexadecenal (2-HE), $C_{16}H_{30}O$, as seen in Figure 4, is a lipid aldehyde. A lipid aldehyde contains a carbon atom bound to a nonpolar R-group, a hydrogen and double-bonded oxygen. Other lipid aldehydes such as 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal, are highly reactive and capable of interfering with physiological processes in the cell concerning insulin resistance, glucose uptake, and inflammation [17, 18].

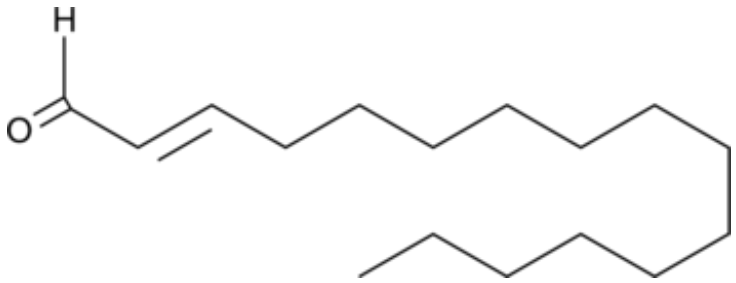

Figure 4: The lipid aldehyde 2-hexadecanal. Lipid aldehydes are known to be highly reactive and to often interfere with processes in the cell.

1.3 Previous Research

There is plenty of previous research regarding T2D. These are especially about what causes the deficient insulin sensitivity and lead to hyperglycemia as well as the development of T2D. According to a study conducted at Karolinska Institutet in Sweden year 2007[19], polyunsaturated fatty acids play a major part in the development of T2D. Fatty acids produce 4-Hydroxy hexenal (4-HHE) that interfere with the pathophysiological processes and thereby insulin resistance. Levels of 4-HHE in T2D patients and rats were twice as high as the levels of non-diabetics. Although it is proved that 4-HHE plays a role in the pathophysiology of T2D, no research has been done on similar lipid aldehydes, for instance 2-HE. Both 2-HE and 4-HHE are derived from the same acids, ω-3-docasahexaenoic and α-linolenic acid [20]. The effect of 2-HE on insulin resistance, lipid oxidation and gene regulation in skeletal muscle cells should therefore be similar to 4-HHE.

Studies have also concluded that patients diagnosed with diabetes have elevated levels

of the gene interleukin 6 (IL6). According to the same study, the expression of this gene is connected to obesity. This implies that the expression of gene IL6 leads to inflammation in tissues in the body, leading to development T2D. However, the main factor that causes the expression of IL6 and therefore causes inflammation remain uncertain. [21]

1.4 Aim of the Study

This study examines the effect the lipid aldehyde 2-HE has on human skeletal muscle cells. The influence 2-HE has on the gene regulation, insulin resistance, glucose-uptake and fatty acid oxidation is examined in order to determine if 2-HE can lead to development of T2D. The results of this study could help broading the knowledge regarding the disease.

2 Method

In order to determine if 2-HE affects gene expression, insulin resistance and the fatty acid oxidation, three different experiments were carried out. One regarding the expression of the gene IL6 and another which examined the efficiency of the glycogen synthesis. The last experiment determined the efficiency of the fatty acid oxidation.

2.1 Gene Expression

The amount of gene IL6-expression was measured in samples with 2-HE, and compared to samples without it. In order to do that, the RNA needed to be extracted and stabilized by reverse transcription to cDNA.

2.1.1 Preparation of the Cells

One six-well plate containing fully differentiated skeletal muscle cells was cleaned in order to preserve them. The medium in the wells was pipetted out. 2 ml of a saline solution (PBS, 0.03%) was then added into each well. All PBS was later aspirated so the cells were left at the bottom of the well. 1 ml medium of DMEM (containing 0.1% glucose)

was pipetted into each well. Solutions containing 2 µmol 2-HE as well as 5 µmol 2-HE and 5 µmol DMSO, were added into two wells each, see Table 1.

Table 1: Added Concentrations of DMSO and 2-HE into wells containing skeletal muscle cells

Well	Concentration	Solution
1	5μM	DMSO
2	5μM	DMSO
3	2μM	2-HE
4	2μM	2-HE
5	5μM	2-HE
6	5μM	2-HE

2.1.2 Lysation

The plate was frozen over night at -20°C, so that the cells did not stagnate. The day after, the cells were washed with 2 µL PBS in each well. 350 µL TRK Lysis Buffer was also added. This was made in order to lysate the cells, making the cell-membrane dissolve, so that the RNA could easily be extracted. Then, the cell membrane was scraped away, so that only RNA remained on the bottom of the wells. The clear supernatant (the top of the medium in the six wells) which contained the RNA, was transfered into test tubes that were frozen at -80°C overnight (see Appendix A).

2.1.3 Protein Concentration

In order to determine the amount of protein in the samples, remaining lysate in the wells was used. Firstly, 25 µL of a medium with known protein concentration was put into the first column of a microplate as a control. Then, 25 µL of medium from each well was pipetted into the remaining columns of the microplate. 100 mL of Reactant Mix (see Appendix B) was mixed thoroughly by pipetting the mixture up and down. 200 µL of this mixture was then added to each well of the microplate. The plate was covered and

incubated at 37°C for 60 minutes. After this, the amount of protein was measured using a spectrometer. The colors of the protein in the wells were then compared with the control The machine used was a spectroscope machine.

2.1.4 Purification of RNA

When the tubes had thawed, 350 µL of 70% ethanol was added and vortexed. 700 µL of each sample were later added to tubes containing a filter, called Hibind RNA Mini Column. The tubes were then centrifuged at 10, 000 rpm for one minute. When the RNA was isolated, the alcohol on the bottom of the tube was removed so that only RNA remained.

In order to purify the RNA, two buffers in the E.Z.N.A TOTAL RNA KIT 1 were used (see Appendix C). 500 µL RNA Wash Buffer 1 was added into each tube. This was then centrifuged at 10,000 rpm for 30 seconds. The filtrate was discarded and the same procedure was done with the same tubes, but this time twice and with Buffer II which was diluted with 100% ethanol. To dry the column and remove all excess alcohol, the tubes were centrifuged at 12, 000 rpm for two minutes. The RNA was transferred to clean 1.5 mL microcentrifuge tubes. After that, 70 µL Nuclease-free Water was added. These tubes were centrifuged at 12, 000 rpm for two minutes and the purified RNA was stored at -70 °C.

