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Addison J. Bond  
Chancellor's Honors Program, abond13@vols.utk.edu

Guoxun Chen Dr.  
gchen6@utk.edu

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The effects of retinoic acid and insulin on glycogenesis in HL1C hepatoma cells

University of Tennessee, Knoxville
Addison Bond
Dr. Chen
UNHO 497 and 498
Abstract

Diabetes prevalence is growing at an alarming rate. This will cause increases of comorbidities including blindness, kidney failure, and limb amputation in diabetic subjects if left untreated. Dietary interventions such as limiting sugars and saturated fats greatly improve the management of the disease. Excess glucose from the diet is stored as glycogen in the liver or muscle through glycogenesis, which is promoted by insulin. Patients with diabetes do not properly create or do not properly respond to insulin, leading to a decrease of glycogenesis and increase in glucose in the blood stream. As a micronutrient, vitamin A has been shown to affect the glucose and lipid metabolism. Recent studies have begun to investigate the role of vitamin A in combination with insulin as a potential regulator in control of glucose metabolism and glycogenesis. The current study is aimed to investigate glycogenesis in the presence of the active form of retinoic acid (RA) without or with insulin. Rat HL1C hepatoma cells were treated with control (vesicle control), 10 µM RA, 10 nM insulin, and 10 nM Insulin + 10 µM RA for 48 hours. After that, the glycogen content in those cells was determined using a glucose release assay to evaluate the effects of RA and insulin on glycogenesis in HL1C cells. The results show that RA did not increase the glycogen content by itself or in the presence of insulin in HL1C cells. We conclude that RA does not affect the insulin-induced glycogenesis in hepatoma cells. This may be helpful to evaluate vitamin A’s effects on diabetes development for patient in the diabetic community.
Introduction

According to the World Health Organization (WHO), obesity in recent years has become a global epidemic affected young children to older adults. Obesity is not a malnutrition problem isolated to high-income countries. Except for in Africa and Asia, there are more people worldwide who are obese than who are underweight. In 2017, it was estimated that around 4 million people worldwide died of obesity or of obesity related health issues. An individual is classified as being obese if his/her body mass index (BMI) is over 30 kg/m$^2$ and is classified as overweight if the BMI is over 25 kg/m$^2$. Obesity is associated with several comorbidities including cardiovascular disease, stroke, and diabetes. There is also a link to several cancers, such as breast, prostate, liver, kidney, and colon cancers specifically. The rise in obesity has played a role in the rise of these comorbidities, which has led to elevated health care costs in obese individuals. Obesity carries with it a vast amount of other health problems, making it an issue that needs to be addressed in order to save the lives of people worldwide.

The association between certain vitamins and minerals and obesity has been a growing field of interest for researchers around the world. Vitamin A (retinol) and its active form retinoic acid (RA) have been studied in the context of obesity, insulin resistance, type 2 diabetes in efforts to find new biochemical bridges between the two elements. As a micronutrient, vitamin A is essential, meaning that it cannot be synthesized in the body. One important source of an individual’s vitamin A comes from carotenoids, which are phytochemicals in colorful and leafy plants and vegetables. Carotenoids are a class of carotenoids including the specific chemical beta-carotene. Beta-carotene is also referred to as pro-vitamin A because it can be converted to retinal then retinol after reduction. Vitamin A exists as retinyl esters in meat-based foods, such as liver and fish. Both beta-carotene and retinyl esters are metabolized to retinol. Retinol undergoes a
two-step conversion to become RA. Retinol is oxidized to retinaldehyde by alcohol dehydrogenases and retinol dehydrogenase. Retinaldehyde is then further oxidized to RA by retinaldehyde dehydrogenase³.

RA’s effects on an individual’s physiology is primarily due to its ability to regulate transcription of its targeted genes⁴. RA is a ligand that binds to retinoic acid receptor (RAR)⁴, and retinoid X receptor (RXR)⁴, which form an RAR/RXR heterodimer or RXR/RXR homodimer. The activation of RAR and RXR by RA causes changes in transcription⁴. These dimers bind to retinoid acid responsive elements (RARE’s) which are present on DNA sequences of promoters of target genes regulated by RA⁴. There are a large number of genes that contain RARE sequences, making them responsive toward RA levels in the body. These include genes coding for embryonic development, neural differentiation, structural proteins, enzymes, receptors, hormones, growth factors, and cytokines⁴.

