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Forrest J. Longway
East Tennessee State University

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Measuring the Effects of CTRP3 and Metformin on H4IIE Hepatocyte Metabolism Using Seahorse Extracellular Flux Analyzer

Thesis submitted in partial fulfillment of Honors

By

Forrest Longway

The Honors College

HID Human Health Program

East Tennessee State University

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Forrest Longway, Author

Dr. Jonathan Peterson, Faculty Mentor

Dr. Gary Wright, Co-Faculty Mentor

Dr. Michael McKamey, Faculty reader

Dr. Burl Williams, Faculty Reader

Abstract:

Non-alcoholic fatty liver disease (NAFLD) results from an unequal uptake/storage and export/oxidation of lipids within the liver and is often a secondary disease to type II diabetes (22). NAFLD causes this imbalance of lipids by altering glucose and lipid metabolism, which corresponds to a decrease in mitochondrial function leading to failure of the liver. One established treatment for type II diabetes and NAFLD is the drug metformin, which has similar properties to the newly discovered CTRP 3 protein which is part of a group of bioactive molecules secreted by adipose tissue, collectively termed adipokines (2-4). Both have similar effects on hepatic glucose and lipid metabolism and both specifically suppress hepatic gluconeogenesis (11, 17, 27, 29). The revolutionary Seahorse extracellular flux analyzer was used to measure the metabolism of H4IIE hepatocytes without use of radiolabeling (1). By detecting the Oxygen Consumption Rate (OCR) of hepatocytes the level of metabolic function within mitochondria can be measured. Once an effective protocol was established using this new technology, hepatocytes treated with metformin had a significantly lower OCR compared to control treated hepatocytes. However, H4IIE hepatocytes treated with metformin and palmitate had a significant increase in OCR and eventually equilibrated with the lower OCR of hepatocytes solely treated with metformin. With similar effect, hepatocytes treated with CTRP3 and palmitate caused a drastic increase in OCR while hepatocytes treated with only CTRP3 had a decrease in OCR. This suggests that CTRP3 increases fatty acid oxidation which decreases lipid concentrations within hepatocytes which could mean future protection of liver against NAFLD. In conclusion, our Seahorse XF analyzer models compare metformin and CTRP3's similarities and suggest the possible liver protective functions of CTRP3. Our results will aid in future research of CTRP3 to further determine its possible uses as a treatment for liver-associated diseases.

Introduction

The emergence of the Seahorse extracellular flux analyzer has uprooted the traditional methods of measuring the metabolic activity of cells. The use of this machine allows one to measure oxygen consumption rate, extracellular acidification rate and pH without the use of radiolabeling molecules. One published paper has demonstrated the accuracy of measuring mitochondrial function by specifically measuring fatty acid oxidation using the extracellular flux analyzer compared to traditional radiolabeling of the fatty acid oxidation end product, ^3HOH (1).

The ability to make measurements of metabolic functions in a wide variety of cells within minutes opens the door to a multitude of research opportunities. One such opportunity is using H4IIE hepatocytes to study the effects of the newly discovered protein, C1q TNF Related Protein 3 (CTRP3), on liver function. The CTRP3 protein is one of 15 CTRP proteins that are part of a group of bioactive molecules secreted by adipose tissue, collectively termed adipokines (2-4). Adipokines play important roles in regulating insulin sensitivity and energy balance, but are noticed to have unusually low and high circulating concentrations in medical cases of diabetes or obesity (4).

The most heavily studied adipokine, adipocyte complement-related protein 30 kDa (Acrp30/adiponectin), has a distribution of receptors throughout the peripheral systems which allows Acrp30/adiponectin the ability to influence a variety of metabolic functions, such as; insulin sensitizing, anti-inflammatory and anti-atherogenic properties (2, 4-10). The C1q/TNR-related protein (CTRP) protein family was discovered by through a bioinformatics analysis of the

human genome based on a structural similarity to Acrp30/adiponectin (3). To date, 15 CTRP family members have been identified, termed CTRP1-CTRP15. CTRP15 is also known as myonectin as it is the only identified member of this protein family to be selectively expressed from skeletal muscle instead of adipose tissue (ref Seldin 2013). Many CTRP proteins have demonstrated unique and tissue specific metabolic functions (3, 11-16).

Out of the family of CTRP, CTRP3 is the one that we have focused on looking more in depth to see its effect metabolic functions. Studies have already proven that CTRP3 has protective qualities within the heart following an ischemia (19-20). In other studies it was found that intraperitoneal injections of CTRP3 caused an acute decrease in blood glucose primarily by reducing hepatic gluconeogenesis (11, 17). CTRP3 also reduces triglyceride accumulation within the liver, but the exact mechanism is still not fully understood (27).

There is great potential for CTRP3 to become a therapeutic drug for liver associated diseases due to how it affects hepatic lipid and glucose metabolism. A prominent liver disease is Non-alcoholic fatty liver disease (NAFLD) that results from an unequal uptake/storage and export/oxidation of lipids within the liver (22). NAFLD causes this imbalance of lipids by increasing glucose and lipid metabolism, which corresponds to a decrease in mitochondrial function. The relationship between liver metabolism function and CTRP3 has not been fully evaluated, but since CTRP3 can drastically reduce hepatic triglyceride accumulation it is likely there are other metabolic processes within hepatocytes that are affected (23, 24, 25).

