

Lipid accumulation and alkaline phosphatase activity in human preadipocytes isolated from different body fat depots

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Abstract

Background: Alkaline phosphatase (ALP) controls intracellular lipid accumulation in human preadipocytes, but it is not known whether ALP is expressed in all body fat depots, or whether it has a similar role at all sites.

Design: Cross-sectional.

Setting and subjects: Subjects undergoing breast reduction and abdominal fat biopsies operations at Charlotte Maxeke Johannesburg Academic Hospital.

Outcome measures: This study compared intracellular lipid accumulation and ALP activity in the presence and absence of ALP inhibitors in preadipocytes that were obtained from different adipose depots. Abdominal and mammary gland preadipocytes were isolated from women and induced to differentiate in culture. ALP activity and intracellular lipid levels were measured at baseline and after 12 days of differentiation in the presence and absence of the ALP inhibitors, histidine and levamisole.

Results: ALP activity was detected in nondifferentiated abdominal (134 ± 7.5 mU/mg protein) and mammary gland (136 ± 9.6 mU/mg protein) preadipocytes. Its activity had increased significantly (p -value < 0.0005 for both) by day 12 of differentiation (388 ± 55 for abdominal and 278 ± 28 mU/mg protein for mammary). Preadipocytes treated with histidine had lower fat accumulation (p -value < 0.0005) and ALP activity (p -value < 0.005) than nontreated cells on day 12, while those treated with levamisole had lower fat accumulation (p -value < 0.005), but elevated ALP activity (p -value < 0.05), compared to nontreated cells. Lipid accumulation (p -value < 0.005) and ALP activity (p -value < 0.05) were higher in abdominal than mammary gland preadipocytes by day 12.

Conclusion: ALP is involved in the control of intracellular lipid accumulation in human preadipocytes that are isolated from both adipose depots. The ability of levamisole to inhibit this process while activating ALP, suggests that this molecule acts via an ALP-independent pathway, while histidine attenuates both lipid deposition and ALP activity.

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Introduction

Alkaline phosphatase (ALP) is expressed in a wide variety of tissues and exists as four different isoenzymes, each coded by a different gene. These four isoenzymes are termed the tissue-non-specific (also known as liver-bone-kidney ALP) intestinal, placental and germ cell forms.¹ The three latter types are often grouped together under the term "tissue-specific ALP". The four isoenzymes can be distinguished from each other via their genes of origin and via their response to specific inhibitors. Thus, tissue-non-specific ALP is inhibited by levamisole, histidine and homoarginine, while the tissue-specific isoforms are not.^{2,3} The tripeptide, Phe-

Gly-Gly, is able to inhibit the tissue-specific ALPs, but not the tissue-non-specific isoform.⁴ Serum levels of ALP have commonly been used for disease diagnosis, with the tissue-non-specific isoforms acting as markers of bone and liver pathology.⁵

All adipose tissue depots are known to contain preadipocytes. Preadipocytes undergo adipogenesis, a finely regulated process of differentiation into mature adipocytes. Involved factors in this process include both positive and negative stimuli that incorporate a variety of hormones, enzymes and nutrients.^{6,7} Adipogenesis can be influenced by fat depot origin in *in vitro* models. For example, preadipocytes that are isolated from the

visceral depot differentiate more slowly, compared with subcutaneous preadipocytes.⁸

Previously, we demonstrated a positive correlation between ALP activity and fat accumulation in murine 3T3-L1 preadipocytic cells⁹ and human mammary gland preadipocytes.¹⁰ The ability of ALP inhibitors to pharmacologically inhibit intracellular lipid accumulation suggests that ALP may play a role in preadipocyte lipid storage.^{9,10} It is not known whether ALP is expressed in other body fat depots, or whether it is involved in the molecular control of adipogenesis at such sites. Abdominal subcutaneous adipose tissue is an important fat storage depot and may influence whole body insulin sensitivity.¹¹ Thus, some, but not all studies, have shown that it is a more important regulator of insulin resistance than the visceral fat depot.^{12,13} It has also been suggested that subcutaneous adipose tissue may play a role as a "reservoir" for triglyceride storage, and may act to limit fat deposition in the visceral depot.^{11,14} Therefore, it is important to understand the molecular events that are implicated in the control of lipid deposition in abdominal subcutaneous preadipocytes and mature adipocytes. Thus, in the current study, preadipocytes were isolated from mammary gland and abdominal subcutaneous fat depots to assess any differences in adipogenesis and ALP activity between these tissue sites.

