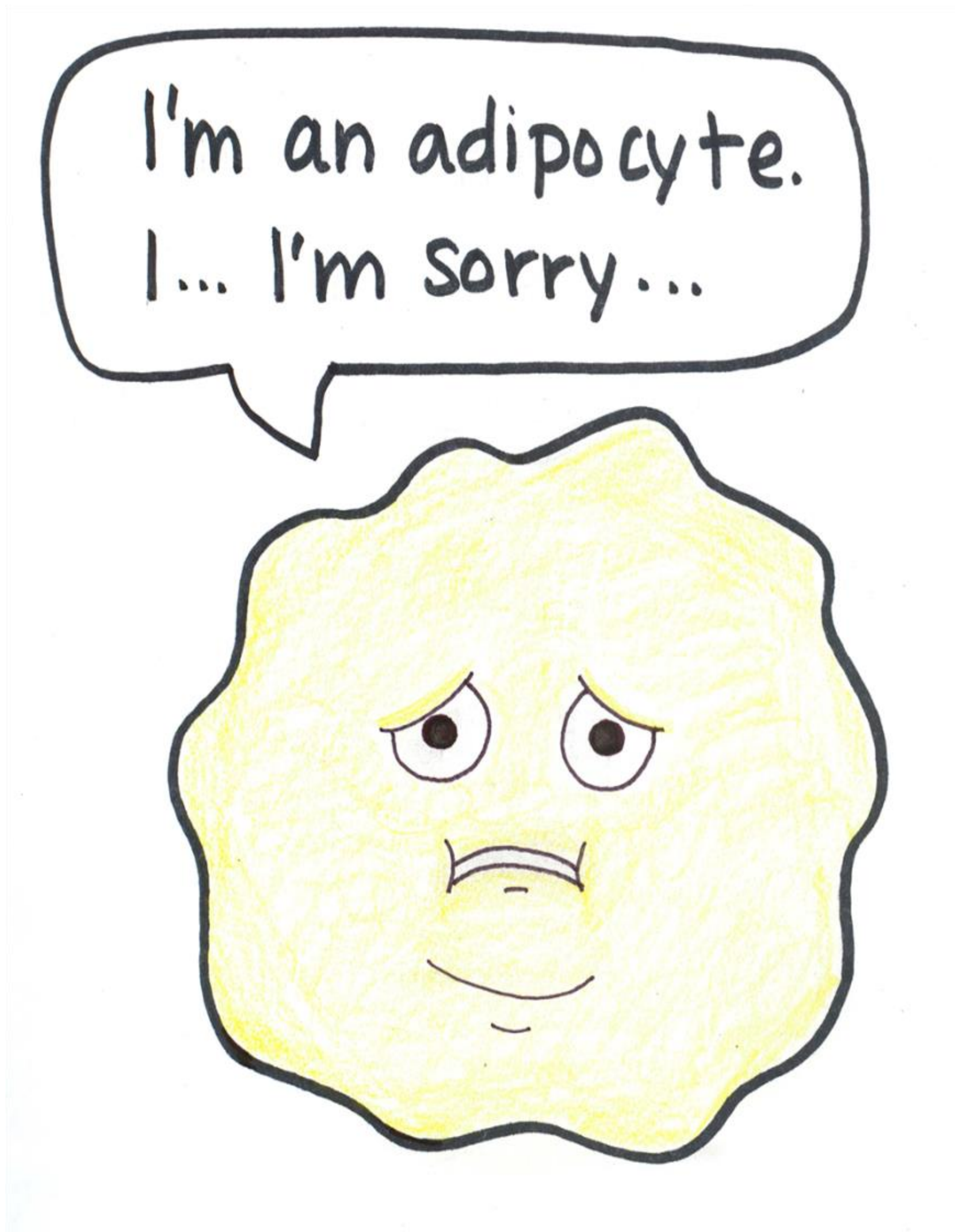
Extracellular cAMP as a Regulator of Lipolysis in Adipocytes

Jonathan Terling 2017 Sahlgrenska Academy



Picture from Beatrice the Biologist

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Extracellular cAMP as a Regulator of lipolysis in Adipocytes

Degree project in medicine, 30 hp
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1. Abstract

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Introduction: Obesity is a major cause of morbidity and mortality globally. Understanding the mechanisms linking obesity to morbidity and the role of adipose tissue in human metabolism is essential to combat this threat. Research shows that the second messenger cyclic adenosine monophosphate (cAMP) can be released by the liver and exert endocrine effects on renal tubule cells causing natriuretic and phosphaturic effects. Extracellular cAMP has also been shown increase fatty acid oxidation in hepatocytes. This urged us to investigate the possible regulatory effect of extracellular cAMP on adipose tissue metabolism.

Objective: To test whether extracellular cAMP affects lipolysis in adipocytes

Methods: 3T3-L1 cells were cultured and differentiated into mature adipocytes using conventional methods and thereafter exposed to different cAMP concentrations for three hours. Effects from blockage of cAMP degradation was tested by adding the ecto-5'nucleotidase inhibitor AMP-CP. Glycerol released into the medium was quantified as a marker of lipolysis. All data were normalized to intracellular protein levels to avoid possible variation from uneven adipocyte distribution between culture wells.

Results: cAMP increased lipolysis at a concentration of 200nM. At lower concentrations of cAMP (between 1-100nM), lipolysis was instead decreased or unchanged. AMP-CP, either alone or in combination with 200nM, had no effect on lipolysis,

Conclusion/Discussion: These results suggest that cAMP both can stimulate and inhibit lipolysis. One possibility is that extracellular cAMP is converted to adenosine, which in turn activates adenosine receptors A1 and A2a expressed on adipocytes. Adenosine receptor A1 inhibits lipolysis whereas A2a stimulates lipolysis. A1 receptors are more abundant in adipocytes and may therefore require lower concentrations to cause this anti-lipolytic effect, while A2a may be more potent overruling the effect of A1 at higher concentrations. Alternatively, cAMP affects lipolysis directly through a yet unknown receptor.

Key Words: cAMP, Lipolysis, Cyclic AMP receptors, 3T3-L1 adipocytes, Obesity.