2.1.5 Stabilization of RNA

The RNA concentrations were firstly counted and normalized using a machine called Nanodrop. This way, all components had the same concentration. They were then left on ice. To stabilize the RNA, it had to go through reverse transcription and amde into double-stringed cDNA. In order to do that, the RNA dilution was added to tubes. They were then centrifuged briefly. A master mix containing the enzyme multiScribe, RNase, housekeeping primers and nucleotides. The enzyme miltiScribe can convert RNA to cDNA, RNase is a stabilizer and housekeeping primers are responsible for housekeeping genes,

which are genes that are required for basic cellular function. 3.4 µL of this master mix as well as 6.6 µL of RNA were added into tubes, which were put on ice and then into a thermocycler. The program run was the High Capacity program. The volume was 10 µL and the temperature was 37 °C. This ran for two hours, and then the cDNA was created.

2.1.6 qPCR

For measurement of the gene expression, 10 µL of RNA-dilution was put in a 96 well plate and 9 µL of RT mix was added into the plates. These were then centrifuged briefly, put on ice and then run through the Taqman program in the PCR (see Appendix) . The program consists of three phases, denaturation, annealing and extension. During the denaturation, the high temperature made the DNA denaturate and split it into two strands. After the temperature had dropped during the annealing, the primers gave the free nucleotides a starting point. This was made so that the free nucleotides could then, during extension, form a new copy of the cDNA and the specific gene is transcribed. This cycle, see Figure 5, was repeated 40 times. The number of transcribed genes were recorded through measuring the amount of fluorescence found in the cDNA. The primers consisted partly of fluorescence, so the greater amount of cDNA sequences that were created, the higher amount of fluorescence.

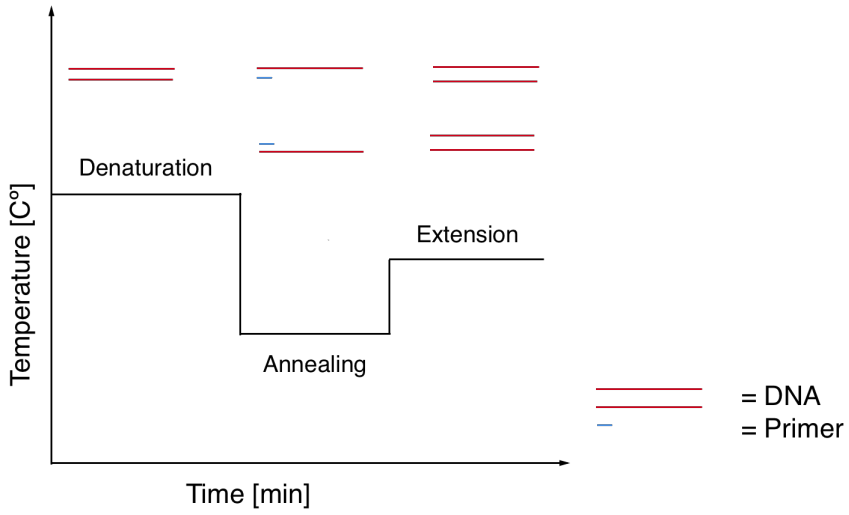

Figure 5: During the Taqman Program in the PCR, the DNA denaturates due to the high temperature in denaturation and seperates into two strands. During annealing, primers bind to these strands. Lastly, new copies of DNA were created during extension.

2.2 Glycogen Synthesis

To determine the effect 2-HE has on insulin resistance, the efficiency of the glycogen synthesis was measured. Glycogen synthesis is one of the two paths that glucose can take after it has entered the cell. The amount of glycogen was measured under the influence of 2-HE. It was then compared to samples without 2-HE.

2.2.1 Preparation of the Cells

The cells were cleaned and prepared following the same procedure as in section 2.1.1. Thereupon, the cells were then put in the incubator and starved for four hours. This was done in order for the cells to absorb glucose more easily after starvation.

2.2.2 Adding of Insulin and Carbon-14

After four hours, the cells were washed clean again following the procedure shown in section 2.1.1. Thereafter, 1 mL of insulin-substance (see appendix D) containing 10 mmol of

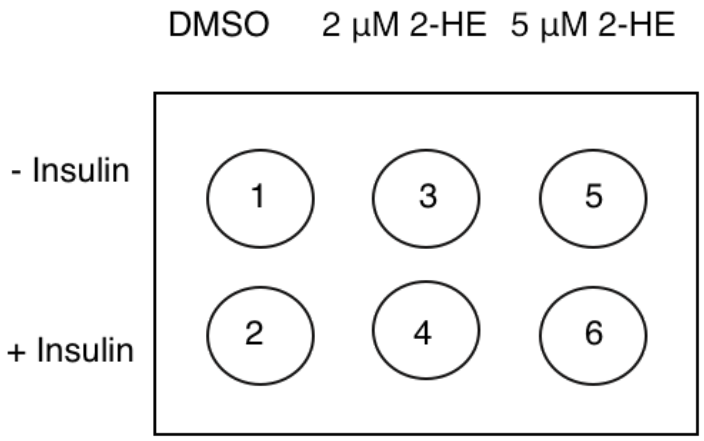

Figure 6: Petri-dish Glucose

insulin was added to each well. This stimulated the cells. After 30 minutes with insulin, 1 mL of media containing radioactive ^{14}C was added (see Appendix D). This solution was then incubated for 90 minutes. After that, the cells were washed and stored in the freezer at -20°C. The reason for the usage of radioactive glucose was so that the amount of radioactive glycogen could be measured through a scintillation counter. Because that radioactive glycogen was made from radioactive glucose, the amount of it could be measured through measuring the amount of radioactivity. This way, it could be determined if the treatment of 2-HE had disrupted the pathway.

2.2.3 Lysation

The medium of the skeletal muscle cells as well as the glycogen were solubilized with Sodium dodecyl sulfate (0.03%, SDS) for three hours at room temperature with a slow shake. SDS was able to destroy cell membranes, so that the glycogen and everything in the cell could be released into the solution. 400 µL of the samples as well as 100 µL of glycogen carrier solution were transferred to Eppendorf tubes. The remaining lysate in the wells were kept for protein concentration determination. These samples were then boiled at 100°C for one to two hours and cooled down on ice. 1.5 mL of 99% methanol was

then added to precipitate the glycogen. After this, the tubes were left at -20°C overnight.