Subjects and method

Reagents

All tissue culture reagents were purchased from Invitrogen Corporation, Scotland, and all other laboratory reagents were purchased from Sigma-Aldrich, South Africa, unless otherwise stated.

Subjects

Human abdominal subcutaneous tissue samples were obtained voluntarily from 13 black women of mean body mass index (BMI) 38 ± 7.4 (range 29.3-53.5) and mean age 46.5 ± 6.5 (range 35-58). Mammary adipose tissue was obtained from 14 black women of mean BMI 25.1 ± 1.6 (range 22-26.7, p -value < 0.0001 vs. abdominal) and mean age 45.7 ± 5 (range 38-55, p -value = 0.77 vs. abdominal). The latter participants underwent elective surgery for mammary gland reduction.

Abdominal subcutaneous fat was collected under local anaesthetic. A transverse skin incision was made in the mid-right abdominal side of each volunteer. An incision length of 10-12 mm was sufficient to collect the required amount of fat. Haemostasis was secured with diathermy and the wound was closed with subcutaneous absorbable sutures. Both groups were admitted to surgery wards in Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg.

All subjects were healthy, as assessed by history and physical examination. No pregnant subjects were included in the study. Informed consent was obtained before surgical intervention. Ethical approval to use the adipose tissue was obtained from the University of the Witwatersrand, Faculty of Health Sciences Human Ethics Committee.

Isolation and culture of preadipocytes from abdominal subcutaneous and mammary gland adipose tissue

Preadipocytes were isolated from adipose tissue using collagenase digestion, as described in a previously published method.¹⁵ The preadipocytes were grown in six well tissue culture plates at a density of 4×10^5 cells per well, in a tissue culture medium, comprising Dulbecco's Modified Eagle Medium (DMEM)-Ham's F12 medium, containing 15 mM HEPES and 2 mmol/l glutamine, supplemented with 10% foetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin. After overnight culture, the cells were washed in DMEM-Ham's F12 medium. Subsequently, the medium was changed every third day, until the cells were confluent. The preadipocytes were stimulated to differentiate over 12 days in differentiation medium supplemented with 100 nmol/l hydrocortisone, 66 nmol/l insulin, 0.5 mmol/l 3-isobutyl-1-methylxanthine and 1 nmol/l triiodo-L-thyronine. The preadipocytes were incubated at 37°C in a humidified atmosphere with 5% CO₂. The culture media were changed every three days.

Alkaline phosphatase inhibitors

Preadipocytes were cultured in the absence or presence of ALP inhibitors. These inhibitors were added at the beginning of each differentiation period. The tissue-non-specific ALP inhibitors, histidine² and levamisole,³ and the tissue-specific ALP inhibitor, Phe-Gly-Gly,⁴ were used at concentrations of 20 mmol/l, 2 mmol/l and 20 mmol/l, respectively. These doses were based on a previous study using the murine 3T3-L1 preadipocyte cell line.⁹

ALP activity and intracellular lipid accumulation were measured before intracellular lipid accumulation was initiated (day 0), and 12 days after stimulation of adipogenesis in preadipocytes isolated from all study subjects. These variables were also measured in the presence of the ALP inhibitors, but not in all subjects. Thus, the effect of histidine was measured in abdominal preadipocytes isolated from 10 subjects and in mammary gland preadipocytes from four subjects. The effect of levamisole was assessed in abdominal preadipocytes isolated from four subjects, and in mammary gland preadipocytes from nine subjects. The effect of Phe-Gly-Gly was measured in preadipocytes sequestered from the mammary tissue of four subjects, but not tested in the abdominal cells.

Measurement of alkaline phosphatase activity

Preadipocyte cell extracts were isolated at baseline (day 0) and 12 days after initiation of adipogenesis, using a previously published method.¹⁶ ALP activity was measured in these cell extracts using an automated colorimetric assay (Roche Diagnostics, Germany). The protein content of the supernatant was analysed using the Bradford method.¹⁷ The ALP activity was calculated as mU of activity per mg of cell protein.