2. INTRODUCTION

2.1. OBESITY

Human ingenuity creates leverage for our inherent behaviours and biological needs such as social interaction, warmongering and eating. We create smoke signals, letters and social media for communication; weapons for war and advanced food systems for creation of food abundance. Not inherently good or bad such leverage cause change in a society. Between the years 1980-2013 the global prevalence of overweight people changed from 28.8% to 36.9% and 29.8% to 38% men and women respectively (1). Addressing the global obesity epidemic, the World Health Organizations (WHO) Global Health Observatory (GHO) writes "*In 2016, 39% men and 40% of women aged 18+ were overweight (BMI ≥ 25 kg/m2) and 11% of men and 15% of women were obese (BMI ≥30 kg/m2)*". WHO also states that both overweight and obesity increase the likelihood of developing the metabolic syndrome and are linked to increased risk for cardiovascular disease, type II diabetes mellitus, malign neoplasms of several kinds, hypertension and cerebrovascular disease. Noticing this worrying trend of increased obesity and its related diseases the WHO in 2013 called for a zero increase in obesity. This goal however is far from achieved as obesity is increasing in spite of implementation of policies promoting healthy foods, physical activity and other obesity combating strategies of some scale worldwide. Policies as of yet show little effect on the obesity pandemic, but clinical interventions against the resulting metabolic syndrome are ever increasing in potency. In the year 2015 obesity contributed to 4 million deaths and 120 million disability adjusted life years, and the leading causes of deaths related to obesity are cardiovascular disease causing 2.7 million deaths per 2015 and type II diabetes mellitus causing 0.6 million deaths per 2015 (2).

Mounting evidence suggests the importance of healthy adipose tissue for normal physiological function and evasion of disease. This is exemplified by both obesity and adipose tissue deficiency being closely tied to cardiovascular disease and the metabolic syndrome (3). Expansion of adipose tissue is mediated through increased size and number of adipocytes, both processes being affected by inflammation. Inflammation, although typically seen as something deleterious, is in its acute form required for angiogenesis and remodelling of extracellular matrix without which adipose tissue will not maintain a functional endocrine and immunological profile. Inability to mount a potent pro-inflammatory response reduces the ability of adipose tissue to expand in response to excess caloric intake. This leads to ectopic fat deposition and chronic inflammation, and such maladaptive adipose tissue favours hypertrophy over hyperplasia (4). Fat deposited in other organs than adipocytes will cause damage depending on location with consequences such as liver steatosis, so called "leaky gut" i.e. defects in tight junctions in the gut allowing less restricted transport between gut and circulation and deteriorated metabolic profile. The consequences of and the risk of developing maladaptive adipose tissue is to a large extent likely dependent on genetic factors

2.2. ADIPOSE TISSUE

Adipose tissue functions as an energy reservoir, endocrine organ (5, 6), producer of factor C3, B and D of the complement system (7) and location for non-shivering

thermogenesis (8). Adipose tissue is distributed in multiple depots around the body and each depot consists of mainly either white, brown and beige adipocytes each with different functions (9). Besides adipocytes, adipose tissue is also constituted by immune cells, nerve cells, extracellular matrix and stroma-vascular cells (10).

Adipose tissue secretes a wide range of hormones such as leptin, adiponectin, Acylation Stimulation Protein and Tumor Necrosis Factor alpha (TNF-α). Leptin is an important regulator of satiety. Adiponectin has antidiabetic, anti-inflammatory and anti-atherogenic effects through e.g. its positive effect on insulin sensitivity, inhibitory effect on smooth muscle cell migration in vascular walls and inhibitory effect on foam cell formation. Acylation Stimulation Protein causes increased cellular intake and esterification of Free Fatty Acids (FFA), causes translocation of Glucose Transporter (GLUT) proteins to the cell surface and increase LPL activity. TNF-α is a pro-inflammatory cytokine that causes insulin resistance (6, 11).

Triacylglycerides (TAG) is the main compound for long-term storage of energy in the mammalian body. Total mass of TAG in adipocytes is dictated by integration of long term energy balance (10) and the balance between processes for fatty acid deposition and mobilization.

Brown adipocytes function mainly as thermogenic cells and are of a different lineage than white and beige adipocytes. Thermogenesis is mediated by mitochondria expressing uncoupling protein 1 (UCP-1) in their inner membranes. UCP-1 uncouples the electron transport chain from ATP-synthesis and instead of ATP heat is generated. Brown adipose tissue (BAT) is primarily present in mammalian newborns as an evolutionary response to their need to maintain body temperature at birth. Since it was discovered much hope has been placed in this tissue as a means to increase passive energy expenditure to reduce obesity (12). The main approach for recruitment of brown adipose tissue is cold exposure that, as of yet, lacks evidence for clinically relevant effects in humans. BAT is however known to be regulated by parathyroid hormone and thereby play a role in cancer cachexia (13).

Browning is a process in which some white adipocytes approach the brown phenotype with increased number of mitochondria and up regulation of UCP-1. Browned white adipocytes are called beige adipocytes and are thought to be of a distinct developmental origin. Several studies show that browning improves whole-body metabolic function through increased uptake of nutrients from the blood stream - although the effect of browning on weight loss may be modest.

These metabolic and endocrine functions make adipose tissue a master regulator of metabolism with capacity for storage and expulsion of lipids, regulation of insulin sensitivity and satiety affecting caloric intake.

2.3. Fatty acid mobilization and deposition

Lipid droplets in adipocytes constitute the main storage facility for TAG, cholesterol and cholesterol esters; bound to the lipid-droplet are lipid droplet-associated proteins of different classes defined by Liu et al. as structural proteins, proteins responsible for lipid metabolism, membrane traffic, signalling and miscellaneous proteins (14). Due to the complexity and wide array of functions the lipid droplet-associated proteins display Liu et al propose a new name for the lipid droplet, the “Adiposome”. That name more closely mirrors the Adiposomes’ function as a metabolic organelle. Proteins in the structural group - such as perilipin - regulate access for TAG lipases to

the substrates stored in the adiposome, thus regulating basal lipolytic rate. When phosphorylated, perilipin will move and grant access to Hormone Sensitive Lipase (HSL) and Adipose Tissue Triglyceride Lipase (ATGL) (15).

Mobilization of Free Fatty Acids (FFA) is initiated by adherence of ATGL or HSL to the adiposome and subsequent hydrolysis of TAG into a diacylglyceride (DAG) in a reaction catalyzed by ATGL or HSL (16). DAG is hydrolyzed into mono-acylglyceride (MAG) by HSL and the last fatty acid is released from MAG by hydrolysis catalyzed by Monoacylglyceride Lipase (MGL), See “figure 1”. This chain of hydrolysis results in three FFA and glycerol. The rate-limiting step in mobilization of fatty acids is hydrolysis of TAG into DAG, which is mainly mediated by ATGL in turn regulated by glucocorticoids among other hormones. Hydrolysis of DAG is 10 to 30-fold higher than hydrolysis of TAG (17). ATGLs’ degree of activity seems to be mainly regulated by access to the adiposome (18).