Patients with NAFLD have been shown to have significantly increased levels of hepatic apoptosis due to continually high concentration of lipids, which leads to an increased severity

of the disease (25, 26). Proteins that function to counteract apoptosis, Bcl-xl and Mcl-1, are shown to be reduced and make hepatocytes more vulnerable to oxidative stress and other acute apoptotic stimulus within NAFLD patients (25). An effective anti-apoptotic therapy might help to lessen the severity of this syndrome of NAFLD patients (26). Further hepatic research has shown that CTRP3 prevents liver lipid accumulation by regulating triglyceride metabolism, which may suggest CTRP3 directly or indirectly prevents hepatic lipoapoptosis (27). This, along with the fact that CTRP3 has been shown to shield cardiac tissue from myocardial infarction induced apoptotic signaling, may indicate CTRP3 as a future protective therapeutic drug for lipoapoptosis of NAFLD patients (20).

This is why it is with great importance that a model protocol be developed with H4IIE hepatocytes using the Seahorse Extracellular Flux Analyzer so that hepatic metabolic effects of CTRP3 can be measured. Since the technology of the extracellular flux analyzer is relatively new, there is little information regarding its use related to H4IIE hepatocyte metabolism. However, two papers have been our main focus to try to replicate and create a model for our specific needs of measuring CTRP3 effects on H4IIE cells. The first paper proposed a protocol to measure fatty acid oxidation within myoblasts using the analyzer (1). The second paper showed a protocol for measuring the effects of the drug metformin on metabolic performance of H4IIE hepatocytes (28).

By combining the protocols of both papers we could develop a well-defined study looking at the effects of metabolism in metformin treated H4IIE hepatocytes. This would help to fine tune a metabolism protocol with the seahorse XF analyzer in which metformin treatment would be

eventually replaced with CTRP3 treatment. Metformin is a great starting point because both metformin and CTRP3 have very similar effects on hepatic glucose metabolism in that they both suppress hepatic gluconeogenesis (11, 17, 27, 29). Metformin is also a wide-spread therapy in the treatment of type 2 diabetes and NAFLD, which directly correlates with the potential therapeutic uses of CTRP3 (11,17,27,29,30). So, profiling the metabolic effects of both CTRP3 and metformin on H4IIE cells may help to show the similarities of function and help CTRP3 to be a new NAFLD therapy.

Materials and Methods

General Methodology

Growth of H4IIE hepatocytes. H4IIE hepatocytes were grown and maintained in cell culture plates with growth media containing 10% fetal-bovine serum in high glucose DMEM via standard aseptic cell culture techniques. Each cell culture dish was given fresh 15 ml of the 10% FBS growth media every 48 hours for the duration of the growth process.

Seahorse XF analyzer Setup. There are two packages that are used with every experiment for the XF analyzer. One is the injection well and must be incubated overnight in a non-CO₂ incubator with 500 µl of XF calibrant in each well. The other package was the cell plate for culturing the H4IIE cells. Once the cells are ready for experimentation and the injection plate

has calibrated overnight, then the injection plate's wells were loaded with the needed chemicals. Using the Seahorse XF analyzer's "assay wizard" an experimental protocol was set up to do a 3 minute session of mixing, a 2 minute session of time delay, and a 3 minute session of measuring. All three of those actions counted as one cycle and were repeated until the desired time is reached for the injections from the injection plate. The Seahorse analyzer then gave step by step directions of what was needed. The XF measurements are non-destructive so the metabolic rate of the same cell population can be measured repeatedly over time without interruption.

Palmitate/BSA preparation. Seahorse provided protocol for preparing correct Palmitate/BSA complex. Two separate 1 L beakers were warmed with 200 ml of tap water in a hot water bath. A separate 250 ml beaker with a stir bar was then warmed in a 37 C water bath. Then, 9 ml of 5M NaCl stock solution was added to 291 ml of dH₂O to make 300 ml of 150 mM NaCl. Ultra fatty acid free BSA was weighed out to a mass of 2.267 g. Then 100 ml of the prepared 150 mM NaCl was transferred to the pre-warmed 250 ml glass beaker with stir bar, which was then placed on a stir plate. The weighed BSA was added to the beaker on the stir plate slowly with continuous stirring. One of the pre-warmed 1L beakers with tap water was placed on a heated stir place. The BSA solution beaker was covered with parafilm and then placed in the 1 liter 37 C⁰ beaker/water bath. The BSA solution beaker was stirred until completely dissolved and then filtered with a vacuum. The filtered BSA solution had 50 ml transferred to a pre-warmed 250 ml beaker, covered with parafilm, and placed within the 1 L beaker. The filtered BSA solution that remained was diluted with 50 ml of 150 mM NaCl solution to make a 0.17 mM stock solution that was aliquoted into 4 ml glass vial at -20 C (this was BSA vehicle control).

Sodium palmitate was weighed out to a mass of 30.6 mg and added to 44 ml of the 150 mM NaCl solution in a 50 ml Erlenmeyer flask. The palmitate flask was placed within the 1 Liter 70 C⁰ water bath. Once solutions achieved correct temperature, 40 ml of palmitate solution was transferred to the BSA solution and stirred 1 hr at 40 C⁰. The final volume of palmitate/BSA solution was adjusted to a volume of 100 ml to achieve a final concentration of 150 mM NaCl to give a 1 mM palmitate solution, pH 7.4. The solution was prepared into 4 ml aliquots in glass vials and stored at -20 C.

CTRP3 protein purification. As previously and routinely performed in the Peterson lab, recombinant full-length mouse CTRP3, containing a C-terminal FLAG *tag* epitope (DYKDDDDK), was produced in mammalian cells (17). Briefly, pCDNA3.1 expression constructs encoding C-terminal FLAG-tagged mouse CTRP3, were used in transient transfections to generate recombinant proteins. HEK 293 cells (Grip-TiteTM cells, Invitrogen) were cultured in DMEM containing 10% (v/v) bovine calf serum supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. Transfections were performed in HEK 293 cells using lipofectamine (Invitrogen) or calcium phosphate method. At 24 h post transfection, media were replaced with serum-free Opti-MEM media (Invitrogen) supplemented with vitamin C (0.1 mg/ml). Supernatants were collected three times, every 48 h, pooled and purified using an anti-FLAG affinity gel according to the manufacturer's protocol (Sigma), and eluted with 150 µg/ml FLAG peptide (Sigma). Purified proteins were dialysed against 20 mM Hepes buffer (pH 8.0) containing 135 mM NaCl in a 10 kDa cut-off Slide-A-Lyzer dialysis cassette (17).

