Sodium acetate decreases phosphorylation of hormone sensitive lipase in isoproterenol-stimulated 3T3-L1 mature adipocytes

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Abbreviations: BSA, bovine serum albumin; cAMP, 3′-5′-cyclic adenosine monophosphate; DMEM, Dulbecco modified eagle medium; NEFA, non-esterified fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; GPR-34, G-protein coupled receptor 43; HCO₃⁻, bicarbonate; HSL(SER563), hormone sensitive lipase; IR, insulin receptor; IRS1, insulin receptor substrate-1; NaAc, sodium acetate; NAD⁺, nicotinamide adenine dinucleotide; PDE3B, phosphodiesterase 3B; pHSL, phosphorylated hormone sensitive lipase; PI3K, phosphatidylinositol 3′ kinase; PKA, protein kinase A; PKB, protein kinase B; SCFA, short-chain fatty acid

Lipolysis, the process of hydrolysis of stored triacylglycerol into glycerol and non-esterified fatty acids (NEFA), is reported to be reduced by short chain fatty acids (SCFA) but the mechanism of this inhibition is poorly understood. The aim of this study was to measure the phosphorylation at serine residue 563 of hormone sensitive lipase with and without exposure to sodium acetate. Using the 3T3-L1 cell line, we identified that stimulating the cells with isoproterenol increased phosphorylated hormone sensitive lipase (pHSL) expression by 60% compared with the basal state. In the presence of the SCFA acetate in stimulated cells, pHSL decreased by 15% compared with stimulated cells alone. These results were mirrored by the NEFA release from stimulated cells that had significantly decreased in the presence of sodium acetate after 60 min (from 0.53 μmol mg⁻¹ protein to 0.41 μmol mg⁻¹ protein, respectively, P = 0.004); and 180 min (1.73 μmol mg⁻¹ protein to 1.13 μmol mg⁻¹ protein, P = 0.020); however, treatment had no effect on glycerol release (P = 0.109). In conclusion, exposure to 4 mM acetate reduced the level of phosphorylation of HSL(SER563) in mature 3T3-L1 adipocytes and led to a significant reduction in NEFA release, although glycerol release was not affected.

Introduction

Over the past 10 years there has been increased interest in the effect of nutritional substrates, such as short chain fatty acids (SCFA), on the metabolic pathway of lipolysis in white adipose tissue. Interest in surface receptor activation and changes to lipolysis, including glycerol and non-esterified fatty acid (NEFA) release have been reviewed in great detail.

Renewed interest in the lipolytic pathway may stem from the findings that adipose tissue, far from being a deposition source for excess fat storage, is actually a multi-functional organ with an ability to generate and secrete adipokines that interact with the surrounding environment. Within the mature adipocyte, lipid droplet formation and degradation is tightly controlled. There are many regulatory proteins and second messengers involved in the regulation of triacylglycerol hydrolysis, contributing to the complexity of lipolysis. On the surface of mature adipocytes, β-adrenergic receptor stimulation results in an increase in release of glycerol and NEFA from stored triacylglycerol. The binding of ligands, e.g., epinephrine, norepinephrine, or glucagon, to these surface receptors initiates a downstream reaction including activation of adenylyl cyclase, increased availability of cAMP, protein kinase A (PKA) phosphorylation of hormone sensitive lipase (pHSL) and perilipin, and subsequent hydrolysis of stored triacylglycerol into diacylglycerol, monoacylglycerol with the consequent release of NEFA and glycerol.

SCFAs can be generated through fermentation of fiber in the colon in humans, and also by the metabolism of alcohol in the liver. SCFA are able to cross the gut lumen although the exact mechanism of membrane transport is still unknown. The presence of HCO₃⁻ or Na⁺/H⁺ exchangers has been proposed allowing the substrates including acetate, propionate, and butyrate, to become bound to serum albumin after entering the circulation. While the majority of butyrate (70–90%) and a proportion of propionate are metabolized through colonocyte oxidation, acetate and the remaining propionate are transported to the liver. The majority of acetate absorbed by the gut and released by the liver is metabolized in mitochondria as an alternative energy source by surrounding tissues including the brain, smooth muscle, and heart.
An inhibitory G protein-coupled receptor (GPR43) for SCFA has been identified in white adipose tissue. Reasons for the presence of a specific receptor for SCFA within white adipose tissue have not been fully identified, but it does suggest that SCFA may play a part in regulation of adipocyte lipolysis. A previous in vivo human study observed a lack of glycerol and NEFA release but that sodium acetate ingestion did not result in a change in NEFA and glycerol and there was a concomitant decrease in fat oxidation. The authors concluded that although acetate appears to have little effect on the basal rate of lipolysis, it did suppress lipolysis during a metabolic alkalosis, i.e., conditions that stimulated lipolysis. The present study therefore aims to identify whether sodium acetate affects adipose tissue lipolysis in vitro. By utilizing the murine cell line 3T3-L1, it was possible to monitor changes in phosphorylation of HSL(Ser563), the enzyme responsible for diacylglycerol hydrolysis, and identify the effect of sodium acetate on adipocyte lipolysis, as a measure of glycerol and NEFA. We hypothesized that in the presence of acetate the rate of NEFA and glycerol release would be reduced in isoproterenol stimulated cells.

