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Enzymes involved in adenosine metabolism in rat white and brown adipocytes

Effects of streptozotocin-diabetes, hypothyroidism, age and sex differences

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1. Adipocytes were isolated from epididymal white fat and interscapular brown fat of male rats, and activities of 5′-nucleotidase, adenosine deaminase and adenosine kinase were measured in cell extracts. 2. 5′-Nucleotidase activity in white adipocytes was increased in streptozotocin-diabetes, decreased in hypothyroidism and increased with age. That activity in brown adipocytes was unchanged in diabetes, decreased in hypothyroidism and increased with age. 5′-Nucleotidase activity was higher in white adipocytes from female rats. 3. Adenosine deaminase activity in white adipocytes was increased in diabetes, decreased in hypothyroidism and increased with age. That activity in brown adipocytes was decreased in diabetes and hypothyroidism. 4. Adenosine kinase activity in both cell types was unchanged in diabetes or hypothyroidism, but increased with age.

INTRODUCTION

There is now a general belief that adenosine may act as a 'local hormone' or 'paracrine agent' in mammalian tissues, showing a diversity of effects. In white adipose tissue, low concentrations of extracellular adenosine act through A_1 adenosine receptors on adipocytes to inhibit adenylate cyclase, lower cyclic AMP concentration and decrease lipolysis (Rodbell, 1980; Londos *et al.*, 1980; Wolff *et al.*, 1981). Adenosine also acts directly to stimulate, or increases sensitivity to insulin of, several other processes in white adipocytes, such as glucose transport, lipid synthesis, pyruvate dehydrogenase activity, leucine oxidation and cyclic nucleotide phosphodiesterase activity (Green, 1983; Honeyman *et al.*, 1983; Joost & Steinfelder, 1982; Schwabe *et al.*, 1974; Smith *et al.*, 1984; Wong *et al.*, 1984, 1985). In addition to these metabolic effects, adenosine is vasodilatory in white adipose tissue (Sollevi & Fredholm, 1981). Effects of adenosine in brown adipose tissue are less fully documented, but it is known that the nucleoside inhibits adenylate cyclase, lowers cyclic AMP concentrations and opposes β-adrenergic stimulation of lipolysis and respiration (Szillat & Bukowiecki, 1983; Schimmel & McCarthy, 1984; Sundin *et al.*, 1984; Woodward & Saggerson, 1986).

Response to adenosine of the various cell types within any tissue in differing physiological states will be determined by the sensitivity of receptor–effector systems to the paracrine agent and by the actual concentration of adenosine in the environment of the receptors. This concentration will in turn be influenced by rates of production and removal by adenosine-metabolizing enzymes. Changes in sensitivity of adipocytes to adenosine is now established in starvation (Chohan *et al.*, 1984), hypothyroidism (Ohisalo & Stouffer, 1979; Malbon & Graziano, 1983; Malbon *et al.*, 1985; Saggerson, 1986; Woodward & Saggerson, 1986), adrenalectomy (Saggerson, 1980) and lactation (Vernon *et al.*, 1983; Vernon & Finley, 1986), and is also observed in diabetes (K. Chatzipanteli & E. D. Saggerson, unpublished work). Meaningful estimates of adenosine concentrations in the environment of adipocyte receptors are not readily available either in whole tissue, because of compartmentation complexities, or in adipocyte incubations, because of the difficulty of measuring the low concentrations.

It has been proposed (Arch & Newsholme, 1978*a*; Green *et al.*, 1981) that adenosine production and utilization in mammalian tissues is primarily dependent on the activity of the producing enzyme 5′-nucleotidase (EC 3.1.3.5) and the two utilizing enzymes adenosine deaminase (EC 3.5.4.4) and adenosine kinase (EC 2.7.1.20). The activity of 5′-nucleotidase in any physiological state may be particularly pertinent, since it is an ectoenzyme in many tissues (DePierre & Karnovsky, 1974; Gurd & Evans, 1974; Trams & Lauter, 1974; Pearson *et al.*, 1980), including white adipose tissue (Newby *et al.*, 1975), and may possibly generate extracellular adenosine from circulating 5′-AMP. One approach towards further understanding of paracrine control by adenosine is to obtain measurements of the maximum activities of adenosine-metabolizing enzymes associated with individual cell types within a tissue in various physiological states. This is relatively simple for white or brown adipocytes, which can be separated easily from other cells and structures within the tissues.