Specific Methodology

1).Developing effect of metformin on H4IIE hepatocyte metabolism model

Cells were split into Seahorse XF 24 well plates to obtain 70% confluence within each of the 20 used wells (A1, C3, 4B and 6D were designated as cell-less temperature controls by XF analyzer). Cells were allowed a day to attach and then were washed with serum-free low glucose DMEM and allowed overnight incubation with serum-free low glucose DMEM. The injection well plate was also incubated overnight with XF analyzer calibrant overnight in a non-CO2 incubator. After the overnight incubation, A 15 ml falcon tube was filled with 10 ml KHB buffer supplemented with 100 μ l of carnitine (0.5M); labeled as “control.” Carnitine is an amino acid that has been found to aid in liver lipid metabolism and protect mitochondrial function (32). A 50 ml falcon tube was filled with 30 ml of KHB buffer supplemented with 300 μ l carnitine (0.5 mM) and metformin (2mM); labeled as “experimental.”

The 24 well plate of cells were washed with 250 μ l of control. The first 3 columns were then filled with 250 μ l of control and the last 3 columns were filled with experimental. The cells were then incubated for 1 hour. During the one hour incubation 1mM decoupler 2,4-dinitrophenol (DNP) was diluted with KHB buffer and added to well A of every injection well (final concentration 100 μ M DNP). The protocol was set to inject well A of the mitochondrial DNP, which is an anti-obesity drug at 104 minutes (31). This was followed by another 48 minutes worth of mix, time delay, and measure periods. Because we were only measuring one variable there is an n=10 for each group. Our model paper (28) was followed exactly at first, but slight variations were made with each experiment treatment to make a better model of

oxygen consumption rates of metformin treated H4IIE hepatocytes, which correlate with mitochondrial/metabolic function.

2). Effect of metformin on H4IIE hepatocyte metabolism in presence of Palmitic acid model

The protocol for examining metformin was followed with adjustments discussed in results-2 section. Palmitic acid was also introduced into the protocol and was added as an injection within the Seahorse XF analyzer, which gave n=5 for each experimental group. Palmitic acid or the vehicle control BSA was added (final concentration 0.2 mM) to metformin treated hepatocytes and vehicle control KHB. Palmitic acid was chosen because it is a saturated fatty acid that is easily taken up and metabolized by the cell and will promote the expression of pro-inflammatory, cytokines, disrupts insulin signaling, and stimulates apoptosis. Excess lipid accumulation leads to increased oxidative stress and mitochondrial dysfunction, which will be noted by the analyzer (24). Palmitic acid treatment will allow us to measure the different rate of palmitate utilization within cells treated with or without metformin, which could show possible protective characteristic of metformin in H4IIE cells.

3). Effect of CTRP3 on H4IIE hepatocyte metabolism in presence of palmitate model

The previous protocol using metformin and palmitate treated cells was followed. However, cells were treated with CTRP3 (5 µg/ml) or without CTRP3 in place of metformin. This gave us n=5 for each treatment group of hepatocytes.

RESULTS

Results-1: Developing model to test the effect of metformin on H4IIE cells

Results-1a: Following the exact procedure described by Logie, et al (28).

