The protective role of hydrogen sulfide from obesity-associated metabolic stress in GLP-1 regulation

by

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Abstract

Circulating palmitic acid (PA) and glycated albumin (GA) are increased in obesity and cause metabolic stress leading to diabetes. This includes the impairment of the glucoregulatory hormone glucagon-like peptide-1 (GLP-1) secreted from intestinal L-cells. Recently, the gasotransmitter hydrogen sulfide (H_2S) has been implicated in the enhancement of GLP-1 secretion. We hypothesized that H_2S can reduce the oxidative stress caused by PA and GA, and play a protective role in L-cell function. In mouse and human L-cell models, PA and GA caused an increase in reactive oxygen species (ROS). The H_2S donor GYY4137 partially blocked PA-ROS induction. In mice, PA-enriched Western diet (WD) elevated body weight in both sexes and elevated fasting blood glucose and lipid peroxidation in males. A single GYY4137 injection improved oral glucose tolerance in WD-fed male mice and also enhanced glucose-stimulated GLP-1 release. To conclude, H_2S reduces oxidative stress in GLP-1 cells and can improve glucose clearance in mice.

Keywords

Palmitic acid, glycated albumin, GLP-1, hydrogen sulfide, oxidative stress

Abbreviations

AGEs: Advanced glycation end-products

BMI: Body mass index

BSA: Bovine serum albumin

CAT: Carnitine acylcarnitine translocase

CPTI: Carnitine palmitoyltransferase 1

CPTII: Carnitine palmitoyltransferase 2

DCF-DA: 2', 7-Dichlorofluorescein diacetate

ETC: Electron transport chain

FAF-BSA: Fatty acid-free bovine serum albumin

FFAs: Free fatty acids

Fig: Figure

GA: Glycated albumin

GI: Gastrointestinal

GLP-1: Glucagon-like peptide-1

GPx: Glutathione peroxidase

H_2O_2: Hydrogen peroxides

H_2S: Hydrogen sulfide

HBSS: Hank's Balanced Salt Solution

IP: Intraperitoneal

MDA: Malondialdehyde

NF-κB: Transcriptional factor nuclear factor-κB

NOx: NADPH oxidases

OGTT: Oral glucose tolerance test

PA: Palmitic acid

RAGE: Receptor for AGEs

ROS: Reactive oxygen species

SOD: Superoxide dismutase

SRB: Sulfate-reducing bacteria

T2DM: Type 2 diabetes mellitus

TBA: Thiobarbituric acid

WD: Western diet

WHO: The World Health Organization

Co-Authorship Statement

Dr. Gagnon provided the general objective of the project and guidance on writing the manuscript. For this research project, I completed all the cell culture and animal work, including blood collections, glucose tolerance tests, and weight measurements. Radhika Nangia provided some cell culture data about the effects of palmitic acid and glycated albumin on NCI-H716 and GLUTag cells and cytotoxicity measurement as part of her 4th year thesis project. Dr. Gagnon established the animal protocol, and Laura Williams and Nicole Paquette provided training on the blood collection and glucose tolerance test techniques.

Acknowledgments

I would like to thank my thesis supervisor, Dr. Jeffrey Gagnon, for his wisdom and guidance, and Dr. Amadeo Parissenti and Dr. Alexander Moise, for their expertise in the field of oxidative stress and nutrition. Special thanks to my fellow M.Sc. candidates, including Laura Williams for her guidance on the animal techniques, Amal Alshehri, Shahnawaz Butt, and Connor Roque, for the occasional help. Additionally, I would like to thank undergraduates Radhika Nangia and Jenna Kennelly for their support during our work together. I also thank Dr. Paul Michael and Ph.D. candidate Eyad Kinkar for their help in troubleshooting, and sharing equipment needed for experiments. Special thanks to Dr. Paul Michael, and Laurentian University's animal care technicians Nicole Paquette and Chris Blomme to provide animal training and take care of my research animals in the facility. I would also like to acknowledge the Natural Sciences and Engineering Research Council (NSERC) for their support of this research project through a scholarship 2019-2020 and a grant to my supervisor Dr. Gagnon which supplied my graduate stipend between 2018-2019. Finally, I must express my very profound gratitude to my family and my friends for providing me with continuous encouragement throughout my years of study.

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1 Introduction

1.1 The widespread problem of obesity

The World Health Organization (WHO) defines obesity as an excessive body fat accumulation in adipocytes, which comes from an imbalance between food intake and energy expenditure (Hall et al., 2011). This diagnosis is based on the measurement of abdominal adiposity and a value of body mass index (BMI) greater than 30 Kg/m^2; extreme obesity is reached when the BMI is greater than 40 kg/m^2. The Public Health Agency of Canada has shown that obesity has tripled in children, adolescents, and adults over the last 30 years. While these statistics demonstrate that this condition is a major public health problem in Canada, the increased proportions of obesity are also observed globally (Jaacks et al., 2019). The excess fat deposition results in a positive energy balance, i.e., food intake is greater than energy expenditure. This positive energy balance is favored by an environment lacking in physical exercise and overconsumption of foods rich in fat and carbohydrates. In humans, obesity appears to be polygenic rather than monogenic (associated with a single gene). The genes involved in obesity code for proteins related to the controls of appetite, food intake, and energy expenditure. Obesity has also been associated with the prevalence of heart disease, stroke and may progress to hyperglycemia, leading to type 2 diabetes mellitus (T2DM) (Lebovitz & Banerji, 2005).

1.2 The pathophysiology of obesity

Obesity is a major risk for metabolic syndrome, which is defined as a cluster of conditions that increases our risk of heart disease, stroke, and T2DM (Magge et al., 2017). These conditions include hypertension, hyperglycemia, and hyperlipidemia (Jepsen, Suvan, & Deschner, 2020). Physical inactivity, genetic predisposition, and poor diet contribute to the pathophysiology of

obesity (Gadde et al., 2018). Visceral fat depot is closely linked with endocrine dysfunctions (Marseglia et al., 2014*)* (Farb & Gokce, 2015) that alter the way hormones function. For example, obesity is often associated with insulin resistance that precedes the onset of T2DM. This insulin resistance is linked to an elevation of lipogenesis and body fat deposition, especially in muscles, due to increased circulating free fatty acids (FFAs) (Malone & Hansen, 2019). This hyperlipidemia also causes adipocyte hypertrophy which causes inflammation by releasing cytokines and adipokines (Farb & Gokce, 2015). This also induces the generation of reactive oxygen species (ROS), producing a process known as oxidative stress (Fernández-Sánchez et al., 2011).

1.3 GLP-1 impairment in obesity

The metabolic dysfunction seen during obesity has been correlated to hormone and glucose homeostasis disturbances. Precisely, anorexigenic (appetite-suppressing) and orexigenic (appetite-stimulating) hormones are released from the gastrointestinal (GI) tract, including oxyntomodulin, PYY, GIP, and glucagon-like peptide-1 (GLP-1) (Martin, Sun, & Keating, 2019). GLP-1 is released in response to food intake. It is secreted by endocrine L-cells found in the colon and the ileum (Hira et al., 2020). It increases and enhances insulin secretion and action, acts as a satiety signal leading to reduced food intake, and slows gastric emptying, hence promoting weight loss (Heppner & Perez-Tilve, 2015). Studies suggest that plasma levels of GLP-1 may be impaired in obese subjects compared to lean ones, and this impairment may help explain the decreased satiety signaling seen during obesity (Madsbad, 2014) (Færch et al., 2015). Since GLP-1 levels were found impaired during obesity, it is then logical to seek for a treatment with GLP-1 analogs to promote weight loss and enhance glucose homeostasis.

1.4 GLP-1 analog to manage obesity

Only a few anti-obesity drugs are currently being used to fight against excessive weight gain. Liraglutide (also called Victoza™) is an analog of GLP-1 that shares 97% homology to native GLP-1(Russell-Jones, 2009) (Knudsen, 2019) and is active for up to 24 h (Knudsen & Lau, 2019) (Bjerre Knudsen et al., 2010). This drug was initially approved for use in Canada to treat type 2 diabetes and, more recently, as a weight-loss medication (Wharton et al., 2019). Liraglutide is well tolerated, with only mild-to-moderate side effects observed, such as nausea, diarrhea, vomiting, indigestion, and constipation (Garber, 2011). It is imperative to find novel therapies against obesity, avoiding any possible detrimental side effects. To do that, we need to fully understand what leads to a reduced GLP-1 level in obesity. Recent studies have suggested that an increased level of systemic oxidative stress observed during obesity damages cells. This leads to cellular dysfunction, which may explain GLP-1 secretion impairment leading to the development of obesity-related complications.

1.5 Obesity and oxidative stress

A study conducted on non-diabetic human subjects demonstrated that an increased level of systemic oxidative stress is closely correlated with fat accumulation (Furukawa et al., 2004). Oxidative stress is the disturbance in the balance between the generation of free radicals, also called reactive oxygen species (ROS), and the body's capacity to neutralize ROS damaging effects with antioxidants (Furukawa et al., 2004). This increase in oxidative stress causes damage to DNA, proteins, and other macromolecules, which leads to the impairment in the function and structure of cells (Asmat, Abad, & Ismail, 2016) (Gerber & Rutter, 2017). This damage may also explain why GLP-1 secretion impairment is seen during obesity (Puddu et al., 2014). Puddu and colleagues have shown that ROS induction by glycated serum in GLUTag, a murine L-cell

model, can suppress basal GLP-1 secretion (Puddu et al., 2014). On a molecular level, obesity may induce systemic oxidative stress through a variety of mechanisms such as oxidative phosphorylation (in the mitochondria) and superoxide generation from the nicotinamide adenine dinucleotide phosphate NADPH oxidases (NOx) (Newsholme et al., 2016). Inducers of oxidative stress in obesity include hyperglycemia, low antioxidant defense, and elevated free fatty acids levels (Manna & Jain, 2015a). In this study, we highlight the lipotoxicity and glucotoxicity associated with obesity.