In order to understand the role of vitamin A and its interactions with glycogenesis, it is first important to gain an understanding of the liver’s role in glycogen synthesis and glucose usage. Glucose is stored as glycogen in animal cells with the largest amount being held in muscle and liver cells⁵. The liver usually contains about 4-7% of its weight as glycogen⁵. The liver is much more complex than the muscle in maintaining glucose levels and its own metabolic energy needs. It is responsible for maintaining blood glucose levels during short fasting periods while also integrating the supply of available fuels with metabolic needs of other tissues⁵. Under the correct physiological conditions, the liver can provide glucose for the whole body⁵. The liver can generate and release glucose to target tissues through gluconeogenesis (the synthesis of glucose from non-carbohydrate sources) and from glycogenolysis (the breakdown of stored glycogen)⁵. Since the liver is involved in providing glucose to other tissues as well as itself, the
rate of glycogenesis is controlled and affected by a number of different physiological conditions in the body.

Glycogenesis is the metabolic pathway in which glucose is stored as glycogen in cells. Excess glucose must be stored in the body for later use. In the liver, glycogen is mainly used for blood glucose homeostasis. Before glucose is stored as glycogen, a series of reactions must take place to convert glucose to stored glycogen. Glycogenesis begins with the phosphorylation of glucose by glucokinase and hexokinases to create glucose-6-phosphate. Glucose-6-phosphate is then converted to glucose-1-phosphate by phosphoglucomutase. Glucose-1-phosphate is next converted to uridine diphosphate glucose (UDP glucose) by UDP-glucose phosphorylase with the cofactor uridine trisphosphate. The resulting UDP glucose is the precursor to glycogen synthesis.

UDP glucose is attached to a tyrosyl residue on the glycogenin protein. Glycogenin initiates the genesis and growth of a chain of glycogen as more UDP glucose is added. The UDP glucose is added to the growing chain by the enzyme glycogen synthase. Once attached to the chain, UDP glucose is referred to as glucan. The chain continues to grow by autocatalysis. Soon branching off of the growing chain begins to occur. On the growing chain, the fourth glucan subunit binds to the first glucan of a chain of 6 glucans bonded in an alpha 1-6 configuration. This is an alpha 1-4 to alpha 1-6 bond facilitated by a branching enzyme. This continues to occur in a rosette-like pattern radiating out from the glycogenin base to form a spherical shape. The activation of the insulin signaling pathway signals for glycogenesis to begin in the liver.

Glycogen breakdown (glycogenolysis) or glycogen synthase (glycogenesis) have been shown to be linked to individual enzymes and key regulators of both pathways as well as vitamin
A. Investigation into this field began as early as the 1930’s. A study in the 1960’s investigated the pathways of glycogen in the body. Singh, Singh, and Dileepan looked at the role of vitamin A toxicity in the hepatic glycolysis pathway in rats. They found that vitamin A toxicity caused an enlargement of the liver, as well as an increase in the levels of lipids, glycogen and vitamin A in the liver. The study also concluded that vitamin A increases the activity of key gluconeogenic enzymes but did not do the same for glycolysis.

Diabetes exists in two types; type 1 or type 2. Type 1 diabetes mellitus (T1DM) is usually first diagnosed in childhood and results in insulin dependence. T1DM patients produce little to no insulin on their own. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes. T2DM results from the body’s resistance to insulin, typically as a result of overproduction and hypersecretion of insulin due to chronic consumption of high sugary or high fat foods. Insulin is an endocrine hormone in the body because it activates a signaling cascade which leads to the cell’s uptake and use of blood glucose (glucose molecules in the blood stream) among many other physiological functions. Serious health consequences can occur in those with either T1DM or T2DM if their insulin levels are not carefully monitored. People with diabetes can increase the overall risk of a premature death as well as cause serious damages to the heart, blood vessels, eyes, kidneys, and nerves.

Studies looking more closely at the relationship between vitamin A and insulin have proven to be promising fields in the future of nutrition related research. Studies from Dr. Guoxun Chen’s lab in the Department of Nutrition at the University of Tennessee made headway to investigate the relationship between the insulin signaling pathway and vitamin A. In a study designed to find how vitamin A status affects obesity, Zhang et al showed that the sterol regulatory element binding protein 1c gene (Srebp-1c) gene in the liver was significantly lower
in vitamin A deficient lean and fatty rats as compared to vitamin A sufficient rats. Srebp-1c is a lipogenic gene, meaning it functions in the creation of fats for storage. It can be expressed as a result of insulin-induced lipogenesis in the liver. The liver X receptor elements on the hepatic Srebp-1c promoter responsible for insulin response are also retinoic acid receptor elements (RARE’s). It is interesting to note that the hepatic Srebp-1c expression level in vitamin A deficient rats is significantly lower than that in vitamin A sufficient rats.