**Results**

Changes in the level of phosphorylated hormone sensitive lipase in control and treated 3T3-L1 adipocytes

In order to identify if sodium acetate and insulin were acting within the lipolytic cascade, changes in the phosphorylation of HSL(Ser563) were analyzed. In the presence of isoproterenol (Iso) the relative volume of pHSL increased 60% compared with the basal state of pHSL, i.e., without stimulation (Fig. 1). Incubating the cells with the addition of Iso+acetate resulted in the relative volume of phosphorylated HSL(Ser563), being reduced by 15% compared with isoproterenol stimulation alone. pHSL was also reduced in the presence of Iso+insulin by 21% and Iso+NaAc+Ins by 24% compared with isoproterenol stimulation.

Changes in the metabolic response to control and treated 3T3-L1 cells in the presence of α-adrenergic stimulation

The mature 3T3-L1 adipocytes were stimulated with a half-maximal dose of isoproterenol over a period of 180 min and this resulted in a significant increase in lipolytic rate, as reflected by the release of both NEFA and glycerol. The authors concluded that although acetate appears to have little effect on the basal rate of lipolysis but that sodium acetate ingestion did not result in a change in NEFA and glycerol and there was a concomitant decrease in fat oxidation. The authors concluded that although acetate appears to have little effect on the basal rate of lipolysis, it did suppress lipolysis during a metabolic alkalosis, i.e., conditions that stimulated lipolysis. The present study therefore aims to identify whether sodium acetate affects adipose tissue lipolysis in vitro. By utilizing the murine cell line 3T3-L1, it was possible to monitor changes in phosphorylation of HSL(Ser563), the enzyme responsible for diacylglycerol hydrolysis, and identify the effect of sodium acetate on adipocyte lipolysis, as a measure of glycerol and NEFA. We hypothesized that in the presence of acetate the rate of NEFA and glycerol release would be reduced in isoproterenol stimulated cells.
significantly higher ($P = 0.014$) compared with isoproterenol alone but treatment with either Iso+acetate and Iso+insulin did not result in any significant change.

In the presence of each treatment there was no significant reduction in the change of glycerol concentration compared with isoproterenol alone. Although glycerol concentration appeared to increase at each time point it was only in the Iso+acetate treatment where glycerol release was observed to increase between 60 to 120 min ($P = 0.006$) and from 120 to 180 min ($P = 0.013$). In neither the Iso+insulin treatment nor Iso+NaAc+Ins, did we observe any change in glycerol concentration between 60 and 120 min. However, during the final 60 min the glycerol concentration increased significantly with Iso+insulin ($P = 0.053$) and Iso+NaAc+Ins ($P = 0.034$).

As both NEFA and glycerol samples were taken from the same well for each treatment, we expected a 3:1 ratio; however this was found not to be the case. At 120 and 180 min the ratio between NEFA release and glycerol release was -1:5:1, i.e., half the release of NEFA that would have been expected.

### Discussion

The aim of this study was to identify whether sodium acetate affects adipose tissue lipolysis in vitro. These data demonstrate that an increase in acetate availability can cause a reduction in the phosphorylation of HSL at the serine 563 residue and supports the significant reduction in NEFA release that was observed in these cells but not our hypothesis regarding the effects on glycerol release.

