Effect of 2-Hexadecenal in Skeletal Muscle Cells on the Development of Type 2 Diabetes

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Abstract

Type 2 Diabetes Mellitus is one of the most prevalent diseases worldwide, however all mechanisms causing the clinical symptoms are not fully understood yet. The aim of this study was to investigate the metabolic effects of 2-hexadecenal on skeletal muscle cells. The metabolic processes studied were glycogen synthesis and palmitate oxidation, which can influence the cell's functionality and resistance to insulin. Gene expressions of the inflammatory marker interleukin-6 were also measured to see if 2-hexadecenal increases cell inflammation. The results indicate that high concentrations of 2-hexadecenal affects the processes investigated negatively, thereby suggesting that 2-hexadecenal may have a role in the development of Type 2 Diabetes Mellitus.

Acknowledgements

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Contents

List of Abbreviations

2-HE 2-Hexadecenal

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl Sulfoxide

PBS Phosphate-buffered Saline

qPCR Quantitive Polymerase Chain Reaction

S1P Sphinosine-1-Phosphate

T2D Type 2 Diabetes Mellitus

1 Introduction

Type 2 Diabetes Mellitus (T2D) is one of the most prevalent diseases worldwide, affecting more than 366 million people. The disease often leads to premature death due to many short- and long-term complications, such as an increased risk for stroke and cardiovascular disease. [1, 2]

To understand the pathogenesis of the disease, it is important to study the uptake of glucose and fat in diabetic cells compared to healthy cells. The metabolic processes in cells are influenced by different molecules. By identifying specific molecules interfering with cell functions such as insulin sensitivity, activity in glycogen synthesis and fat oxidation, it is possible to develop treatments that target these molecules. Since obesity and a high Body Mass Index are linked to T2D, it is relevant to study the molecules that are elevated among obese diabetic patients to determine whether these can cause impaired metabolic functions and T2D. [3, 4]

1.1 Background

The diagnosis criteria for T2D is a fasting blood glucose level above 7.0 mmol/l, with fasting being defined as no calorific intake at least 8 hours prior to the test. T2D is caused by evolving insulin resistance [5]. Insulin resistance is defined as an impaired response to the hormone insulin by insulin receptors on the cell surface [6].

β-cells are located in the pancreas where they produce insulin post-prandially (post-meal) as a response to an influx of glucose in the body [7]. If the β-cells produce enough insulin, the body is able to maintain normal blood glucose levels (euglycemia) despite increased insulin resistance. As the disease develops, the β-cells cannot further increase the amount of insulin which leads to reduced glucose uptake by cells, causing hyperglycemia (high blood sugar) [7]. There is also evidence that insulin resistance itself eventually leads to β-cell failure [8].

Skeletal muscle cells play a critical role in the post-prandial uptake of glucose and is

one of the primary sites in the body where insulin resistance can be detected. Skeletal muscle cells are also essential for maintaing normal glucose homoeostasis in the body since 80% of glucose is taken up in skeletal muscle tissue post-prandially. [7]

Insulin resistance can be affected by many different metabolic syndromes in the body, one of which being lipotoxicity. Lipotoxicity is the harmful accumulation of lipid deposits in other tissues than adipose (fat) tissue [9]. High levels of a lipid group called complex sphingolipids have been shown in clinical studies to correlate positively with insulin resistance and impaired ability to maintain euglycemia (normal blood sugar) [6]. Elevated levels of ceramides, a subgroup of sphingolipids, in blood plasma correlates with both insulin resistance and obesity which could explain the positive correlation between obesity and T2D [10].

The sphingolipid sphinosine-1-phosphate (S1P) is a bioactive lipid responsible for many signalling processes in the body. One product of the degradation of S1P is the lipid reactive aldehyde 2-hexadecenal (2-HE), see Figure 1 [11]. It is produced when the enzyme sphingosine-1-phosphate lyase acts upon S1P in various cells in the body [12]. Reactive aldehydes are considered to contribute to the signalling of free fatty acids and may cause inflammation and oxidative stress, which in turn is associated with different metabolic syndromes [13, 14, 15]. Furthermore, increased levels of inflammation are also associated with T2D [16].

