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Enzymological characterization of a putative canine analogue of primary hyperoxaluria type 1

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This paper concerns an enzymological investigation into a putative canine analogue of the human autosomal recessive disease primary hyperoxaluria type 1 (alanine:glyoxylate/serine:pyruvate aminotransferase deficiency). The liver and kidney activities of alanine:glyoxylate aminotransferase and serine:pyruvate aminotransferase in two Tibetan Spaniel pups with familial oxalate nephropathy were markedly reduced when compared with a variety of controls. There were no obvious deficiencies in a number of other enzymes including D-glycerate dehydrogenase/glyoxylate reductase which have been shown previously to be deficient in primary hyperoxaluria type 2. Immunoblotting of liver and kidney homogenates from oxalotic dogs also demonstrated a severe deficiency of immunoreactive alanine:glyoxylate aminotransferase. The developmental expression of alanine:glyoxylate/serine:pyruvate aminotransferase was studied in the livers and kidneys of control dogs. In the liver, enzyme activity and immunoreactive protein were virtually undetectable at 1 day old, but then increased to reach a plateau between 4 and 12 weeks. During this period the activity was similar to that found in normal human liver. The enzyme activities and the levels of immunoreactive protein in the kidneys were more erratic, but they appeared to increase up to 8 weeks and then decrease, so that by 36 weeks the levels were similar to those found at 1 day. The data presented in this paper suggest that these oxalotic dogs have a genetic condition that is analogous, at least enzymologically, to the human disease primary hyperoxaluria type 1.

Introduction

Primary hyperoxaluria can be divided into two genetically and enzymologically distinct diseases, namely type I (PH1, McKusick 25990) and type 2 (PH2, McKusick 26000). Both are rare autosomal recessive conditions characterized by hyperoxaluria, and also concomitant hyperglycolic aciduria (in PH1 only) or hyper L-glyceric aciduria (in PH2 only). The excessive synthesis of oxalate in PH1 and PH2 and the low solubility of calcium oxalate lead to deposition in the kidney as calculi or nephrocalcinosis [1]. PH1 is caused by a deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase/ serine:pyruvate aminotransferase (AGT, EC 2.6.1.44; SPT, EC 2.6.1.51) [2,3]. PH2 is caused by a deficiency of the non-tissue-specific cytosolic enzyme D-glycerate dehydrogenase/ glyoxylate reductase (DGDH, EC 1.1.1.29; GR, EC 1.1.1.26/79) [4,5].

A feline analogue of PH2 has been described [6] and characterized enzymologically [7], but so far there have been no reports of an animal equivalent of PH1. Jansen and Arnesen [8] have recently identified a familial oxalate nephropathy in a Tibetan Spaniel population. The present paper concerns the enzymological characterization of the disease in two of these dogs, the results of which suggest this to be analogous to PH1.

Materials and Methods

Animals. The parents of the oxalotic dogs were Tibetan Spaniels from a relatively inbred Norwegian population. However, there was no close familial relationship, although there were common ancestors in the 4th and 5th generations [8]. The first litter contained three pups, two of which (one male and one female) suffered from oxalate nephropathy [8]. Remating produced a litter of four pups, one of which (female) had oxalate nephropathy (unpublished). Oxalosis due to secondary

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causes was, as far as possible, excluded. The oxalotic dogs presented in this study, which were obtained one from each litter, were both female aged 6 and 9 weeks.

The clinical condition and pathology of the oxalate nephropathy in the two animals from the first litter have been described previously [8]. Briefly, these consisted of a variety of non-specific symptoms, such as vomiting, slight diarrhea, poor appetite, retardation of growth and anaemia. Signs of polyuria and polydipsia were also observed. Histological examination of the kidneys revealed marked interstitial fibrosis. The proximal tubules were dilated and contained degenerate epithelial cells, dystrophic calcified cellular debris and numerous crystals characteristic of calcium oxalate [8].

The control dogs, all Tibetan Spaniels unless otherwise stated, were as follows: two animals (one male and one female) aged 1 day suffering from cleft palate, one (a Bichon Frise male) aged 10 days with *Escherichia coli* sepsis, a male aged 4 weeks with unspecified enteric disease, an 8-week-old male with obstructive uropathy due to struvite stones, a 12-week-old male with hydrocephalus internus, another 12-week-old male with trauma and a 36-week-old female with chronic tubulointerstitial nephritis. The effects of any of these conditions on hepatic or renal AGT/SPT in the dog are unknown, but none of the controls suffered from primary liver disease or, with the exception of the 36 week control, primary kidney disease. The two 1-day-old controls were second generation offspring from one of the parents of the oxalotic dogs mated with an unrelated dog. Therefore, assuming an autosomal recessive mode of inheritance, these animals would each have a 1/4 chance of being heterozygotes. The other controls were unrelated to the oxalotic dogs. With the exception of the 10 day and 4 week controls which died of their disease, the animals were killed by intraperitoneal injection of phenobarbital. In all cases the tissues were removed within 2 h of death and stored at −70 or −20 °C until required.