1.6 Palmitic acid and oxidative stress

Lipotoxicity refers to the structural and functional damage of high concentrations of fatty acids to cells (Ertunc & Hotamisligil, 2016). Palmitic acid (PA or palmitate, 16:0) is the major circulating saturated fatty acid in humans that accounts for 20-30% of total fatty acids in membrane phospholipids, and adipose triacylglycerols (Carta et al., 2017). PA can be extracted from diet or synthesized endogenously via de novo lipogenesis (Carta et al., 2017), and plays an important role in cellular energy production. Energy is produced when PA enters cells through CD36, a fatty acid transporter. Briefly, the fatty acid gets activated by long-chain acyl-CoA synthetases, then enters the matrix of mitochondria via carnitine palmitoyltransferase 1 (CPTI), carnitine acylcarnitine translocase (CAT) and carnitine palmitoyltransferase 2 (CPTII), after which it undergoes β-oxidation to generate acetyl-CoA. The acetyl-CoA then activates the electron transport chain (ETC) to produce ATP (Ly et al., 2017) (Fig 1.). Unfortunately, an excess of PA can lead to cellular complications which can involve multiple pathways such as the activation of inflammatory pathways through transcriptional factor nuclear factor-κB (NF-κB) activation, oxidative stress elevation by ROS production, and mitochondrial and endoplasmic

reticulum stress by constantly activating the β-oxidation pathway (Egnatchik et al., 2014) (Ly et al., 2017) (Fig 1.)

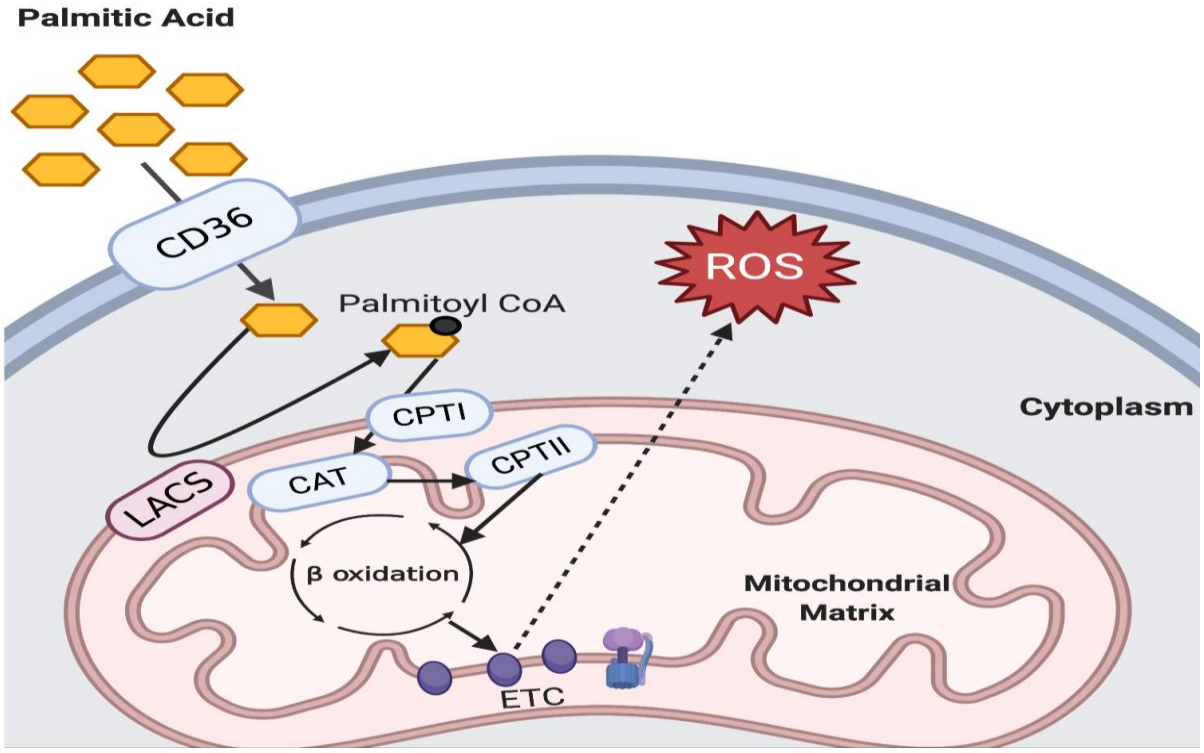

Figure 1: Palmitic acid induces ROS production.

PA enters cells through the fatty acid transporter CD36, then gets activated by long-chain acyl-CoA synthetases (LACS). Palmitoyl CoA enters the matrix of mitochondria via carnitine palmitoyltransferase 1 (CPTI), carnitine acylcarnitine translocase (CAT) and carnitine palmitoyltransferase 2 (CPTII), then undergoes β-oxidation to generate acetyl-CoA, which activates the electron transport chain (ETC) to produce ATP. Excessive amounts of PA increases ROS production by constantly activating this β-oxidation pathway (Image adapted from Ly et al., 2017, Created with BioRender.com).

1.7 Glycated proteins and oxidative stress

Glucotoxicity refers to the harmful effects of chronic hyperglycemia on cells (McClain, 2004). It is known that high blood glucose promotes the attachment of sugars to proteins to form ketoamines through a process called protein glycation (Welsh et al., 2016). More specifically, the initial product of this reaction is called a Schiff base, which spontaneously rearranges itself into

an Amadori product, also known as irreversible ketoamines (Siddiqui et al., 2019). This group of ketoamines formed by glycation of proteins is also called fructosamine, which can undergo further modifications to form advanced glycation end-products (AGEs) (Ravichandran et al., 2019). It takes weeks and even years to end up producing these endogenous AGEs. Additionally, blood AGEs have exogenous origin from food intake, where 10%–30% of total ingested AGEs are absorbed in the gut (Baye et al., 2017).

Both endogenous and exogenous AGEs were found to be toxic to cells (Delgado-Andrade, 2016), and their accumulation in cells has also been implicated in metabolic stress (Gaens et al., 2013). Precisely, AGEs induce oxidative stress in cells through the interaction with a receptor for AGEs (RAGE), which in turn activates NADPH oxidases (NOx) that ultimately leads to the generation of ROS (Cepas et al., 2020) (Fig 2.). Moreover, this RAGE activation leads to the activation of the transcription factor NF-κB, which is involved in the pro-inflammatory signaling pathway (Cohen et al., 2003) (Bierhaus & Nawroth, 2009). Chronic AGE-rich nutrition has been shown to decrease the life span of mice (Cai et al., 2007).

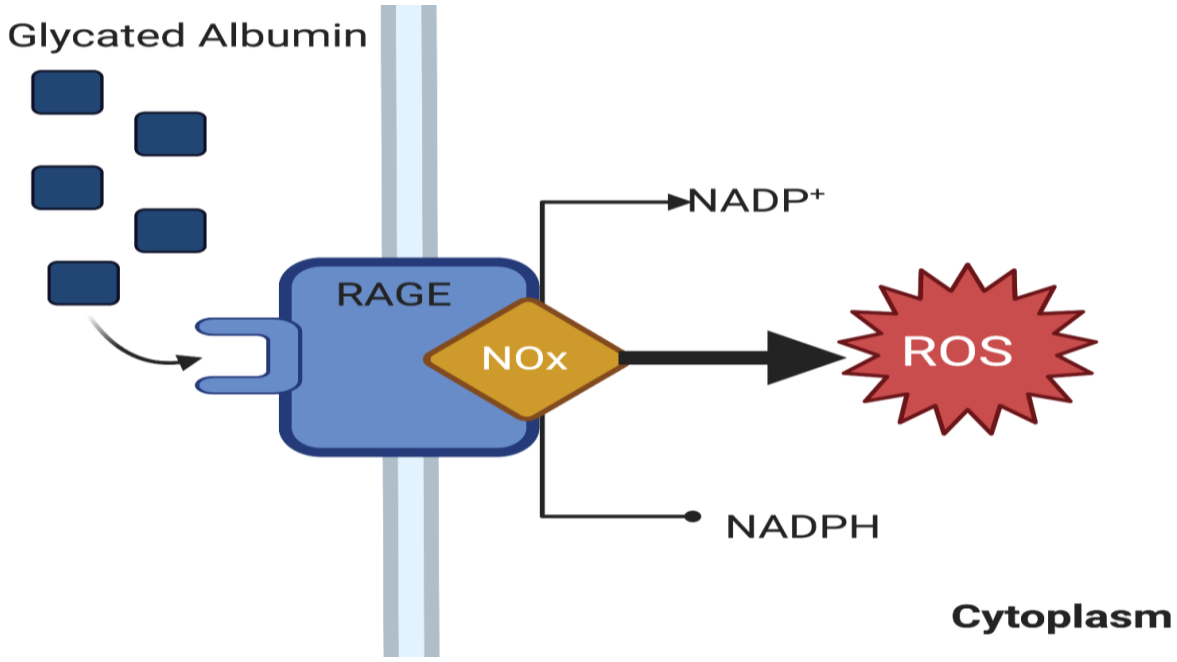

Figure 2: Glycated albumin induces ROS production.

Glycated proteins bind to its receptor RAGE which, in turn, activates NOx that generates ROS (Image adapted from Cepas et al., 2020, created with BioRender.com).