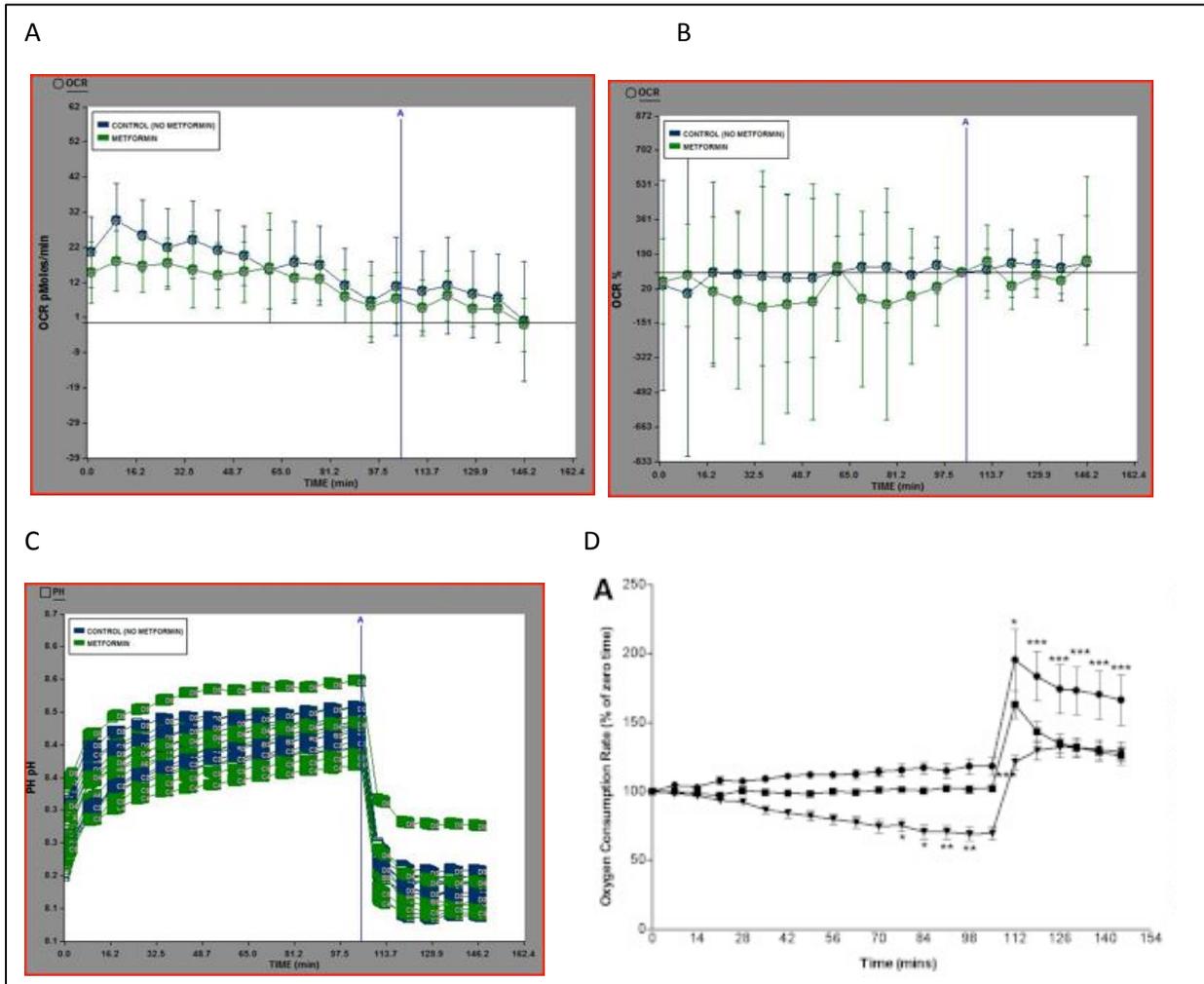


Figure 1. H4IIE hepatocytes treated as described by Logie et al (28), where time 0 is 1 hour incubation with metformin. Hepatocytes were treated with metformin (green) or vehicle control (blue). The vertical line represents the injection of mitochondrial decoupler, DNP. **A**, Oxygen Consumption Rate (OCR) presented in pMol/min. **B**, OCR presented as percent change compared to basal. When OCR is increased/decreased over time it is shown a percent increase/decrease according to basal OCR rate (100%). **C**, Shows the increasing pH levels of both group types. **D**, Published paper by Logie et al (28) OCR graph stimulation without (squares) or with metformin (inverted triangles). OCR, Oxygen Consumption Rate; Vehicle control, KHB.

The protocol within paper (28) was followed exactly and shown in figure 1D, but as can be seen in figure 1B we had very different results. The cells continually decrease in oxygen consumption rate (OCR) until the H4IIE hepatocytes reached 0% OCR as shown in figure A, which indicates the cells were dying. The cells dying would explain why the decouple DNP did not work after injection. Figure 1C shows that the pH of the cells increased as high as 8.6, which is most likely the reason for the cell death and steadily decreasing OCR. This experiment was repeated, but changing the overnight incubation by decreasing to 17 hours. However, the results were exactly the same as shown in figure 1 and with the same pH change as noted in figure 1C. Our results did not match the published work by Logie et al (28), therefore the next series of experiments were performed.

