Changes in Serum Alkaline phosphatase Isoenzymes associated with Diabetes Mellitus, Fasting and Aging in the Rat.

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Abstract

We have determined the effect of diabetes mellitus and starvation/re-feeding on serum alkaline phosphatase (ALP) isoenzymes in the rat. Both ketotic (DKA) and non-ketotic (NKDM) diabetic rats showed elevated total ALP activity, primarily due to an increase in intestinal and bone/liver ALP isoenzyme in the NKDM and DKA groups, respectively. There was a decrease in intestinal ALP activity in 3 day starved rats which was reversed after re-feeding for 5 days. Aging was associated with a fall in the bone/liver isoenzyme. This latter change was mainly attributed to a fall in the “bone fraction” of the bone/liver ALP isoenzyme. Since in humans bone/liver and intestinal ALP activity is altered by diabetes mellitus and feeding may increase intestinal ALP, the rat models may be useful in the study of the variables that influence ALP.

Keywords: Diabetes Mellitus; Starvation; Alkaline phosphatase isoenzymes; rats.

Introduction

Alkaline phosphatase (ALP) activity is increased in the plasma of diabetic rats. In contrast to diabetes mellitus (DM), starvation is associated with a decrease in ALP activity which is reversed by re-feeding.

In order to obtain more information on the mechanisms underlying these changes we examined ALP isoenzyme changes in DM and starvation using the rat. Since elevated ALP activity is also seen in the plasma from diabetic humans, these studies may have relevance to human diabetes and provide a model for its study.

Materials and Methods

Male Sprague Dawley rats were selected for this study. All the animals that received food were fed ad libitum (rat and mouse No. 1 Modified Maintenance Diet, SDS, Witham, Essex, UK). All animals were allowed free access to water. All animals were housed individually in wire-bottomed cages to minimize coprophagia. We have previously demonstrated a circadian rhythm in plasma ALP activity in anaesthetized control rats, between 0800 and 1800 h. Therefore, in the present study, all samples were collected at 1100 h for ALP measurements.

Design of studies

Study 1
Rats (8 weeks old; n=8) were rendered diabetic (non-ketonuric: NKDM) by a single intravenous injection (tail vein) of streptozotocin (STZ: Sigma Chemical Company, Poole, Dorset, UK), 65 mg per kg body weight. The STZ was dissolved in 0.5 ml citrate buffer, pH 4.5. Control rats received an injection of vehicle only. Animals in this group were maintained for 62 days before sampling. The rats selected to receive STZ were approximately 12 g heavier than those selected as controls because DM is associated with decrease in body weight. This slight difference in starting weight was not, however, associated with differences in total ALP activity. Urine sampled 24 h after the induction of diabetes was tested with Multistix (Ames Division, Miles Laboratories, Stoke Poges, Bucks, UK) and revealed glycosuria ( > 6.0 mmol/l).

Study 2

Rats (8 weeks old; n= 14) were rendered diabetic (diabetic ketoacidosis: DKA) with a higher dose of STZ (120 mg per kg body weight). The sampling time for these animals was 48 h after STZ, since we have previously shown that many DKA rats do not survive beyond this time.10 Six ketogenic rats were treated by subcutaneous administration of insulin (2-4 units human Actrapid and 1-4 units human Monotard MC; Novo Nordisk, Crawley, W.Sussex, UK) at 09.00 h and 18.00h, daily. The first insulin injection was given at 18.00 h on day 1, 7 h after inducing diabetes with STZ. The final injection was given at 09.00 h on day 3, 2 h before sampling. The dose of insulin administered was adjusted so as to reduce the glycosuria and ketonuria to trace amounts. Urine sampled 24 h after the induction of diabetes were tested with Multistix and revealed glycosuria (> 6.0 mmol/l) with ketonuria (> 15.0 mmol/l).

Study 3

Normal healthy rats were maintained from birth for 7 weeks (n=5), 16 weeks (n=8) and 42 weeks (n=12) before sampling.

Study 4

Normal healthy rats (9 weeks old; n=10) were fasted for 3 days before sampling. Five of these rats were re-fed for 5 days before sampling.