Measurement of adipogenesis

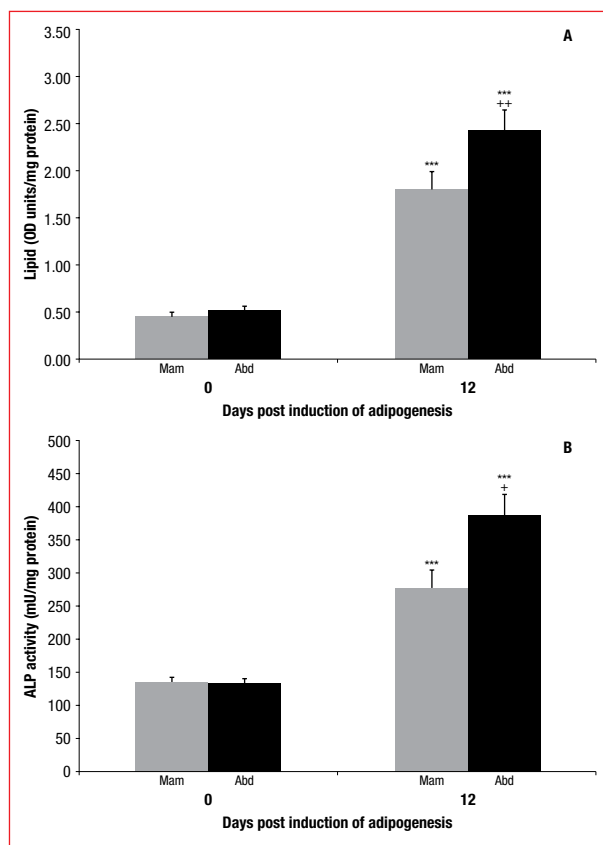
Intracellular fat accumulation, the hallmark of adipogenesis, was measured on day 0 and day 12 using the Oil Red O technique.¹⁸ This method depends on the ability of the lipid droplets in the adipocyte to absorb the red dye, which is then extracted from the cells using isopropyl alcohol, and the absorbance measured at 510 nm. Lipid levels were expressed as optical density (OD) units per mg of cell protein.

Statistical analysis

ALP activity and adipogenesis levels were expressed as mean \pm standard error of mean (SEM). Differences in ALP activity or cellular fat accumulation within different treatment regimens were analysed using Student's paired t-test. Analysis of covariance (ANCOVA), with adjustment for BMI, was used to compare cellular lipid and ALP levels between the preadipocytes isolated from the abdominal subcutaneous and mammary gland adipose tissue. The relationship between the change in ALP activity and the change in intracellular lipid levels over the 12-day incubation period was assessed by calculating the percentage change in both variables, and by implementing a univariate Pearson correlation analysis. This was performed on combined data from the abdominal and mammary gland preadipocytes and on data from each individual body site. Pearson correlation was also used to analyse the relationship of intracellular lipid and ALP levels with BMI. All statistical analyses were carried out using Statistica® version 9 (StatSoft, USA).

Results

ALP activity was detected in undifferentiated human preadipocytes from both mammary gland and subcutaneous abdominal adipose depots. Enzymatic activity in these cells increased during the 12-day differentiation period and was paralleled by an increase in cellular fat accumulation. Significant differences (p -value < 0.0005) for both these variables were detected between differentiated preadipocytes on day 12, and undifferentiated preadipocytes on day 0 (Figure 1). On day 0, lipid and ALP activity levels were similar in the two different preadipocyte cell populations. However, on day 12, both lipid (p -value