FIGURE 1. MOBILIZATION OF FREE FATTY ACIDS

The only DAG lipase identified in adipocytes is HSL, which is tightly regulated by phosphorylation. HSL can be phosphorylated at three sites which will each increase HSLs hydrophobic area and thus increase binding of hydrophobic substrates (19). HSL is phosphorylated by Protein Kinase A (PKA), which is activated by intracellular cAMP derived from activated Adenylyl Cyclase (AC). AC activity is increased by activation of GsPCRs such as the β3-adrenergic receptor or glucagon receptor and decreased by activation of GiPCRs such as the A1 adenosine receptor. Stimulation of the insulin receptor will activate Protein Phosphatase 2A (PP2A) that dephosphorylates HSL and phosphodiesterase 3B (PDE3B) that will degrade intracellular cAMP, thus decreasing activation of HSL via PKA.

Adipose tissue has low capacity for *de novo* lipogenesis and thus fatty acids deposed in adipocytes originate either from lipogenesis in the liver or ingestion with subsequent transportation to the adipose tissue via lipoproteins (20). However, TAG in adipocytes grown in vitro is mainly derived from lipogenesis. Ingested lipids are taken up in the intestine and transported to the liver via chylomicrons. Lipoproteins are synthesized by the liver and carry TAG and cholesterol between liver and other tissues. From the liver TAG-rich VLDL is secreted into the inferior vena cava and enters the circulation. Lipoprotein lipase (LPL) bound to cellular membranes of, among other cell types, adipocytes and endothelial cells, catalyze hydrolysis of lipoprotein bound TAG and release FFAs from the glycerol backbone of TAG. FFAs are then transported through the cellular membrane; See “figure 2”.

Transport of fatty acids across the cell membrane is either mediated by proteins or via the “flip-flop” mechanism. The proteins are split into two groups mediating either constitutive uptake - like the protein FAT/CD36 (21) - or induced uptake like the protein FATP4 (22). The flip-flop mechanism means incorporation of a fatty acid in the outer layer of the cell membrane followed by a turnover in the phospholipid bilayer placing the fatty acid in the inner layer. From there the fatty acid is free to enter the cell (23).

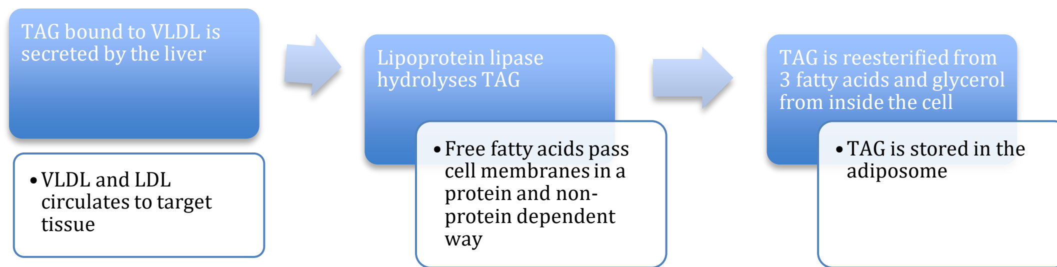

FIGURE 2. FATTY ACID TRANSPORT AND STORAGE

Lipogenesis is a process mainly occurring in the liver where several molecules of acetyl-CoA are fused and made into acyl-CoA. Acetyl-CoA is derived from carbohydrate catabolism directly and protein catabolism via citrate production in the mitochondria. Citrate can pass from mitochondria to the cytoplasm where it will be metabolized into acetyl-CoA and oxaloacetate. Seven acetyl-CoA will react via acetyl-CoA Carboxylase to create malonyl-CoA, which will bind to Acyl Carrier Protein (ACP); this molecule can further react to create fatty acids of different length.

FFA in cells can 1) act as ligands for transcription factors regulating metabolic pathways and 2) be used catabolically in β-oxidation or 3) anabolically for reesterification to TAG and subsequent storage in Adiposomes, synthesis of membrane lipids or signalling molecules (23). β-oxidation will yield 1 acetyl-CoA + 1 NADH + 1 FADH2 per cycle resulting in approximately 19 ATP per cycle.

In concentrations as low as 90pmol/L insulin inhibits half of fatty acid mobilization compared to 200pmol/L needed to cause a 50% decrease in glucose mobilization (24). Insulin will cause increased activity in LPL, dephosphorylation of HSL via activation of PP2A, increased reesterification of fatty acids through activation of acetyl-CoA carboxylase and Fatty Acid Synthase (FAS) (23), increased hydrolysis of cAMP through activation of PDE3B, increased glycolysis resulting in increased amounts of substrates for lipogenesis and cause translocation of FATP1/4 and FAT/CD36 to the cellular membrane (21, 22).

2.4. cAMP

Since 1958 when the first article describing cAMP as an intracellular signal transducer (25) was published, the role of cAMP within the cell has been thoroughly researched. cAMP has risen to be recognized as a universal regulator of cell function with effects dependent on location in the organism as well as concentration. Active processes for efflux of cAMP from cells in general as a response to AC activation have been known since 1963 (26) and is known in adipocytes (27). These processes have mainly been thought of as means to control intracellular cAMP levels or initiate the cAMP-adenosine pathway (28). Several articles have shown effects from extracellular cAMP on various tissues such as proximal renal tubule epithelial cells (29) and hepatocytes (30). This urged us to explore the role of extracellular cAMP in the regulation of adipocyte metabolism.

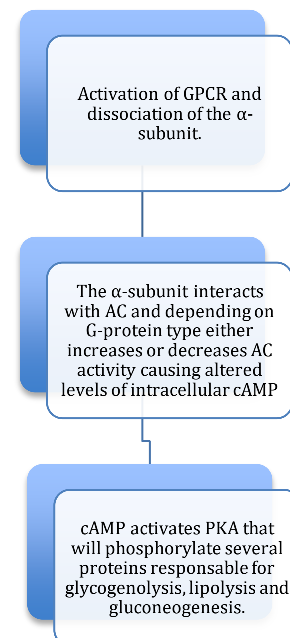

FIGURE 3 CAMP PATHWAY

cAMP is produced by AC of nine membrane bound varieties that are regulated by G-Protein Coupled Receptors (GPCR), see figure 3. GPCRs are internally bound to either stimulatory G-proteins (Gs) increasing cAMP production or inhibitory G-proteins (Gi) inhibiting cAMP production via regulation of ACs. Gs and Gi proteins are heterotrimeric consisting of α-, β- and γ-subunits. In trimeric form the α-subunit is bound to GDP that will be replaced by GTP following activation of associated GPCR, causing dissociation of the subunit and its subsequent interaction with AC. Gs and Gi proteins will each counteract the effects of the other (31). cAMP either interacts with targets inside the cell with subsequent degradation by PDE3B and is reused in ATP synthesis or leave the cell via Multidrug Resistance-associated Protein 4 (MPR4) (32). Outside the cell Ecto-phosphodiesterase (Ecto-PDE) and Ecto-5'nucleotidase sequentially convert extracellular cAMP into adenosine. Adenosine either binds A2 receptors leading to an increase in cAMP production or A1/3 receptors attenuating cAMP production making expulsion of cAMP important in autocrine and paracrine signalling (28). Phosphodiesterases are found in close proximity to cells but only in trace amounts in the blood, making cAMP in circulation stable (29). Physiological concentration of cAMP in mammalian plasma ranges between 10-50 nM (29).