Livers / kidneys. After thawing, the tissues were treated differently depending on the enzyme assayed. For D-glycerate dehydrogenase/glyoxylate reductase, the tissues were homogenized in a ground glass mortar and pestle by hand in ice-cold 154 mmol/l KCl, containing 10 mmol/l mercaptoethanol and 100 mmol/l $MnCl_2$. The homogenate was dialysed overnight at 4 °C against 50 mmol/l sodium acetate buffer (pH 6.0) containing 10 mmol/l mercaptoethanol [5,7]. For the other enzyme assays and the immunoblotting experiments, tissue samples were sonicated in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 100 umol/l pyridoxal phosphate [3]. In both cases any insoluble or fibrous material was spun off and the supernatant used for the assays.

Enzyme assays. Alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44), serine:pyruvate aminotransferase (SPT, EC 2.6.1.51), glutamate:glyoxylate aminotransferase (GGT, EC 2.6.1.4), alanine:2-oxoglutarate aminotransferase (AlOT, EC 2.6.1.2), aspartate:2-oxoglutarate aminotransferase (AsOT, EC 2.6.1.1) D-amino acid oxidase (DAO, EC 1.4.3.3) and lactate dehydrogenase (LDH, EC 1.1.1.27) were assayed as described previously [3]. D-Glycerate dehydrogenase (DGDH/OHP, EC 1.1.1.29) was assayed in both directions (i.e., using either hydroxypyruvate/NADPH (OHP) or D-glycerate/NADP (DGDH) as substrates) while glyoxylate reductase (GR, EC 1.1.1.26/79) was assayed using glyoxylate/NADPH as described by Danpure et al. [7].

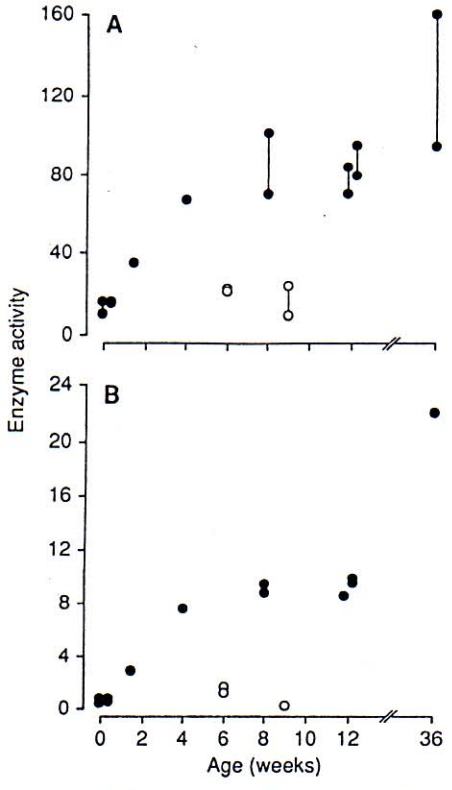

Fig. 1. Activities of AGT (A) and SPT (B) in dog livers at various ages. Solid circles = unaffected dogs; open circles = oxalotic dogs. All dogs were Tibetan Spaniels except the 10-day-old control which was a Bichon Frise. Units of enzyme activity = nmol/min per mg protein. Where possible duplicate assays were performed on *different* parts of the liver. These values are joined by vertical lines.

Immunoblotting. Sodium dodecyl sulphate-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously [9,10] using affinity-absorbed rabbit anti-human liver AGT IgG, and then development with goat anti-rabbit IgG conjugated to horseradish peroxidase.

Results

Unlike the other enzymes studied, there was a considerable variation in the levels of activity of AGT and SPT in the livers and kidneys of the control dogs depending on their postnatal age (Figs. 1 and 2). In the liver both AGT and SPT activity increased from 1 day,