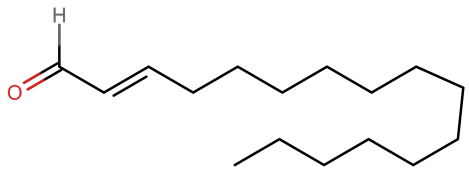

Figure 1: 2-Hexadecenal

1.1.1 Metabolic Cell Functions

Glycogen synthesis is a process in which glycogen is produced using glucose. The glycogen is then stored in the liver and skeletal muscles. If the process is faulty hyperglycemia

occurs, which is a characteristic of T2D. [17]

Palmitate oxidation is a form of fatty acid oxidation. The end products of the reaction are H_2O and CO_2. It is an alternate way for the cell to obtain energy other than glycolysis (breaking down of glucose), producing energy in the form of heat. [18]

Gene expression is the process by which a gene is expressed to make a protein. The Central Dogma is the process assembling protein from DNA via RNA. DNA is transcribed into RNA which is then translated into protein [19]. Interleukin-6 is a protein linked to chronic inflammation, which is a hallmark of T2D. It is coded by the gene *IL6*. [16, 20]

1.2 Background to Methods

To measure gene expression, RNA analysis was performed using the technique Quantitive PCR and Taqman primers.

1.2.1 Quantitive Polymerase Chain Reaction (qPCR)

RNA is replicated in a temperature cycle using Quantitive PCR and the enzyme polymerase. At 90°C, the cDNA (complementary DNA) strands melts and split into two halves of separate strands, allowing the Taqman primers to join their complementary sequences. At 60°C the enzyme polymerase is activated, adding a complementary strand to both of the two half separated strands, making two whole strands and releasing Taqman signals. The two whole separated strands then melt into four separate half strands again when the temperature is 90°C.

The amount of strands thus increases by a factor of 2 each cycle, with 1 whole starting strand in the first cycle becoming 2 in the next, then 4, 8, 16, 32... etc, see Figure 2. qPCR is used to accurately determine the original number of RNA strands through inverse calculation.

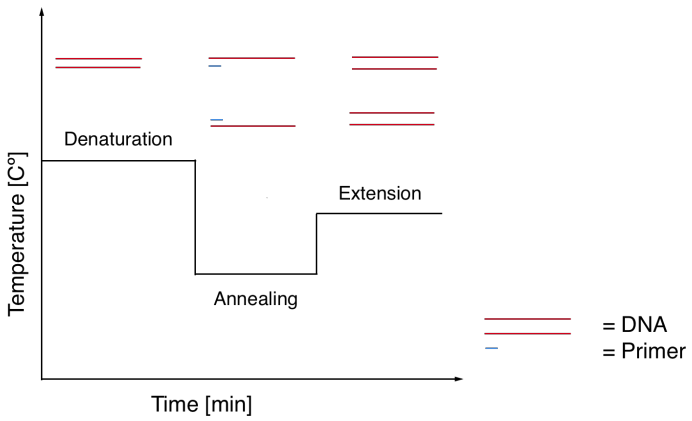

Figure 2: The PCR Temperature Cycle

1.2.2 Taqman Primers

Taqman primers are used to perform Quantitive PCR by silencing all genes except for the gene tested. The primers detect their complementary strand formed out of free nucleotides and bind to them. When the enzyme polymerase passes through that region, the probe is liberated and a fluorescence signal is released and detected by the thermocycle. The amplification can then be measured by a thermocycle. If the amount of replicated strands increase, the signal gets stronger, making it possible to calculate the amount of RNA at the end.

1.3 Previous Research

Previous research has shown that 2-HE causes cytoskeletal reorganisation, leading to apoptosis (programmed cell death) [12]. TD2 has also been shown to be an inflammatory disease in the body [21]. Another lipid aldehyde, 4-hydroxy-2-hexenal, induces skeletal muscle insulin resistance [21]. In a study by Pillon NJ and Croze ML et al. the lipid aldehyde 4-hydroxy-2-nonenal was found to impair insulin signalling functions [22].