G-protein coupled cAMP receptors (CaR1-CaR4) have been identified in the Dictyostelium discoideum amoeba but no homologous protein has been found in mammalians. Sequence similarities between CaR1-CaR4 and the mammalian secretin receptors are however apparent of which the parathyroid hormone receptor (PTH1R) is the most similar (29). Activation of PTH1R causes lipolysis, browning and change of preferred energy substrate from carbohydrates to fats in adipocytes (33). It is thus possible that cAMP through PTH1R can cause similar effect.

Hepatocytes stimulated by glucagon will increase cAMP production and efflux leading to increased levels of cAMP both intra- and extracellularaly. Glucagon is known to increase transcription of gluconeogenic genes such as Glucose-6 phosphatase (G6Pase) and Phosphoenolpyruvate carboxykinase (*Pepck*) via intracellular cAMP activating PKA, in turn phosphorylating cAMP-responsive element-binding protein (CREB). However, glucagon stimulation also induces expression of the genes for Carnitine Palmitoyltransferase 1a (Cpt1a) and Acyl-Coenzyme A oxidase (Aox) via extracellular cAMP. Mechanisms for efflux of cAMP are up-regulated leading to increased extracellular cAMP that is subsequently metabolized into adenosine. Adenosine will pass through the cell membrane via the transporter CNT2 and activate AMP-Activated Protein Kinase (AMPK) which in turn activates PPARα (30). AMPK regulates genes associated with cellular metabolism causing effects including inhibition of lipogenesis and stimulation of hepatic and muscular gluconeogenesis and fatty acid oxidation. Among up-regulated genes are Cpt1a and Aox (34). Cpt1a helps transfer fatty acids into the mitochondria for oxidation and Aox mediates a step in fatty acid oxidation. Activation of these genes thus leads to increased fatty acid oxidation giving more substrate for gluconeogenesis. By inducing fatty acid metabolism *in vivo* cAMP injections reduced development of obesity induced liver steatosis in mice (30). This is an example of the importance of the cAMP-adenosine pathway.

In the kidney, liver-derived extracellular cAMP inhibits reabsorption of phosphate and sodium in the proximal tubule (29). The mechanism for this effect is unknown, but cAMP has been shown to adhere to cell membranes of proximal tubule cells, as well as other cell types (35), opening the possibility of cAMP-receptors being present (36). The phosphaturic and natriuretic effects suggest inhibition of the Na-P_i

cotransporter that is also inhibited by parathyroid hormone (PTH). In contrast to the proposed mechanism for extracellular cAMP in liver, cAMP degradation into AMP is not extensive in the kidney thus making it unlikely that the effect is mediated via adenosine (37). Indeed, renal tissue perfused with adenosine shows altered reabsorption while addition of cAMP causes increased diuretic effect even in such a setting. This implies that extracellular cAMP can exert direct effects that are independent on AMP/adenosine.

2.5. Parathyroid hormone

The main physiological role of Parathyroid Hormone (PTH) is regulation of calcium and phosphate concentrations in the blood via effects on bone formation, gut absorption and renal tubular uptake. PTH is regulated by calcium concentration in blood.

PTH has been shown to activate HSL via the PKA pathway (39) and increases in PTH are linked to metabolic disease such as type II diabetes, cardiovascular disease and cachexia in cancer and chronic kidney disease. Moreover PTH has been shown to induce browning in adipocytes causing increased expression of UCP-1 (13). Taken together, PTH increases lipolysis and thermogenesis in adipose tissue in addition to its well-established effects on calcium and phosphate metabolism.

2.6. Aim

This study aims to test whether extracellular cAMP affects lipolysis in adipocytes.

3. Materials and methods

3.1. Cell culture

3T3-L1 cells were differentiated into adipocytes using conventional protocols (40) and were used in all experiments in this study. Cells were grown in 12-well plates and experiments were carried out 1-3 days after full differentiation. A functional adipocyte phenotype is suggested by a pro-lipolytic action of β3 adrenergic receptor agonists and an anti-lipolytic action of insulin.

3.2. cAMP stimulation

The aim of the experiment is to run a cAMP dose-response experiment, exposing 3T3-L1 adipocytes to cAMP at different concentrations in an insulin- and adenosine-deprived setting. 12 hours prior to addition of cAMP, cells were cultured in serum free DMEM 10% BSA in order to negate any anti-lipolytic effect from insulin present in the fetal bovine serum in the regular growth medium. After rinsing wells with PBS, the cells were incubated at 37C° with cAMP at concentrations ranging from 1-200 nM, β3 adrenergic receptor agonists (CL316,243, 1µM) or control in 300 µL KRHG buffer without adenosine for 180 minutes 100µL of culture media was aspirated after 180 minutes and cells were lyzed by adding homogenisation buffer.

3.3. Co-stimulation with insulin or AMP-CP

The aim of the experiment is to stimulate 3T3-L1 cells with cAMP + AMP-CP/insulin in an otherwise insulin- and adenosine-deprived setting. 12 hours prior to addition of

cAMP and co-stimulants, cells were cultured in serum free DMEM 10% BSA in order to negate any anti-lipolytic effect from insulin present in the fetal bovine serum in the regular growth medium. After rinsing cells with PBS, KRHG buffer without adenosine was added to the wells. The ecto-5'nucleotidase inhibitor AMP-CP (100 uM) or insulin (0.1 uM) was added to the medium prior to addition of cAMP (200nM). After 180 minutes incubation at 37C° 100uL of culture media was aspirated and cells were lyzed by adding homogenizsation buffer.

3.4. Glycerol and protein measurements

Samples from all wells were analysed in duplicates on 96 well half-area microplates from Corning.

To assess the lipolytic effect of extracellular cAMP, glycerol was measured by "Free glycerol reagent" in the cell culture medium. Samples were diluted 1:2 in KRHG and incubated with reagent for 30 minutes. An absorbance analysis was carried out at 540nM in a spectrometer (SpectraMax i3x). Duplicates with internal deviation larger than SD=0.05 were excluded. See Figure 7 in "Figures and appendices" section for a typical standard graph.

In the cAMP "dose-response" experiment, glycerol values were corrected for protein content of cell lysates to negate differences in glycerol concentration due to varying number of cells in the wells. Cell lysates were produced by adding homogenisation buffer to wells followed by scraping with a cell scraper and aspiration to tubes. Tubes were spun at 4 °C, 10G for 10 minutes to remove debris. Protein was measured in the supernatant using the "PierceTM BCA Protein Assay Kit". Protein measurement was not done in the AMP-CP or insulin co-stimulation experiments as protein samples went missing in the lab freezer.