The day after, the samples were spun in a centrifuge at 10, 000 rpm for 15 minutes at 4°C. A pellet was formed, and the present alcohol removed. 70% ethanol was added and the samples were then re-spun for 15 more minutes at 4°C. Then, 300 µL of dH_2O was added and left at room temperature for six hours. After that, the solution was transferred to 4 mL scintillation vials, together with a control and one empty console. The protein concentration was later counted in a liquid scintillation counter.

2.2.4 Protein Concentration Determination

The protein concentration needed to be determined. That was done using a spectrometer, following the procedure in section 2.1.3.

2.3 Fatty Acid Oxidation

In order to determine the efficiency of the fatty acid oxidation, radioactive tritium (^3H) was added to the wells. Through the fatty acid oxidation, radioactive water was later on produced and the amount of it was measured using a scintillation machine.

2.3.1 Preparation of the Cells

A plate with six wells containing fully differentiated skeletal muscle cells were cleaned following the procedure in section 2.1.1. 1 mL of a solution containing 1 mL of a mixture of ^3H and regular H (see Appendix E) was added to each well. The plate was then put in the incubator for six hours.

2.3.2 Protein Concentration Determination

The media that contained radioactive tritium (^3H), water (H_2O) and Tritium-palmitate (^3H-Palmitate) was transferred into new tubes. The plate with the cells was then washed and kept in the freezer for protein determination. The protein concentration was measured following the procedure as in section 2.3.1.

2.3.3 Adding of Charcoal

After six hours in the incubator, charcoal and water were put on a magnetic stirrer for 30 minutes. 0.8 mL of the slurry was then transferred to 2 mL roundbottomed Eppendorf Tubes. A 0.2 mL sample from each cell supernatant was added to the slurry. As a control, the same amount of medium was added to a tube as well. These samples were shaken every five minutes for 30 minutes, as well as centrifuged for 15 minutes. Thereafter, 0.2 mL of supernatant (as demonstrated in Figure 7) from each tube was pippetted out carefully into scintillation vials. 2.8 mL of scintillation liquid was added into the vials, as well as 20 µL of medium and 0.2 mL water as a control. The levels of radioactive water that had formed was counted using a scintillation machine.

During the final calculations, the protein concentration was included as well as the amount of radioactive water. However, results from another researcher was also used to achieve a more precise result (see Appendix F).

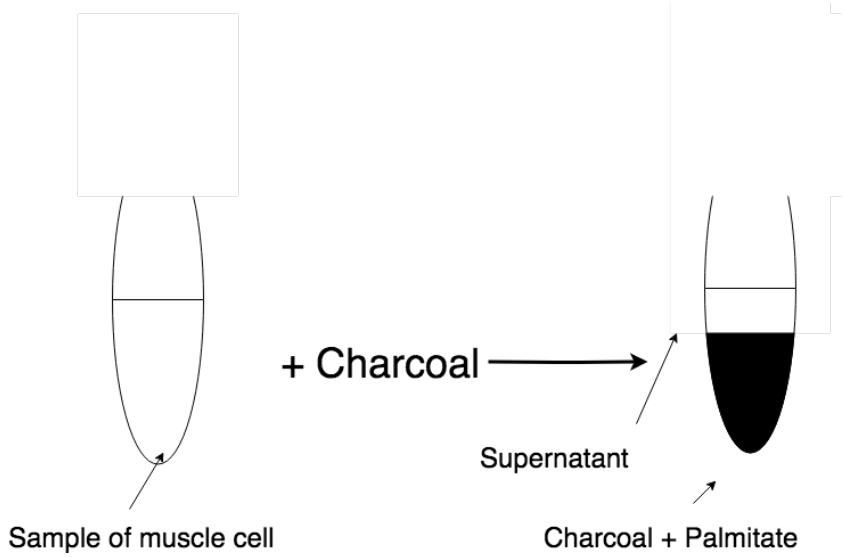

Figure 7: Charcoal Experiment

3 Results

The results from the experiment regarding gene expression implied that the higher the concentration of 2-HE, the higher level of expression of the gene IL-6. 2-HE also had a

negative impact on the insulin-related glucose uptake. Likewise it affected the fatty acid oxidation negatively.

3.1 Gene Regulation

As seen in figure 8, the higher concentration of 2-HE, the higher level of expression of IL6. The sample containing solely DMSO had a gene expression equal to 0.6904 scaled units. The one with 2 µmol 2-HE had 1.063 scaled units. That is an increase of 54%. The concentration of 5 µmol 2-HE had the highest gene expression, equal to 1.246 scaled units. That is an increase of 80%.

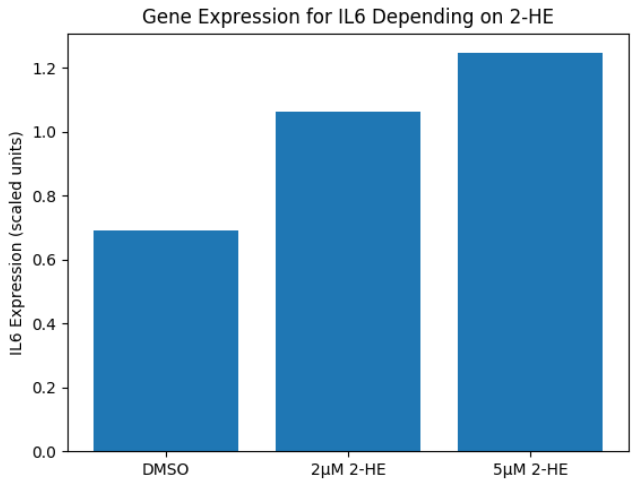

Figure 8: Gene Expression of IL6

3.2 Glycogen Synthesis

As seen in figure 9, the samples without insulin had a lower level of glucose-uptake. The control was set to one, the sample with 2 µmol 2-HE had an efficiency of 0.9564 cpm/mg(counts per minute per milligram) of protein and the sample with 5 µmol 2-HE had a higher uptake, approximately 1.123 cpm/mg of protein. Samples with insulin had higher uptake of glucose. The one containing DMSO had an uptake of 1.6435 cpm/mg of protein. 2 µmol 2-HE had an efficiency at 1.492 cpm/mg of protein. That is an decrease of 9.2%, compared

to the sample with insulin and DMSO. The sample with 5 µmol 2-HE was 1.553 cpm/mg of protein. The decrease of that compared to the sample with insulin and DMSO was 11%.