1.4 Aim of Study

The aim of the study was to examine how the lipid aldehyde 2-HE affects the metabolic pathways of skeletal muscle cells. Skeletal muscle cells with and without added concentrations of 2-HE were investigated regarding their ability to synthesise glycogen from glucose, their ability to oxidate palmite fat, and their sensitivity to insulin. The purpose of RNA extraction from skeletal muscle cells was to study whether *IL6* gene expression-related inflammation was affected by 2-HE or not.

2 Method

The effect of 2-HE on three different cell functions was investigated. Glycogen synthesised in reference cells and cells with added 2-HE could be compared by measuring levels of radioactive glycogen anabolized (constructed) from ^{14}C glucose. Palmitate oxidized in reference cells and 2-HE cells was likewise compared by measuring the amount of radioactive palmitate that had been metabolized into H_2O after palmite oxidation. 2-HE's influence on cell inflammation was researched measuring expressions of the gene *IL6*, an inflammatory marker.

2.1 Measuring Glycogen Synthesis

Two cell plates were used, each containing six wells. Skeletal muscle cells were washed with Phosphate-buffered saline (PBS), and one of three different mediums was added to each well. Mediums were made of Dulbecco's Modified Eagle's Medium (DMEM), containing 0.1% glucose and concentrations of either Dimethyl Sulfoxide (DMSO) or 2-HE. Two wells were used per solution, whose concentrations were 5 µmol DMSO (reference sample), 2 µmol 2-HE, and 5 µmol 2-HE. For the set-up, see Figure 3.

After starving the cells for 4 hours, insulin was added to half of the wells. Glucose containing radioactive isotope ^{14}C was added to all the wells after 30 min. The cells were then incubated, washed and stored, see Appendix A, Figure 8.

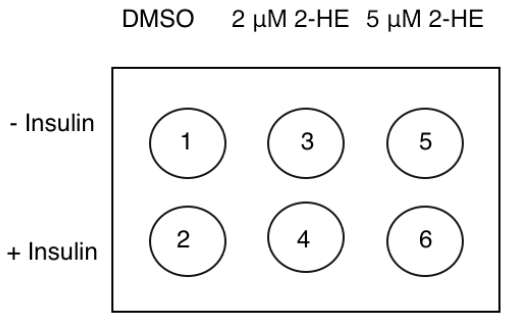

Figure 3: One of the Two Plates for Measuring of Glycogen Synthesis

The samples were put in a slow shaker with added sodium dodecyl sulfate, in order to break down the cell membranes and prevent further formation of glucose into glycogen. Lysates (the mediums containing broken down cells) from all wells were then transferred into twelve Eppendorf tubes. Some of the lysate was saved for determination of protein concentration later. The samples were boiled to dissolve glycogen, and ethanol was added to each Eppendorf tube. The tubes were then shaken with a vortex machine to mix their contents, and stored overnight, see Appendix A, Figure 8.

In order to separate the glycogen pellets with the supernatants (liquids floating above the precipitates), the samples were spun in a centrifuge. The supernatants was then removed. Ethanol was used to wash the glycogen pellets to ensure removal of possible glucose, and then the pellets were respun in the centrifuge for purification. After drying the pellets, distilled water was added to the pellets to dissolve them, see Appendix A, Figure 8.

To measure the amount of glycogen, the samples containing distilled water and glycogen were vortexed. They were then transferred to vials containing scintillation liquid. The scintillation liquid was added in order for a scintillation counter machine (measures radioactive decay) to be able to measure the radioactive decay from the ^{14}C in the glycogen pellets. For reference counting, the original glucose solution was also distilled with water and measured in the scintillaion counter, see Appendix A, Figure 9.

The remaining lysate saved before pelletization was used to determine protein con-

centration per well, and therefore number of cells in each well. The normalization process made it possible to adjust the emission data from each of the six wells to the different amount of cells in each well. It was performed using dye mixed from two reagents, and a spectroscope machine, where the sample changes colour depending on the amount of protein, see Appendix B, Figure 10.