3.5. Reagents and chemicals

For cell culture, 12-well plates were acquired from Sarstedt. Cells of the 3T3 cell line were acquired from other members of the lab. "Dulbeccos' modified eagles' medium" (DMEM) was acquired from Thermo-Fisher, Dexamethasone from Sigma, insulin (Actrapid) from Novo Nordic, BIX from Sigma, Newborn calf serum from Gibco, fetal bovine serum from Hyclone. For cell stimulation experiments KRHG buffer without adenosine was made from stock solutions, BSA was acquired from Sigma, AMP-CP was acquired from Tocris, insulin was acquired from Sigma, cAMP was acquired from Sigma, CL316,243 (CL) from Tocris and cell scrapers from Sarstedt. Homogenisation buffer including phosphorylation inhibitors (Phosstop and Complete) was freshly made from stock solutions. For quantification of glycerol "Free glycerol reagent F6428" was acquired from Sigma-Aldrich. Protein measurement was performed with "PierceTM BCA Protein Assay Kit". The spectrometer used for glycerol and protein quantification was a SpectraMax i3x (Molecular Devices).

3.6. Defining the protocol

Several repeats of different experimental setups were performed to optimize our protocol for the purpose of this study i.e. measuring the effect of cAMP on lipolysis. Initially culture medium was sampled three times during a 180 minutes period of incubation leading to successively reduced volumes of medium left to dilute metabolic waste products from cells. Possibly this could cause feedback interfering

with the results even when changed volumes are corrected for mathematically. Next the 12-hour period of serum free growth prior to stimulation was established as Fetal Bovine Serum (FBS) used in cell culture containing insulin, which effectively reduce lipolysis for a considerable time. Minor changes to stimulation and extraction procedures were added continuously with alterations such as tight sealing of cell plates during incubation and thorough cleansing of cell scraper between wells instead of changing of scraper. All experiments presented were performed under the same conditions using the protocol described above.

3.7. STATISTICS

Results are reported as ratios of glycerol in intervention samples to control samples ± SEM. In the dose-response experiment differences between control and intervention groups were assessed using Mann - Whitney U test as our values likely will not assume normal distribution. In co-stimulation experiments with AMP-CP or insulin all groups were compared with each other using the same Mann – Whitney U test. A *P*-value of <0.05 was considered statistically significant. The number of wells stimulated and analysed for each concentration is noted as "n" in the tables.

Power analysis was done retrospectively as pre-test estimation of variance was not possible. For the dose-response experiment, power analysis was performed using the website "openepi.com" using the power analysis of mean difference. Confidence interval was set to 95% and each intervention groups' mean, amount of samples and standard deviation was put in manually and compared to the control group (0nM). For costimulation with insulin experiments the same procedure was used but all groups were compared against each other. Power analysis for costimulation with AMP-CP was not performed as no significant differences were found.

4. ETHICS

All experiments were performed on 3T3-L1 cells. No ethical conflicts were identified.

5. RESULTS

5.1. EXTRACELLULAR cAMP BOTH REDUCED AND INDUCED LIPOLYSIS

Extracellular cAMP significantly reduced lipolysis at a concentration of 1nM and 100nM though power analysis showed a power of 78% for the 100nM group. In contrast, lipolysis was significantly increased when adipocytes were exposed to 200nM; See table 1 and figure 4. 1uM β3 adrenergic receptor agonist (CL) induced lipolysis most potently and served as a positive control indicating that the adipocytes have functional pathways for lipolysis. Significant results were not present when glycerol was not normalised to protein.

TABLE 1 GLYCEROL IN GROWTH MEDIUM PRESENTED AS RATIOS OF GLYCEROL IN SAMPLE TO NEGATIVE CONTROL.

cAMP concentration	Average	CI 95%	P-value against control	n	power
0nM	1.00	0.92 - 1.07		18	
1nM	0.69	0.67 - 0.72	0.0105	3	100.00%
10nM	0.77	0.64 - 0.90	0.0797	3	87.58%
30nM	0.9	0.67 - 1.13	0.2317	9	12.84%
50nM	0.82	0.68 - 0.97	0.0704	8	58.13%
100nM	0.79	0.66 - 0.92	0.0124	9	78.63%
150nM	1.07	0.87 - 1.26	0.7211	6	8.90%
200nM	1.35	1.15 - 1.56	0.0019	6	90.45%
b3	1.89	1.56 - 2.22	<0.0001	9	99.95%

GROUPS MARKED WITH RED ARE NOT SIGNIFICANTLY DIFFERENT FROM 0NM CAMP. POWER VALUES MARKED WITH RED ARE BELOW 80%. CAMP (CYCLIC ADENOSINE MONOPHOSPHATE). "N" IS THE NUMBER OF WELLS ANALYSED.

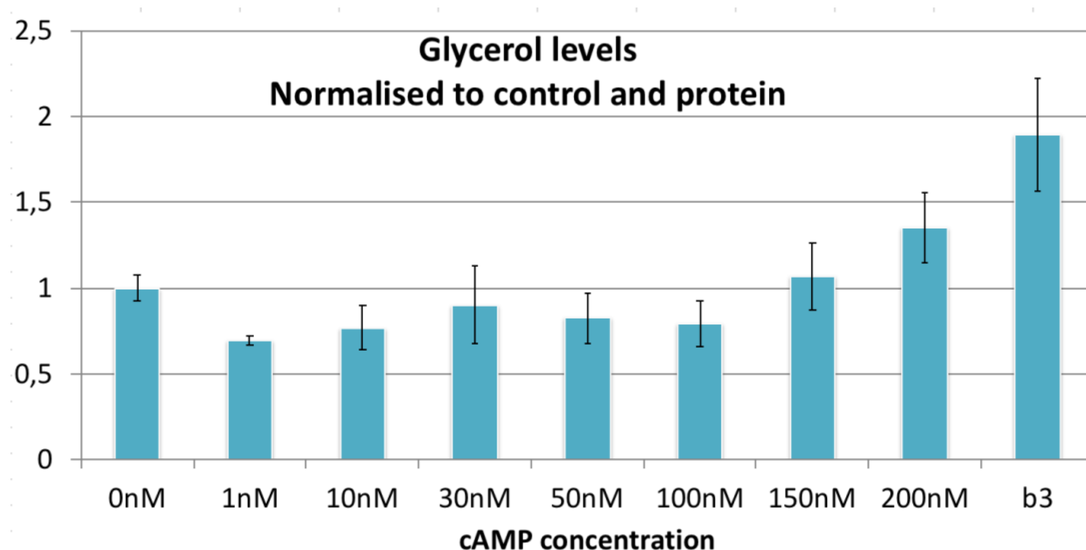

FIGURE 4. CONCENTRATIONS OF GLYCEROL IN GROWTH MEDIUM OF CELLS EXPOSED TO INCREASING CONCENTRATIONS OF CAMP AS RATIOS OF GLYCEROL IN CONTROL. B3 STIMULATION (CL) IS A POSITIVE CONTROL. VALUES ARE NORMALISED TO PROTEIN AND CONTROL. CAMP (CYCLIC ADENOSINE MONOPHOSPHATE). SIGNIFICANT CHANGE IN RELATION TO CONTROL IS SEEN IN GROUPS 1NM (DECREASE), 200NM (INCREASE) AND B3 (INCREASE).