Results-1b: First series of modifications

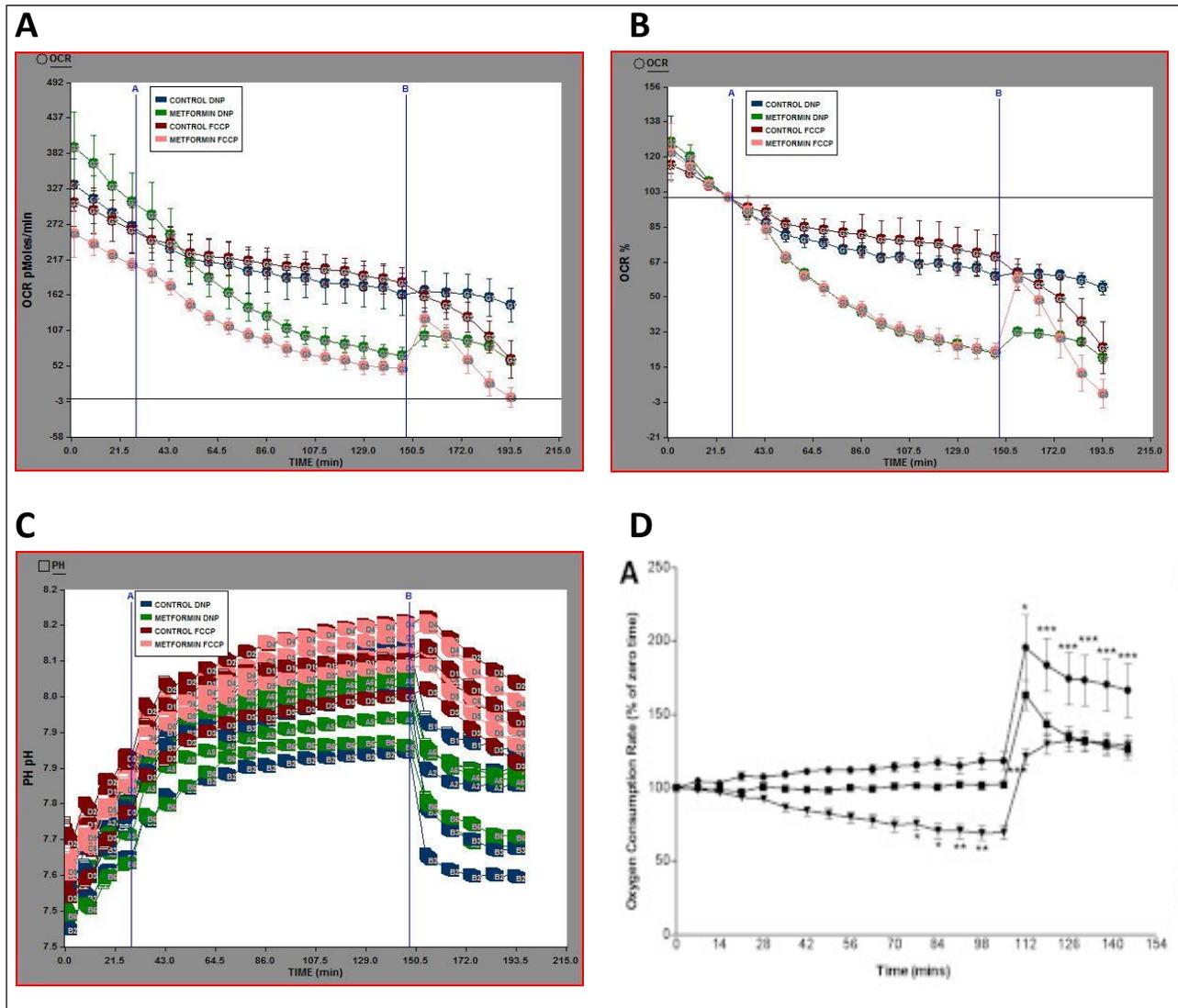


Figure 2. H4IIE hepatocytes treated as described in published paper by Logie et al (28) with adjustments described in results. Hepatocytes were treated with either metformin and DNP (green), metformin and FCCP (pink), vehicle control and DNP (blue), or vehicle control and FCCP (red). The vertical line with letter A represents the injection of metformin or vehicle control. The vertical line with letter B represents the addition of either DNP mitochondrial decoupler or FCCP mitochondrial decoupler. **A**, Oxygen Consumption Rate (OCR) presented in pMol/min. **B**, OCR presented as percent change compared to basal. When OCR is increased/decreased over time it is shown a percent increase/decrease according to basal OCR rate (100%). **C**, Shows the increasing pH levels of both group types. **D**, Published paper by Logie et al (28) OCR graph in which we are trying to model stimulation without (squares) or with metformin (inverted triangles) (28). Vehicle control, KHB.

This attempt at making a better model changed many different variables from the original protocol. There was no longer any overnight incubation of serum free DMEM or use of krebs buffer within the H4IIE hepatocytes. Instead, cells were split into XF 24 well plate and allowed over 4 hours to properly attach with 100 ul of normal cell media (10% FBS DMEM) within each well. Well volume was brought to 250 an hour before experiment begins, after allowing at least 4 hours to attach. Metformin was not added to the media before experiment, but was instead weighed out to a mass of 0.01 g and dissolved within 5 ml of KHB buffer. The metformin was then made to be the first injection (final concentration in well 2mM) within the seahorse wells after 24 minutes of basal cell metabolism readings. The second injection was then made to be either DNP or FCCP to further investigate the possibility of a better performing mitochondrial decoupler. The pH in figure 2C still showed an increase, but was found to be significantly lower than the pH increase in figure 1C. Overall cell metabolism was significantly improved within this protocol as shown within figure 2A and 2B. Metformin treated H4IIE hepatocytes caused a decrease in OCR when compared to vehicle control KHB, which mimics the effects shown within our model pape shown in figure 2D (28). FCCP also showed to be a slightly better mitochondrial decoupler when compared to DNP. This was established as our protocol for measuring the effect of metformin on H4IIE hepatocyte metabolism, except FCCP was decided to be the designated mitochondrial decoupler injected. Our results did not match the published work by Logie et al (28), therefore the next series of experiments were performed.

Results-2: Developing model to test effect of metformin in presence of Palmitic acid

Results-2a:

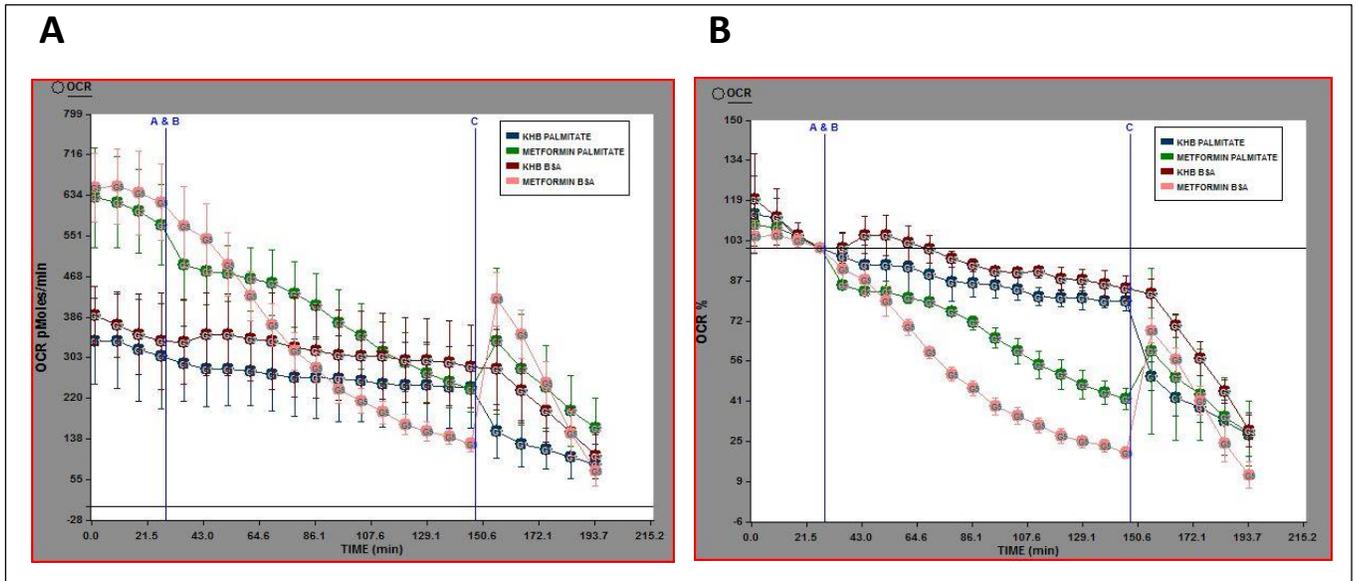


Figure 3. H4IIE hepatocytes were treated with metformin and palmitate (green), metformin and BSA (pink), KHB and palmitate (blue), or KHB and BSA (red). Vertical line letter A is injection either metformin or vehicle control, vertical line B is either Palmitate or vehicle control injection, vertical line letter C is injection of FCCP **A**, Oxygen Consumption Rate (OCR) presented in pMol/min. **B**, Represents figure 3A's data as OCR percent where baseline rate data is set at 100%. Metformin vehicle control, KHB; Palmitate vehicle control, BSA

The experiments described for figure 2 were duplicated, except (100uM) FCCP was the only mitochondrial decoupler. Further, the addition of the free fatty acid palmitate was also included. Thus, well A injected every cell with either metformin or vehicle control (KHB) and well B injected every cell with either palmitate or the palmitate vehicle control (BSA). This model shows how the hepatocytes first react to the injection of metformin followed by how they react over the next 130 minutes. Metformin treated H4IIE cells differentiated themselves from the metformin vehicle control hepatocytes by having significantly lower OCR% rates.

Results-2b

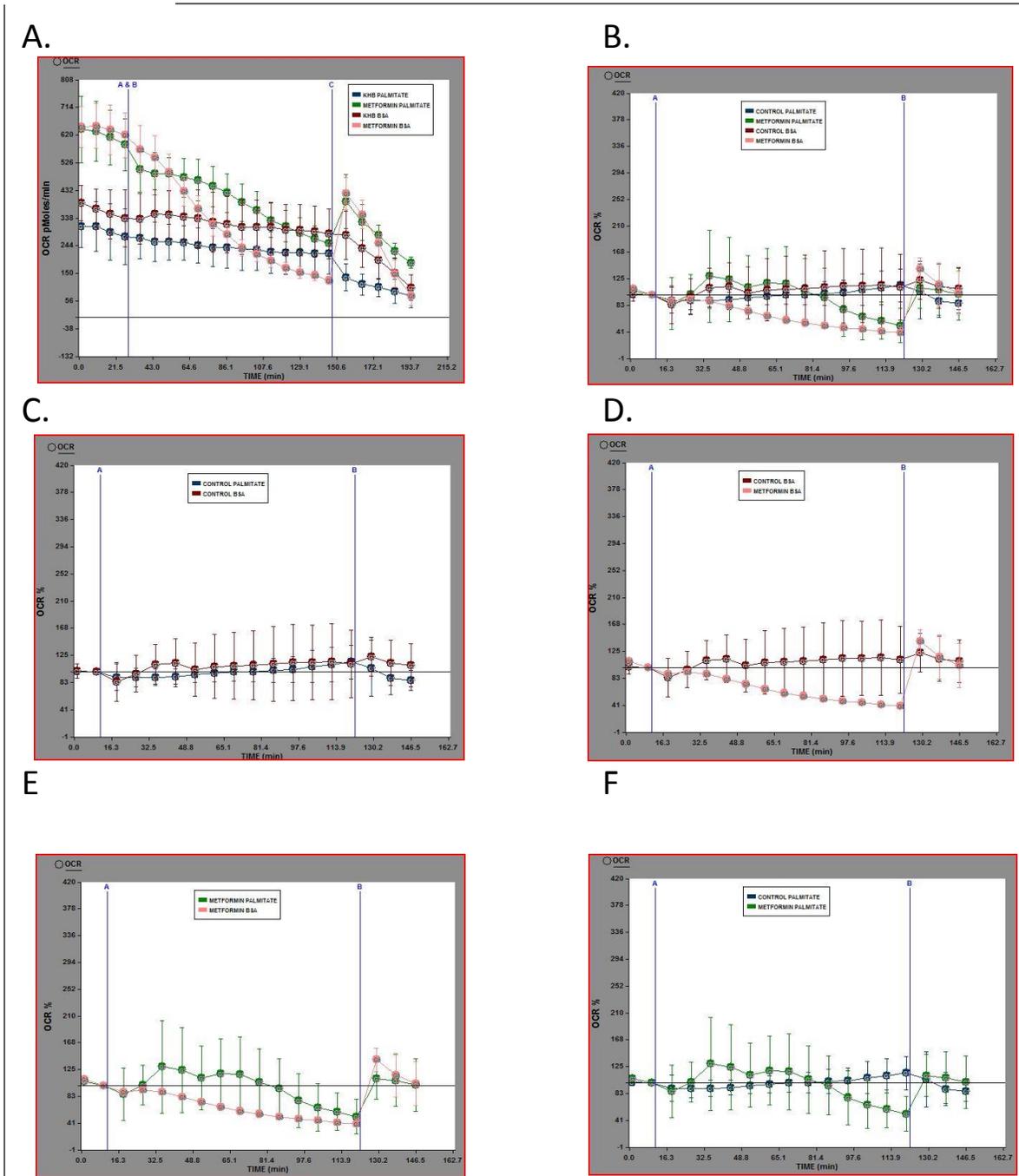


Figure 4. Time zero represents 1 hour incubation with either metformin or metformin vehicle control. Injection well A represents the injection of either palmitate or palmitate vehicle control. Injection well B represents the injection of mitochondrial decoupler FCCP to all cells. **A**, Oxygen Consumption Rate (OCR) presented in pMol/min. Rest of graphs present OCR% where baseline rate data is set at 100%. **B**, OCR% all experimental groups. **C**, H4IIE hepatocytes treated with metformin vehicle control and palmitate (blue) or metformin vehicle control and palmitate vehicle control (red). **D**, H4IIE hepatocytes treated with metformin and palmitate vehicle control (pink group) or metformin vehicle control and palmitate vehicle control (red). **E**, H4IIE hepatocytes treated with metformin and palmitate (green) or metformin and palmitate vehicle control (pink). **F**, H4IIE hepatocytes treated with metformin and palmitate (green) or metformin vehicle control and palmitate (blue). Metformin vehicle control, 10% FBS DMEM; Palmitate vehicle control, BSA