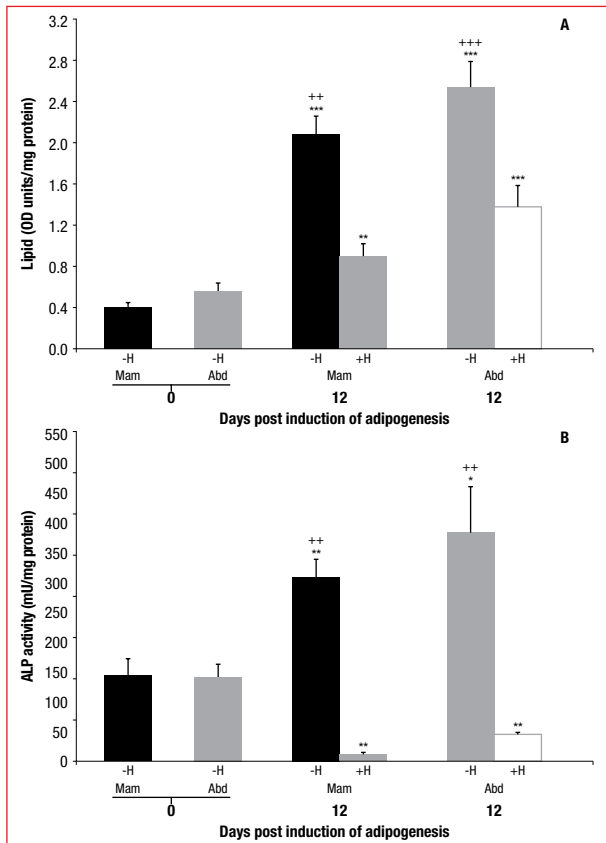


Data are shown as a mean \pm standard error of mean
 *** p -value < 0.0005 vs. day 0, ** p -value < 0.05 , *** p -value < 0.005 vs. mammary gland
 Abd: abdominal, ALP: alkaline phosphatase, Mam: mammary gland, OD: optical density

Figure 1: Comparison of (a) lipid accumulation and (b) alkaline phosphatase activity in human preadipocytes isolated from mammary gland ($n = 14$, light bars) and abdominal subcutaneous ($n = 13$, dark bars) adipose tissue

< 0.005 , Figure 1 a) and ALP activity (p -value < 0.05 , Figure 1 b) levels were higher in the abdominal than those in the mammary preadipocytes.

Figure 2 shows the effect of histidine treatment on intracellular lipid levels and ALP activity in abdominal and mammary gland preadipocytes. With histidine treatment, intracellular lipid levels were significantly higher for all preadipocyte populations on day 12, compared to day 0 (Figure 2 a). Histidine treatment led to lower levels of lipid (p -value < 0.005 for mammary and p -value < 0.0005 for abdominal) on day 12 in treated, compared to untreated, cells. The level of ALP activity on day 12 was significantly higher (p -value < 0.005 for mammary and p -value < 0.05 for abdominal), than that observed on day 0 for preadipocytes that were not treated with histidine. However, with histidine treatment, ALP activity decreased significantly (p -value < 0.005 for both) from day 0 to day 12 in both preadipocyte populations (Figure 2 b). The activity of ALP in both abdominal depot- and mammary gland-derived cells was significantly lower in histidine-treated preadipocytes (p -value < 0.005), compared to untreated preadipocytes on day 12 (Figure 2 b).

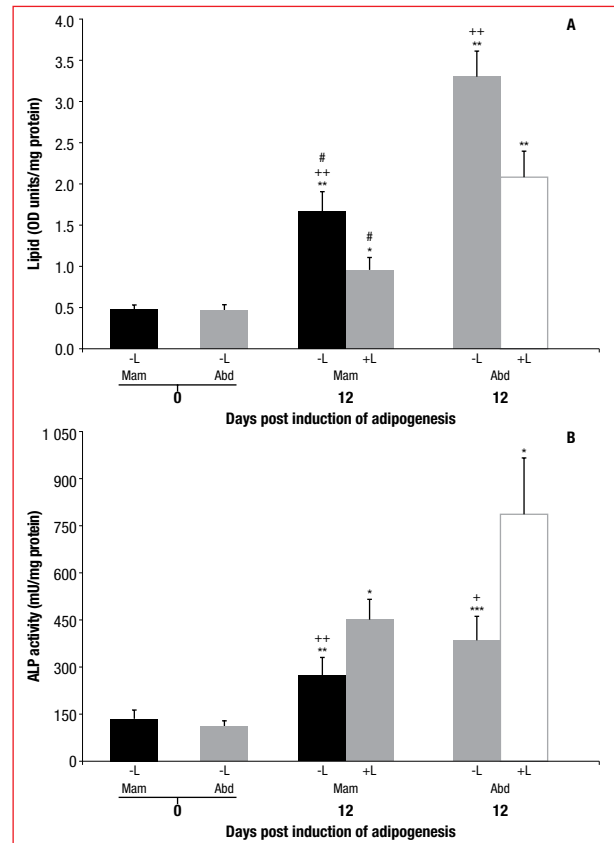


Data are shown as a mean \pm standard error of mean
^{*}p-value < 0.05, ^{**}p-value < 0.005, ^{***}p-value < 0.0005 vs. day 0; ^{**}p-value < 0.005, ^{***}p-value < 0.0005 vs. preadipocytes treated with histidine
 Abd: abdominal, ALP: alkaline phosphatase, -H: those not treated with histidine, +H: those treated with histidine, Mam: mammary gland, OD: optical density