6. BLOCKADE OF CAMP METABOLISM DID NOT ALTER LIPOLYSIS

No significant differences in lipolysis were seen when blocking cAMP degradation with AMP-CP, see table 3 and figure 5. In this experiment 200nM cAMP did not show increased lipolysis compared to control. Furthermore, these data were not normalized to protein levels.

TABLE 2. THE AVERAGE GLYCEROL VALUE FOR EACH GROUP NORMALISED TO H2O IS DISPLAYED. CI 95% DISPLAYS THE 95% CONFIDENCE INTERVAL. N DISPLAYS NUMBER OF SAMPLES IN EACH GROUP.

Additive	Average	CI 95%	n
cAMP + AMP-CP	1.01	0.93 - 1.11	3
cAMP 200nM	0.99	0.96 - 1.03	3

H2O AMP-CP	0.96	0.89 - 1.03	3
H2O	1.00	0.95 - 1.04	3

"N" IS THE NUMBER OF WELLS ANALYSED.

TABLE 3. P-VALUES CALCULATED WHEN COMPARING EACH GROUP TO EACH OF THE OTHER GROUPS.

P-value	cAMP 200nM + AMP-CP	cAMP 200nM	H2O AMP-CP	H2O
cAMP 200nM + AMP-CP		0.7	0.4	0.7
cAMP 200nM			>0.9999	>0.9999
H2O AMP-CP				0.7
H2O				

VALUES ARE P-VALUES.

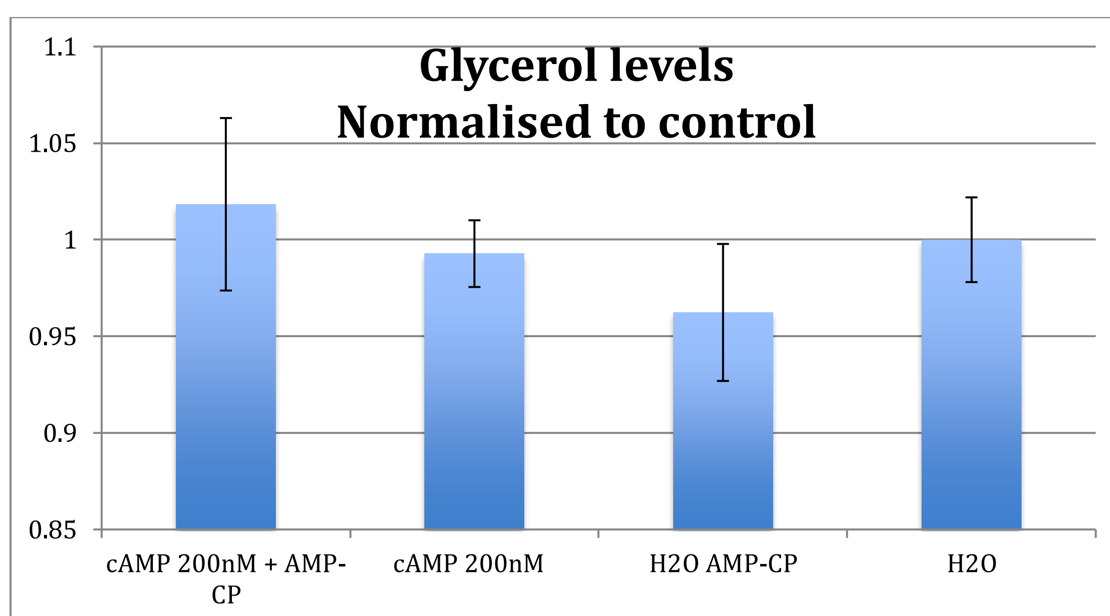

FIGURE 5. LEVELS OF GLYCEROL IN EACH GROUP. VALUES ARE DIVIDED BY THE MEAN OF THE H2O GROUP. NO SIGNIFICANT DIFFERENCES WERE FOUND.

6.1. Co-stimulation with insulin

No significant differences in lipolysis were seen when co-stimulating with insulin, see table 5 and figure 6. These data were not normalized to protein levels.

TABLE 4. THE AVERAGE GLYCEROL VALUE FOR EACH GROUP NORMALISED TO H2O IS DISPLAYED. CI 95% DISPLAYS THE 95% CONFIDENCE INTERVAL. N DISPLAYS NUMBER OF SAMPLES IN EACH GROUP.

Additive	Average	CI 95%	n
cAMP 200nM + insulin	0.98	0.85-1.12	3

cAMP 200nM	1.04	1.01 - 1.08	3
H2O + insulin	0.91	0.90 - 0.92	3
H2O	1	0.96 - 1.03	3

"N" IS THE NUMBER OF WELLS ANALYSED.

TABLE 5. P-VALUES CALCULATED WHEN COMPARING EACH GROUP TO EACH OF THE OTHER GROUPS.

P-value	cAMP 200nM + insulin	cAMP 200nM	H2O insulin	H2O
cAMP 200nM + insulin		0.7	0.7	0.7
cAMP 200nM			0.1	0.2
H2O insulin				0.1
H2O				

RED BOXES INDICATE INSIGNIFICANT DIFFERENCE BETWEEN COMPARED GROUPS. GREEN BOXES INDICATE SIGNIFICANT DIFFERENCE BETWEEN COMPARED GROUPS. VALUES ARE P-VALUES.

TABLE 6 POWER CALCULATIONS CAMP-INSULIN COSTIMULATION

Power	cAMP 200nM + insulin	cAMP 200nM	H2O insulin	H2O
cAMP 200nM + insulin		0.1204	0.203	0.02774
cAMP 200nM			100%	0.4717
H2O insulin				99.91%
H2O				

POWER VALUES ABOVE 80% ARE MARKED WITH GREEN.