The protocol followed is described in results 2a except that cells were incubated with 2mM metformin one hour prior to beginning seahorse XF analyzer. So, the only injections in the Seahorse XF analyzer were palmitate or BSA vehicle control and FCCP mitochondrial decoupler. Logie et al (28), reported that it took one hour exposure to metformin in order to show its effects. So we concentrated on what happens to hepatocyte metabolism specifically after that hour incubation with metformin and in the presence of palmitate. Metformin without palmitate had a lower OCR% when compared to all the other groups. However, metformin and palmitate had a sharp OCR% increase right after palmitate injection and eventually equilibrated with the lower OCR% of solely metformin treated hepatocytes. Hepatocytes treated with the metformin vehicle control (10% FBS DMEM) and palmitate had a lower OCR % than the double control hepatocytes.

Results-3: Developing model to test the effect of CTRP3 with palmitate on H4IIE cells

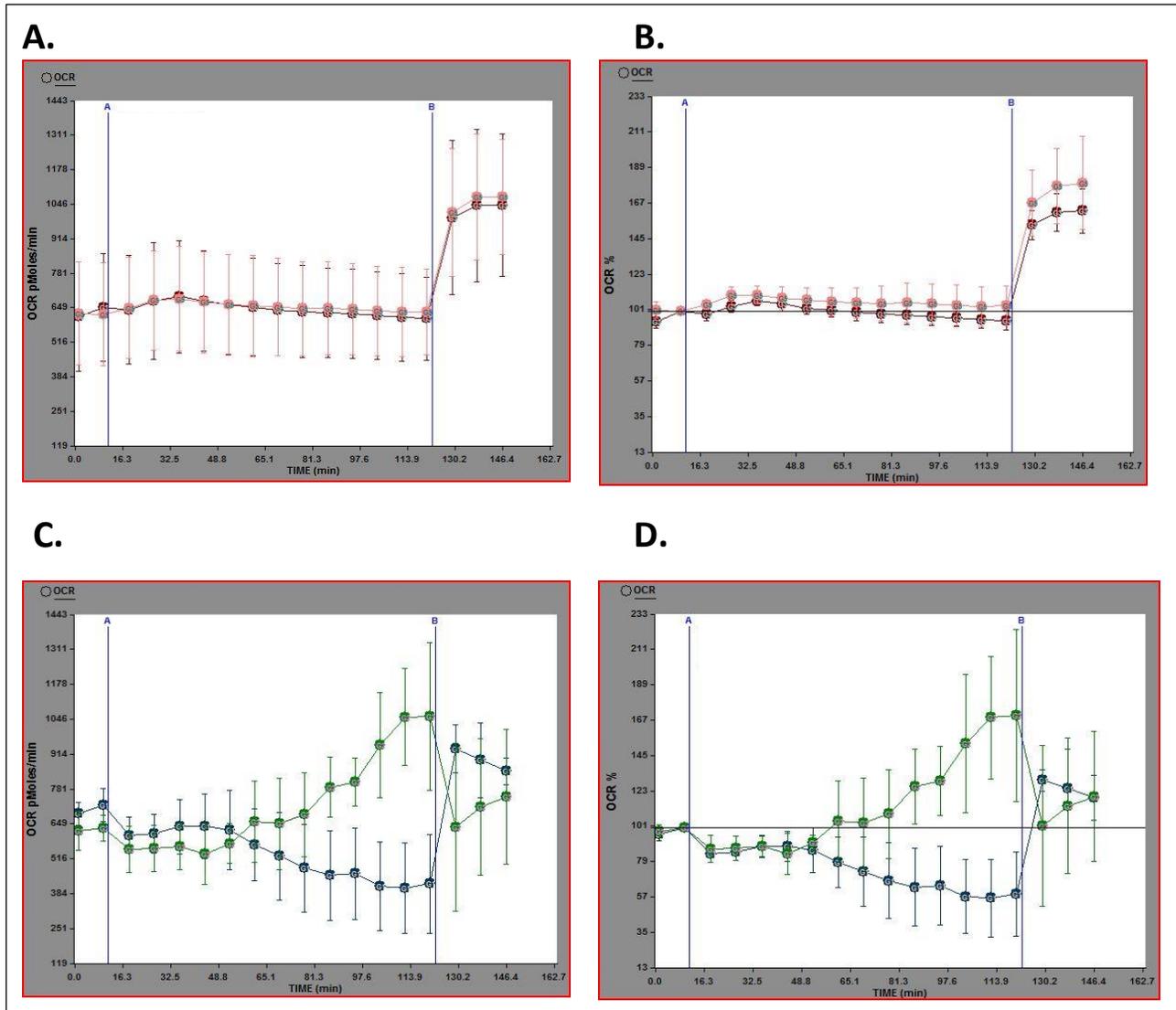


Figure 5. Time zero represents one hour incubation of H4IIE hepatocytes with or without CTRP3. Vertical line with letter a represents injection of palmitate or palmitate vehicle control, and letter b represents injection of FCCP. **A**, OCR measured in pmol/min of CTRP3 treated (pink) and no CTRP3 (red) without presence of palmitate. **B**, OCR% baseline measure of figure 5A data. **C**, OCR measured in pmol/min of CTRP3 with palmitate (green) or no CTRP3 with palmitate (blue). **D**, OCR% baseline measure of figure 5C data.