5′-Nucleotidase and adenosine deaminase activities have been found in both the adipocyte and the stromal cell fractions on disaggregation of white adipose tissue with collagenase (Green & Newsholme, 1981; Vernon *et al.*, 1983). Measurements of the three enzyme activities have been reported for whole white adipose tissue in several pathophysiological states (Green *et al.*, 1981; Vernon *et al.*, 1983; Newsholme *et al.*, 1985) and for brown adipose tissue in starvation (Newsholme *et al.*, 1985). In addition, male/female differences have been discussed (Green *et al.*, 1981; Vernon *et al.*, 1983). However, except for measurements made in white adipocytes during pregnancy and lactation (Vernon *et al.*, 1983), studies of adaptive changes in these three enzyme activities do not appear to have been made in the adipocyte populations derived from white and brown adipose tissues. Here we undertook a study of these three enzymes in adipocytes in hypothroidism and streptozotocin-diabetes. These conditions were chosen since adipose-tissue metabolism in general is greatly altered by changes in thyroid-hormone and insulin status, and particularly because these states are associated with alterations in responsiveness of adipocytes to adenosine (see above). In addition we note some effects of age, sex and cell size.

MATERIALS AND METHODS

Chemicals

These were obtained and treated as described by Woodward & Saggerson (1986). In addition, radiochemicals were from Amersham International, Amersham, Bucks., U.K., and *erythro*-9-(2-hydroxy-3-nonyl)adenine was a gift from Burroughs Wellcome Co., Triangle Park, NC, U.S.A.

Animals

Male Sprague–Dawley rats bred at University College London were used throughout. These were maintained on Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.). Male diabetic rats and their controls were selected at 160–180 g body wt. (aged 6 weeks). Age-matched female animals weighed 150–180 g. Diabetes was induced by a single subcutaneous injection on day 1 of streptozotocin (100 mg/kg) dissolved in 0.2 ml of 50 mM-citrate buffer (pH 4.0) containing 0.15 M-NaCl. On day 3 animals were selected as diabetic if showing a strongly positive 'Clinistix' test for urinary glucose [$>0.5\%$ (w/v) glucose]. These animals showed similar weight loss to those described by Chatzipanteli & Saggerson (1983). Insulin-treated diabetic rats received 20 units of protamine zinc bovine insulin (Weddel Pharmaceuticals, London E.C.1, U.K.)/kg subcutaneously on days 3 and 4 and were killed on day 5. These animals regained body weight (Chatzipanteli & Saggerson, 1983). For study of hypothyroidism, male rats were selected at age 5 weeks (110–120 g) and then maintained for 4 weeks on a low-iodine version of the No. 3 Breeding Diet with drinking water containing 0.01% (w/v) 6-n-propyl-2-thiouracil (Chohan *et al*., 1984; Saggerson & Carpenter, 1986). Euthyroid controls for these animals were also selected at age 5 weeks and then maintained for a further 4 weeks on the No. 3 Breeding Diet, by which time their body weights were 240–260 g. All rats were maintained at approx. 21 °C on a 13 h-light/11 h-dark cycle, with light from 06:00 h to 19:00.

Isolation of adipocytes

White adipocytes were isolated from the epididymal adipose tissues of male rats and the perigenital adipose tissues of female rats (three or four animals in each case) by disaggregation in collagenase (1 mg/ml) essentially as described by Rodbell (1964). Brown adipocytes were isolated from the interscapular depot of three or four male rats (Woodward & Saggerson, 1986) by the procedure originally described by Fain *et al*. (1967) and elaborated by Nedergaard & Lindberg (1982).