1.8 Oxidant/antioxidant balance

Systemic oxidative stress occurs when an imbalance between the generation of free radicals and the body's capacity to neutralize oxidation's damaging effects with antioxidants (Furukawa et al., 2004). Many compounds can act as antioxidants, which are of two types: exogenous and endogenous. Exogenous antioxidants are molecules that are provided from food intake, which include vitamin C and E, while endogenous antioxidants are produced internally (Abdali, Samson, & Grover, 2015). These include the enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Guerra-Araiza et al., 2013). Given the evidence suggesting that oxidative stress contributes to the pathogenesis of obesity and T2DM (Manna & Jain, 2015b) (Oguntibeju, 2019), antioxidants may be useful to prevent these conditions.

1.9 Hydrogen sulfide as an antioxidant

The chronic detrimental effects of PA and AGEs disturb the balance between the generation of ROS and the body's ability to neutralize their damaging effects with antioxidants, which will eventually lead to cellular dysfunction. It is then crucial to seek to counteract this imbalance by reducing these oxidants and increasing antioxidants. H_2S is a gas that acts as a signaling molecule and as an antioxidant (Shefa, Kim, Jeong, & Jung, 2018). It is produced endogenously from L-cysteine by the enzymes cystathionine gamma-lyase and cystathionine beta-synthase (Chiku et al., 2009). Moreover, H_2S is produced in a significant amount in the gut by the intestinal microbiome, mainly by a variety of sulfate-reducing bacteria (SRB) (Singh & Lin, 2015) of the distal ileum and colon. SRB are also found in the same niche as GLP-1 secreting L-cells (Hira, et al., 2020). This gasotransmitter has numerous physiological effects on cells (Filipovic et al., 2018). It acts as a vasodilator (Materazzi et al., 2017), a relaxant of smooth muscle (d'Emmanuele di Villa Bianca et al., 2009), and was found to stimulate GLP-1 secretion (Pichette et al., 2017). Most recently, H_2S has emerged to protect against neuronal degeneration (Kang et al., 2020) and cardiovascular disease (Shen et al., 2015) by playing an anti-inflammatory role, and by upregulating antioxidant enzymes such as GPx and SOD (Bian et al., 2016). While lower levels of H_2S can neutralize ROS, such as hydrogen peroxides (H_2O_2), high concentrations of that gas can be toxic and are known to cause chronic intestinal inflammation (Linden, 2014). It is therefore essential to work within the physiological range of H_2S levels to profit for its beneficial action.

1.10 Hypothesis

Since increased circulating palmitic acid and glycated albumin (GA) during obesity are known to cause oxidative stress which impairs normal cell functions, the gasotransmitter H_2S can oppose ROS production through its antioxidant ability. We hypothesized that elevated amounts of circulating PA and GA during obesity result may increase ROS production and impair GLP-1 secretion, while H_2S may play a protective role by neutralizing ROS and restoring GLP-1 secretion.

2 Materials and Methods

2.1 Cell culture

The human NCI-H716 cell line was obtained from American Type Culture Collection (ATCC). The mouse GLUTag cell line was obtained from Dr D Drucker (Lunenfeld-Tanenbaum Research Institute). Cell lines were maintained in 10 cm plates containing Roswell Park Memorial Institute (RPMI 1640) medium for the NCI-H716 cells and low-glucose Dulbecco's Modified Eagle's Medium (DMEM) for GLUTag cells. Both media were supplemented with 1% penicillin-streptomycin (Pen Strep; Life Technologies), and 10% fetal bovine serum (FBS) (Life Technologies) and incubated at 37 °C in 5% CO_2. Experiments were performed in twenty-four-well plates, which were seeded with cells at a density of 200,000 cells per well for NCI-H716 cells and 250,000 cells per well for GLUTag cells and allowed to proliferate for 48 hours. Matrigel (Corning Life Sciences) was used to secure the NCI-H716 cells to a basement membrane matrix (Hughes, Postovit, & Lajoie, 2010).

2.2 PA/FAF-BSA conjugation preparation

This study used the fatty acid preparation method of Cousin (Cousin et al., 2001). Stock 5 mM PA/fatty acid-free bovine serum albumin (FAF-BSA) was prepared as follows. 100mM PA (Sigma) stock solution was dissolved in 0.1 M NaOH and heated at 70℃ in a water bath. Simultaneously, a 10% (wt/vol) fatty acid-free FAF-BSA (EMD Millipore Corp) solution was prepared in deionized H_2O in a 55 ℃ water bath. To prepare a 5mM PA/10% FAF-BSA stock solution, 0.5mL of the 100mM PA was added to 9.5 mL 10% BSA solution, which was then heated at 55℃ in a shaking water bath for 10 minutes before it was vortex-mixed for 10 seconds and then incubated for an additional 10 minutes in a 55℃ water bath. The PA/FAF-BSA complex solution was cooled to room temperature before being sterile filtered using a 0.45μm pore size filter. A control solution for this complex was prepared in the same way by using just regular BSA (GE Healthcare Life Sciences). The prepared 5mM PA/FAF-BSA and regular BSA aliquoted solutions were stored at -20 ℃, where they were stable for three weeks. The stored PA/FAF-BSA and its control stock solutions were heated for 15 minutes at 55 ℃ and cooled to room temperature before the study.

2.3 Intracellular ROS detection

Levels of intracellular ROS were measured using the cell-permeable dye, 2', 7-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich). DCF-DA is oxidized in the presence of ROS to produce dichlorofluorescin, which is a highly fluorescent compound. After 48 hours, the cells were washed with phenol red-free Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) and incubated in 1mL of HBSS (1x) with 100μM DCF-DA for 10 minutes and 30 minutes for the NCI-H716 and GLUTag cells, respectively. NCI-H716 cells showed more success in detecting ROS when incubated with the DCF-DA dye for 10 minutes as opposed to the

recommended 30 minutes (Yang & Chan, 2018). As DCF-DA is sensitive to light and air, the plates were covered with aluminum foil at all times.

Commercially prepared 10mg/mL glycated albumin (EMD Milipore, Canada) was added to phenol red-free HBSS to make 10, 50, 200 μg/mL treatment solutions. Regular BSA (GE Healthcare Life Sciences, U.S.A) was dissolved in water and added to HBSS to match glycated albumin concentrations for control.

The stock 5mM PA/FAF-BSA was diluted in phenol red-free HBSS to make various treatment concentrations. For control solutions, regular BSA stock solutions were added to HBSS to match the concentrations of BSA in the PA/FAF-BSA solutions. After the 10-minute or 30-minute dye incubation, the cells were washed with HBSS, and the treatments and controls were added. The cells were then returned to the incubator. Fluorescence was measured using the Fluostar Optima plate reader (BMG Labtech) at 15 minutes then at 30-minute intervals for two hours. These readings were measured with the excitation filter set to 485 nm and emission filter at 520 nm.

2.4 Cytotoxicity assessment of palmitic acid

To check for the cytotoxic effect of PA, a Neutral Red assay was conducted using the protocol outlined by Repetto (Repetto, del Peso, & Zurita, 2008) with slight modifications. 0.020mL of Neutral Red dye was diluted in 5mL of each treatment or control solution before 1mL of the solutions was added to each well. The cells were then returned to the incubator. After 2 hours, treatments and dye were aspirated before cells were washed with a wash fixative, composed of distilled water with 1% calcium chloride and 0.37% [vol/vol] formaldehyde (Sigma-Aldrich). An extraction solution composed of distilled water with 50% [vol/vol] bonded ethanol and glacial acetic acid 1% [vol/vol] (Sigma-Aldrich) was then added. Cells were gently agitated on a

platform shaker for 5 minutes to allow for the complete extraction of cells. Readings were measured with absorbance set to 600 nm.

2.5 Animals

Male and female C57BL/6 mice (5 or 6 weeks old) were purchased from Charles River Laboratories (St. Constant, Quebec). Animals were group-housed in standard cages on a 12-hour light/dark cycle in the Paul Field Animal Care Facility at Laurentian University. The Laurentian University Animal Care Committee approved protocols following guidelines of the Canadian Council for Animal Care (CCAC, Ottawa, ON: Vol. 1, 2nd edition, 1993: Vol. 2, 1984).

2.6 *In vivo* experimental design

Upon arrival in the facility, 15 female and 15 male mice were randomly divided into two groups according to diets. Mice had access to water and food *ad libitum* and were either fed a Western diet (Research Diets, Inc. New Brunswick, NJ, USA, 43% Carbohydrates, 40% fats: 31.32% PA, 17% proteins and 14.5% salt (energy percent)) or a chow diet (Envigo Teklad, Madison WI, 40% Carbohydrates, 5.5% fats: 0.7% PA, 22% proteins and 0.4% salt (energy percent)) ad libitum for 20 weeks. Blood was collected every two weeks after an oral glucose gavage (OGTT) for glucose and GLP-1 measurements.

2.7 Oral glucose tolerance test and GLP-1 determination

An oral glucose tolerance test (OGTT) was conducted at week 16 of the study. Mice were first injected intraperitoneally (IP) with 30 mg/kg of the H_2S donor GYY4137 or a vehicle then immediately were fasted overnight for 16 hours before receiving oral gavage of D-(+)-glucose (2g/kg body wt). Blood glucose was measured from the lateral saphenous vein during the

experiment using a glucometer (OneTouch Verio) at 0, 5, 30, 60, and 90 minutes after glucose administration. For GLP-1 measurement, blood was collected from the lateral saphenous vein into EDTA coated capillary tubes (Sarsdedt) at 0, 5, and 30 minutes after glucose gavage. Diprotin A (DPP-4 inhibitor) and aprotinin (a protease inhibitor) were added to the capillary tubes (10% v/v) to prevent degradation of the target hormone. The capillary tubes were centrifuged at 5000*g for 5 minutes at 4 °C. 10μL of plasma was examined for total GLP-1 using a commercial competitive ELISA kit (Millipore).