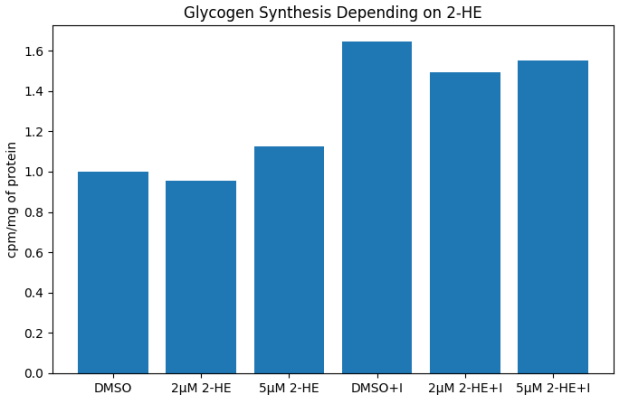

Figure 9: The Efficiency of the Glycogen Synthesis, I = Insulin

3.3 Fatty Acid Oxidation

As seen in Figure 10, the efficiency of the fatty acid oxidation was the highest in the sample with no added 2-HE. That efficiency was 476.5 $\mathrm{Pmol\,mg^{-1}\,h^{-1}}$ of H_2O. The efficiency of the sample with a concentration of 2 µL 2-HE was 442.2 $\mathrm{Pmol\,mg^{-1}\,h^{-1}}$ of H_2O. That is equal to a decrease of 7.2%. The efficiency of the sample with a concentration of 5 µL 2-HE was 344.9 $\mathrm{Pmol\,mg^{-1}\,h^{-1}}$ of H_2O. This indicates a lower efficiancy of 28%.

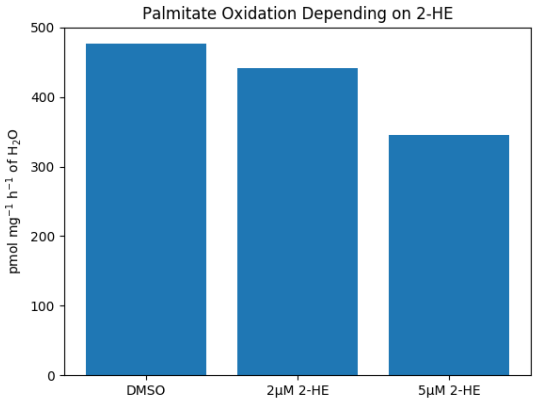

Figure 10: The Efficiency of the Fatty Acid Oxidation

4 Discussion

According to the results, a high concentration of 2-HE can lead to development of type II diabetes (T2D). A high concentration of 2-HE results in an impaired fatty acid oxidation, which leads to less 2-HE being decomposed and to more 2-HE accumulating in the tissues in the body. The higher concentration of 2-HE, the higher the risk of developing insulin resistance as well as inflammation in the body. These are both characteristics of T2D.

4.1 Fatty Acid Oxidation

The higher the concentration of 2-HE, the less efficient the fatty acid oxidation becomes. In comparison to the control containing DMSO, skeletal muscle cells with 2 µmol 2-HE has 34.3 $\mathrm{Pmol\,mg^{-1}\,h^{-1}}$ of H_2O less efficient fatty acid oxidation, which is reasonable, due to 2-HE being produced by the cell itself. A small concentration of 2-HE should therefore not be harmful for the cell. Cells with 5 µmol 2-HE has a decrease in efficiency at 131.6 $\mathrm{Pmol\,mg^{-1}\,h^{-1}}$ of H_2O. This is most likely because the concentration of 2-HE is higher than the cell is used to. A high concentration of 2-HE results in less palmitate oxidizing to CO_2 and H_2O. This leads to fatty acids, among 2-HE, accumulating in the cell. Consequently, it becomes a vicious circle.

4.2 Gene Regulation

According to the result, the higher concentration of 2-HE, the higher level of gene expression of IL6. The expression of the gene IL6 is proven to be the first step of development of inflammation in the body, which results in development of T2D. Consequently, the higher concentration of 2-HE, the higher the risk of fulfilling the first demand of inflammation in the body, which leads to T2D. However, this study only regards the effect 2-HE has on the cellular level and not the tissue in the body, which is the predominant place of inflammation. Furthermore, the study does not examine what exact concentration of 2-HE that leads to a specific expression of the IL6-gene. Additionally it does not examine how

long time it takes for the inflammation to develop.

4.3 Glycogen Synthesis

2-HE has a negative impact on insulin-related glucose-uptake. The difference between the efficiency of glycogen synthesis in the controls with and without insulin is 0.6435 cpm/mg (counts per minute per milligram). Consequently, without added insulin, the glucose uptake is minimal. The difference between the control and 2 µmol 2-HE is 0.0436 cpm/mg and the difference between the control and 5 µmol 2-HE is 0.123 cpm/mg. It is unlikely that a higher concentration, which is 5 µmol of 2-HE, lead to an increase of glycogen synthesis. That is the opposite effect of the lower concentration (2 µmol. Further studies should be done to confirm this result. Furthermore, because of the low glucose uptake level and small differences between the samples, it is difficult to differentiate the exact effect of 2-HE without the influence of insulin. However, this study focuses on T2D and therefore, the importance lays on insulin-related glucose uptake. Consequently, the result of the samples with no insulin can be seen as a control. They can be used to compared with the samples with insulin. The efficiency of the sample with DMSO and insulin was increased by 0.123 cpm/mg, implying that insulin increases the glucose uptake. But under the influence of 2 µmol 2-HE, the efficiency of glycogen synthesis was decreased by 0.1515 cpm/mg. That was a much larger decrease than in the same sample without insulin. With insulin as well as 5 µmol 2-HE, the decrease in efficiency was 0.061 cpm/mg. That is unlikely since it is the opposite reaction compared to the result regarding 2 µL of 2-HE. Further studies should be done in order to affirm this result.

When the result is looked at as a whole, it implies that 2-hexadenenal affects insulin resistance, and therefore later can lead to development of T2D. The difference in the glucose uptake is more significant than in the samples with insulin. This implies that 2-HE affects the insulin resistance negatively. 2-HE is therefore harmful for patients with hyperinsulinemia.