Collection of samples

Rats were anaesthetized with pentobarbitone (90 mg per kg body weight) intraperitoneally (Sagatal; May and Baker Ltd., Dagenham, Essex, UK), as previously described.11 Blood was collected by cardiac puncture (performed only once for each animal) and placed in fluoride-oxalate bottles for glucose estimation. Blood was also collected and serum samples stored at 40°C for the determination of serum total ALP activity and the analysis of ALP isoenzymes (intestinal and bone/liver) within 48 h.

Determination of plasma glucose concentration.

Plasma glucose concentration was determined using an enzymatic method (glucose oxidase) on a Beckman analyzer (Beckman Ltd., High Wycombe, Bucks, UK).
Determinination of Serum ALP activity

Total ALP activity was measured at 37°C, using a Boehringer test kit (Buffer catalogue No. 782874, Catalogue No. 782858; Boehringer Mannheim Ltd., Lewes, East Sussex, UK) on an Encore analyzer (Baker Instruments Corporation, Allentown, Pennsylvania, USA) and using the recommended method of the International Federation of Clinical Chemistry. Serum intestinal ALP activity was determined using the Encore analyzer from the percent activity remaining after p-bromotetramisole (pBT) inhibition of bone and liver ALP activity using a procedure previously applied to the examination of human sera and plasma. Using butanol extracts of rat tissues pBT (0.1 mmol/l) was found to produce 95% inhibition of rat bone and liver (B/L) ALP compared to 6% inhibition of rat intestinal ALP activity. The equation used to calculate intestinal ALP activity takes into consideration the incomplete blockade of the B/L isoform and the slight inhibition of intestinal ALP.

\[
\text{Intestinal ALP} = \frac{\text{Total ALP} + \text{pBT} - 0.05 \left(\text{Total ALP} - \text{pBT}\right)}{0.89}
\]

Electrophoretic analysis

Serum samples from all animals were electrophoresed on cellulose acetate membrane (Sephaphore III, Gelman Instruments Co., Ann Arbor, Mich, USA) using Tris-barbitone buffer (Gelman high resolution buffer pH 8.8, ionic strength 0.05) at 250 v for 60 min at 4°C. The volume of sample applied to the membrane was 1µl for samples of total ALP activities < 200 U/l. For samples with higher activities the volume of sample was correspondingly reduced. Alkaline phosphatase activity was demonstrated by incubating the membrane for 60 min at 37°C with a chromogenic enzyme substrate, p-toluidinium- 5 - bromo-4 - chloro-3- indoxylphosphate (Sigma Chemical Co., Poole, UK), final concentration 1.25 mmol/l, incorporated together with 2- amino-2- methyl propanediol buffer, 1 mol/l, pH 10.2 magnesium sulphate, 1 mmol/l and polyethylene glycol 10g/l in a 10g/l agar gel. The membrane was then fixed with 5% v/v acetic acid, washed with distilled water and dried overnight. The membrane was stained with an indigogenic substrate and the isoenzymes qualified by reflectance densitometric scanning at 600 nm using a Corning 720 Densitometer (Corning Medical, Medfield, Mass, USA) Fig (1&2). The detection limit for ALP activity using this method is <5 u/l.

Statistical analysis

Results are presented as median and (range) and were compared using two-tailed Mann Whitney (for unpaired values) and Wilcoxon (for paired values) tests.

Results

Alkaline phosphatase activity in NKDM rats (Study 1): table 1.

The NKDM rats started at a significantly higher weight than their corresponding control group. After 62 days, the weight of the control rats had doubled, whereas there was no significant change in the weight of the NKDM rats. At 62 days, the median weight of the NKDM rats was less than half that of the controls.
The significant increase in total ALP activity in NKDM was similar to previous studies.\textsuperscript{1,2} Most (approximately 90\%) of the increase in total ALP activity was

**Table 1:** Alkaline phosphatase activity (U/I) and weight (g) of 62-day non-ketonuric diabetic (NKDM) and control rats.