2.8 *In vivo* oxidative stress analysis

Levels of circulating peroxidated lipids were examined to assess oxidative stress in the Western diet and chow diet mice plasma (20μL) of both sexes. The end product during lipid peroxidation, malondialdehyde (MDA), was measured in blood plasma in a 96-well plate with a colorimetric assay kit (Abcam, Cambridge MA). Plasma MDA reacts with thiobarbituric acid (TBA), producing the MDA-TBA complex quantified at 532 nm using a microplate absorbance reader.

2.9 GLP-1 receptor antagonism

Male mice were pretreated with the H_2S donor GYY4137 (30mgl/kg) and immediately were fasted overnight for 16 hours before receiving an injection of GLP-1 receptor antagonist exendin-49–39 (50 nmol/kg, Cayman Chemical Company) or a vehicle 5 minutes before an OGTT. Blood glucose was measured from the lateral saphenous vein during the experiment using a glucometer (OneTouch Verio) at 0, 5, 30, 60, and 90 minutes after glucose administration.

2.10 Statistical analysis

All data are expressed as mean ± standard error of mean. Studies with multiple doses of the same treatment were analyzed by one-way ANOVA, followed by a Bonferroni post hoc test. Studies with two or more independent variables (i.e., time and treatment) were analyzed by two-way ANOVA, followed by Bonferroni post hoc test at individual time points. A two-tailed paired Student's t-test analyzed studies comparing two groups. $P<0.05$ was considered statistically significant.

3 Results

3.1 Palmitate stimulates ROS production in both NCI-H716 and GLUTag cells

First, we developed a protocol for ROS detection and examined the effect of PA on ROS production in NCI-H716 and GLUTag cell lines. In NCI-H716 cells, PA increased ROS in a time- and dose-dependent manner. The 500μM PA solution, relative to its control, stimulated ROS production by threefold at 120 minutes (3.655 ± 0.186-fold over the control, $p<0.0001$, Fig 3.A) (3.655 ± 0.186-fold of control, $p<0.0001$, Fig 3.A). In the GLUTag cell line, all the doses tested showed significant ROS stimulation compared to the corresponding BSA controls by at least one fold at 120 minutes (2.074 ± 0.183 fold of control, $p<0.0001$, Fig 3.B). To determine cytotoxicity when cells were treated with treatments of PA, cell viability was assessed via the neutral red assay. Relative to BSA controls, 500μM PA was not toxic to NCI-H716 cells (Fig 3.C). However, a modest but significant reduction in cell viability was observed in GLUTag cells (0.915, ± 0.090-fold of control, $p<0.05$, Fig 3.D).

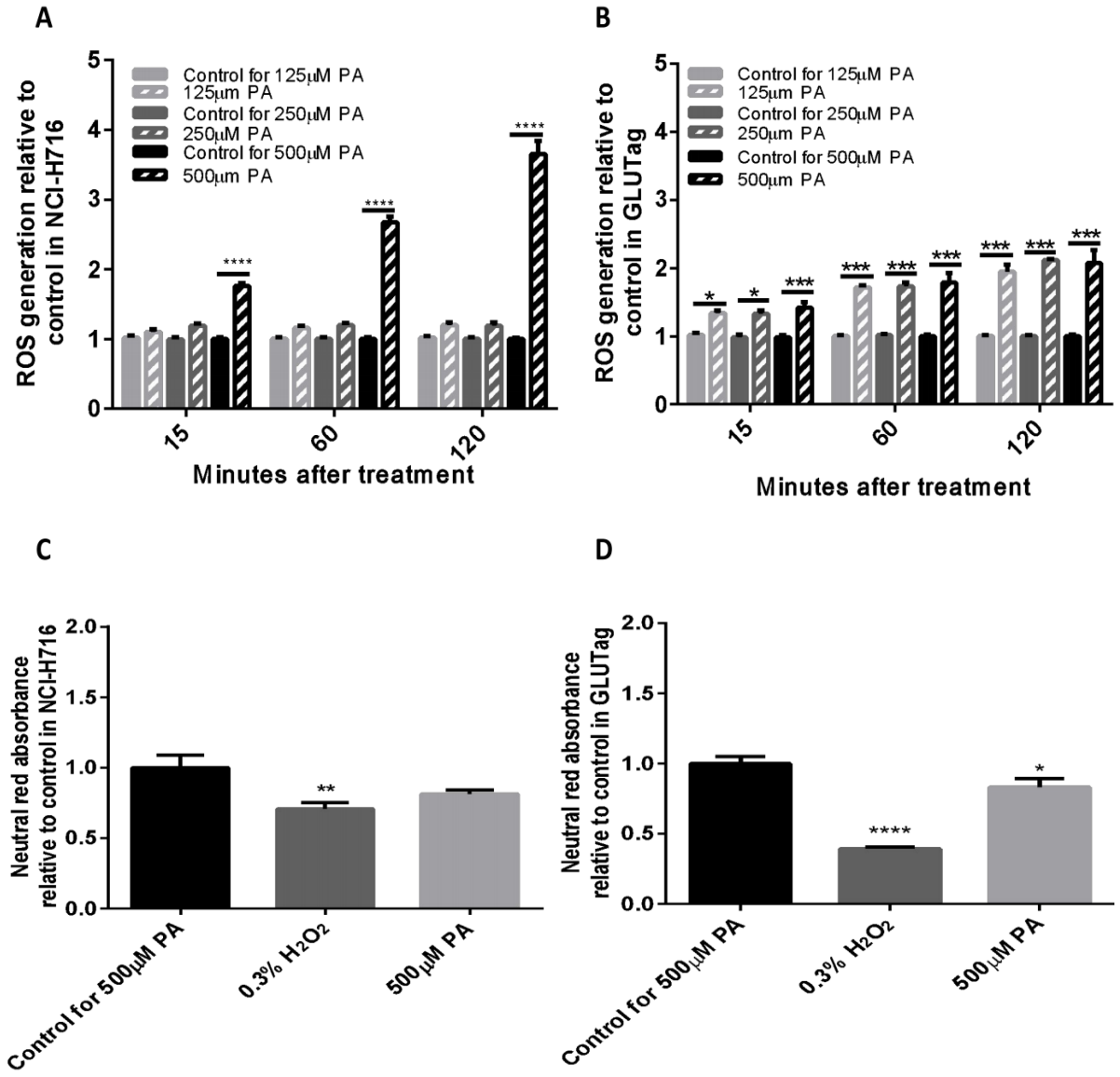

Figure 3: Palmitate stimulates ROS production in both NCI-H716 and GLUTag cells with minimal effect on cell viability.

ROS generation (A and B) was determined after a 2-hour incubation with 2',7'-DCF-DA, and palmitic acid treatment and regular BSA as control. Cell viability (C and D) was assessed via Neutral Red absorbance after a 2-hour incubation with Neutral Red dye and indicated treatments. Data were analyzed using a Two-way ANOVA followed by a Bonferroni post hoc test, *=$p<0.1$, **= $p<0.01$, ***=$p<0.001$, ****=$p<0.0001$. Results are expressed as means ±SEM, n= 8.

3.2 H_2S reduces basal and palmitate-induced ROS in NCI-H716 cells

To investigate the effect of H_2S on ROS, NCI-H716 cells were treated with a slow-releasing H_2S donor, GYY4137. GYY4137 at a 1mM concentration significantly reduced basal ROS by half after 120 minutes of treatment (0.593 ± 0.022 fold of control, $p<0.0001$, Fig 4.A). 500µM PA-induced ROS was significantly reduced by 25% by co-incubating cells with 1mM GYY4137 (1.219 ± 0.036-fold with GYY4137 versus 1.478 ± 0.036-fold without GYY4137, $p<0.0001$, Fig 4.B).

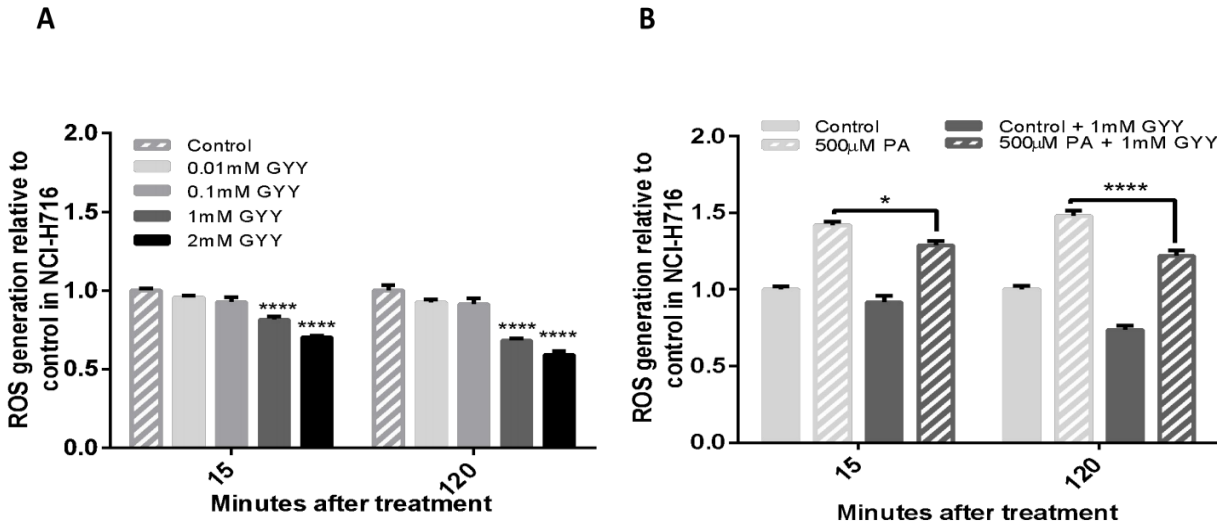

Figure 4: GYY4137, a slow-releasing H_2S donor, reduces basal and palmitate-induced ROS in NCI-H716 cells.