Preparation of adipocyte fractions

Preparations of white or brown adipocytes were washed twice in 10 ml of buffer (45 mM-Tris/HCl/45 mM-β-glycerophosphate, pH 7.4). The cells were then re-suspended in 5–10 ml of the same, ice-cold, buffer and the cells were broken by agitation on a vortex mixer (Martin & Denton, 1970). The homogenate was decanted from the floating fat, which was then washed by similar agitation with a further 5–10 ml of the same buffer. The two fat-free homogenates were pooled and samples taken for measurement of DNA by the method of Switzer & Summer (1971). An initial centrifugation was performed (4 °C) for 1 min at 3000 $g_{av.}$ to remove nuclei and cell debris, and the resulting supernatant was then re-centrifuged for 30 min at 30000 $g_{av.}$ to yield a 'soluble protein' supernatant and a 'particulate protein' pellet, which was resuspended in 1 ml of the Tris/β-glycerophosphate buffer. These extracts were stored at −40 °C before assay, and protein contents were measured by the method of Lowry *et al*. (1951), with bovine albumin as a standard.

Enzyme assays

All assays were performed at 37 °C. Essentially all assayable 5′-nucleotidase activity was in the 30000 g particulate fraction, whereas essentially all of the adenosine deaminase and adenosine kinase activities were in the 30000 g supernatant.

Samples of white-adipocyte (20–50 μg) or brown-adipocyte (40–80 μg) particulate protein were assayed for 5′-nucleotidase as described by Newby *et al*. (1975).

Portions (10–20 μg) of white- or brown-adipocyte soluble protein were assayed for adenosine deaminase and adenosine kinase essentially as described by Arch & Newsholme (1978*b*), with some modifications. Deaminase was assayed over a time course of 3–15 min in a 0.04 ml final volume containing 77 mM-phosphate buffer (pH 7.4), 3.3 mM-sodium citrate, 4 mM-$MgCl_2$, 0.4 mM-EDTA and 200 μM-[2-^{3}H]adenosine (50 μCi/μmol). The reaction was terminated by the addition of 7 μl of 2 M-$HClO_4$, containing adenine, adenosine, inosine and hypoxanthine (all approx. 5 mM). The reaction products, inosine and hypoxanthine, were separated by t.l.c. (Arch & Newsholme, 1978*b*) and quantified by liquid-scintillation counting. The assay procedure for the kinase was similar to that for the deaminase, except that the final concentration of [2-^{3}H]adenosine was 2 μM (8.8 μCi/nmol), and 4 mM-ATP was also present. In addition, *erythro*-9-(2-hydroxy-3-nonyl)adenine (10 μM) was also added to kinase assays to inhibit the deaminase completely, since, in preliminary experiments, rapid loss of the adenosine substrate via the deaminase caused significant interference in the kinase assay. *erythro*-9-(2-Hydroxy-3-nonyl)adenine had no effect on the assay of adenosine kinase itself. Again, the products of the assay were separated by t.l.c. (Arch & Newsholme, 1978*b*), and radioactivity in the nucleotide region of the chromatogram was quantified by scintillation counting.

Measurement of mean adipocyte dimensions

The masses of samples of white adipocytes were determined either by drying portions of cells at 70 °C until constant weight was obtained or by extracting cell lipids into chloroform (Folch *et al*., 1957) and drying the chloroform extracts to constant weight. These two

Table 1. Effects of hypothyroidism, streptozotocin-diabetes and age on enzyme activities in brown and white adipocytes

Activities are expressed as nmol/min per 100 μg of adipocyte DNA, and are means ± S.E.M. for the numbers of separate cell preparations indicated in parentheses. Statistical significance is indicated as follows: for comparison against the appropriate control, a, b, c, d indicate $P < 0.05$, < 0.02, < 0.01, < 0.001 respectively; for effect of insulin administration to diabetic animals, e, f, g indicate $P < 0.05$, < 0.01, < 0.001 respectively; for comparison of euthyroid controls (9 weeks old) with 'normal' controls (6 weeks old), h, i, j indicate $P < 0.05$, < 0.01, < 0.001 respectively.

