Enzymes involved in adenosine metabolism in rat white and brown adipocytes

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

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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, lipoprotein 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 \(\beta\)-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(Vernon et al., 1983; Vernon & Finley, 1986), and is also observed in diabetes (K. Chatzipanteli & E. D. Saggerson, unpublished work).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, 1978a; 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
enzymes in adipocytes. These conditions are 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 Chazipanteli & 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 (Chazipanteli & 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 perirenal 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, to remove nuclei and cell debris, and the resulting supernatant was then re-centrifuged for 30 min at 30000 g, 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 −80 °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 3000 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 (1978b), 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-MgCl₂, 0.4 mM-EDTA and 200 μM-[2-3H]adenosine (50 μCi/μmol). The reaction was terminated by the addition of 7 μl of 2 m-HClO₄, containing adenine, adenosine, inosine and hypoxanthine (all approx. 5 mM). The reaction products, inosine and hypoxanthine, were separated by t.l.c. (Arch & Newsholme, 1978b) 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 [2-3H]adenosine was 2 μM (8.8 μCi/μmol), 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, 1978b), 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 euglycemic controls (9 weeks old) with 'normal' controls (6 weeks old), h, i, j indicate *P < 0.05, < 0.01, < 0.001 respectively.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lipocytes</th>
<th>5'-Nucleotidase</th>
<th>Adenosine deaminase</th>
<th>Adenosine kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.8±2.7</td>
<td>7.9±0.8</td>
<td>13.4±0.7</td>
<td>27.3±3.9</td>
</tr>
<tr>
<td>Diabetic</td>
<td>65.2±8.2b</td>
<td>7.7±0.9</td>
<td>27.5±1.9d</td>
<td>15.1±0.8a</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>23.8±1.3b,f</td>
<td>8.0±1.0</td>
<td>20.9±0.8d,e</td>
<td>25.9±3.6c</td>
</tr>
<tr>
<td>diabetic</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Euthyroid control</td>
<td>100.0±13.7l</td>
<td>27.5±2.0j</td>
<td>23.8±2.1l</td>
<td>25.2±2.9</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>49.2±7.6a</td>
<td>8.9±0.6d</td>
<td>10.0±1.0c</td>
<td>8.8±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Enzymes of adenosine metabolism in adipocytes

METHODS

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 (Chan-dramouli & 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
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

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

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>5'-Nucleotidase activity (nmol/min per mg of particulate protein)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>47.6±13.5</td>
<td>60.9±7.5</td>
<td>86.9±7.5</td>
</tr>
<tr>
<td>9</td>
<td>240-260</td>
<td>54.4±4.3</td>
<td>54.4±4.3</td>
</tr>
</tbody>
</table>

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.
Enzymes of adipocytes in 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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REFERENCES


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