Additionally, the expression level of hepatic expression of Gck (glucose kinase gene) and Pklr (liver type pyruvate kinase gene) mRNA are significantly lower in vitamin A deficient rats than in vitamin A sufficient rats. This suggests a relationship between vitamin A status and glycolysis. It suggests that decreasing the amount of vitamin A in the diet leads to a decrease in the enzymes needed for glycolysis. A recent study in 2018 provided the momentum for the construction of this thesis. Dr. Chen and other researchers investigated the role of vitamin A’s effects on metabolic abnormalities in rats fed a high fat-diet. The research results indicated that vitamin A status altered the expression of levels of proteins required for glucose metabolism. Dr. Chen et al. concluded that vitamin A status contributes to the regulation of hepatic glucose metabolism and could regulate hepatic carbohydrate metabolism.

The interconnection between vitamin A status and hepatic carbohydrate metabolism is complex upon peering into the depths of the current literature. It is for this reason, and under the guidance of Dr. Chen, that this study was constructed. The goal of this thesis is to investigate further the interconnection between RA and insulin in liver cells with hopes to find data indicating promising areas of research moving forward. In order to implement this research into a two-semester time frame, the study was focused on the effects of RA and insulin on
glycogenesis in HL1C hepatoma cells. Based off of the current literature, it is Dr. Chen’s hypothesis that an interaction exists between RA and insulin in glycogenesis in hepatoma cells.


**Materials and Methods**

**Treatment Groups**

Four different treatment groups were created for this experiment. The groups were control (vesicle control), 10 µM RA, 10 nM Insulin, and Insulin + 10 µM RA. Fourteen plates were subcultured for each round of experiments. Of the 14 plates used, 12 were treated with different media corresponding to the different control and test groups while the remaining 2 were used to continue the cell line via subculture. These 2 plates underwent a 1 plate to 7 plate subculture, resulting in 14 plates for more rounds of the experiment. The 12 plates were divided evenly into 4 groups of 3 plates. Of the 3 plates in each group, 2 were used to assess glycogen content and the remaining plate was used for protein analysis. Prior to treatment, the medium of the cells was switched from containing 5% FBS to contain 0.5% FBS. After two days of incubation with the treatment media, the plates were removed for analysis. This process was repeated two additional times for a total of 3 experimental rounds.

**Cell culture**

The tissues used for this experiment were obtained from the same lineage as outlined in a previous study by Dr. Chen and Dr. Meredith Howell. The DMEM medium used for cell growth was supplemented with 5% FBS and 1% PS. Beginning with 2 cell plates, the cells were subcultured upon reaching 70-80% confluence on the plate. Cells were allowed to reach the appropriate confluence in an incubator at 37 degrees Celsius with 5% CO₂.

Appropriate subculturing procedure followed the same standard procedure for each subculture. The cells were removed from the incubator and placed inside the fume hood to minimize the risk of contamination. All materials (pipets, new plates, media) was either autoclaved or sterilized with ethanol spray prior to entering the hood. The media were extracted
from the plates via pipet suction and discarded. The cells were incubated within 1 milliliter of trypsin solution for 2-3 minutes to detach them from the bottom of the plate and from each other. The duration of the trypsin wash varied by each subculture and each plate’s individual confluence level. After the removal of trypsin, the plates were washed with media to mix the cells with the media. The cells and media from the plate were then placed in the same tube to assure even displacement of the cells throughout the medium. This ensured that the same number of cells was inoculated onto each subculture plate. Three mL of medium containing cells were seeded into each plate and placed in the incubator for five days.

**Glycogen extraction**

The plates were placed on ice and had their media vacuumed out. Four hundred microliters of ice-cold PBS were added to each plate. The plate was scrapped, and the content was transferred in a 1.5 microcentrifuge tube. The tube was centrifuged at 1,000 x RPM at 4 degrees Celsius for 5 minutes. Residual liquid was removed as much as possible, and the remaining mass of cells was then weighed. 30% KOH solution was added to reach 10x the volume derived from the cell mass. This meant that if the average cell mass of all the tubes was 40 mg, then 400 mL of 30% KOH was added to the tubes. The tubes were then placed in a 100 degrees Celsius water bath for 30 minutes. Once removed, ice cold ethanol was added to equal 60% of the final volume of the precipitate previously recorded. The tubes were next centrifuged for 15 minutes at 4 degrees Celsius at 19,000 RPM. The supernatant was then removed, and the remaining precipitate containing extracted glycogen was resuspended in 70 microliters of double distilled water and placed in the -80 degrees Celsius freezer until being used later.

When resuming and after thawing the tubes, 37 % HCl was added to each of the test tubes to equal to 3M final concentration. The tubes were next placed in a 100 degrees Celsius
water bath for 2 hours for hydrolysis of the glycogen to glucose. After this, the glucose concentration was measured using a glucose assay kit.