Condition	Adipocytes . . .	5′-Nucleotidase		Adenosine deaminase		Adenosine kinase	
		White	Brown	White	Brown	White	Brown
Control		32.8 ± 2.7 (4)	7.9 ± 0.8 (5)	13.4 ± 0.7 (4)	27.3 ± 3.9 (3)	0.54 ± 0.01 (3)	0.24 ± 0.04 (3)
Diabetic		65.2 ± 8.2[b] (3)	7.7 ± 0.9 (4)	27.5 ± 1.9[d] (3)	15.1 ± 0.8[a] (4)	0.58 ± 0.04 (4)	0.24 ± 0.06 (3)
Insulin-treated diabetic		23.8 ± 1.3[a,f] (4)	8.0 ± 1.0 (4)	20.9 ± 0.8[d,f] (4)	25.9 ± 3.6[e] (4)	1.26 ± 0.04[d,g] (4)	0.63 ± 0.03[c,f] (3)
Euthyroid control		100.0 ± 13.7[i] (3)	27.5 ± 2.0[j] (3)	23.8 ± 2.1[i] (4)	25.2 ± 2.9 (4)	0.86 ± 0.10[h] (3)	0.60 ± 0.06[h] (3)
Hypothyroid		49.2 ± 7.6[a] (3)	8.9 ± 0.6[d] (3)	10.0 ± 1.0[c] (3)	8.8 ± 0.9[c] (4)	1.20 ± 0.12 (3)	0.60 ± 0.02 (3)

methods were in good agreement (see Table 2) and were taken as a reasonable estimate of cell mass, because the intracellular water space of white adipocytes is extremely small. DNA determinations were performed on portions of the same cell preparations. Assuming 7.5 pg of DNA per nucleus in a diploid cell (Johnston *et al.*, 1968) and that the adipocytes are spheres of density 0.9 g/ml, mean cell diameters and surface areas were calculated from the cell dry-weight values.

Statistical methods

Statistical significance was evaluated by Student's *t* test for unpaired samples.

RESULTS AND DISCUSSION

General

In the present study, values for adenosine kinase activity in both white and brown adipocytes were consistently found to be substantially lower than those of 5′-nucleotidase or adenosine deaminase. This is similar to the observations of Vernon *et al.* (1983) and Newsholme *et al.* (1985) in whole tissues, but differs from the studies of Green *et al.* (1981) in whole tissue and Vernon *et al.* (1983) in white adipocytes, where 5′-nucleotidase and adenosine deaminase activities were generally only 3–6 times that of the kinase. The reasons underlying these differences between studies are unclear at present.

Effect of diabetes

5′-Nucleotidase and adenosine deaminase activities relative to white-adipocyte DNA were doubled in streptozotocin-diabetes (Table 1). Green *et al.* (1981) observed a 50% increase in adenosine deaminase activity relative to whole white-adipose-tissue protein, but found no change in 5′-nucleotidase activity. By contrast, in brown adipocytes, diabetes had no effect on 5′-nucleotidase activity and decreased deaminase activity by 45%. Differences between cell types in the direction of change of 5′-nucleotidase in diabetes is also exemplified by the decrease in this activity noted in liver plasma membranes from streptozotocin-diabetic rats (Chandramouli & Carter, 1975). Adenosine kinase activity in both adipocyte types was unchanged in diabetes. Administration of two relatively large daily doses of insulin to diabetic animals partially or totally restored the changes in 5′-nucleotidase and the deaminase, and also increased the activity of the kinase in both cell types by approx. 2.5-fold over the normal value (Table 1).