4.4 Future Studies

Although this study promotes a broader knowledge on the role of 2-HE connected to metabolic disturbances, further studies are required. Studies should include skeletal cells from multiple donors with different genetic backgrounds, age, gender and diet. Furthermore, studies regarding what concentration of 2-HE that cause an impaired fatty acid oxidation and to what extent should be done. Along with how long this defect lasts. Moreover, further studies should include how high expression of IL-6 is required in order to develop inflammation. Also, how 2-HE affects tissues in rats and in the human body, not only on a cellular level. Results from the fatty acid oxidation and the glycogen synthesis should be confirmed. The exact concentration related to bodyweight of 2-HE that is harmful for patients with hyperinsulinemia should also be examined. Finally, future studies should include what foods that contribute for an increase of 2-HE in the body.

5 Conclusion

According to the results in this study, 5 µmol of 2-hexadecenal (2-HE) caused the fatty acid oxidation to become 28 % less efficient. This means that a high concentration of 2-HE in the body causes a higher amount of lipid aldehydes to accumulate in the cell. An example of a lipid aldehyde is 2-HE. 5 µmol of 2-HE caused gene IL6 to express 80% more. Subsequently, the higher the concentration 2-HE, the higher the risk of inflammation, which is a characteristic feature of Type II Diabetes (T2D). Furthermore, 5 µmol of 2-HE lowered insulin-related glucose uptake with 11%. This indicates that 2-HE has a negative impact on insulin-related glucose uptake. Therefore, patients diagnosed with T2D should avoid a diet containing 2-HE. Consequently, a high concentration of 2-HE plays a role in the development of metabolic disturbances. Because of that, a high concentration of 2-HE can lead to development of T2D. However, more research should be done on this subject in order to gain further knowledge on it.

References

[1] Taylor SI. Deconstructing type 2 diabetes. *Cell*. 1999;97(1):9–12.

[2] Association AD, et al. Diagnosis and classification of diabetes mellitus. *Diabetes care*. 2010;33(Supplement 1):S62–S69.

[3] Association AD. 2. Classification and Diagnosis of Diabetes. *Diabetes Care*. 2015;38(Supplement 1):S8–S16. Available from: `https://care.diabetesjournals.org/content/38/Supplement_1/S8`.

[4] Organization WH, et al. Screening for type 2 diabetes: report of a World Health Organization and International Diabetes Federation meeting. . 2003;.

[5] Gæde P, Tarnow L, Vedel P, Parving HH, Pedersen O. Remission to normoalbuminuria during multifactorial treatment preserves kidney function in patients with type 2 diabetes and microalbuminuria. *Nephrology Dialysis Transplantation*. 2004;19(11):2784–2788.

[6] Vijan S, Hofer TP, Hayward RA. Estimated benefits of glycemic control in microvascular complications in type 2 diabetes. *Annals of internal medicine*. 1997;127(9):788–795.

[7] DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes care*. 2009;32(suppl 2):S157–S163.

[8] Goldberg RB. Lipid disorders in diabetes. *Diabetes care*. 1981;4(5):561–572.

[9] Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, et al. An estimation of the number of cells in the human body. *Annals of human biology*. 2013;40(6):463–471.

[10] Janne Karlsson BOMPOWGB Thomas Krigsman. *Biologi 1*. 4th ed. Liber; 2011.

[11] Shapiro JA. Revisiting the central dogma in the 21st century. *Annals of the New York Academy of Sciences*. 2009;1178(1):6–28.

[12] Lillioja S, Mott DM, Howard BV, Bennett PH, Yki-Järvinen H, Freymond D, et al. Impaired glucose-tolerance as a disorder of insulin action. *New England Journal of Medicine*. 1988;318(19):1217–1225.

[13] DeFronzo RA. The triumvirate: β-cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes*. 1988;37(6):667–687.

[14] Nohturfft A, Losick R. Fats, flies, and palmitate. *Science*. 2002;296(5569):857–858.

[15] Park JW, Park WJ, Kuperman Y, Boura-Halfon S, Pewzner-Jung Y, Futerman AH. Ablation of very long acyl chain sphingolipids causes hepatic insulin resistance in mice due to altered detergent-resistant membranes. *Hepatology*. 2013;57(2):525–532.

[16] Engin AB. What is lipotoxicity? In: Obesity and Lipotoxicity. Springer; 2017. p. 197–220.

[17] Merrill Jr AH. Sphingolipids. In: Biochemistry of lipids, lipoproteins and membranes. Elsevier; 2008. p. 363–397.

[18] Yadav U, Ramana KV. Regulation of NF-B-induced inflammatory signaling by lipid peroxidation-derived aldehydes. *Oxidative medicine and cellular longevity*. 2013;2013.

[19] Pizzimenti S, Ciamporcero ES, Daga M, Pettazzoni P, Arcaro A, Cetrangolo G, et al. Interaction of aldehydes derived from lipid peroxidation and membrane proteins. *Frontiers in physiology*. 2013;4:242.

[20] Grúz P, Shimizu M, Sugiyama Ki, Honma M. Mutagenicity of ω-3 fatty acid peroxidation products in the Ames test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2017;819:14–19.

[21] Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Jama*. 2001;286(3):327–334.

E.Z.N.A.® TOTAL RNA KIT I

Appendix A

Revision No: 3.0

Quick Guide

Please visit www.omegabiotek.com for a downloadable user manual containing additional protocols, troubleshooting tips, and ordering information.

Product	R6834-00	R6834-01	R6834-02
Purifications	5	50	200
HiBind® RNA Mini Columns	5	50	200
2 mL Collection Tubes	10	100	400
TRK Lysis Buffer	5 mL	40 mL	150 mL
RNA Wash Buffer I	5 mL	50 mL	200 mL
RNA Wash Buffer II	5 mL	12 mL	50 mL
Nuclease-free Water	2 mL	30 mL	60 mL

Supplied by user:

- Microcentrifuge capable of at least 14,000 x *g*
- RNase-free pipette tips and 1.5 mL microcentrifuge tubes
- 100% ethanol
- 70% ethanol
- Homogenization equipment
  - Omega Homogenizer Columns (HCR003)
  - Needle and Syringe
  - Mortar and pestle
  - Glass Beads
  - Rotor-stator Homogenizer
- Optional: 14.3M 2-mercaptoethanol (β-ME)

Before starting:

- Optional: Add 20 µL β-ME per 1 mL TRK Lysis Buffer.
- Prepare RNA Wash Buffer II according to the directions on the bottle

RNA Extraction and Purification from Tissue

LYSE

1. Determine the proper amount of starting material. Homogenize and disrupt the tissue according to the table below. For homogenization techniques, please refer to the downloadable product manual from www.omegabiotek.com.