**Glucose liquicolor kit**

Four additional empty 1.5 mL tubes were used to serve as controls for the liquicolor kit. They were a hydrolysis glycogen control, a hydrolysis negative control, a glucose standard with 100 microliters of glucose, and a negative kit control tube. Seven hundred microliters of the glucose reagent provided in the kit was added to each empty tube corresponding to each of the experimental group. Seven microliters of the sample of one tube was then added to the corresponding tube containing the 700 microliters of the glucose reagent. This was repeated for each of the tubes. A 96-well plate was then loaded with the samples and the controls for absorbance reading in the Glomax machine. The optical density each plate was recorded and corresponded to the amount of glucose present in the plate. The data were compiled into tables and graphs for statistical analysis.

**Statistical Analysis**

A full table of results containing numerical data can be found at the end of this report (Figure 2). All data were analyzed using ANOVA statistical analysis. The difference between data sets was considered statistically significant at p< 0.05.

**Results**

*HL1C Cells treated with 10 nM Insulin and 10 nM Insulin + 10 µM RA released more total glucose from glycogen*

As shown in Figure 1, a significantly greater amount of glucose was released from glycogen in the cells treated with 10 nM Insulin, or 10 nM Insulin + 10 µM RA as compared to that from the control group. The glucose released from the 10 µM RA group was not significant
from that of the control group. Although there was no significant difference between the control and 10 μM RA group, there was still a trend of increase of glucose released from glycogen in cells in the 10 μM RA group than that in the control group. There was not a significant difference in total glucose released between the 10 μM RA and the 10 nM Insulin + 10 μM RA group, even though there seems to be a trend that the amount of glucose released was greater in the 10 nM Ins. + 10 μM RA group.

**Figure 1** shows the data collected from the total glucose released from HL1C cells treated with the control, RA, insulin, and Insulin + RA groups for 48 hours.

**Discussion**

By using the glucose assay kit and measuring the amount of glucose in each of the four sample groups, an overview of the amount of glycogen in each of the groups can be made. There
will be a correlation between the amount of glucose released and the amount of glycogen which was initially stored in the cells. This logic is due to the fact that the glycogen was hydrolyzed to glucose as outlined in the *Materials and Methods* section above. Significantly more glucose was released from cells treated with 10 nM Insulin and 10 nM Insulin + 10 µM RA (Figure 1). The trend of the graph showed that the control group had the lowest amount of glucose released, followed by the RA group, a sharp rise in the Ins and the RA + Ins groups. In Figure 1, the lack of additional significant increase in the amount of glucose present in the 10 nM Insulin + 10 µM RA group in comparison to the 10 nM Insulin group does not support the idea that the insulin synergized with RA to induce glycogenesis in HL1C hepatoma cells.

This experiment shows how the combination of insulin and RA can affect glucose stored as glycogen. From the data gathered and shown in Figure 1, it appears that RA alone is not sufficient for increasing the glycogen content in HL1C cells. Once in the presence of insulin, however, it is shown to significantly increase of the amount of glycogen.

The significance found within the insulin groups was to be expected. Insulin promotes glycogenesis to store the glucose as glycogen. This finding is significant to the health and physiology of an individual. The effects of 10nM Insulin and 10 µM RA seen in Figure 1 can be used to modify the diet in those suffering from glycogen storage diseases such as von Gireke’s Disease, Pompe’s disease, and McArdle’s disease. Supplementing the diet with vitamin A may not lead to a better storage of glucose as glycogen and decrease symptoms of certain diseases.

Further research into this field will need to be conducted in order to identify more connections between vitamin A and glycogenesis to assist those suffering from glycogen storage diseases, diabetes, and other health problems.
These findings are important to all those with health problems relating to glucose metabolism, most notably those with diabetes. The lack of either insulin production in T1DM or the lack of the body’s response to the presence of insulin in T2DM indicates that the glucose will not be readily stored as glycogen under these diseases’ conditions. Management of the glucose metabolism is there for very important for individuals with both T1DM and T2DM. Consuming vitamin A with a meal has the potential to help with the control of glucose in the body. While not significant, the insulin + RA group also had a notably greater amount of glucose released compared to the RA group alone in HL1C cells. This shows that RA in the presence of insulin at least does not reduce the storage of glucose as glycogen.

This experiment is one that needs further investigation. While similar studies have been conducted in muscle tissue, this is the first to investigate the role of RA on glycogenesis in hepatocytes. Upon further research and study, more information can be discovered to link vitamin A to the control of glycogenesis and glucose metabolism.

Acknowledgements

I would like to thank Dr. Chen for his generous time, resources, and willingness to aid the future of research in the next generation. A very special thanks goes to Aaron Armstrong who selflessly gave time and instruction to ensure success and completion of this project. Additional acknowledgments go to Tiannan Wang for her help for initiating cell culturing of HL1C cells for the project.

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References


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**Figure 2.** Data from glycogen harvest and optical density reading from spectrometer.