The physiological role(s) of 5′-nucleotidase remains uncertain. One proposed use of the enzyme is in scavenging nucleoside from extracellular 5′-AMP in close association with nucleoside transport, as found in some cell types (Fleit *et al.*, 1975; Frick & Lowenstein, 1978; Dornand *et al.*, 1979). If this were applicable to white adipocytes, then it would be predicted that conversion of extracellular 5′-AMP through to inosine would be accelerated in white adipocytes in diabetes. Another proposed use of 5′-nucleotidase is for generation of extracellular adenosine from circulating 5′-AMP. If this were so, white adipocytes releasing more fatty acid in an insulin-deficient state would also produce more vasodilatory adenosine, and thereby facilitate fatty acid distribution by blood flow. It is noteworthy that this is accompanied by diminished metabolic responsiveness of white adipocytes to adenosine in the diabetic state (K. Chatzipanteli & E. D. Saggerson, unpublished work), thereby ensuring that the enhanced adenosine production would not simply shut off lipolysis and promote re-esterification.

It is also pertinent that insulin administration *in vivo* decreases blood flow in rat white adipose tissue, and it has been hypothesized that this is achieved by decreased production of adenosine (Madsen & Malchow-Moller, 1983). At present it is not possible to apply any physiological interpretation to the changes seen in brown adipocytes.

Effect of hypothyroidism

In this state, 5′-nucleotidase activity was decreased by approx. 50% and 70% in white and brown adipocytes respectively (Table 1). Likewise adenosine deaminase activity was decreased by 60–65% in both cell types, but the kinase activity was unchanged. The physiological

Table 2. 5′-Nucleotidase activity in white adipocytes from age-matched male and female rats

Values are given as means ± S.E.M. for the numbers of separate cell preparations indicated in parentheses. Significant differences between males and females are given by a, b, indicating $P < 0.05$, < 0.01 respectively.

Sex	Age (weeks)	Body wt. range (g)	5′-Nucleotidase activity (nmol/min per mg of particulate protein)	5′-Nucleotidase activity (nmol/min per 100 μg of DNA) (*A*)	Cell dry wt. (ng/cell)	Cell lipid (ng/cell)	Mean cell diameter (μm)	Mean cell surface area (μm²) (*B*)	10^3 × Ratio *A*/*B*
Male	6	160–180	47.6 ± 13.5 (3)	17.8 ± 4.7 (3)	7.6 ± 1.7 (3)	7.5 ± 1.7 (3)	25.0 ± 1.9 (3)	1986 ± 303 (3)	9.0
Female	6	150–180	86.9 ± 7.5 (3)	51.7 ± 2.2[b] (4)	15.8 ± 2.7[a] (5)	13.4 ± 0.4[a] (3)	31.8 ± 1.7[a] (5)	3221 ± 359[a] (5)	16.1
Male	9	240–260	54.4 ± 4.3 (3)	32.6 ± 2.7 (3)	–	–	–	–	–

consequences of such changes are uncertain at present. If 5′-nucleotidase activity were particularly important in setting the extracellular adenosine concentration (see above), then it can be predicted that this would be decreased in hypothyroidism in both tissues. However, receptor-mediated effects of adenosine on the adipocytes would be maintained, because both brown and white adipocytes have increased sensitivity to adenosine in hypothyroidism (Saggerson, 1986; Woodward & Saggerson, 1986).

Effect of age

The animals used as controls for the hypothyroid state were approx. 3 weeks older than those used as controls for the diabetic animals, and it is apparent that, except for adenosine deaminase in brown adipocytes, all three enzyme activities increase with age relative to the cell DNA content (Table 1). This effect of age was most pronounced for 5′-nucleotidase activity, which is increased by approx. 3-fold in both cell types in the older animals. These results indicate the importance of careful age-matching of animals when comparing these activities in different pathophysiological states.