The process of lipolysis is undoubtedly complex with many factors influencing this pathway; these include activation of inhibitory/stimulatory G protein-coupled receptors, β-adrenergic stimulation, insulin/glucagon signaling, and the interactions between these factors. The regulation of lipolysis is therefore tightly controlled but also depends on the energy state of the cell and the availability of substrates and hormones, e.g., glucose and insulin. In this study we were able to identify an intracellular change to lipid metabolism, from the basal to stimulated state, through examination of the phosphorylation status of HSL. Previously it has been shown that the phosphorylation status of HSL is critical in the catabolism of triacylglycerol. Using both an in vitro model and intact cell model Fredrikson et al. demonstrated that the phosphorylation of HSL was associated with the availability of cAMP and that the enzyme activity of HSL increased 2-fold in the presence of the catalytic subunit of protein kinase and cAMP. Under basal conditions of lipolysis, there was continuous low level phosphorylation of HSL. Upon stimulation with the β-adrenergic receptor agonist isoproterenol, the level of phosphorylation increased 60% and this observation supports previous work showing an increase in pHSL in the presence of isoproterenol and similar increases in glycerol release in the presence of isoproterenol. In this study we further demonstrated that the presence of sodium acetate reduced the phosphorylation of HSL by 15% compared with isoproterenol stimulated cells alone. It is clear that there are other serine residues (for example 659 and 660) that are also linked to the activity of HSL and that future work should consider examining the phosphorylation status of these serine residues. In addition, the effects of acetate on the phosphorylation of serine residue 563 is also warranted because phosphorylation at this site is linked to the inhibition of HSL activity and hence it might increase phosphorylation in a reciprocal manner to the decrease in HSL. To the best of our knowledge these novel findings demonstrate the ability of the short chain fatty acid, acetate, to decrease the level of activity of one of the key enzymes, HSL, in the lipolytic pathway.

The most common endogenous inhibitor of the lipolytic pathway is insulin and activation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) is known to interact with the lipolytic pathway in adipocytes. For a review see reference 2. It is well documented that under conditions of excess glucose availability, insulin not only increases glucose uptake through the GLUT4 transporter but also stimulates the phosphatidylinositol 3′ kinase (PI3K) pathway. The subsequent downstream signaling process is unresolved regarding the mechanism by which insulin decreases adipocyte lipolysis; however, protein kinase B (PKB) dependent and -independent pathways have been proposed to operate. Under conditions of high lipolytic stimulation, the phosphorylation of PKB on Ser473, initiates the activation of phosphodiesterase 3B (PDE3B), which in turn hydrolyses cAMP into 5′-AMP. This conversion to 5′-AMP results in a decrease in available cAMP and therefore reduces the ability of PKA to phosphorylate HSL and perilipin, both of which are essential for hydrolysis of stored triacylglycerol. Our results support these findings since in the presence of 1.34 μmol insulin, there was an observed 21% reduction in pHSL compared with isoproterenol stimulated pHSL alone (Fig. 1). There was no evidence for an additive effect in the presence of both acetate and insulin in the stimulated cells as pHSL had only decreased by 24% compared with isoproterenol stimulated cells.

Short chain fatty acids have an affinity to G protein-coupled receptors with acetate exhibiting specific affinity for GPR-43. Other studies have shown that activation of G protein-coupled receptors.
receptors present in the cell membrane of adipocytes results in a reduction in the lipolytic rate as determined by the release of NEFA and/or glycerol. In this study we have demonstrated that incubating mature 3T3-L1 adipocytes with supra-physiological levels of sodium acetate also reduces the rate of lipolysis, as measured by the rate of NEFA release, although glycerol release appeared to remain unaffected in the presence of sodium acetate. These data agree with previous reports showing changes in lipolysis, expressed as a reduction in the level of glycerol and/or NEFA release, in the presence of ligands of G protein-coupled receptors containing a small carboxylic acid tail region similar to that of sodium acetate. The relationship between NEFA and glycerol release was also found to be lower than expected. It may, however, be that re-esterification of fatty acids into the adipocyte and an increase in mitochondrial oxidation within adipocytes had taken place, which would account for the reduction in NEFA to glycerol ratio.

In conclusion, these data support our hypothesis that increased availability of the short chain fatty acid, acetate, reduces the level of phosphorylation of HSL (SER563) in mature 3T3-L1 adipocytes, and lead to a significant reduction in NEFA release, but not of glycerol, into the surrounding environment.