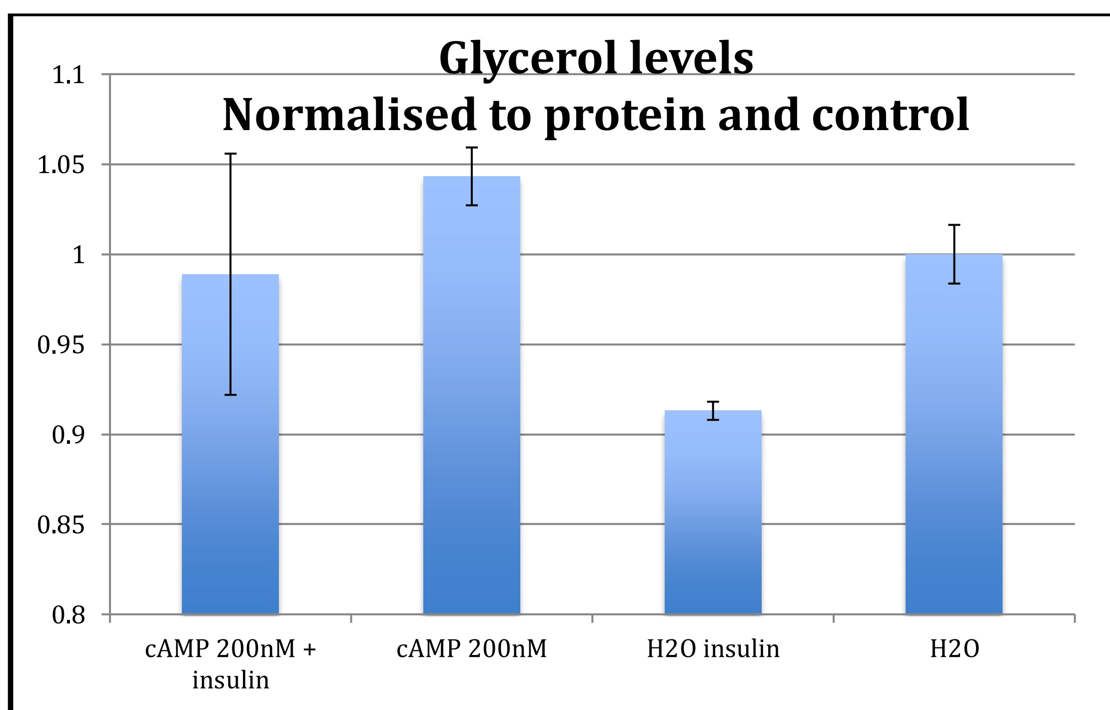

FIGURE 6. LEVELS OF GLYCEROL IN EACH GROUP. VALUES ARE DIVIDED BY THE MEAN OF THE H2O GROUP. THE H20 + INSULIN GROUP IS SIGNIFICANTLY DIFFERENT FROM GROUPS H2O AND CAMP 20NM.

7. DISCUSSION AND CONCLUSION

The pattern where low concentrations of cAMP (1nM and tendencies at 100 nM) decrease while high concentrations (200 nM) increase lipolysis might be due to several superimposed mechanisms. Lipolysis inhibition might be mediated via adenosine receptor A1, while the lipolysis stimulatory effect might be due to stimulation of adenosine receptor A2a, uptake of adenosine via CNT2 or a yet unidentified membrane bound receptor with affinity for cAMP, possibly the PTHR.

7.1. ADENOSINE RECEPTORS

Adenosine has emerged as an important factor in the development of the metabolic syndrome in obesity with effects on glucose homeostasis, adipogenesis, insulin sensitivity and inflammation (41). Different concentrations of adenosine are required to activate different receptors in different tissues based on relative receptor abundance. This makes the response to adenosine both location and concentration dependent. WAT expresses A1, A3, A2a and A2b (from highest to lowest expression) of which A1 inhibits lipolysis and A2a increases lipolysis (41). This likely means that lower concentrations of adenosine are required to induce the effects of A1 than A2a giving net lipolysis inhibition at low concentrations. With increasing concentrations of adenosine, A2a receptors may be increasingly activated, overriding the effects of the A1 receptor and thereby leading to lipolysis stimulation. Experiments in primary cell cultures of human white adipocytes show that raising adenosine concentration above 1000 μM or overexpressing the A2a receptor results in lipolysis but lower concentrations inhibit lipolysis (42). Which concentrations of adenosine is required for net lipolysis in 3T3-L1 cells is unknown. In our experiments we added at a maximum 200 nM of cAMP making it very unlikely that a concentration of 1000 μM adenosine is reached through conversion of the added cAMP. However, stimulation with cAMP might induce a cascade leading to expulsion of cAMP out of the cells. Dose-response experiments with adenosine on 3T3-L1 cells and measuring of cAMP and adenosine in growth medium after stimulation have to be performed to rule out A2a stimulation as the mechanism for lipolysis increase.

To differentiate between effects from adenosine and cAMP at least two approaches can be applied. Either blocking conversion of cAMP to adenosine or blocking adenosine receptors would eliminate adenosine interference. cAMP conversion into adenosine occurs in two steps of which the second can be blocked with AMP-CP reducing the formation of adenosine and possibly maintaining higher concentrations of cAMP for a longer time. Our minor AMP-CP experiment generated however no significant results. An alternative approach is using DPSPX, a pan adenosine receptor antagonist which we have yet to test.

7.2. ADENOSINE UPTAKE

Lv et al demonstrated that in the vicinity of hepatocytes extracellular cAMP is converted into adenosine that is taken up via CNT2 causing up-regulation of CPT1a and Aox leading to increased fatty acid oxidation. Such mechanism does not require the presence of a cAMP receptor (30). Perhaps this mechanism exists and interferes with lipolysis in adipocytes. However, expression of genes involved in fatty acid oxidation and lipolysis and their proteins have not been quantified in our experiments. CNT2 can be blocked to rule out this mechanism as the reason for increased lipolysis.

7.3. CAMP RECEPTORS

In case a mammalian receptor for extracellular cAMP exists and promotes lipolysis, low concentrations of cAMP are likely rapidly degraded leading to minimal cAMP binding to the receptors and larger activation of inhibitory adenosine receptors. In a setting with higher cAMP concentration complete degradation takes longer to occur leaving more time for cAMP to act on hypothesized receptors causing lipolysis. However, results in this study when pharmacologically blocking cAMP degradation with AMP-CP does not support this hypothesis.

There is yet no evidence regarding presence of membrane bound cAMP receptors in mammals or that cAMP binds the PTHR (29). Methods to validate the existence of such a receptor could use binding assays with radiolabeled cAMP on PTHR-null cells or reverse genetics looking for reduction of cAMP binding to/interaction with cells in certain knock-outs. cAMP broken down in cells is reused in ATP production in order to reduce the need for energy demanding purine synthesis. Only smaller parts of cAMP transported out of cells is retaken - while the rest is secreted via urine - making it an energy demanding process. This suggests that extracellular cAMP has an important function.

7.4. 3T3-L1 AS A MODEL OF ADIPOCYTES

The 3T3-L1 cell line is a widely used model of adipocytes used in research in vitro. They have adipocyte-like capabilities such as adiponectin production as well as presence of adipocyte-specific organelles. However, 3T3-L1 adipocytes are generally not as sensitive to stimuli as primary adipocytes.