Fluorescence was analyzed after a 2-hour incubation with 2',7'-DCF-DA and treatments. (A) 1mM and 2mM GYY4137 significantly reduces basal ROS by nearly half. (B) 500µM PA-induced ROS was significantly reduced by co-incubating cells with GYY4137 at 1mM. Data were analyzed using a Two-way ANOVA followed by Bonferroni post hoc test, *= $p<0.01$, ****=$p<0.0001$). Results are expressed as means ±SEM n=8.

3.3 Western diet elevates body weight in both sexes and elevates fasting blood glucose and lipid peroxidation in male mice

To investigate the effect of elevated PA *in vivo*, mice were fed either a chow diet or a Western diet rich in PA. The Western diet elevated body weight in both sexes with a pronounced weight increase in males at 16 weeks (43.445 ± 0.761 grams for WD-fed mice compared to 33.050 ± 1.485 grams for chow diet-fed mice, $p<0.0001$, Fig 5.B) that appeared much earlier (4 weeks) than in the females (20 weeks) (31.145 ± 1.896 grams for WD-fed mice versus 23.950 ± 0.866 for chow diet-fed mice at 16 weeks, Fig 5.A). No significant elevation in fasting blood glucose was seen in females at 16 weeks (7.775 ± 0.795 mM for WD-fed mice versus 6.475 ± 0.226 mM for chow diet-fed mice, Fig 5.C) while a much greater elevation was seen in males (10.550 ± 0.572 mM for WD-fed mice versus 7.900 ± 0.593 mM for chow diet-fed mice, $p<0.0001$, Fig 5.D). Circulating lipid peroxidation was assessed by the MDA assay and levels were only elevated in the WD-fed males (184.515 ± 2.011 nmol/ml for WD-fed mice versus 112.660 ± 1.845 nmol/ml for chow diet-fed mice, $p<0.0001$, Fig 5.E).

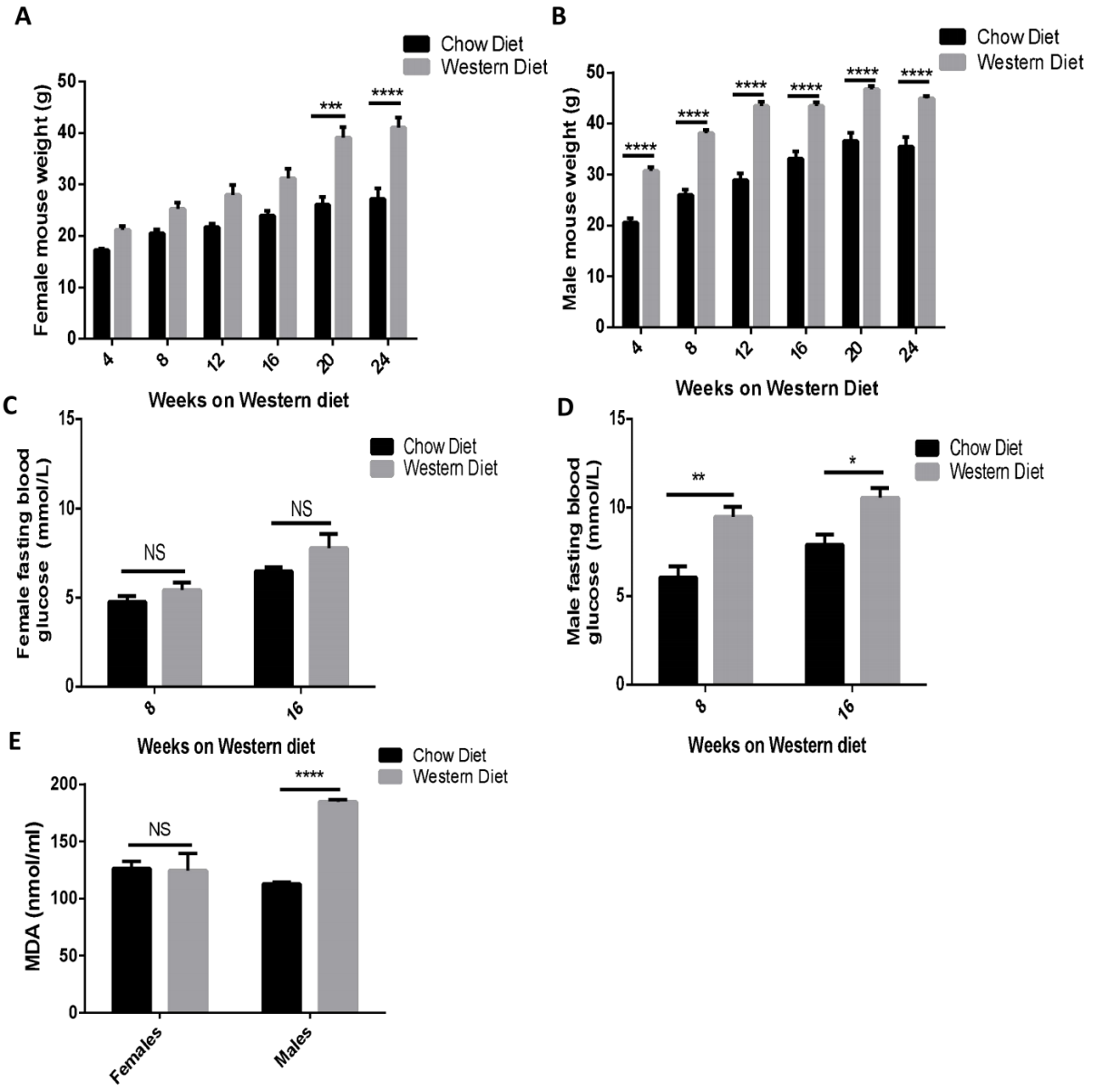

Figure 5: Western diet elevates body weight in both sexes and elevates fasting blood glucose and lipid peroxidation in male mice.

Bodyweight was examined in female (A) and male (B) mice maintained for 24 weeks on a Western or chow diet. Fasting blood glucose at 8 and 16 weeks were examined in female (C) and male mice (D) on a Western and chow diet. (E) Circulating lipid peroxidation was examined in both sexes after 24 weeks on a Western or chow diet. Data were analyzed using a Two-way ANOVA followed by Bonferroni post hoc test, *= $p<0.1$, **= $p<0.01$, ***=$p<0.001$, ****=$p<0.0001$). Results are expressed as means ±SEM n= 11 Western diet, n=4 chow diet.

3.4 H_2S improves oral glucose tolerance in Western diet-fed male mice

After an oral glucose challenge (2g/kg), blood glucose response was examined in fasted mice given an IP injection of the H_2S donor GYY4137 (30 mg/kg) or saline at -16 hours. No significant change was observed in females injected with GYY4137 compared to control mice injected with saline (Fig 6.A-B). Glucose tolerance was significantly improved in males injected with the H_2S donor ($p<0.01$ for treatment effect, Fig 6.C-D).