Comparisons between brown and white adipocytes

In nearly every case, the values were obtained in brown- and white-cell preparations made simultaneously from the same animals. There was a tendency for adenosine deaminase activity to be higher and for the kinase activity to be lower in the brown adipocytes, although these differences were less apparent in the older rats (Table 1). However, 5′-nucleotidase activity showed considerable differences between the two cell types. At either 6 or 9 weeks of age, this activity in brown cells was only approx. 25% of that in white cells. Newsholme *et al.* (1985) noted qualitatively similar inter-tissue differences when the three enzyme activities were measured in extracts from whole adipose tissues. In general, therefore, it would be predicted that steady-state concentrations of adenosine in incubations of brown adipocytes would be lower than those in incubations of comparable numbers of white adipocytes. In support of this, it is observed that addition of exogenous adenosine deaminase to incubations of brown adipocytes has only a slight effect on the responsiveness of the cells to noradrenaline (Woodward & Saggerson, 1986), whereas this addition has a very large effect on the noradrenaline-sensitivity of white adipocyte incubations (Fernandez & Saggerson, 1978).

Sex differences in 5′-nucleotidase activity

Green *et al.* (1981) reported that 5′-nucleotidase activity relative to tissue protein was approx. 6-fold higher in the perigenital white fat of female rats than in the epididymal depots of male rats. It is not clear whether the two sexes were age-matched in that study. Vernon *et al.* (1983), however, reported that 5′-nucleotidase activity per quantity of male adipocytes was not different from the value for cells from pregnant or lactating female rats and that, relative to DNA content, this activity did not differ in whole tissues taken from males or from lactating or pregnant females. Since Green *et al.* (1981) reported that 5′-nucleotidase activity was the same in whole white adipose tissue from pregnant and non-pregnant females, Vernon *et al.* (1983) concluded that the activity of 5′-nucleotidase (and of the deaminase and the kinase) does not show any sex difference. This interpretation is open to some doubt. First, the male and female rats used by Vernon *et al.* (1983) were not age-matched. Second, Vernon *et al.* (1983) made no direct comparisons in adipocytes, on the one hand, between virgin and pregnant or lactating females or, on the other hand, between males and females. Third, activity changes (or lack of changes) seen in whole tissues may not reflect those seen in the adipocyte population, because of the contribution of non-adipocyte cells. Table 2 shows that, relative to cell DNA, 5′-nucleotidase activity in female white adipocytes was 3 times that in cells from age-matched males. This difference was less apparent when the activity was expressed relative to protein in the 30000 ***g*** pellet. The white adipocytes in this study were substantially smaller than those examined by Vernon *et al.* (1983), but a clear sex difference in cell size was observed, with male and female cells having mean volumes of 8.5 ± 1.9 pl and 17.5 ± 3.0 pl respectively. Since female cells are larger and 5′-nucleotidase is a plasma-membrane enzyme, it was considered possible that the sex difference in activity simply reflects the larger plasma-membrane area of the female cells. However, Table 2 shows that 5′-nucleotidase activity in female cells was still higher, even when expressed relative to cell surface area. The 5′-nucleotidase activities presented in Tables 1 and 2 were obtained at different times of the year, and the lower activity for 6-week-old male rats in Table 2 is taken to reflect a seasonal change. A seasonal change in 5′-nucleotidase activity in preparations from 9-week-old male animals was also apparent, but the effect of age noted above was still apparent.

Concluding remarks

This study demonstrates for the first time changes in these three enzyme activities within the adipocyte populations of brown and white adipose tissues. Differences in absolute activity and in the nature of adaptive changes are apparent between these two cell types. Activities are affected by thyroid-hormone and insulin status, by age and, in the case of 5′-nucleotidase, by sex. Further work should attempt to elucidate the extent to which changes reflect acute control or alterations in enzyme synthesis and degradation. Such studies could be particularly interesting in the case of 5′-nucleotidase, since as a cell-surface glycoprotein it undergoes various stages of post-translational processing (Wada *et al.*, 1986; van den Bosch *et al.*, 1986), appears to circulate between the cell surface and an intracellular pool (Stanley *et al.*, 1980; Wilcox *et al.*, 1982; Widnell *et al.*, 1982), possibly interacts with elements of the cytoskeleton (Mannherz & Rohr, 1978; Carraway *et al.*, 1979), and is attached to the plasma membrane as a short-stalked integral membrane protein (Baron *et al.*, 1986), possibly through an unusual hydrophobic anchor involving an inositol lipid (Low *et al.*, 1986).

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