Figure 2: The effect of histidine on (a) lipid accumulation and (b) alkaline phosphatase activity in human preadipocytes (n = 4 for mammary and n = 10 for abdominal). The darker paired bars represent preadipocytes not treated (-H), while the lighter bar represents those treated (+H) with histidine for mammary gland and abdominal preadipocytes on day 12

Figure 3 shows the effect of levamisole treatment on intracellular lipid levels and ALP activity in abdominal and mammary gland preadipocytes. Preadipocytes from both fat depots, treated or nontreated with levamisole, had higher lipid levels on day 12 than on day 0 (Figure 3 a). Treatment with levamisole caused the cells to accumulate fewer lipids by day 12 (p-value < 0.005 for preadipocytes from both depots), compared to nontreated cells (Figure 3 a). Abdominal preadipocytes, whether treated or not treated with levamisole, had higher lipid levels than preadipocytes from the mammary gland by day 12 (Figure 3 a). In the presence or absence of levamisole, and irrespective of fat depot source, ALP activity was higher in preadipocytes on day 12 than on day 0 (see Figure 3 b). On day 12, ALP activity was higher (p-value < 0.005 for mammary and p-value < 0.05 for abdominal) in levamisole-treated, than in nontreated, cells (Figure 3 b).

Treating the collected preadipocytes from the mammary gland with the tissue-specific ALP inhibitor,



Data are shown as mean \pm standard error of mean
^{*}p-value < 0.05, ^{**}p-value < 0.005, ^{***}p-value < 0.0005 vs. day 0; ^{*}p-value < 0.05, ^{**}p-value < 0.005 vs. preadipocytes treated with levamisole; [#]p-value < 0.05 vs. abdominal preadipocytes

Abd: abdominal, ALP: alkaline phosphatase, -L: not treated with levamisole, +L: treated with levamisole, Mam: mammary gland, OD: optical density

Figure 3: The effect of levamisole on (a) lipid accumulation and (b) alkaline phosphatase activity in human preadipocytes (n = 9 for mammary and n = 4 for abdominal). The darker paired bars represent preadipocytes not treated with levamisole (-L), while the lighter bar represents those treated (+L) with levamisole for mammary gland and abdominal preadipocytes on day 12

Phe-Gly-Gly, showed no effect on alkaline phosphatase activity (322 ± 29 vs. 321 ± 25 mU/mg protein for nontreated, and treated, cells, respectively) or lipid accumulation (1.96 ± 0.10 vs. 1.90 ± 0.09 OD units/mg protein for nontreated, and treated, cells, respectively) by day 12. Comparing the results on day 12 with those on day 0 (lipid level was 0.42 ± 0.01 OD units/mg protein and ALP activity was 143 ± 11 mU/mg protein), it was found that there was a significant increase in both lipid accumulation (p-value < 0.005 for both treated and nontreated cells) and ALP activity (p-value < 0.005 for both treated and nontreated cells).

Correlation analysis on combined data from both body sites demonstrated a strong positive relationship ($r = 0.66$, p-value < 0.0001, n = 27) between the percentage change in ALP activity and the percentage change in intracellular lipid levels in preadipocytes that were not treated with ALP inhibitors. When this analysis was performed separately on preadipocytes taken from the mammary gland ($r = 0.86$, p-value < 0.0001,

n = 14) and the abdominal depot (r = 48, p-value = 0.09, n = 13), a stronger correlation was noted in the former preadipocyte population. Pearson correlation showed that BMI did not relate to intracellular lipid or ALP levels in either abdominal (r = -0.37, p-value = 0.21; r = -0.46, p-value = 0.11, respectively) or mammary gland (r = -0.37, p-value = 0.21; r = -0.16, p-value = 0.59, respectively) preadipocytes after 12 days of culture. When data for these two adipose depots were combined, there was still no correlation of BMI with lipid (r = 0.30, p-value = 0.13) or ALP (r = 0.10, p-value = 0.64) levels.

Discussion

The present study is the first to show that ALP is expressed in preadipocytes isolated from human abdominal subcutaneous fat tissue, and that the level of intracellular lipid accumulation is higher in subcutaneous abdominal preadipocytes, compared to that in preadipocytes taken from the mammary gland.