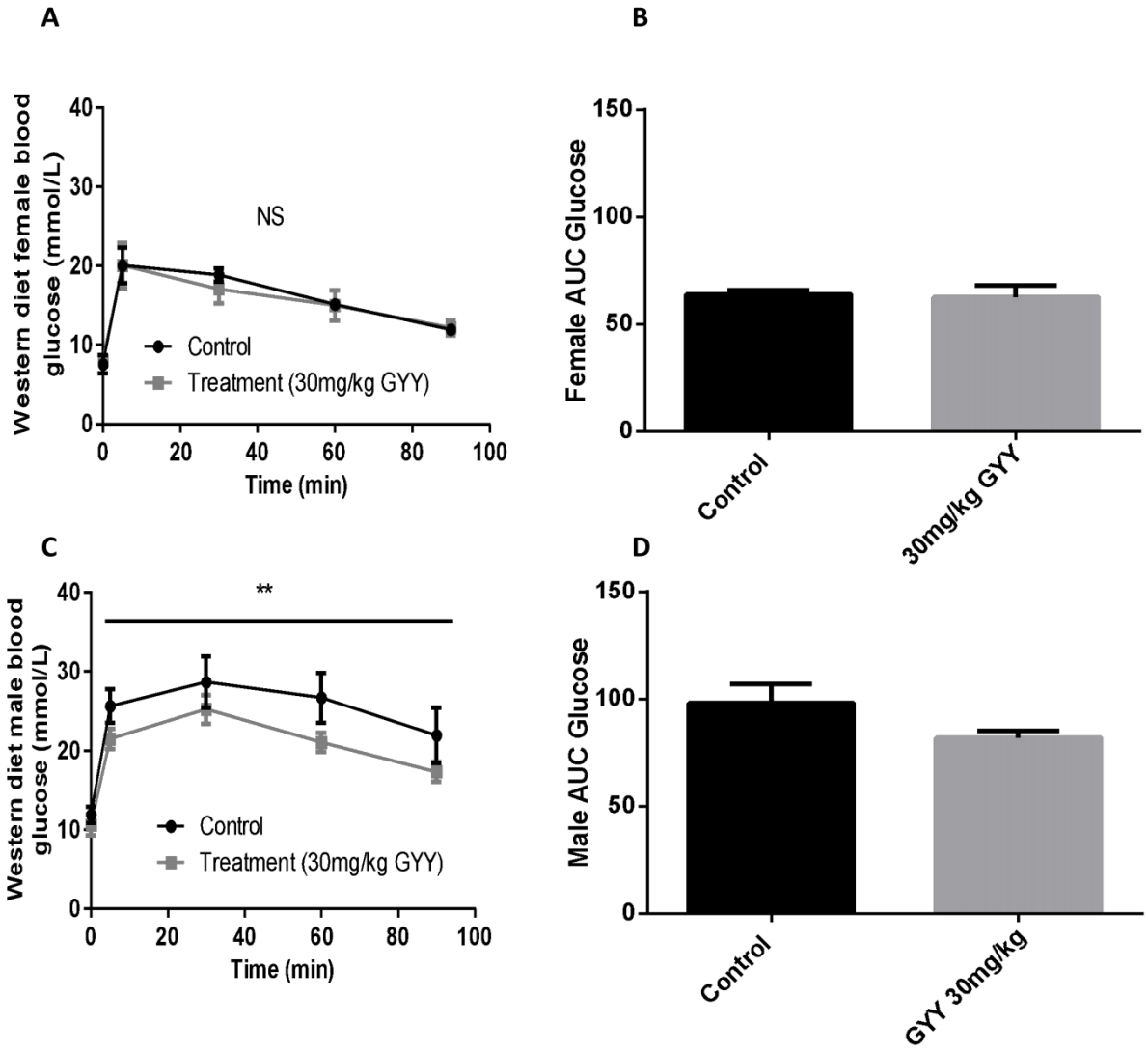

Figure 6: The H_2S donor, GYY4137, improves oral glucose tolerance in Western diet-fed male mice.

Plasma glucose levels in response to an oral glucose challenge were examined in fasted obese mice given an IP injection of GYY4137 (30mg/kg) or saline at -16h. Glucose concentration and area under the curve (AUC) was assessed in female (A and B) and male (C and D) mice. Results are expressed as absolute glucose means ± SEM over time after an oral glucose challenge (2g/kg). Data were analyzed using a Two-way ANOVA, **= $p<0.01$ effect for the treatment. WD-fed mice were divided into two groups, n= 6 treatments and n=5 controls.

3.5 H_2S enhances glucose-stimulated GLP-1 release in Western diet-fed mice

Plasma GLP-1 response to an oral glucose challenge was examined in WD-fed mice given an IP injection of GYY4137 (30 mg/kg) or saline at -16 hours. Animals (combined data from both male and female mice) receiving GYY4137 had a greater elevation (delta) in GLP-1 release (12.774 ± 4.402 for GYY4137-treated mice compared to 2.041 ± 3.205 for control, $p<0.05$, Fig 7.A). The enhancement in GLP-1 was much more pronounced in male mice as WD-fed males had completely lost their GLP-1 response (peak of 16.728 ± 7.015 for GYY4137-treated mice compared to -0.626 ± 5.991 for control mice, $p<0.05$, Fig 7.C). A much smaller improvement was observed in females (peak at 6.843 ± 1.673 for GYY4137-treated mice compared to 4.709 ± 2.602 for control mice, Fig 7.D).

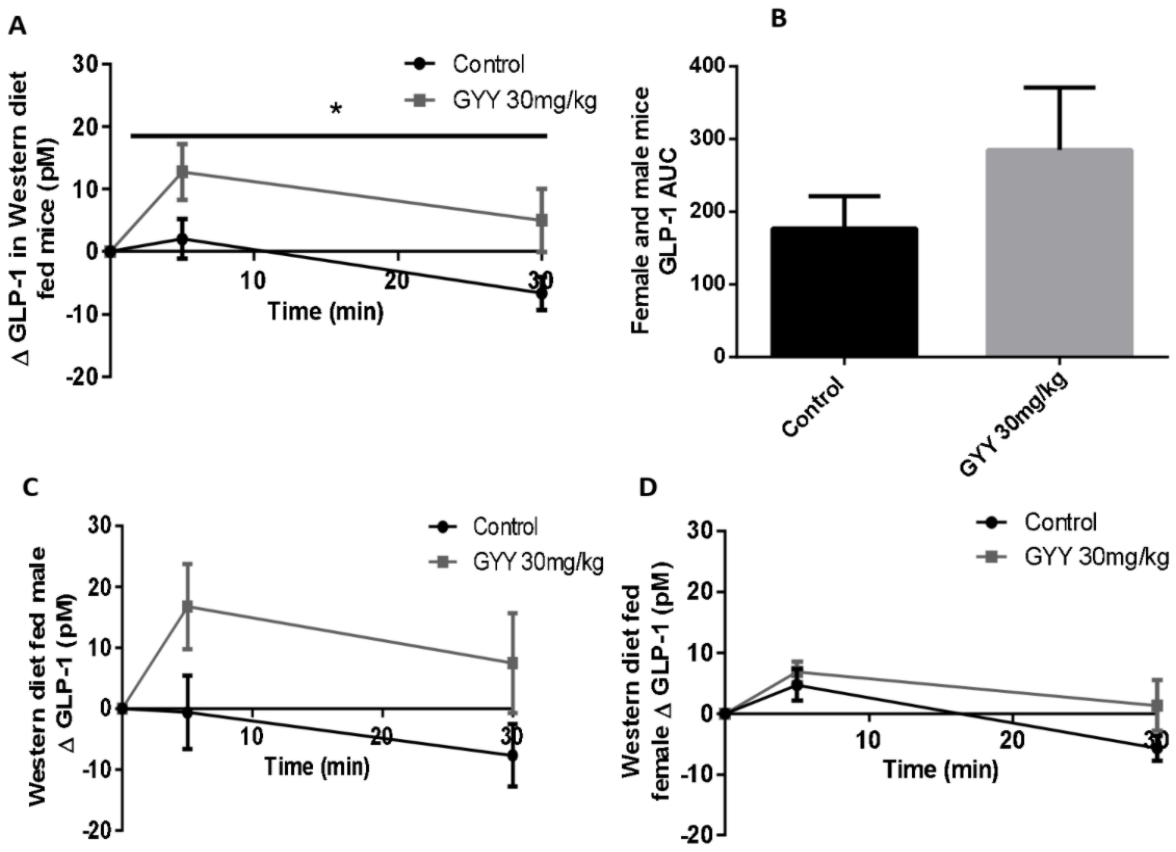

Figure 7: The H_2S donor, GYY4137, enhances glucose-stimulated GLP-1 release in Western diet-fed mice.

Plasma GLP-1 response to an oral glucose challenge was examined in fasted mice given an IP injection of GYY4137 (30mg/kg) or saline at -16h. GLP-1 response was assessed combining data from both male and female mice (A) and its corresponding AUC (B), male mice (C), and female mice (D). Results are expressed as the change in GLP-1 from baseline in means ±SEM over time after oral glucose challenge (2g/kg). Data were analyzed using a Two-way ANOVA, *= $p<0.05$ effect for the treatment. n= 12 treatments and n=10 controls in (A)and (B) and n= 6 treatments and n=5 controls in (C) and (D).

3.6 GLP-1 antagonism impairs glucose tolerance in GYY treated male mice

As H_2S may alter glycemia in a GLP-1 independent manner, we next examined the impact of the GLP-1R antagonist Exendin9-39 in mice treated with the H_2S donor GYY4137. After an oral glucose challenge (2 g/kg), blood glucose response was examined in fasted mice given an IP injection of GYY4137 (30 mg/kg) at -16 hours and a 50 nmol/kg Exendin-9–39 or vehicle at -5 minutes. GLP-1 antagonism led to a significant increase in blood glucose relative to vehicle control ($p<0.05$ for Exendin9-39 effect, Fig 8. A-B).

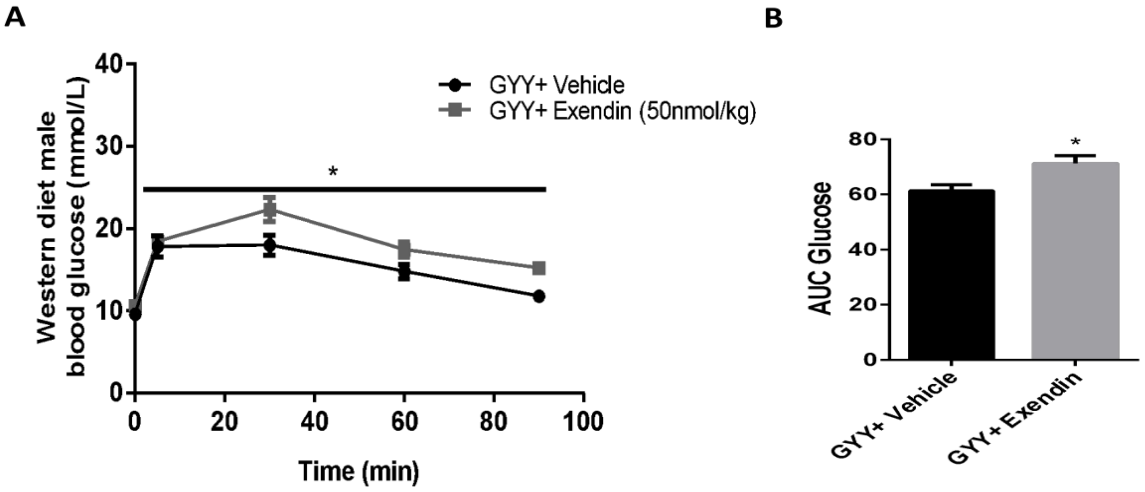

Figure 8: GLP-1 antagonism impairs glucose tolerance in GYY treated obese male mice.