Amount of Tissue	Amount of TRK Lysis Buffer
≤ 15 mg	350 µL
20-30 mg	700 µL

Note: For samples stored in RNALater® use 700 µL TRK Lysis Buffer.
Optional: Add 20 µL β-ME per 1 mL TRK Lysis Buffer. Store for up to 4 weeks at room temperature.

2. Centrifuge at maximum speed (≥12,000 x *g*) for 5 minutes.
3. Transfer the cleared supernatant to a clean 1.5 mL microcentrifuge tube (not supplied). Do not transfer any fatty upper layer that may have formed as it may reduce RNA yield or clog the column.

BIND

4. Add 1 volume 70% ethanol. Vortex to mix thoroughly. Do not centrifuge. If any sample has lost its volume during homogenization, adjust the volume of ethanol accordingly.
5. Insert a HiBind® RNA Mini Column into a 2 mL Collection Tube.
6. Transfer 700 µL sample (including any precipitate that may have formed) to the HiBind® RNA Mini Column.
7. Centrifuge at 10,000 x *g* for 1 minute. Discard the filtrate and reuse the Collection Tube.
8. Repeat Steps 6-7 until all of the sample has been transferred to the column.

OPTIONAL: This the starting point of an optional on-membrane DNase I Digestion protocol. If an RNA removal step is required, please continue to the DNase I Digestion Protocol on the reverse page. (See DNase I Digestion Set, Cat# E1091 for more information). If DNase I digestion is not required, proceed to Step 9.

WASH

9. Add 500 µL RNA Wash Buffer I. Centrifuge at 10,000 x *g* for 30 seconds. Discard the filtrate and reuse the collection tube.
10. Add 500 µL RNA Wash Buffer II diluted with 100% ethanol (see bottle for instructions). Centrifuge at 10,000 x *g* for 1 minute. Discard the filtrate and reuse the collection tube.
11. Repeat Step 10 for a second RNA Wash Buffer II wash step.
12. Centrifuge the empty HiBind® RNA Mini Column at maximum speed for 2 minutes to dry the column. This step is critical for removal of trace ethanol that may interfere with downstream applications.

ELUTE

13. Transfer the HiBind® RNA Mini Column to a clean 1.5 mL microcentrifuge tube (not provided).
14. Add 40-70 µL Nuclease-free Water. Centrifuge at maximum speed for 2 minutes. Store eluted RNA at -70°C.

RNA Extraction and Purification from Cultured Cells

LYSE

1. Determine the proper amount of starting material. Harvest and disrupt cells (do not use more than 1 x 10^7 cells) with TRK Lysis Buffer according to the table below. Vortex or pipet up and down to mix thoroughly. For cell harvesting techniques, please refer to the downloadable product manual from www.omegabiotek.com.

Number of Cells	Amount of TRK Lysis Buffer
< 5 x 10^6	350 µL
5 x 10^6 - 1 x 10^7	700 µL

Optional: Add 20 µL β-ME per 1 mL TRK Lysis Buffer. Store for up to 4 weeks at room temperature.

2. Homogenize the cells accordingly to one of the following methods:

- Syringe and Needle: Shear high MW DNA by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.
- Homogenizer Mini Column (HCR003): Load the lysate into a Homogenizer Mini Column inserted into a 2 mL Collection Tube. Centrifuge at maximum speed (≥12,000 x *g*) for two minutes to collect the homogenized lysate.

Note: Incomplete homogenization of the sample may cause the column to clog resulting in decreased yields.

3. Proceed to Step 4 of the RNA EXTRACTION AND PURIFICATION FROM TISSUE protocol on the reverse page.

DNase I Digestion Protocol

1. For each HiBind® RNA Mini Column, prepare 75 µL DNase I stock solution (73.5 µL DNase I Digestion Buffer + 1.5 µL DNase I (20 Kunitz/µL)). See DNase I Digestion Set, Cat# E1091 for more information.
2. Add 250 µL RNA Wash Buffer I. Centrifuge at 10,000 x *g* for 1 minute. Discard the filtrate and reuse the Collection Tube.
3. Add 75 µL DNase I digestion mixture directly onto the surface of the membrane of the HiBind® RNA Mini Column. DNA digestion will not be complete if some of the mixture is retained on the wall of the HiBind® RNA Mini Column.
4. Let sit at room temperature for 15 minutes.
5. Add 250 µL RNA Wash Buffer I. Let sit at room temperature for 2 minutes.
6. Centrifuge at 10,000 x *g* for 1 minute. Discard the filtrate and reuse the Collection Tube.
7. Proceed to Step 10 of the RNA EXTRACTION AND PURIFICATION FROM TISSUE protocol above.

8 Appendix B

Experiment no.	EXP-18-CE9641
Author	Ingermo Katrin (KBERGD)
Date Started	20 Jun 2018 14:21 (UTC + 2)
Title	METHOD: Protein determination using Pierce BCA Protein assay
Project:	Methods

Protein determination using Pierce BCA Protein assay

The BCA Protein Assay combines the well-known reduction of Cu2+ to Cu1+ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu1+) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction.

The reaction that leads to BCA color formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein. However, unlike the Coomassie dye-binding methods, the universal peptide backbone also contributes to color formation, helping to minimize variability caused by protein compositional differences.

Previous versions

Nov 2009 Inger Kuhn

EXP-12-BA8523 Eva Palmer

9 Appendix B

HAZARDOUS ACTIONS

General:
Use eye protection, gloves and lab coat.

Read the safety data sheet for chemicals used in the method.

In the event of a chemical spill, clean up only if it's considered safe, use appropriate protective equipment and absorbing material

If flammable material is spilled, immediately warn everyone

Reagent B of the kit:
Very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment.
Leftovers have to go as chemical waste.

For details regarding risks and waste handling see the Risk Assessment.

Risk assessment

Protein determination BCA.pdf

SHA512 checksum
iwF4g8MZ5vuEwo7rpCGj7YnJYYvJ6AkOu+RnOqRbw2N3We3FewYyC8RZ9KRNCkaIOnTGIWXcu+hEZJL/0uT+Ew==

Procedure:

Prepare standard solutions from albumin stock (2000 µg/ml) in the same diluent as your samples. First take 400µl of standard + 1.6 ml diluent, then make twofold dilutions of this solution in order to obtain 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml. As last standard use only diluent. The standard can be stored in a freezer.