Figures 5A and 5B show that there is no difference between hepatocytes without palmitate treatment, even when hepatocytes were treated with CTRP3. However, palmitate treated cells had significantly different responses depending on whether or not hepatocytes were treated with CTRP3. H4IIE hepatocytes that were treated with CTRP3 and palmitate had a drastic increase in OCR due to increased fatty acid oxidation. However, cells that were treated with CTRP3 without palmitate treatment had a drastic decrease in OCR, due to decrease in mitochondrial metabolic activity. Interestingly, mitochondrial decoupler FCCP caused a sharp OCR decrease only with CTRP3 and palmitate treated hepatocytes but can't be explained currently. Due to the complexity of extracting CTRP3 and diminished stock supply this was the only experimental run performed.

DISCUSSION

We were unable to replicate the data presented by Logie et al, (28) published protocol for measuring metabolic activity of H4IIE hepatocytes using the seahorse XF analyzer. The cells all appeared to die as noted by the extremely low oxygen consumption rate (OCR) that eventually gets down to 0. One theory is that since the seahorse XF analyzer does not regulate CO₂ levels the cell media would have continually dropping levels of acidic CO₂ solution, creating a basic solution. In figure 1C it is shown that the pH got as high as 8.6 which is too basic for cells to survive. The lightly buffered KHB solution that the cells were kept in during the use of the Seahorse XF analyzer thus did not properly keeping the solution at a neutral pH. It was also thought that incubating the cells overnight in serum free cell media may have starved the cells to the point of death. Since all of the cells were unhealthy and dying the effects of metformin could not be properly measured and changes were needed for the protocol used.

Adjustments were made to eliminate the overnight incubation with serum free cell media and discontinue the use of KHB. It was found that by allowing 4 hours for the H4IIE hepatocytes to properly attach after splitting cells into Seahorse XF 24 well plate and keeping the cells within 10% FBS cell media, even when running within Seahorse XF analyzer, created a healthier environment for the cells. Figure 2C shows that the pH levels with the adjusted protocol rose to a maximum of an 8.2 pH, which is significantly lower than the 8.6 pH shown in figure 1C. Figure 2A also shows that OCR started out at approximately 300 which was significantly higher

than the OCR of 30 shown in figure 1A. This indicates that the cells were in a healthier environment, which allowed them to survive and present a model of the effects of metformin.

We now have similar data and figures when comparing the OCR % graph in figure 2B to published OCR% graph Logie et al in figure 2D (28). By analyzing figures 2A and 2B, H4IIE hepatocytes treated with metformin are shown to have significantly lower oxygen consumption rates. Metabolic respiration is an aerobic process that requires oxygen as the final electron acceptor. So, hepatocytes that have a lower rate of oxygen consumption directly translate to a lower rate of metabolic respiration. These results are supported by research suggesting that metformin (similar to CTRP3) alters hepatic glucose metabolism by reducing hepatic gluconeogenesis (11, 17, 27, 28, 29). The exact mechanism of how metformin reduces gluconeogenesis is still heavily debated. However, it is currently suggested that metformin reduces mitochondrial respiration by inhibiting complex I and by decreasing hepatic energy state, which is directly expressed in our data figures 2A and 2B by the lower OCR (27, 33,34).

Two models were created in studying the effects of hepatocytes treated with metformin and palmitate. Figure 3 shows the effects of metformin the instant it was injected. Figure 4 specifically demonstrates on the effects of metformin after 1 hour long incubation. Both figures show that metformin treated H4IIE hepatocytes have consistently lower OCR. However, hepatocytes treated with metformin and palmitate show a significant increase in OCR after palmitate injection compared to the lower OCR of hepatocytes without palmitate. This can be explained by the fact that metformin reduces mitochondrial respiration by inhibiting complex I, which in return increases the activation of AMP-activated protein kinase (27, 33, 35). An

increase in AMP-activated protein kinase increases the rate of fatty acid metabolism and mitochondrial biogenesis, which would require the hepatocytes to consume more oxygen (36, 37). This elevation of OCR following palmitate injection is then most likely due to the elevated utilization of palmitate through fatty acid oxidation by metformin treated H4IIE hepatocytes. It appears that palmitate was then continually depleted, thus reducing fatty acid oxidation, and explains the following decline in OCR until equilibrating with the lower OCR of hepatocytes solely treated with metformin.

Although only one experimental group measuring CTRP3's metabolic effect on H4IIE hepatocytes was performed, there was very interesting results. Both hepatocyte groups without the injection of palmitate behaved exactly the same, showing CTRP3 did not have an effect in this case. However, CTRP3 with palmitate drastically increased OCR while hepatocytes that were only treated with palmitate drastically decreased OCR. Even though the exact mechanism is still not known, this models the fact that CTRP3 reduces triglyceride accumulation within the liver (27). The sudden increase after palmitate injection suggests that CTRP3 drastically increased fatty acid oxidation in order to utilize the abundance of fatty acid palmitate. This closely mimics the effects of metformin with palmitate on H4IIE hepatocytes, as shown in figure 4, and gives a great starting point for further research on liver-protective characteristics of CTRP3. In conclusion, Our Seahorse XF analyzer data allows the comparison of metformin and CTRP3's similarities and suggest the possible liver protective functions of CTRP3. Our results will aid in future research of CTRP3 to further determine its possible uses as a treatment for liver-associated diseases.

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