It has previously been shown that ALP is expressed in both the 3T3-L1 murine preadipocyte cell line and human preadipocytes from the mammary gland, and that ALP activity increases in conjunction with increased cellular fat accumulation.^{9,10} The present study has confirmed the parallel relationship between elevated ALP enzyme activity and cellular fat accumulation in abdominal subcutaneous adipose tissue. The intracellular deposition of lipid is the defining feature of the maturation of preadipocytes into adipocytes, a process termed "adipogenesis". Therefore, it is possible that ALP may be a modulator of adipogenesis. Other studies have shown that ALP, which is a ubiquitously expressed enzyme, plays a pivotal role in tissue development,¹⁹ cellular differentiation²⁰ and osteoblast cell mineralisation.²¹

Treatment of human preadipocytes with the tissue-non-specific ALP inhibitor, histidine,² blocked both intracellular lipid accumulation and ALP activity. Paradoxically, treating human preadipocytes with levamisole, which is also a tissue-non-specific ALP inhibitor,³ decreased intracellular lipid accumulation, but stimulated alkaline phosphatase activity. When human preadipocytes were incubated with a tissue-specific ALP inhibitor, Phe-Gly-Gly,⁴ no inhibition of either fat accumulation or ALP activity was observed. The inhibition of ALP activity by histidine, but the lack of inhibition with Phe-Gly-Gly, would suggest that the enzyme isoform that is present in both fat depots is of the tissue-non-specific variety. Results from 3T3-L1 cells using ALP inhibitors and PCR demonstrate that it is the tissue-non-specific isoform that is expressed in this cell type.⁹ The ability of levamisole to increase

ALP activity in human preadipocytes has been reported previously,¹⁰ and a study of rat hepatocytes, which are known to express the tissue-non-specific ALP isoform, also showed a similar effect.²² It should be noted that levamisole inhibits ALP activity in cell extracts of human preadipocytes.¹⁰ This confirms that it is the tissue-non-specific form. Thus, levamisole only activates ALP activity in human preadipocytes with intact membranes,¹⁰ suggesting that this process is modulated by factors residing in the cell membrane system, the identities of which are currently unknown.

The ability of levamisole to block intracellular lipid accumulation, but at the same time to increase ALP activity, counters the hypothesis that functional ALP is required for cellular lipid accretion. However, it is possible that in human preadipocytes, levamisole inhibits intracellular lipid accumulation at a point downstream of ALP, and this may block any feedback inhibition of ALP by factors downstream of this point, thus leading to the rise in ALP activity that was observed in this study. The fact that levamisole is able to inhibit ALP activity in murine 3T3-L1 cells suggests either that this cell line does not express the molecule responsible for mediating the ALP stimulatory effect, or that the murine homologue is not bound by levamisole. The mechanism by which levamisole inhibits adipogenesis in human adipocytes is not known, but investigating its method of action may be invaluable for a more complete understanding of the molecular events that are implicated in the adipogenic pathway.

Despite the effects of levamisole on ALP activity and adipogenesis that were observed in the current study, there is good evidence that ALP plays an important role in intracellular lipid accumulation. Thus, a strong positive correlation between the percentage change in ALP activity and the percentage change in cellular lipid accumulation was observed in our study. It is also important to note that ALP is expressed in preadipocytes isolated from different species and tissues, including rat adipose tissue,²³ human preadipocytes,¹⁰ adipocyte precursor cells in human bone marrow,²⁴ rabbit preadipocytes,²⁵ murine bone marrow preadipocytes²⁶ and adipocytes from chicken bone marrow.²⁷ Previous studies have shown that ALP is located on lipid droplets in 3T3-L1 cells,⁹ human preadipocytes¹⁰ and HepG2 cells.²⁸ The latter is a human hepatocyte cell line, that like primary human hepatocytes, is able to accumulate lipid within a membrane-bound droplet, similar to that observed in adipocytes.²⁹ These data demonstrate that ALP is expressed in cells that are able to accumulate lipid within specialised organelles, and its location on the surface of these lipid droplets is further evidence of the input of ALP into cellular lipid storage.