<table>
<thead>
<tr>
<th></th>
<th>Starting weight</th>
<th>Final Weight</th>
<th>Total ALP</th>
<th>IALP</th>
<th>%I</th>
<th>B/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON Rats</td>
<td>241</td>
<td>490+</td>
<td>185</td>
<td>80</td>
<td>46</td>
<td>106</td>
</tr>
<tr>
<td>(230-249)</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>NKDM 62 days</td>
<td>253*</td>
<td>237*</td>
<td>614*</td>
<td>464*</td>
<td>78*</td>
<td>143*</td>
</tr>
<tr>
<td>(249-256)</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
</tbody>
</table>

Statistical Analysis: Control v NKDM using the Mann-Whitney test.
* p = <0.005; ** p = <0.001.
Starting weight v Final weight using Wilcoxon test.
+ p = <0.01.
Abbreviations:
ALP = Alkaline phosphatase activity
IALP = Intestinal alkaline phosphatase
%I = Intestinal ALP expressed as a percentage of total ALP activity
B/L = Bone and Liver ALP activity
n = Number of animals sampled

**Fig.1:** Representative examples of the electrophoretic pattern of ALP isoenzymes from control, long-term diabetic (NKDM) and ketotic (DKA) rats.
No. 1&2 control rats, No. 3&4 NKDM rats, No. 5&6 DKA rats.
attributable to the intestinal isoenzyme.

Electrophoretic analysis showed the principal ALP isoenzyme to migrate at a position identical with that of ALP from rat intestinal extract. Electrophoresis also revealed a more anodically migrating ALP with mobility similar to that of ALP extracted from rat liver. These results indicate that the liver fraction of B/L ALP is increased in addition to the intestinal fraction in NKDM (Fig 1, N. 3&4).

The median plasma glucose concentration was 32.5 mmol/l (range: 27.5 –36.5) in the NKDM group which was significantly higher (p < 0.001) from that of the control animals (12.4; 9.1- 13.5 mmol/l).

**Alkaline phosphatase activity in DKA rats (Study 2): table 2.**

The starting weight of both the untreated and insulin-treated DKA groups were very similar. However, after 2 days the weight of the insulin-treated group was significantly greater than that of the untreated DKA group. Both groups showed a decrease in weight; this decrease was greater for the untreated group.

The DKA group showed a significantly elevated total ALP activity which was normalized by insulin treatment. The isoenzyme findings indicate that most of the increase in total ALP activity in DKA rats is attributed to the B/L ALP isoenzyme.

The expected total ALP activity in normal rats of similar weight is 428 (372-482)U/l with 66% of the activity attributed to the B/L isoenzyme. Thus, the ALP values in the insulin-treated animals resemble those of the normal controls. Electrophoretic analysis confirmed that the principal fraction in DKA is B/L ALP. The electrophoretic mobility of this fraction correspond to that of ALP extracted from rat bone (Fig1, No. 5&6).

**Table 2:** Alkaline phosphatase activity (U/l) and weight(g) of untreated and insulin-treated diabetic ketoacidotic (DKA)

<table>
<thead>
<tr>
<th></th>
<th>Starting weight</th>
<th>Final Weight</th>
<th>Total ALP</th>
<th>IALP</th>
<th>%I</th>
<th>B/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKA Rats</td>
<td>257 (240-261)</td>
<td>216++ n=8</td>
<td>667  n=8</td>
<td>91   n=8</td>
<td>18</td>
<td>544</td>
</tr>
<tr>
<td>2 days</td>
<td>(194-236)</td>
<td>(444-1016)</td>
<td>(62-237)</td>
<td>(10-31)</td>
<td>(382-804)</td>
<td></td>
</tr>
<tr>
<td>DKA+ Insulin</td>
<td>253 (240-263)</td>
<td>236+* n=6</td>
<td>351** n=6</td>
<td>102  n=6</td>
<td>31</td>
<td>247**</td>
</tr>
<tr>
<td>2 days</td>
<td>(218-244)</td>
<td>(164-416)</td>
<td>(67-136)</td>
<td>(21-60)</td>
<td>(104-329)</td>
<td></td>
</tr>
</tbody>
</table>