Fasted male mice maintained for 20 weeks on a Western diet were injected 5 min before oral glucose challenge with 50 nmol/kg Exendin (9-39) or vehicle 16 hours after they got a single injection of GYY the night before. Animals had access to water ad libitum. Blood glucose in males is significantly different, suggesting that GLP-1R antagonism prevents GLP-1 effects on glycemia (A and B). Data were analyzed using a Two-way ANOVA followed by Bonferroni post hoc test, *= $p<0.05$. Results are expressed as means ±SEM n= 6 treatments and n=5 controls.

3.7 GA-induced ROS production in NCI-H716 cells

ROS levels were measured using DCF-DA in the cells that were treated for 2 hours with various doses of GA and regular BSA as control in NCI-H716 cells. GA increased ROS in a time and

dose-dependent manner. The 200µg/ml GA, relative to its control, caused the most significant ROS generation by a fold change of 0.5 at 120 minutes (1.492 ± 0.089fold of control, p<0.0001, Fig 9.A). To determine cytotoxicity when cells were treated with GA treatments, cells were assessed via Neutral Red assay. Relative to its BSA control, the 200µg/ml GA was not toxic to NCI-H716 (Fig 9.B).

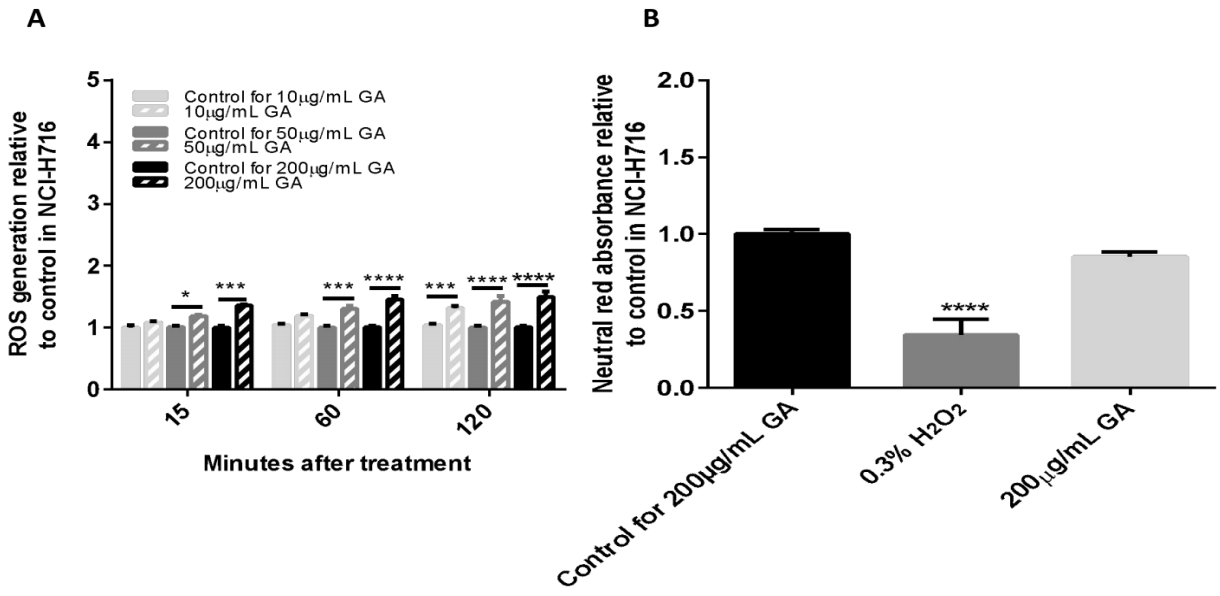

Figure 9: Glycated albumin stimulates ROS production and does not affect cell viability in NCI-H716 cells.

Fluorescence was analyzed after a 2-hour incubation with 2',7'-DCF-DA, and glycated albumin treatment and absorbance was measured after a 2-hour incubation with Neutral Red dye and the treatment. (a) Glycated albumin at 10, 50, and 200µg/mL has significant effect on ROS production compared to control in NCI-H716 cells at 120min. (b) Cell viability was assessed via Neutral Red absorbance after a 2-hour incubation with Neutral Red dye and indicated treatments. Data were analyzed using a Two-way ANOVA followed by Bonferroni post hoc test, *= p<0.1, ***=p<0.001, ****=p<0.0001). Results are expressed as means ±SEM, n= 8.

4 Discussion

Obesity is associated with elevated circulating free fatty acids, including PA, a highly abundant saturated fatty acid found in high-fat diets (Arner & Rydén, 2015). The excessive dietary

amounts of PA have been implicated in metabolic stress resulting from oxidative stress elevation by constant ROS production (Ly et al., 2017). Oxidative stress causes cytotoxicity that eventually results in cell dysfunction, leading to apoptosis when those cells show a low antioxidant defense and cannot counteract the excess amount of ROS production (Ly et al., 2017). Therefore, PA-induced oxidative stress may explain GLP-1 secretion impairment during obesity. While current studies recognize the cytotoxicity that may arise from elevated levels of PA *in vitro* in chronic conditions (Vasu et al., 2015) (Filippello et al., 2018), they do not determine to what extent this cytotoxicity impairs L-cells, and whether this oxidative stress can be reversed.

To explore the effects of acute PA treatment in physiological and pathophysiological simulations, three doses of PA (125μM, 250μM, and 500μM) were used. These doses were selected as the physiological concentration of PA in human plasma was found to range between 0.3 to 4.1 mmol/L compared to 0.03 to 3.2 mmol/L for oleic acid which is the second most abundant free fatty acid (Abdelmagid et al., 2015).To mimic circulating plasma PA during obesity, lipotoxic conditions can be simulated by incubating cells with 500μM of palmitate (Filippello et al., 2018). We found the 500μM PA caused the most significant ROS production by over threefold in NCI-H716 cells and twofold in GLUTag cells. This is in agreement with previous work in GLUTag's demonstrating PA induced ER stress (Filippello et al., 2018). Interestingly, in our work, the NCI-H716 cells required the 500μM dose of PA to induce ROS, whereas the GLUTag's had significant induction of ROS at all the tested doses. Although NCI-H716 and GLUTag cell lines are both enteroendocrine L-cell models, they vary considerably from one another, and this may explain the differences seen in dose-dependent ROS generation

(Kuhre et al., 2016). For example, GLUTag cells secrete a variety of peptides compared to the NCI-H716 cells, while NCI-H716 cells secrete larger amounts of the expressed peptides (Kuhre et al., 2016). Another possible explanation is that the levels of endogenous antioxidants may vary between the two cell lines. There may be higher levels of antioxidants present in the NCI-H716 cells, which can counteract the lower does PA-induced levels of ROS. Indeed, when the cells were examined for PA-induced cytotoxicity using the neutral red test, a significant reduction in viability was observed in GLUTag and not NCI-H716 cells. Additional experiments exploring the antioxidant capacity of each of these cell models may resolve this discrepancy. It is important to recognize that *in vivo*, a variety of fatty acids and cell stress inducers would be present and that an animal model of high fat diet-induced oxidative stress will better recapitulate the fatty acid environment of L-cells.

In the cell experiments, we then demonstrated the antioxidant capacity of the H_2S donor GYY4137. On its own, and in PA-induced ROS generation, H_2S was able to reduce ROS levels. H_2S is well established as an antioxidant gas. Hydrogen sulfide exhibits antioxidant properties in two ways. First, H_2S has a weak reducing propriety, which can react chemically with the superoxide anion (Al-Magableh, Kemp-Harper, & Hart, 2015). Second, H_2S can upregulate antioxidant enzymes such as glutathione peroxidase (Cheung & Lau, 2018) (Bian et al., 2016) and Superoxide dismutase (Sun et al., 2012) through its S-sulfhydration action (Mustafa et al., 2009) (Filipovic et al., 2018).

Next, we explored the effects of the PA-rich Western diet (WD) oxidative stress, GLP-1 regulation, and glucose regulation in C57BL/6 mice. As expected, WD elevated body weight in

both female and male mice (Baena et al., 2017). However, males had a higher increase in body weight at an earlier point in the study and exhibited a significant elevation in fasting blood glucose relative to females. Importantly these indications of metabolic dysfunction aligned with the degree of circulating peroxidated lipids found in males (measured as MDA levels). While MDA does not provide a clear sense of the oxidative stress within the L-cells, it does serve as a proxy for the degree of oxidative stress in an animal (Yonny et al., 2016). It is possible that the females were less sensitive to WD-induced metabolic dysfunction due to the elevated levels of estrogen. Estrogen is known to induce lipid oxidation for use as an energy source (Mauvais-Jarvis et al., 2013) and also plays a beneficial role in glucose homeostasis, as low estrogens can lead to insulin resistance (Mauvais-Jarvis et al., 2013). Since our experimental design included both male and female mice, we were able to detect this difference in WD sensitivity. By knowing that metabolic homeostasis is regulated differently in males and females, it is then imperative to continue to perform studies on both sexes and develop a new experimental design to better mimic and represent the actual physiological state of the women.