Furthermore, a previous study has shown that treating 3T3-L1 and HepG2 cells with small interfering RNA designed specifically to knock out ALP mRNA translation leads to reduced intracellular lipid accumulation.³⁰ Additionally, in mice that harboured a tissue-non-specific ALP gene knockout, severe bone pathology, as well as an almost complete lack of adipose tissue, were observed.³¹ Lastly, in humans, a recent study demonstrated that polymorphisms in the tissue-non-specific ALP gene are associated with body fat distribution.³²

Intracellular lipid accumulation and ALP activity were only measured at baseline and on day 12 of the differentiation period. The latter represents a time point at which all the proteins that are characteristic of mature adipocytes are already being expressed.³³ Therefore, from the current experiments it is not possible to determine whether ALP function is required for the initiation of the adipogenic process, or is only necessary at the later stages of this pathway. The intracellular location of the ALP molecule at the surface of the lipid droplet suggests that it may assist in the formation of this organelle, a process which occurs at the terminal section of the differentiation pathway, the early portion of which (within the first hour) is characterised by a cascade of transcription factor gene expression.³⁴ Evidence that ALP acts at a later stage of adipogenesis derives from experiments performed in 3T3-L1 and HepG2 cells, whose treatment with the ALP inhibitors, histidine and levamisole, demonstrated that although intracellular lipid accumulation was reduced, the expression of peroxisome proliferator-activated receptor γ , a transcription factor that plays a key role in initiating adipogenesis, was not altered.²⁸ In order to determine the exact point at which ALP functions within the adipogenic pathway, further experiments are required in which ALP expression is monitored immediately after adipogenesis has been initiated, and ALP inhibitors are added at specific time points.

The present study is the first to demonstrate that intracellular lipid accumulation and ALP activity are higher in abdominal than mammary gland preadipocytes. This makes physiological sense as the abdominal subcutaneous adipose depot is a major storage site for lipid, whereas the mammary gland is not. Previously, depot-specific differences in adipogenesis have been observed, with preadipocytes isolated from visceral adipose tissue differentiating more slowly than those isolated from the subcutaneous abdominal depot.⁸ No studies have assessed the ALP activity within preadipocytes from the visceral fat depot. It is important to study the regulation of adipogenesis in the different body fat depots because it is known that visceral adipose tissue is more strongly associated with adverse metabolic risk factors than subcutaneous

adipose tissue,^{35,36} whilst gluteofemoral adiposity is protective.³⁷ Thus, an understanding at the molecular level of the regulation of fat storage in these adipose depots may have implications for the treatment of obesity-associated metabolic disorders.

The principal drawback of this study is that the agents that were used to inhibit ALP activity were not ALP-specific. Each of the inhibitors has effects other than those on ALP, which may mediate their ability to block intracellular lipid accumulation. However, it should be noted that these inhibitors are sufficiently specific to have been routinely used to differentiate between the tissue-specific and tissue-non-specific isoenzymes of ALP.²⁻⁵ In addition, and as noted previously, there is excellent evidence from other studies^{9,10,28,30-32} of the involvement of ALP in intracellular lipid accumulation.

A further weakness of this study was that the two groups of subjects from whom abdominal or mammary gland adipose tissue were isolated were not matched for BMI. This was countered by including BMI as a covariate within the ANCOVA model when comparing ALP activity and intracellular lipid levels between the preadipocytes that were isolated from the two body fat depots. Furthermore, no correlation was observed between BMI and preadipocyte lipid or ALP levels. Therefore, it is unlikely that the observed differences in these two variables after 12 days of tissue culture were because of the higher BMI of the subjects from whom the abdominal preadipocytes were isolated.

Some of the performed experiments using ALP inhibitors were repeated on only four different preadipocyte preparations. However, statistically significant effects were still observed, suggesting that the experiments were not compromised by the low *n* number. Furthermore, a previous study that used 3T3-L1 cells also showed that the effects of the ALP inhibitors were very strong. Significant inhibition was observed after only three repeats.⁹ However, it should be noted that in the current study, correlation analyses performed using data from the individual body sites with *n* numbers of only 13 or 14 may not have been sufficiently powered to detect truly significant correlations, so these results should be interpreted with some caution.

Conclusion

The differences in observed lipid accumulation between abdominal subcutaneous preadipocytes and preadipocytes from mammary gland adipose tissue may relate to differences in intracellular ALP activity. The ability of histidine to inhibit both ALP activity and intracellular lipid accumulation suggests that the antiobesity properties of this amino acid are mediated by both central and peripheral effects. However, the peripheral effects of histidine must be confirmed in

animal models. The observed relationship between ALP activity and cellular fat accumulation suggests that ALP plays a pivotal role in cellular fat accumulation in human preadipocytes that are isolated from adipose tissue at both body sites.

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This paper is published in tribute to the memory of Janice Paiker, whose knowledge, leadership, humour, perspicacity and caring nature, are missed by all.

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