Presence of lipolytic pathways and expected response to stimuli are considered markers for healthy cells and we tested this through insulin and β3 adrenergic receptor stimulation. Insulin stimulation decreased lipolysis and β3 adrenergic receptor stimulation increased lipolysis as expected.

7.5. ERRORS

The sample size of several groups in this study was very small making the results brittle. Sample sizes for both co-stimulation experiments and the groups 1 nM and 10 nM in the dose-response experiment are 3 per group. Retrospective power analysis shows low power for several groups (10nM among them) but 100% power for 1nM group.

In the dose response experiments results are not present when glycerol is not normalized to protein. In the co-stimulation experiments glycerol levels are not normalized to protein meaning a difference could perhaps be identified should values be normalized to protein. The protein normalization compensates for uneven cell density in wells and possibly also for uneven adipocyte differentiation. This study generated significant results only when protein normalization was applied, possibly indicating uneven distribution of cells.

Not all cells included in the dose-response experiments were stimulated the same time after differentiation. Time for stimulation ranged between 1-3 days after full differentiation of adipocytes and this time is not noted nor analysed in the results. This difference in time could cause cells to change their susceptibility of lipolytic signals based on energy rich substrate concentrations in growth medium during time from differentiation to stimulation. Abundance of energy could cause cells to accumulate more fat and to be more susceptible to lipolytic signals.

Effects from cAMP stimulation on 3T3-L1 cells might differ between long term and acute stimulation. Incubation time of 180 minutes might be to long.

7.6. Closing remarks

Whatever the future has in store for the mammalian-CaR hypothesis one will never cease to be amazed by new capacities of old discoveries. cAMP has been at the center of massive amounts of laboratory and theoretical work for decades and yet this molecule manages to surprise us. As always, more research is needed.

8. Acknowledgements

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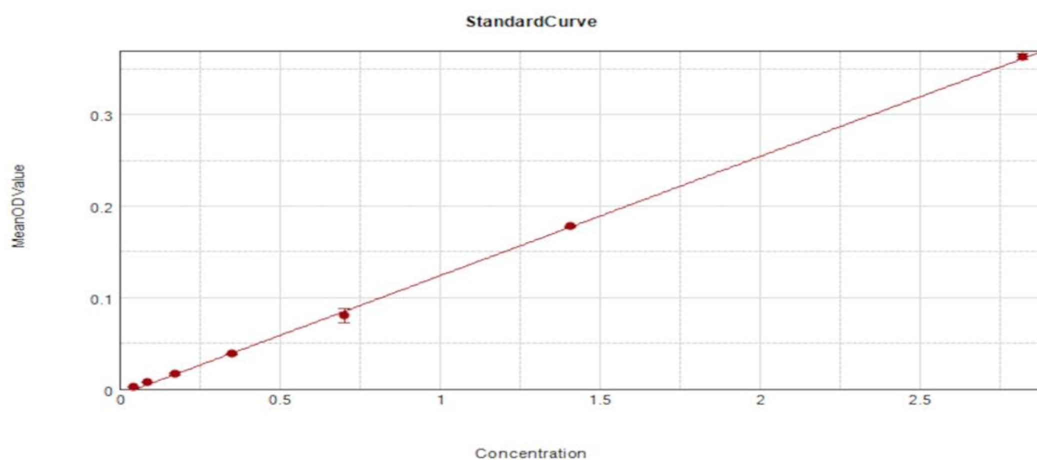
Tables, Figures and Appendices



	Parameter	Estimated Value	Std. Error	Confidence Interval
STD01 $R^2 = 1.000$	A	-0.007	0.001	[-0.010, -0.003]
	B	0.131	0.001	[0.127, 0.134]

FIGURE 7 TYPICAL GLYCEROL STANDARD CURVE

Kan molekylen cAMP hjälpa dig bränna fett?

Fettväv är inte de flesta människors favoritorgan. Fettväven bär dock på många hemligheter, som att den finns i 2 huvudsakliga typer och är viktig för att bevara god hjärt-kärl hälsa. Vit fettväv är en aktiv del i reglage av aptit, påverkar insulinkänslighet i kroppen samt insulinsekretion från bukspottkörteln vilket gör fettväven till ett viktigt hormonutsöndrande organ. Vitt fett producerar dessutom komponenter i komplementsystemet som ingår i immunförsvaret. Komplementsystemet är ett infektionsförsvar där proteiner lösta i blodet binder bakterier och hjälper till att bryta ned dem. Brunt fett finns huvudsakligen hos nyfödda och små däggdjur för att reglera kroppstemperatur.

Frisättning av energi från fettväven är under kontroll av nervsystemet som samverkar med fettvävens egna reglage vilket baseras på blodsockernivåer och andra tecken på energibehov i kroppen. När väven växer blir den totala utsöndringen av hormon större och förändrad, vissa hormon minskar och andra ökar. Både hos personer med för mycket fettväv, som vid fetma, och för lite, som vid grav malnutrition, uppstår omställningar i kroppen som gör det mer sannolikt att utveckla diabetes, åderförfettning, blodfettsrubbningar[*] och högt blodtryck; denna omställning kallas sammantaget för metabola syndromet vilket i sin tur ökar risken för stroke, hjärtinfarkt och fönstertittarsjuka. Gener är av stor betydelse för vilka effekter fetma ger men påverkar inte lika starkt sannolikheten att utveckla fetma.

cAMP är en molekyl som sedan länge är känd som signalmolekyl inuti celler med effekt på flertalet biologiska funktioner. Rollen cAMP spelar utanför celler är relativt okänd men man har funnit effekter på både lever och njurar. Leverns nedbrytning av fettsyror ökaar när den utsätts för cAMP. Detta gav oss idén att undersöka ifall cAMP kan påverka fettväv. En ökad inblick i vita fettvävens hantering av fettsyror och den bruna fettvävens värmealstrande kan hjälpa oss förstå hur fetma kopplas till sjukdom och hur man kan motverka utveckling av fetma och övervikt.

För att testa om cAMP påverkar fettvävens nedbrytning och frisättning av fett odlade vi i labb fram fettceller som vi utsatte för cAMP. Sedan mättes koncentrationen restprodukter från fettnedbrytning i odlingsvätskan cellerna omges av. Detta visade att cAMP i låga koncentrationer motverkar och i högre koncentrationer stimulerar fettnedbrytning. Mekanismen för dessa effekter är dock oklar så vägen är lång till ett cAMP preparat som hjälper människor undvika eller reducera fetma och dess effekter.

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[*] Blodfetter syftar till triglycerider (som är fett) och transportproteiner för triglycerider och kolesterol kallade high density lipoprotein (HDL) och low density lipoprotein (LDL) som alltså är proteiner, inte fetter. LDL anses dock spegla kolesterolnivåerna i kroppen.