We then tested the effect of a single injection of the H_2S donor GYY4137 on WD fed mice. We selected GYY4137 as it releases H_2S over a longer period of time relative to other H_2S donors (Li et al., 2008) (Lee et al., 2011). GYY4137-treated male mice had a significant improvement in glucose tolerance and GLP-1 secretion relative to control male mice, whereas glucose tolerance in female mice was largely unaffected by GYY4137. It is important to note that female mice, despite being on the WD for 16 weeks, did not exhibit the same degree of impaired glucose tolerance and impaired GLP-1 secretion compared to males. Indeed, the GLP-1 response to oral glucose was completely lost in WD-fed male mice. To determine whether H_2S has the ability to

rescue WD-induced metabolic impairment in females, future studies may require a longer diet duration relative to the one used in males.

Since administering an H_2S donor (via IP injection) would likely affect targets other than gut endocrine cells, we explored the impact of GLP-1 receptor antagonism given after the H_2S donor. As expected, mice receiving the H_2S donor and GLP-1 R antagonist had an impaired glycemic response relative to the H_2S donor alone. This suggests that the GLP-1 pathway, at least partly, mediates the action H_2S on glycemia. To fully confirm that the H_2S mediates its effects through GLP-1R, a separate GLP-1R antagonist alone would have been required, as well as a determination of the effects of GLP-1 R antagonist along on glucose tolerance. However, as this study was designed to investigate the effect of H_2S on WD-fed mice, there were insufficient animals to explore an additional GLP-1R antagonist alone group.

The enhanced GLP-1 secretion in mice receiving GYY4137 is in line with our labs' previous work in both L-cells and in mice (Pichette et al., 2017). In that study, GYY4137 enhanced GLP-1 secretion by nearly twofold compared with vehicle-treated GLUTag cells. In mice, H_2S levels were increased by administering a probiotic diet that enhanced sulfate-reducing bacteria. While this previous work provided a novel mechanism using the gut microbiome to enhance H_2S and GLP-1, the current study demonstrated a role for H_2S to protect from fatty acid-induced metabolic stress. Indeed, the potential role of H_2S as a therapeutic treatment is promising as trials are underway exploring its anti-inflammatory properties (Magierowski et al., 2017).

In addition to our in-depth analysis of PA, we also investigated another elevated circulating factor in obesity, glycated albumin. Albumin is the major circulating protein in human plasma (~80% of proteins in circulation) (Freitas et al., 2017). In humans, albumin concentrations range between 35-55 mg/mL, with over 20% of albumin being glycated in individuals with T2DM (Freitas et al., 2017). For this project, the highest concentration of glycated albumin used was 200μg/mL, as an estimation of how much GA is the L-cells exposed to during obesity. Exploring the actual plasma concentration of GA during obesity and manufacturing more glycated albumin should be done to ensure physiological relevance for the enteroendocrine L-cells. As shown in Figure 9, GA at 10, 50, and 200 μg/mL caused a statistically significant increase in ROS production. This finding is similar to other studies that have explored the effects of glycated albumin on ROS-induction using similar detection techniques on other cell types (Zhang Min et al., 2006) (Rodiño-Janeiro et al., 2010). Future work will also explore the elevation of GA in obese models, and whether glycated albumin can induce impaired GLP-1 secretion.

5 Extended discussion

5.1 Cytotoxic effect difference between the NCI-H716 and GLUTag cell lines

500μM PA was chosen to further analyze the fatty acid's effect on cell toxicity, as this concentration resulted in the highest increase in ROS production for both cell lines. When treated with 500μM palmitate, the GLUTag cells demonstrated a significantly lower neutral red uptake compared to its corresponding BSA control. This suggests that 500μM palmitate is toxic to mouse GLUTag cells (Fig 3.D). On the other hand, NCI-H716 cells showed a lower but not significantly reduced uptake of neutral red dye than its corresponding BSA control (Fig 3. C). As

the Neutral Red assay determines cells' ability to incorporate and retain the Neutral Red dye in lysosomes, this assay estimates toxicity leading to cell apoptosis (Repetto et al., 2008) (Almaguel et al., 2010). Excess in PA-induced ROS production in the mitochondria can result in lysosomal membrane perforation, thereby decreasing lysosomal stability and enhancing apoptosis (Almaguel et al., 2010). Nevertheless, additional assays that examine mitochondrial activity (MTT assay), apoptosis (cleaved caspase assay) and DNA content (propidium iodide) would further clarify this.

5.2 *In vitro* GLP-1 secretion by palmitic acid

The body of literature that explores the effect of palmitate on GLP-1 secreting cells is somewhat conflicting. On the one hand, as PA is a fatty acid and fats are known to stimulate GLP-1 secretion, several studies have demonstrated a stimulatory effect in cells. However, one group demonstrated that a 24-hour chronic exposure of palmitate reduced GLP-1 secretion and induced ER stress leading to GLUTag cell apoptosis (Filippello et al., 2018). In our preliminary experiments, we were unable to impair GLP-1 release using PA incubations, as most cells did not survive an overnight incubation in various doses of PA (data not shown). Ultimately, we decided a better model to explore the chronic effects of PA toxicity would be an *in vivo* PA fed mouse model. Future studies may explore *in vitro* if dose/incubation time optimization can be done to induce cell stress, without killing the cells, so that GLP-1 secretion studies can be examined. *In vivo*, L-cells could also be collected from intestinal tissue to explore GLP-1 secretion in primary culture. Indeed, one study has shown that obese mice on a high-fat diet had a reduction in GLP-1 expression (Sun et al., 2012).

5.3 Physiological hydrogen sulfide concentration and its role as an antioxidant

When investigating the antioxidant ability of H_2S on PA-induced ROS, it is important to work with colonic gas concentrations within the physiological range. According to Rose and his colleagues, the concentration of colonic H_2S ranges from 0.2 to 1 mmol/L and reaches as high as 3.4 mmol/L in human feces (Rose et al., 2005). In culture media, 400 µM GYY4137 released less than 20 µM of H_2S for a week (Lee et al., 2011), and another study run by Li and his colleagues, determined that GYY4137 (1 mmol/L, i.e., 100 nmol incubated) slowly releases about 4–5 nmol of H_2S per 25 min both *in vitro* and *in vivo* (Li et al., 2008). In comparison, 400 µM NaHS, a slow-releasing H_2S donor, generated almost 400 µM of H_2S that only lasted for an hour (Li et al., 2008). The plasma of mice injected intraperitoneally with GYY4137 (133 µmol/kg) showed H_2S increased during the first 30 minutes and returned to normal after 8 hours (Cheung & Lau, 2018). The administration of GYY4137 needs to be addressed when studying the effect of H_2S targeting of the L-cell. When injecting the compound, H_2S will diffuse into all cells not only the liver and kidneys (Cheung & Lau, 2018) but also to the intestinal villi (Drucker et al., 2019), where L-cells are located. Briefly, Drucker and colleagues have found that intestinal ischemia-induced mice, which have received IP injections of GYY4137, showed a relatively normal villus structure compared to control, thus reducing intestinal injury (Drucker et al., 2019). For these specific H_2S release rates, the study was done by using the slow H_2S donor that releases the gas within the physiological range. Our lab showed that H_2S could stimulate GLP-1 secretion both *in vitro* and *in vivo* (Pichette et al., 2017). GYY4137 increased GLP-1 secretion by nearly twofold compared with vehicle-treated GLUTag cells (Pichette et al., 2017). C57BL/6 male mice were fed a diet supplemented with prebiotic chondroitin sulfate, which has increased the abundance of SRB and colonic H_2S levels and an enhanced GLP-1 secretion

(Pichette et al., 2017). This study does not confirm whether H_2S had a direct effect on GLP-1 secretion. To explore that, we designed the animal experiment to involve direct injection of an H_2S donor intraperitoneally.

The Antibe team is testing ATB-346, a derivative of naproxen, which releases H_2S. They have successfully shown that ATB-346 counteracts the harmful effect of non-steroidal anti-inflammatory drugs on the GI tract (Magierowski et al., 2017), and now the company is working on a late Phase II clinical trial. Hydrogen sulfide has also emerged to have antioxidant properties. One possible mechanism behind this ability is described to involve S-sulfhydration, also called persulfidation (Mustafa et al., 2009) (Filipovic et al., 2018). In the aorta, Cheung and his colleagues showed that IP injected H_2S in mice induced persulfidation of GPx and reduced lipid peroxidation. Moreover, persulfidation has been found to dissociate Keap1/Nrf2 complex and enhance Nrf2 effect on activating the expression of antioxidant proteins that protect against oxidative (Guo et al., 2014) (Filippello et al., 2018).

5.4 H_2S protective effect on mitochondria from excessive PA

Excessive PA continuously activates β-oxidation that disturbs mitochondrial function, reducing its fatty acid uptake and increasing ROS production (Egnatchik et al., 2014), resulting in lipotoxicity. At the mitochondrial level, H_2S has been found to reduce ROS by inhibiting mitochondrial complex IV in cardiomyocytes under ischemia/reperfusion conditions (Sun et al., 2012). A study done by Giangregorio in 2016 showed that H_2S regulates the carnitine/acylcarnitine carrier that plays a role in the transport of acylcarnitines into mitochondria for β-oxidation (Giangregorio et al., 2016). This might imply a possible interaction between the gas and palmitate and could be a new mechanism either to explain a new antioxidant effect pathway for H_2S or to show that H_2S interferes with PA.

6 Conclusion

In summary, both PA and GA can increase ROS production in L cell lines. H_2S has been shown to have the ability to reduce oxidative stress induced by PA in L-cells, and it has been shown to enhance GLP-1 secretion and improve glucose clearance in mice. This study lays the foundation for future work, exploring how bacterial products such as prebiotics, probiotics, and postbiotics may be used as a treatment for obesity complications.

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