* P = < 0.04; **p =< 0.001
Starting weight v Final weight using Wilcoxon test.
+ p = < 0.03; ++ p = < 0.01
Table 3: Alkaline phosphatase activity (U/l) and weight (g) in 7 weeks, 16 weeks and 42 week-old non-diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
<th>Total ALP</th>
<th>IALP</th>
<th>% I</th>
<th>B/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 week old rats</td>
<td>203</td>
<td>371</td>
<td>92</td>
<td>33</td>
<td>279</td>
</tr>
<tr>
<td>(7W)</td>
<td>(192-211)</td>
<td>(268-576)</td>
<td>(89-283)</td>
<td>(25-49)</td>
<td>(177-293)</td>
</tr>
<tr>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>16 week old rats</td>
<td>490</td>
<td>185</td>
<td>80</td>
<td>46</td>
<td>106</td>
</tr>
<tr>
<td>(16 W)</td>
<td>(434-509)</td>
<td>(144-239)</td>
<td>(46-129)</td>
<td>(29-57)</td>
<td>(80-130)</td>
</tr>
<tr>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>42 week old rats</td>
<td>671</td>
<td>118</td>
<td>66</td>
<td>59</td>
<td>50</td>
</tr>
<tr>
<td>(42W)</td>
<td>(607-751)</td>
<td>(90-142)</td>
<td>(42-109)</td>
<td>(44-77)</td>
<td>(27-64)</td>
</tr>
<tr>
<td>n=12</td>
<td>n=12</td>
<td>n=12</td>
<td>n=12</td>
<td>n=12</td>
<td>n=12</td>
</tr>
</tbody>
</table>

Weight: 7W v 16W = p<0.002; 7W v 42 W = p<0.001; 16W v 42W = p<0.001
Total ALP: 7W v 16W = p<0.002; 7W v 42W = p<0.001; 16W v 42W = p<0.001
IALP: 7W v 16W = NS; 7W v 42W = p<0.01; 16W v 42W = NS
%I: 7W v 16W = NS; 7W v 42W = p<0.002; 16W v 42W = p<0.01
B/L: 7W v 16W = p<0.002; 7W v 42W = p<0.001; 16W v 42W = p<0.001

Fig. 2 Representative examples of the electrophoretic pattern of ALP isoenzymes from 7 week, 16 week, 42 week old control rats and 3 day fasted rats.
No. 1&2 7 week old rats, No. 3&4 16 week old rats, No. 5&6 42 week old rats, No.7 7 week old rat, No. 8 3 day fasted rat.

The median plasma glucose concentration was 21.8 mmol/l (range: 17.4-25.9) in the DKA group. Corresponding values for the insulin-treated animals were: 4.1 mmol/l (2.7-10.1). These values were significantly different (p<0.001). Corresponding glucose values in healthy rats are listed in the next section, below.

Alkaline phosphatase activity in rats of different ages (Study 3): table 3.

ALP activity was assessed in 7,16 and 42 week old rats. There was a progressive and significant fall in total ALP activity with increasing age. This fall was mainly attributed (90 to 93% of the total change) to a decrease in the B/L ALP isoenzyme. The changes in intestinal isoenzyme were very small (6 to 10% of the total decrease).
Analysis of the electrophoretic patterns (Fig. 2, No. 5&6) showed that in the 42 week old rats, the loss of B/L fraction (by inhibition) is associated with the diminution of the bone fraction with retention of activity in the liver position.

The median plasma glucose concentration were 9.6 (8.3- 9.8) mmol/l for the 7 week animals, 12.4 (9.1-13.5) mmol/l for the 16 week animals and 9.3 (8.1-10.8) mmol.l for the 42 week animals. These values did not differ significantly.

**Alkaline phosphatase activity in starved/re-fed rats (Study 4): table 4.**

Starvation, resulted in a significant fall in body weight and refeeding resulted in a significant increase in body weight of rats. Total ALP activity was markedly and significantly greater in the re-fed rats. This increase was exclusively attributed to a change in the intestinal isoenzyme.

The activity of this latter isoenzyme was actually 5 fold greater in refed rats compared with starved animals. The B/L isoenzyme values were similar in the starved and refed rats.

The median plasma glucose concentration was 4.7 mmol/l (range : 3.9-6.9) in the 3-day starved group. Corresponding values for the refed animals were: 7.3 (6.6-9.3). These values were significantly different (p < 0.005).

Table 4: Alkaline phosphatase activity (U/l) and weight (g) in 3-day starved and 5-day re-fed non-diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>Starting weight</th>
<th>3 day starved weight</th>
<th>5 day Re-fed weight</th>
<th>Total ALP</th>
<th>IALP</th>
<th>%I</th>
<th>B/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-day starved rats</td>
<td>267+ (266-274)</td>
<td>214</td>
<td>237</td>
<td>34</td>
<td>15</td>
<td></td>
<td>175</td>
</tr>
<tr>
<td>n=5</td>
<td></td>
<td>n=5</td>
<td>(193-256)</td>
<td>(23-64)</td>
<td>(12-27)</td>
<td>n=5</td>
<td>(159-219)</td>
</tr>
<tr>
<td>3 day starved +5 day refed rats</td>
<td>262**# (261-265)</td>
<td>215**@ (210-220)</td>
<td>286**$ (282-297)</td>
<td>314*</td>
<td>174*</td>
<td>48**</td>
<td>159</td>
</tr>
<tr>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>(252-378)</td>
<td>(93-205)</td>
<td>(37-66)</td>
<td>n=5</td>
<td>(106-204)</td>
</tr>
</tbody>
</table>