2.2 Measuring Palmitate Oxidation

One cell plate with six wells was used for measuring the amount of palmitate oxidized to form H_2O. A solution was prepared by mixing 15 mL glucose-free DMSO with 75 mL palmitic acid and 15 mL radioactive palmitic acid containing ^3H. The solution was first mixed with DMSO as a reference sample. Different amounts of 2-HE were added to two other solutions, resulting in 2 µmol respective 5 µmol concentrations of 2-HE.

From each of the concentration samples, 1 mL was added to 2 wells respectively immediately after aspiration and washing, the same procedure as in section 2.1. The cells were then incubated for 4 hours at 37°C. The medium was kept in tubes for further measuring and the cells were washed with PBS and kept in the freezer at -20°C.

Then, active charcoal mixed with Tris-HCI was put in a magnetic stirrer to make a slurry. The slurry was transferred to six Eppendorf tubes. The samples were thawed and supernatant from each well was pipetted into the Eppendorf tubes. A tube without charcoal, but with the same amount of medium added served as a negative reference. The samples were incubated and mixed, during which time the charcoal bound to the remaining palmitate that had not been oxidized. Afterwards, the samples were centrifuged and then stored, see Appendix C, Figure 11.

The supernatant containing the products of the reaction was pipetted out and the remaining palmite and charcoal pellet were discarded, see Figure 4. Supernatant from each tube was mixed with scintillation liquid, making it possible for the scintillation counter to measure the amount of ^3H H_2O. See Appendix C, Figure 11.

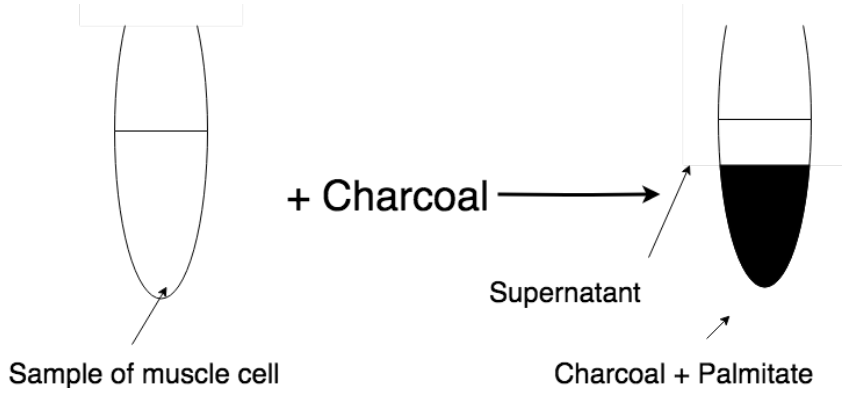

Figure 4: Supernatant and Charcoal Pellet

The protein from each cell well was measured in order to normalize the amount of ^{3}H water to the amount of protein per well. The normalization process was performed using the same procedure as the protein measuring process during glucose synthesis. See Appendix B, Figure 10.

2.3 Gene Expression

Gene expression analysis was performed in order to investigate whether 2-HE affected gene expression in skeletal muscle cells. The analysis was done on *IL6*, an inflammatory marker. A plate with six wells was used for extraction of RNA. The cells were washed with PBS and starved in 1 mL of DMEM containing 0.1% glucose. Solutions were prepared with concentrations of 5 µmol DMSO (reference sample), 2 µmol 2-HE, and 5 µmol 2-HE, the same procedure as for measuring glycogen synthesis and palmite oxidation.

After four hours, the DMEM was aspirated and the cells were washed with PBS. TRK Lysis buffer was added, causing cells to lysate, dissolving their membranes, and freeing RNA. The remaining cell parts were scraped off the plate walls, leaving only the cleared supernatant containing RNA. Then the samples were frozen at -80°C. When thawed, 500 µL of 70% alcohol was added to each test tube tube. The tubes were then vortexed to make the RNA percipitate.