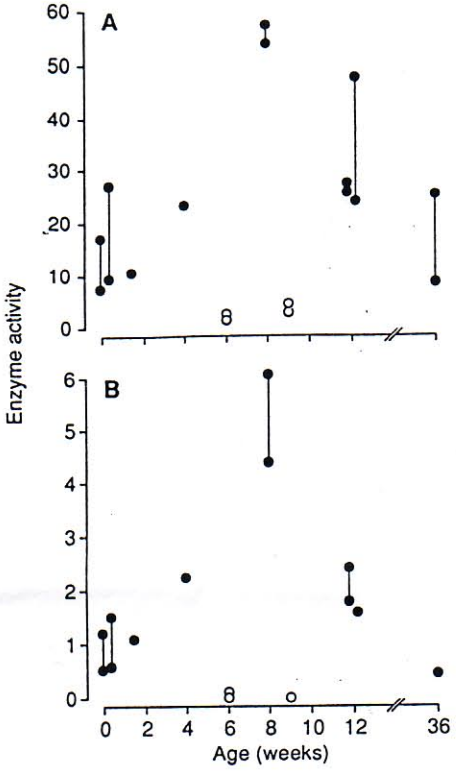

Fig. 2. Activities of AGT (A) and SPT (B) in dog kidneys at various ages. Solid circles = unaffected dogs; open circles = oxalotic dogs. All dogs were Tibetan Spaniels except the 10-day-old control which was a Bichon Frise. Units of enzyme activity = nmol/min per mg protein. Where possible duplicate assays were performed on *different* parts of the kidney (mainly cortex). These values are joined by vertical lines.

TABLE I

Enzyme activities in livers from two oxalotic dogs compared with controls

OHP, GR, DGDH = activities of D-glycerate dehydrogenase/glyoxylate reductase using hydroxypyruvate/NADPH, glyoxylate/NADPH and D-glycerate/NADP as substrate, respectively; GGT = glutamate:glyoxylate aminotransferase; AlOT = alanine:2-oxoglutarate aminotransferase; AsOT = aspartate:2-oxoglutarate aminotransferase; DAO = D-amino acid oxidase; LDH = lactate dehydrogenase; AGT = alanine:glyoxylate aminotransferase; SPT = serine:pyruvate aminotransferase. For all the controls (except for the AGT and SPT assays) $n = 6$; for AGT and SPT $n = 4$. For all assays (except AGT and SPT) there was no obvious relationship between enzyme activity and age in the control dogs. For these enzymes the controls, aged between 1 day and 9 months, were all unaffected Tibetan Spaniels in colonies related (1-day-old controls only) or unrelated to the oxalotic dogs. Because of the marked relationship between AGT and SPT enzyme activity and age in the control dogs (see Fig. 1), only those aged between 4 and 12 weeks have been included in this table. They consisted of four Tibetan Spaniels. Both oxalotic dogs were Tibetan Spaniels (ages 6 and 9 weeks).

Enzyme	Enzyme activities (nmol/min per mg protein)			
	control dogs		oxalotic dogs individual values	
	mean	(range)		
OHP	92	(76–117)	147,	153
GR	81	(45–107)	98,	129
DGDH	3.8	(1.1–6.0)	8.7,	1.8
GGT	7.03	(2.83–10.5)	7.33,	6.17
AlOT	66.4	(53.0–80.7)	89.5,	48.0
AsOT	677	(343–1218)	1243,	787
DAO	1.80	(0.82–3.08)	2.47,	1.58
LDH	573	(407–775)	996,	592
AGT	71.8	(67.3–79.7)	21.5,	9.7
SPT	8.67	(7.52–9.83)	1.22,	0.18

when it was virtually undetectable, up to 4 weeks when it reached a plateau which lasted at least until 12 weeks. In the kidney, on the other hand, AGT/SPT activity increased in the controls to a maximum at 8 weeks and then decreased to very low levels by 36 weeks. However, the AGT/SPT activity in the kidney of the 36 week control, which suffered from chronic tubulo-interstitial nephritis, should be treated with some caution, as the effect of this condition on enzyme activity is unknown. These developmental profiles of enzyme activity were matched by similar changes in the levels of immunoreactive AGT protein (Fig. 3). The large differences in AGT and SPT activity between some of the duplicate samples taken from different parts of the same organ indicated considerable heterogeneity with respect to the distribution of these enzymes within liver and kidney.

In both livers and kidneys of the two oxalotic dogs (6 and 9 weeks old) the activities of AGT and SPT were markedly reduced compared to age-matched controls (4–12 weeks old) (Tables I and II, Figs. 1 and 2).

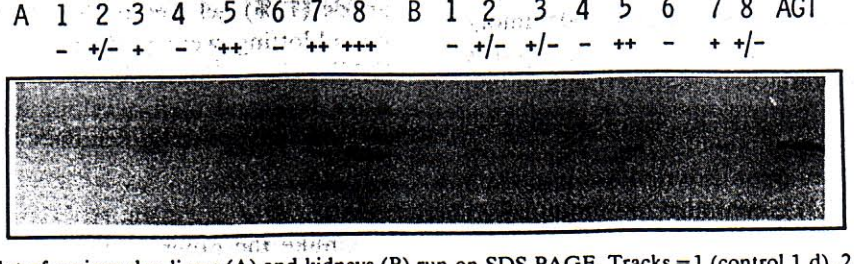

Fig. 3. Anti-AGT immunoblot of various dog livers (A) and kidneys (B) run on SDS PAGE. Tracks = 1 (control 1 d), 2 (control 10 d), 3 (control 4 w), 4* (oxalotic 6 w), 5 (control 8 w), 6* (oxalotic 9 w), 7 (control 12 w), 8 (control 36 w), AGT = purified human liver AGT standard. All dogs were Tibetan Spaniels except the 10-day-old control which was a Bichon Frise. For the liver and kidney samples 50 μg tissue protein was loaded, and for the AGT standard 80 ng was loaded. A subjective assessment of the relative amount of immunoreactive AGT protein was made (+++ = most, +/− = just detectable, − = undetectable). No other bands were detectable in other regions of the gel.