For each assay 200µl of reagent is needed. Prepare reagent directly before use by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (i.e. for one microplate with 96 wells 20 ml A is mixed with 0.4 ml B).

1. Pipette 25 µl of each standard into column 12 (last column) in the microplate. Pipette 25 µl of unknown sample into other wells

2. Add 200 µl of the reagent mix to each well and mix plate thoroughly

3. Cover plate and incubate at 37°C for 60 minutes.

4. Measure the absorbance at or near 562 nm on a plate reader. (in emergency cases, if the reader does not work, use a flatbed scanner with TMA adaptor to make an image of the plate in resolution 150 dpi)

Notes:
- Wavelengths from 540-590 nm can be used.
- If lower or higher measurement values are desired, incubation time can be from 30 minutes to up to 2 hours.

10 Appendix C

Experiment no.	EXP-18-CE9640
Author	Ingermo Katrin (KBERGD)
Date Started	20 Jun 2018 13:19 (UTC + 2)
Title	METHOD: cDNA synthesis using High Capacity cDNA Reverse Transcription Kit
Project:	Methods

HAZARDOUS ACTIONS

The risk for this method is low

General
Use eye protection, gloves and lab coat.

Read the safety data sheet for chemicals used in the method.

In the event of a chemical spill, clean up only if it's considered safe, use appropriate protective equipment and absorbing material.

If flammable material is spilled, immediately warn everyone.

RNase Zap
Care must be taken when handling RNAse Zap since it is irritating to eyes, the respiratory system and the skin.

For more details regarding risks and waste handling see the Risk Assessment.

Risk assessment

cDNA High Capacity.pdf

SHA512 checksum
XoNNMMTmb++9H6gTqt6ZJ0jgW6owLnHfIGqdw1YxtZMiyG3vZd6tFLCvTrMU8hQnIg4ncLb/V6Xy4cnuwWXJ9Q==

Materials

High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems #4368813

RNA samples:

- Free of inhibitors of reverse transcription and PCR.
- Dissolved in PCR-compatible buffer or water.
- Free of RNase activity *NB: If you suspect that the RNA contains RNase activity, add RNase Inhibitor to the reverse transcription reaction at a final concentration of 1.0U/µl.*

11 Appendix C

Protocol overview

- Turn on the thermocycler.

- Allow the kit components to thaw on ice, but leave the MultiScribe enzyme and the RNase inhibitor in the freezer until you need them. Keep them on ice and put them back immediately after use.

- Prepare the RT 2x master mix on ice. Also prepare -RT Master Mix (same mix but no enzyme) for some of the samples, these will serve as controls for genomic DNA contamination in the qPCR experiment.

- **Dilute your RNA samples on ice to the same concentration**. This is done in order to be able to validate the endogenous control in the qPCR experiment, that is make sure that it is expressed at constant levels in your study. To be able to do this it is crucial to have all the cDNAs at the same concentration.

- Make 10 µl of RNA dilution in PCR tubes.

- Add 10 µl of 2 x RT mix into the tubes.

- Centrifuge briefly.

- Keep on ice until you are ready to load the thermocycler.

- Run the High Capacity program:

25°C 10 min
37°C 120 min
85°C 5 min

Set the volume to 20 µl.

12 Appendix D

HAZARDOUS ACTIONS

The risk for this method is acceptable

General
Use eye protection, gloves and lab coat.

Read the safety data sheet for chemicals used in the method.

In the event of a chemical spill, clean up only if it's considered safe, use appropriate protective equipment and absorbing material

Ethanol
Flammable. Don't store large amounts in the lab.

Scintillation liquid
Optiphase Hisafe has a risk of serious damage to eyes. Both Optiphase Hisafe and Ultima Flo M are harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
Scintillation liquid must be handled with care, it must not be poured into the drain. Used scintillation vials must be placed in chemical waste boxes. Use protective glasses when handling the scintillation liquid.

Human cells
Human cells must be treated with precaution, care must always be taken not to transfer live cell material to skin or eyes. See EXP-18-CE9635 for more on cell culture.

SDS
Toxic to inhale, weigh out in a ventilated hood. Use SDS in solution if possible.

For more details regarding risks and waste handling see the Risk Assessment.

Risk Assessment

Glycogen synthesis in cell cultures.pdf

SHA512 checksum
xBp+aJdAVjCJg2Gz9I2XNhk+5+zH3Z1ILIgbOLPfxOppq4V7rtaYL5skufizeUngIhROa9AXc3Ht6mDffOtciQ==

13 Appendix D

Materials and method:

Materials

- **DMEM** (#21885, 1 g/l) for human skeletal muscle cells / **MEM alpha** (1 g/l) for L6 cells:
  No FBS, PEST and Fungizone added to media in this assay.
- **Glucose:** D-[U-14C]-glucose (Perkin Elmer, NEC042B005MC; 1 mCi/ml; 250-360 mCi (9.25-13.3 GBq)/mmol))
  - Final concentration: 1 µCi/ml
  - Dilute 1:50 in DMEM (1 µl D-[U-14C]-glucose + 49 µl DMEM per ml medium)
- Ice cold **PBS** 1X
- 0.03% **SDS**
- **Carrier glycogen** solution, 20 mg/ml (Glycogen from bovine liver, Type IX - Sigma- catalogue # G0885)
- 98% ethanol
- 70% ethanol
- Scintillation vials and scintillation liquid
- **Insulin** (Actrapid 100 IE/ml) Dilute 1:100 in DMEM (10 µl insulin + 990 µl DMEM)

1. Seed 1500 myoblasts/well (approx 3 x 6-well plates from one T-150 flask) in 6-well plates and differentiate for 7 days at 70-80% confluence. Do at least 3 wells for basal and 3 wells for insulin stimulation, per subject and condition

Day 1 of experiment

2. Serum starve the cells with DMEM for 4h.
3. Change to exactly 1 ml of fresh DMEM with or without insulin/BSA/inhibitor/compounds etc according to your experiment setup. 20 µl of insulin diluted 1:100 is added per ml of medium => 20 mU/ml (120 nM). Incubate for 30 min at 37°C.
4. Add 50 µl/ml medium (1 µl isotope + 49 µl DMEM) of the glucose isotope solution and incubate for 90 min @ 37°C. Save some isotope solution in the freezer.
5. Wash wells 4 times with cold PBS on ice.
6. Freeze directly overnight at -20°C.