To extract the RNA, E.Z.N.A TOTAL RNA KIT I was used, see Appendix D, Figure 12 and Figure 13. Lysates were put into HiBind RNA Mini Columns with filters. When

centrifuged, the filter separated the RNA from the rest of the lysate. The DNA was also purified using the same kit by adding wash to the RNA and centrifuging again, which further separated the RNA from impurities. In order to store the RNA, it was mixed with distilled water and frozen to prevent degeneration. To normalize the RNA concentrations, adjusting for different amounts of cells in each well, the protein content was measured by a NanoDrop machine.

2.3.1 Reverse Transcription and Quantitive PCR

In order to stabilize RNA, reverse transcription was performed to synthetically reverse RNA into cDNA. A thermocycler machine (used to amplify RNA and cDNA through polymerase chain reaction for easier measurements) and the enzyme Multiscribe containing polymerase was used for this process. A solution was made using Multiscribe mixed with RT 2x master mix containing nucleotides, primers, and stabilizers (RNAse inhibitors destroying RNA). The stabilizers were added in order to create stable cDNA.

After diluting the RNA samples in order to get the same starting concentration of RNA, 6.6 µL of RNA was added to 3.4 µL of the RT 2X master mix (containing enzymes and free nucleotides). The solution was mixed, placed on ice and then the thermocycler was run at a high capacity program at 37°C for two hours with the volume on the thermocycler set to 10 µL. See Appendix D, Figure 14

To calculate the amount of copies of the different RNA samples (already reverse transcripted into cDNA) Quantitive PCR was used. The temperature cycle was repeated 40 times in total. Taqman primers (TaqMan™ Array Human IL6 Pathway) were used to perform Quantitive PCR in a thermocycle with cycling temperatures of 90°C and 60°C.

Due to fluorescencent substances in the Taqman primers, fluorescence increased with the number of cDNA strands. The fluorescence could then be detected in the thermocycle machine, making it possible to calculate the original number of strands. The value was calculated by the Thermocycle using ct values (numbers of cycles per sample to reach a certain level of expression) and from there the gene expression was calculated. The

amount of RNA in the respective samples containing 2-HE and without 2-HE was then compared to a DMSO reference sample to see if the RNA sequences targeted had been more or less expressed in the 2-HE samples.

3 Results

For plotting the glycogen synthesis and palmite oxidation tables, additional data from lab members working at the same project was used, see Appendix F. All graphs show the difference between samples with added 2-HE and control (DMSO) samples. The glycogen synthesis graph shows the glycogen formed with half the samples containing insulin. For palmitate oxidation, the graph shows the amount of water and therefore palmitate oxidized. The gene expression graph shows the number of IL6 expressions scaled.

3.1 Glycogen Synthensis

Figure 5 shows the amount of ^{14}C cpm/mg (counts per minute per mg protein) formed from glycogen syntensis. Counts per minute were measured in the respective finished glycogen pellet in the reference sample (5 µmol DMSO) and in samples with 2 µmol respectively 5 µmol amounts of 2-HE. The data was normalized with regards to protein for an easier comparison, where the DMSO control sample without added insulin had the value of 1 cpm/mg of protein. The counts per minutes were ^{14}C decay.