Statistical Analysis: 3- Day starved v 3- Day starved + 5 day re-fed using the Mann-Whitney test.
*p = <0.02; ** P = <0.008.
Starting Weight v 3-Day starved weight using Wilcoxon test.
+P = <0.04; #p = <0.04.
Starting Weight v 3-Day starved following 5-Day re-feeding weight using Wilcoxon test. $p= <0.04.

Discussion

The present findings confirm the previous reports of altered total ALP activity in the DM, starved and starved-re-fed rat model. The main source of ALP activity in the NKDM model, is the intestinal isoenzyme as previously described. This was confirmed by electrophoresis which showed the principal ALP isoenzyme was intestinal with a smaller liver fraction. These results indicate that the liver fraction of B/L ALP is increased in addition to the intestinal fraction in NKDM (Fig. 1, No. 3&4). In the DKA model, the corresponding principal fraction is B/L. ALP. The electrophoretic mobility of this fraction corresponded to that of ALP extracted from
rat bone (Fig.1, No. 5&6). Moreover, the rise in B/L ALP in DKA is reversed by insulin administration. This latter observation is important because it indicates that the ALP elevation cannot be attributed to the known hepatotoxicity of STZ.\textsuperscript{18}

The pathogenesis of the elevation in B/L ALP in DKA is likely to be related to the acidosis and other severe metabolic changes which occur in this situation.\textsuperscript{19,20} In contrast, the elevation in intestinal ALP in NKDM may relate to the hyperphagia and gut hypertrophy which is known to occur in those animals.\textsuperscript{21,22,23} NKDM rats eat throughout the day, unlike healthy age-matched controls, which are nocturnal feeders. Healthy rats show a circadian pattern for total ALP activity, highest in the morning, soon after cessation of feeding and lowest levels in the late afternoon shortly before feeding resumes.\textsuperscript{6}

The present study confirms our previous work\textsuperscript{6} indicating that starvation diminishes and re-feeding augments total ALP. Here, we show that the isoenzyme influenced by these changes in the dietary regime is of intestinal origin. Interestingly, Street & Highman (1978),\textsuperscript{16} found an 18 h fast was associated with a 39\% fall in total ALP activity; we found a similar fall in ALP activity following 3 day starvation. These findings indicate that the starvation-induced fall in ALP activity is no time dependent (between 18 h and 3 days).

The increase in serum ALP activity in 3 day starved rats that have been re-fed points to a relationship between food intake and increased intestinal ALP activity.\textsuperscript{24} The fat content of the meal may also be relevant, since studies in the rat and man have shown that the magnitude of the rise in intestinal ALP is dependent on the fatty acid chain length.\textsuperscript{25,26} The present findings are similar to those observed in man since the activity of intestinal ALP in diabetic patients has been shown to fall following fasting.\textsuperscript{27}

The present study shows that aging in male rats is associated with a fall in total ALP. This is mainly attributable to a decrease in the B/L ALP. In both the rat and man, examination of tissue extracts indicate that liver ALP migrates slightly faster than the bone ALP, with some overlap. The reason for the fall in the bone isoenzyme with age is unclear, but may relate to a reduction in bone growth.

In man, elevated plasma total ALP has been reported in DKA\textsuperscript{9} and NKDM.\textsuperscript{9,28} Detailed isoenzyme analysis in NKDM has been reported and has revealed an increase in intestinal, bone and liver isoenzymes.\textsuperscript{2,9,22,27,28}

The increase in plasma glucose concentration in both the NKDM and DKA groups is well established both in human and animal studies. The starvation-induced fall in plasma glucose concentration and the reversal following re-feeding were as previously described.\textsuperscript{11}

There are similarities between the ALP changes in the DM rat and in human diabetes. It is likely that poor diabetic control may relate to increases in B/L ALP, whereas hyperphagia may mainly increase intestinal ALP. The diabetic rat may therefore be a useful model for the study of these influences.
References