MICRO-METHOD (standard assay in our lab)

Day 2 of experiment

7. Solubilize with 0.5 ml 0.03% SDS for 1-3 hours at RT (slow shake). Transfer 0.4 ml of the lysate from the wells to 2 ml Eppendorf tubes and add 100 µl (2 mg) carrier glycogen solution (20 mg/ml). Remaining lysate in the wells is kept for protein concentration determination (BCA Protein Assay).
8. Boil samples in a heating block for 1 hr with the lid loose (you can also have the tubes in a water bath in an oven at 90-100°C for 1-2 hrs).
9. Leave tubes to cool on ice (make sure that the lids are always loose, do not make holes, since you will add alcohol which together with you sample might leak out of the tube).
10. Add 1.5 ml 98-99% ethanol to precipitate the glycogen.
11. Close tubes properly, vortex, and leave tubes at -20°C o/n.

Day 3 of experiment

12. Spin the samples at 4°C. Spin for 15 min at 10 000 rpm, turn tubes upside down to remove ethanol.
13. Wash pellet once carefully with 1 ml ice cold 70% ethanol.
14. Re-spin the glycogen at 4°C and aspirate ethanol off - leave to dry for 30-90 min.
15. Add 300 µl dH_2O. It takes time to dissolve the pellet and it is best to leave it o/n at RT (it usually takes >6 h).

Experiment by KB: The pellets were dissolved with water for 1h and then counted. New water was added and incubated o/n. Only 2% of the cpm was left after incubation with water o/n compared to after 1h. According to this experiment incubation for 1h is enough, see graph below and EXP-12-BA1321)

Day 3 continued or Day 4 of experiment

14 Appendix E

Materials

1. Stock solution of 2% (w/v) fatty-acid-free albumin. Adjust pH to ~7.5, filter and store @ -20°C in 5-10mL aliquots.
2. Stock solution (5mM) of cold palmitate in BSA (see preparation of palmitate protocol)
3. Serum and antibiotic-free DMEM (DMEM 0%, cat. #21885)
4. Hot palmitate (1mCi/mL in ethanol) 9-10(n)-^{3}H palmitic acid (product TRK760, GE Healthcare, stored in Cell lab freezer)
5. Insulin (120nM): calculate a final volume of 10µl insulin/mL medium and make a 1:50 dilution in DMEM 0% (if wanted)
6. AICAR (2Mm) (if wanted)
7. Activated charcoal (now or on the day you want to process the samples)

- Prepare 0.02M Tris-HCl, pH 7.5
- Weigh out ~0.1g activated charcoal powder per sample (Sigma-Aldrich cat # C4386). Make sure to add a generous margin of error.
- Add Tris-HCl and mix ~5min. Leave to sediment and remove the supernatant. Repeat 2-3 times to get rid of the “floaters”. You can also filter-vaccum the charcoal solution. Fill up to final volume with Tris-HCl. You will need 0.8mL of charcoal solution per sample. Store @ +4°C for up to two weeks. For longer storage times, the solution must be autoclaved.

15 Appendix E

Body text

Experiment (charcoal extraction)

1. Remove old medium and wash once with sterile PBS. Add the hot and cold palmitate to the 0% DMEM, dispense 1ml medium/well in 6 wells plates or 0.5mL/well in a 12 wells plate.
2. If desired, add stimulus and incubate for 6h.
3. Put the charcoal slurry on a magnetic stirrer and transfer 0.8mL of the charcoal slurry to 2 mL roundbottomed Eppendorf tubes (use the special charcoal pipette and cut of the top of the pipette tip).
4. Collect 0.2mL sample from each cell supernatant and add to the slurry. Also add the same volume of labeled medium (negative control) to a tube. DO NOT add charcoal to the controls!!!
5. Incubate @RT for 30 min; mix every 5 min. Do not exceed 1h or the palmitate might redissolve from the charcoal.
6. Centrifuge at 13,000 rpm for 15 min. Then withdraw 0.2 ml of supernatant **carefully** without disturbing the charcoal phase (if disturbed – recentrifuge the sample) and add to 2.8mL of scintillation liquid in 4mL scintillation vials and count. Also include 20 ul of labeled medium and 0.2 ml Tris-HCl or water (blank).

IF YOU CANNOT PERFORM THE CHARCOAL EXTRACTION DIRECTLY, FREEZE THE SAMPLES.

Notes from the bench:

- To interpret the data, normalization to protein content is necessary: therefore make sure you harvest wells for protein.
- To interpret the data, don't forget to save an aliquot of the hot/cold palmitate in solution (several experiments=several aliquots). They represent the "max" counts that you will get.
- Insulin and AICAR concentrations (respectively 120nM and 2mM) are considered to be max concentrations for this assay. If you want to use submax concentrations, you need to decrease those.
- Incubation time for the assay can be decreased to 4 hours.
- Add supernatant from a culture of Pseudomonas aeruginosa grown in MEM (positive control; stock solutions of this can be found in the workshop freezer) to an Eppendorff tube. Positive control is only nessecary if you use a new charcoal slurry

Remaining questions:
What is the pH of BSA, does that affect the total reaction mixture?
How much lipid is present in serum?
AICAR and the adenosine kinase as control.
FCCP can be used as a positive control
Adding carnitine?

16 Appendix F

Table 2: Total Glycogen Synthesis Data

Concentration	Dataset1	Dataset2	Dataset3	Average
Control DMSO	1	1	1	1
2uM	1.00200317	1.066468701	0.8008134089	0.9564284267
5uM	1.254814411	1.336735979	0.7774673114	1.1230059
DMSO + I	1.536758797	1.750191159	1.643400847	1.643450268
2uM + I	1.490800072	1.552750653	1.431634727	1.491728484
5uM + I	1.663637924	1.740891661	1.253278245	1.55260261

*I = Insulin

Table 3: Total Palmite Oxidation Data

Concentration	Dataset1	Dataset2	Dataset3	Average
Control DMSO	451.651715	551.4027097	426.4259339	476.4934529
2uM	465.1988375	438.4327536	422.8809134	442.1708348
5uM	387.274108	285.8390106	361.6215911	344.9115699