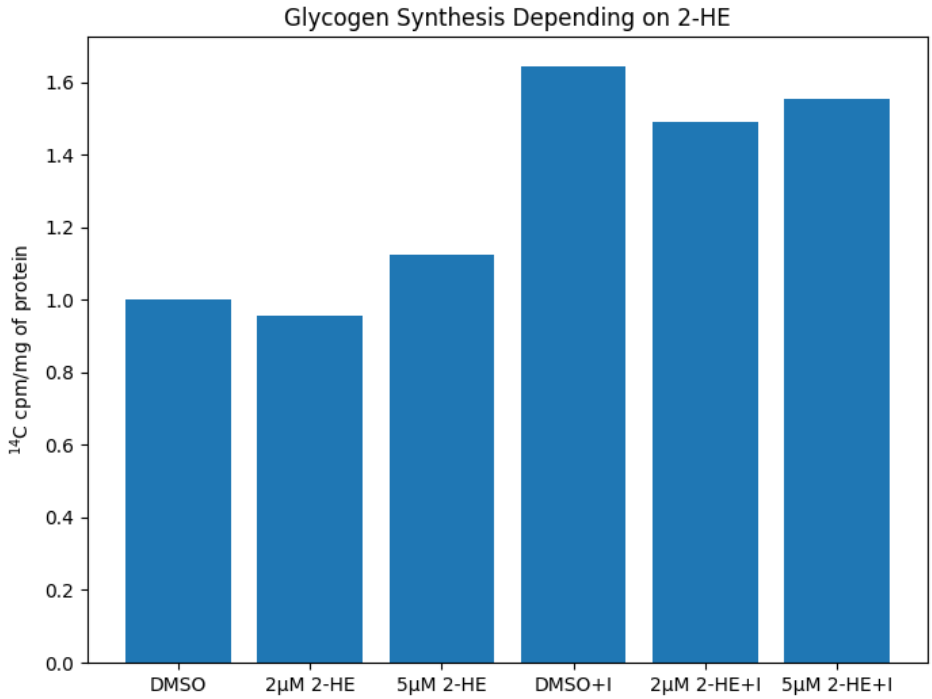

Figure 5: Glycogen Synthesis Depending on 2-HE (I = insulin)

Scaled to the control sample of 1 cpm/mg of ^{14}C, the results were 0.96 for the 2 µmol 2-HE sample and 1.12 for the 5 µmol 2-HE sample without insulin. With insulin added, the DMSO sample results was 1.64, the 2 µmol 2-HE sample was 1.49 and the 5 µmol 2-HE sample was 1.55. All units were in cpm/mg (counts per minute/mg) of protein.

3.2 Palmitate Oxidation

The graph shows the amount of radioactive H_2O per hour formed with ^{3}H during palmite oxidation in the reference sample (5 µmol DMSO) and in samples with 2 µmol respectively 5 µmol amounts of 2-HE.

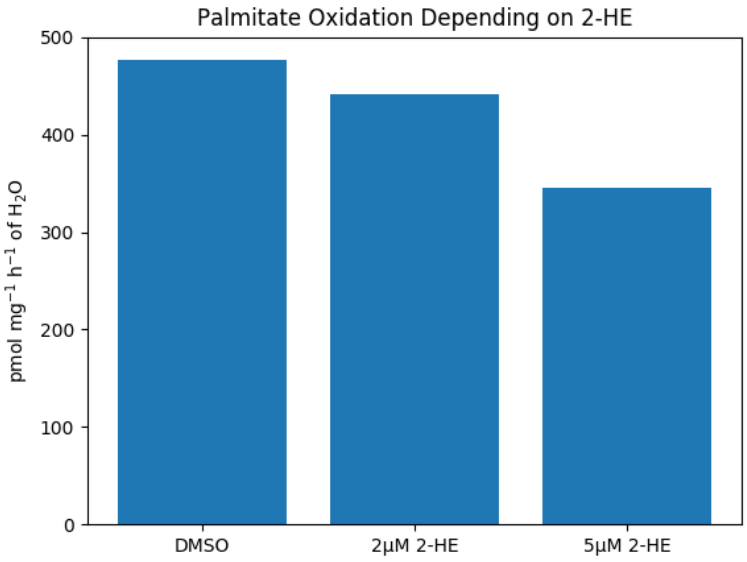

Figure 6: Palmitate Oxidation Depending on 2-HE

The amount of H_2O in the reference DMSO sample was 476.49 pmol mg^{-1} h^{-1}, 442.17 pmol mg^{-1} h^{-1} in the 2 µmol sample and 344.91 pmol mg^{-1} h^{-1} in the 5 µmol sample.

3.3 Gene Expression

The graph shows the amount of RNA expression for the gene IL6 that was measured in each respective sample, scaled for easier comparison. According to the scaled data, the RNA expressions for the DMSO (reference sample) was 0.69, 1.06 in the 2 µmol 2-HE sample and 1.24 in the 5 µmol sample.