Materials and Methods

Cell culture

Mature 3T3-L1 cells were cultured in a 12-well plate format for all experiments. Un-differentiated 3T3-L1 cells were plated in PM-1-L1 media (Zen Bio) and fed every 2 d until they reached confluence. The cells were left for a further 48 h to initiate growth arrest before the media was changed to DM-2-L1 differentiation media (Zen Bio). Cells were incubated in DM-2-L1 for 72 h before the media was gradually changed to AM-1-L1 media (Zen Bio), in accordance with the feeding manual published by Zen Bio. Cells were fed every other day until day 9 post-differentiation when cells were subjected to the various experimental treatments. Cells were treated in phenol red-free high glucose (4.5 g/L) DMEM (Sigma Aldrich) supplemented with 2% fatty acid free BSA (PAA Laboratories), subsequently referred to as treatment media. For cells that were treated with sodium acetate (Sigma Aldrich), 4 mM sodium acetate was added to the culture media 30 min prior to the start of lipolysis analysis, and was present in treatment media for each subsequent time point. After the 30 min pre-incubation, cells were washed in 37 °C 1× PBS and treated with or without 5 μM isoproterenol, 4 mM sodium acetate (Iso+acetate), 1.34 μmol insulin (Iso+insulin) (Invitrogen) or both acetate plus insulin (Iso+NaAc+Ins). The half-maximal dose of 5 μmol as a stimulating dose of isoproterenol was determined in a separate study (unpublished results) using a titration of isoproterenol against NEFA release in 3T3-L1 cells at the same stage of differentiation as the present study.

Metabolic analysis

Cells were incubated for a total of 180 min in treatment media. Every 60 min, samples were collected from each treatment media and analyzed for both NEFA and glycerol concentration. Samples were analyzed for NEFA using WAKO NEFA-HR(2) kit according to the manufacturer’s instructions (Alpha Laboratories) and also for glycerol concentration using the method by Boobis and Maughan. Briefly, stock solutions of the reaction mixture were prepared, including glycerol buffer, glycerol dehydrogenase, and NAD+ (Sigma Aldrich); standards were prepared from a stock solution of 2 mM glycerol (Sigma Aldrich). Samples and standards were dispensed into a 96 plate and reaction mixture was added to each well. The plate was mixed well and left to incubate for 4 h. The reaction was stopped by the addition of sterile distilled H2O before the plate was read fluorometrically at excitation 340 nm and emission 460 nm, on a SpectraMax M5 microplate reader. Samples not analyzed on the same day were stored at −20 °C. NEFA and glycerol samples were normalized to total protein.

Western blotting

Cells were cultured and treated in a 12-well plate format. At the end of the 120 min incubation, cells were washed twice with ice cold 1× PBS, before the addition of lysis buffer (Cell Signaling Technology supplied by New England Biolabs) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Cells were incubated on ice for 5 min, and then scraped into suspension. lysates were sonicated briefly in 1 s bursts for 10 s, and then centrifuged at 10,000 × g for 10 min at 4 °C. Due to the presence of free triacylglycerol, supernatants were aspirated and centrifuged a second time. Protein concentration was calculated using the Bradford method, using BSA as the standard. Protein samples of 15 or 25 μg were then separated by 4–15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). Each membrane was blocked overnight in 5% skimmed milk and then incubated with pHSL (SER563) and GAPDH primary antibodies (1:1000) (New England Biolabs), followed by horseradish peroxidase conjugated secondary IgG (1:3000) (New England Biolabs). Visualization of immunoreactive proteins occurred using the chemiluminescence detection kit LumiGLO with peroxide reagent (New England Biolabs). Images were acquired using ChemiDoc XR+ and analyzed using ImageLab 3.0 software. First, the relative volumes of both pHSL and GAPDH were calculated before subtracting for background. The volume of pHSL and GAPDH was initially measured by determining the number of pixels within a standardized area for each sample from the recorded western blot image. Using isoproterenol as the positive control and standardizing the loading against GAPDH, the remaining samples were standardized against the internal control. Furthermore the pHSL from the positive control was normalized against GAPDH and expressed as 100% from which all the remaining samples were expressed as a percentage of this maximum stimulated condition.

Statistical analysis

All experiments were performed at least three times in triplicate. Statistical analysis was performed using SPSS Statistics 20 software. All data were analyzed for normality using the Shapiro–Wilks test. The glycerol Delta-glycerol and Delta NEFA data were found to be normally distributed thus analyzed using a two-way ANOVA followed by a post-hoc Tukey test. In contrast the NEFA data were not normally distributed and thus
were analyzed using a Kruskal–Wallis test followed by a pairwise Mann–Whitney. A P value of less than 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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