TABLE 2

Enzyme activities in kidneys from two oxalotic dogs compared with controls

Details as for Table I.

Enzyme	Enzyme activities (nmol/min per mg protein)			
	control dogs		oxalotic dogs individual values	
	mean	(range)		
OHP	36	(18–67)	71,	62
GR	15	(0–44)	18	
DGDH	1.1	(0.2–2.3)	0.3,	4.9
GGT	6.86	(5.33–10.2)	5.00,	3.50
AlOT	6.6	(0.8–12.0)	2.5,	1.3
AsOT	475	(360–720)	335,	233
DAO	4.90	(2.82–7.42)	3.60,	1.50
LDH	983	(801–1387)	1014,	924
AGT	31.5	(23.2–53.5)	1.3,	4.5
SPT	3.08	(1.58–6.05)	0,	0

Hepatic AGT was reduced to 14–30% of the mean normal control level and renal AGT to 4–14%. Similarly hepatic and renal SPT were reduced to only 2–14% and 0%, respectively. In the liver, the activities of a variety of other enzymes, including those deficient in human PH2 [5] and the feline analogue of PH2 [7], were near normal or somewhat elevated (Table I). In the kidneys, a number of other enzymes also showed slight reductions in activity, but not as much as the depletion in AGT/SPT activity (Table II). Decreases in enzymes, such as AlOT, DAO, AsOT and GGT, were probably more indicative of a poorer state of preservation of the kidneys and the difficulty of obtaining representative tissue samples, as the spread of values in the controls was very wide. Immunoblotting of an SDS-PAGE gel confirmed the enzymological results by demonstrating a marked deficiency of both hepatic and renal immunoreactive AGT protein in the two oxalotic dogs (Fig. 3).

Discussion

The enzyme deficiencies (i.e., AGT/SPT) found in the livers and kidneys of the oxalotic dogs are the same as those found previously in the livers of human PH1 patients [2,3]. In addition the activities of OHP, DGDH and GR, which are the deficient enzymes in human PH2 [5] and cat PH2 [7] were normal. This suggests that this familial condition found in a Tibetan Spaniel colony is a good candidate for being an analogue of human PH1.

There are a number of significant differences in the characteristics of expression of AGT in the dog compared to that found in the human that might limit its usefulness as a clinical model for the human disease. Firstly, there appear to be marked differences in the post-natal developmental expression of AGT in dog liver compared to human liver. In the latter the level of AGT 2 days post-natally is little different to that found in adulthood (Danpure and Jennings, unpublished observations), whereas AGT in 1-day-old dog liver is virtually undetectable, even though by 4 weeks it has reached levels very similar to those found in humans.

Secondly, the tissue-specific expression of AGT appears to be different in the two species. The rather limited evidence available [11] indicates that AGT is liver-specific in humans. The specific activity of AGT in the kidneys of human adults was less than 2% of that found in the livers [11]. On the other hand, in the dog the level of AGT enzyme activity and immunoreactive protein was very similar in the liver and kidney at 8 weeks. To our knowledge AGT has not been determined in neonatal human kidney, so whether it is transiently expressed is unknown.

Thirdly, in human liver, at least, the intracellular compartmentation of AGT is very important in the aetiology and pathogenesis of PH1. In normal human liver, AGT is entirely peroxisomal [12], whereas in up to one-third of all human PH1 patients the disease appears to be caused, at least in part, by the mis-routing of AGT from the peroxisomes to the mitochondria [10,13]. The dog, on the other hand, has an exclusive mitochondrial localization for its AGT [14]. It has been suggested that, although enzymically active, mitochondrial AGT can not fulfil its metabolic role (glyoxylate detoxification) properly in human liver [10]. The efficiency with which mitochondrial AGT in dog liver can detoxify glyoxylate, or whether it is important for it to do so in order to prevent hyperoxaluria and oxalosis, remains to be seen.

Further studies will be needed to determine the metabolic consequences of the AGT deficiency and the long term pathology in these oxalotic dogs before it can be ascertained whether their oxalotic nephropathy will be clinically useful as a model for PH1. However, in any case, the dog may turn out to be a very interesting system in which to study the control of the developmental and tissue-specific expression of AGT.

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