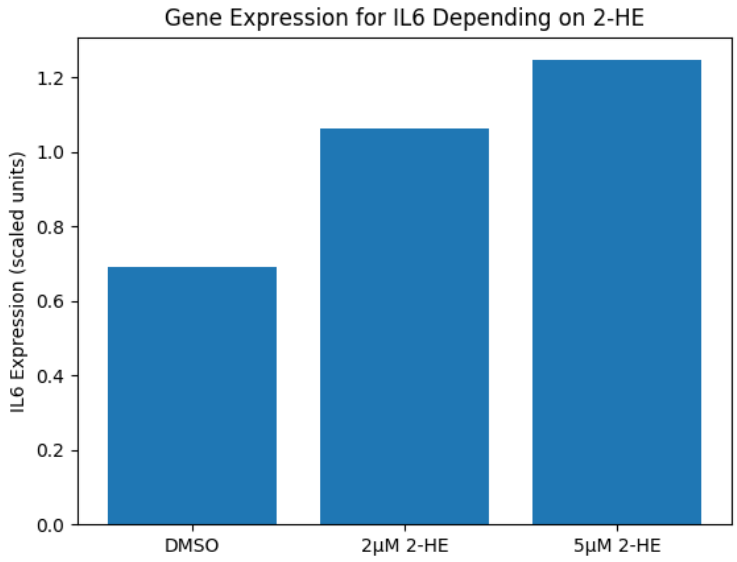

Figure 7: Gene Expression for IL6 Depending on 2-HE

4 Discussion

The results from the glycogen synthesis and palmitate oxidation indicate that 2-HE impacts these functions negatively. 2-HE seems to increase the expression of the inflammatory marker *IL6*.

4.1 Glycogen Synthesis

Glycogen synthesis seemed to be affected by 2-HE though the results were not expected. In the samples without insulin, the 2 µmol sample of 2-HE showed a decrease in the amount of glycogen synthesised, while the 5 µmol sample of 2-HE showed an increase. The 2-HE results in the samples without insulin were expected to be similar to the DMSO reference sample since insulin has a key role in glycogen synthesis, thus 2-HE might not affect glucose synthesis negatively without the presence insulin. It is possible that 2-HE only targets glucose processes activated with insulin present.

In the samples with added insulin, there was a decrease in glycogen synthesis in both the 2 µmol sample and the 5 µmol sample, indicating that 2-HE has an effect on the

insulin receptors or pathway. Insulin enables glucose to enter the cell, which theoretically leads to an increased amount of synthesised glycogen. It was not dose determined since the 2 µmol sample had produced less glycogen in comparison with the 5 µmol sample.

4.2 Palmitate Oxidation

Palmitate oxidation was affected by 2-HE. The 2 µmol and the 5 µmol 2-HE sample showed a decrease in the amount of palmitate oxidized compared to the control DMSO sample. Furthermore, in the 5 µmol 2-HE sample the cells oxidized less palmitate than in the 2 µmol, showing that the effect on palmite oxidation is dose dependent.

The palmitate oxidation data indicates that 2-HE disturbs the palmitate oxidation mechanisms in skeletal muscle cell at elevated levels $\leq$ 2 µmol. More studies with varying concentrations of 2-HE need to be done to confirm this indication. If high levels of 2-HE impair palmite oxidation, it could lead to adipose desposits in the body with more 2-HE formed as a result of synthesis in the new fatty tissues. The increased 2-HE could possible contribute to other negative effects in the body, such as increased insulin resistance and abnormal RNA replication, leading to a self-perpetuating cycle.

4.3 Gene Expression

The RNA analysis indicates that 2-HE increases gene expressions of IL6, which is a sign of inflammation in the cells. The data also shows that the level of inflammation is dose dependent since the 5 µmol 2-HE sample showed a higher increase than the 2 µmol 2-HE sample. This was expected since IL6 had been previously shown to cause chronic inflammation (see section 1.1.1).

4.4 Further Studies

The glucose synthesis data deviated from the rest of the data by not indicating a clear link between concentration of 2-HE and impaired functions. If the data was indeed correct,

more experiments could be performed to study why glucose synthesis is not as affected by 2-HE as other metabolic functions. Regarding RNA expression and inflammation, it could be valuable knowing which specific parts of the cell that 2-HE target to better understand better how the inflammation process affected by *IL6* works.

Further studies should be performed in order to determine what concentration of 2-HE becomes harmful in tissues, in order to be able to screen for abnormal levels in a population. Studies could also be done on how to prevent harmful levels of 2-HE in medicine, but also lifestyle causes.

5 Conclusion

Based on the data regarding the effect of 2-HE on glycogen synthesis, palmitate oxidation and gene expression, elevated doses of 2-HE seem to affect the metabolic processes in the body regarding RNA expression and palmite oxidation. The data for glycogen synthesis does not have the same correlation between increased doses of 2-HE and decreased synthesis. It is shown though that 2-HE affects the insulin pathway. 2-HE thus affects some of the metabolic pathways of the skeletal muscle cells, contributing to T2D.

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A Glycogen Synthesis

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 dH2O. 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

Figure 8: Glycogen Synthesis

16. Vortex
17. Transfer the solution to 4 ml scintillation vials containing 2.7 ml of scintillation liquid and count in a liquid scintillation counter.
18. Controls: Dilute the D-[U-14C]-glucose solution used (1 µl + 49 µl DMEM) 1/100 with H2O, add 20 µl of this to scintillation vials (triplicate).
19. Determine protein concentration and use the calculation template available in the common tools to calculate values.

Reference

L. Al-Khalili, A.V. Chibalin, K. Kannisto, B. B. Zhang, J. Permert, G. D. Holman, E. Ehrenborg, V. D. H. Ding, J. R. Zierath and A. Krook, Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content CMLS, Cell. Mol. Life Sci. 60 (2003) 991-998.

File Attachment

Sameer glycogen synthesis template.xlsx

SHA512 checksum
Rak3Akn3yslsR5R49HomMJKGU0zLJjQpGT+7uYgrB9xBhxqdp7qkcZhbX+YLSpupGc8Owc3TEmIrlStJ+q0rkg==

GS_lnger.xlsx

SHA512 checksum
2mzmhGS2EMq1adSILSJYVKHF1ufytY4B9MuD0fEIDx9G7wMZAEoNbSY5s6S0VOEN+r7C8ezwjgTge4iMTUTEUg==

GS_lnger_simplified.xlsx

SHA512 checksum
JP0UjiU2BNWOtnI2NaWlAhm222UdIAgVTDOiUG996XvxXVdDFCpocQLPz0uN2UrttYy7/UVGVHNZAfaqxaMNWw==

Results graph - about 2% is left after o/n incubation. See EXP-12-BA1321

sheet1.xlsx

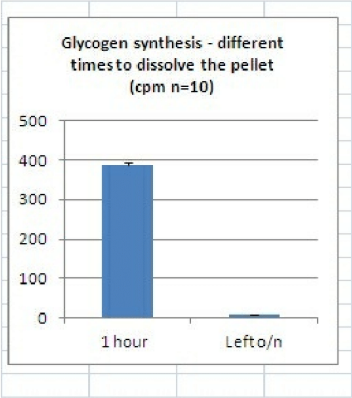

Figure 9: Glycogen Synthesis

B Protein determination

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.

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Figure 10: Protein Determination

C Palmitate Oxidation

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?

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Figure 11: Palmitate Oxidation

D RNA Extraction

E.Z.N.A.® TOTAL RNA KIT I

Revision No: 3.0

omega BIO-TEK

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.

Figure 12: RNA Extraction

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.

Figure 13: RNA Extraction

E cDNA Synthesis

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.

Figure 14: DNA Synthesis

F Data for Graphs

Table 1: Total Glycogen Synthesis Data (^{14}C cpm/mg of protein)

Concentration	Dataset 1	Dataset 2	Dataset 3	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 2: Total Palmitate Oxidation Data (pmol mg^{-1} h^{-1} H_2O)

Concentration	Dataset 1	Dataset 2	Dataset 3	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