Tissue and Organ Expression of Catalase in Acatalasemic Beagle Dogs

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Abstract: Acatalasemic Beagle dogs which were maintained in our laboratories showed no sign of catalase activity at all in the erythrocytes, and glutathione peroxidase and superoxide dismutase were at normal levels. Immunoblotting analysis demonstrated that no catalase protein is detectable in their erythrocytes. On the other hand, catalase activity was detected in other tissues and organs, albeit at varying, lower levels than in normal dogs. Quantitative immunoblotting analysis consistently demonstrated that the catalase protein is expressed in the liver and kidneys of acatalasemic dogs in proportion to the activity in these organs. The catalase mRNA expressions in the blood, liver and kidneys in acatalasemic dogs were almost the same as those in normal dogs. These results suggested that catalytically normal catalase protein is translated from mRNA in the tissues and organs including erythrocytes, but in erythrocytes this enzyme protein is disposed of by an unknown mechanism.

Key words: acatalasemia, Catalase, dog, glutathione peroxidase, superoxide dismutase

Introduction

In 1948, Takahara and Miyamoto [17] first reported acatalasemia, which is the genetic deficiency of catalase activity in erythrocytes, in Japanese. A peculiar progressive gangrenous disease in the oral cavity, named Takahara's disease, was characteristically found along with the Japanese-type acatalasemia, and provided a clue for the discovery of this disease [17]. In the 1960s, acatalasemia was found in the Swiss [1], and an acatalasemic mouse strain, which was experimentally

generated by X-ray irradiation [4], was also reported. The biochemical and enzymatic differences between the Japanese-type acatalasemia and the mouse acatalasemia have been well characterized [13]. Fukuda *et al.* [6] reported spontaneous and hereditary acatalasemia in our Beagle dog colony. Interestingly, the acatalasemic dogs occasionally exhibit signs of an oral gangrenous disease very similar to Takahara's disease [17] and were therefore regarded as a possible animal model for the Japanese-type acatalasemia but the biochemical and molecular biological characterization of dog

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acatalasemia has not been performed so far.

In the present study, we investigated whether dog acatalasemia is associated with the presence of inactive catalase protein in the erythrocytes or with the complete absence of this enzyme protein, by using a specific antibody raised against catalase purified from normal Beagle dog erythrocytes [9]. In addition to the erythrocytes, we also investigated other tissues and organs for the expression of catalase protein and mRNA.

Materials and Methods

Animals: Three normal male Beagle dogs, three male acatalasemic Beagle dogs and a male hypocatalasemic Beagle dog, which was obtained by crossing a normal dog with an acatalasemic homozygote [18] were used (Body weights: 10–12 kg). All animals were maintained under regular animal care conditions in the Laboratory Animal Science and Toxicology Laboratories, Sankyo Co., Ltd. prior to use. Peripheral blood samples were collected from the cephalic vein of the foreleg with a heparinized syringe. The dogs were killed by exsanguination from the carotid artery under pentobarbital anesthesia, and the liver, kidneys, heart, lungs, spleen, pancreas, small intestine, brain and muscle were collected and stored frozen at -80°C until use.

Preparation of hemolysate and tissue and organ homogenates: All procedures were carried out at 4°C . Hemolysates were prepared by osmotic shock according to the method described previously [9]. To each type of solid tissue and organ, four volumes of 10 mM phosphate buffer (pH 7.4) containing 1.15% potassium chloride were added. Homogenization of tissues and organs was conducted by means of a Polytron homogenizer (Kinematica GmbH, Switzerland). The supernatant fractions of the homogenate after centrifugation at 9,000 g were used for measurement of the catalase activity.

Measurement of the anti-oxidant enzymes: Catalase activity was measured spectrophotometrically at 240 nm according to the method of Abei [2]. One unit of the catalase activity was defined as the activity required to destroy 1 μmole hydrogen peroxide in 30 sec at 25°C in 50 mM phosphate buffer (pH 7.0).

Glutathione peroxidase (GPx) activity was measured according to the method of Kraus *et al.* [7]. One unit of the GPx activity was defined as the activity required

to oxidize 1 μmole of glutathione in 1 min.

Superoxide dismutase (SOD) activity was measured as the activated autooxidation of tetracyclic catechol with a BIOXYTECH SOD-525 Assay Kit (OXIS International, Inc., USA). The assay was carried out according to the manufacturer's instructions. The SOD activity was expressed U-525 units [11].

Hemoglobin content was determined according to the method of Drabkin and Austin [3]. Protein concentrations were determined by means of a DC Protein Assay Kit (Bio-Rad Laboratories, USA) with bovine serum albumin (Sigma Inc., USA) as the standard.

Immunoblot analysis: SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [8]. Hemolysate containing 100 μg of protein from normal, hypocatalasemic and acatalasemic dog erythrocytes that showed catalase activity of 7.2, 4.2 and 0 units/mg protein, respectively, were applied to a 10% SDS-polyacrylamide gel, and electrophoresis was carried out. Immunoblotting analysis with anti-dog catalase IgG and alkaline phosphatase was carried out according to the method described previously [9]. For quantitative immunoblotting analysis of the catalase protein in the liver and kidneys, 2 μg of the hepatic homogenates from each of the three normal and three acatalasemic dogs and 5 μg of the renal homogenates from the three normal dogs and 10 μg of the renal homogenates from the three acatalasemic dogs were subjected to the immunoblotting analysis according to the above method. In this case, the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech Inc., UK) was used as the detection system. In the standard experiment for calibration, the bovine liver catalase (40 to 250 ng) was used in duplicate. This quantitative immunoblotting analysis showed a good linearity, with a correlation coefficient of 0.998. The catalase bands visualized on Hyperfilm-ECL (Amersham Pharmacia Biotech Inc., UK) were quantified with an imaging densitometer (Model GS-670, Bio-Rad Laboratories, USA) and the intensity of each catalase band relative to that of 1 μg bovine liver catalase was calculated for normalization.

RNA analysis: Total RNA was extracted from the liver and kidneys with ISOGEN (Nihon Gene Inc., Japan) and from serum-free blood with ISOGEN LS (Nihon Gene Inc., Japan) according to the manufacturer's instruction manual. The cDNA clone of normal dog catalase,

named pTCCAT-8 [10], was labeled with [α - 32 P] dCTP at a specific radioactivity of about 5×10^8 cpm/ μ g with the Random Primer DNA Labeling Kit ver. 2 (Takara Shuzo Inc., Japan). For Northern blot analysis, total RNA (30 μ g) from the blood, liver and kidneys were denatured in a mixture of formaldehyde and formamide [16] and electrophoretically separated on formaldehyde containing 1.2% agarose gel. The samples were blotted onto Hybond-N membrane (Amersham Pharmacia Biotech Inc., UK) and UV-crosslinked (Crosslinker XL-1, Taitec Inc., Japan). The membrane was prehybridized in a prehybridization solution (50% formamide, $5 \times$ SSPE, $5 \times$ Denhardt's solution, 0.1% SDS and denatured sonicated salmon sperm DNA) at 42°C for 2 hr. The hybridization reaction was done at 42°C for 16 hr in a prehybridization solution containing 32 P-labeled catalase DNA probe (1×10^6 cpm/ml). After hybridization, the membrane was washed twice with $2 \times$ SSC-0.5% SDS at room temperature for 15 min and twice with $1 \times$ SSC-0.1% SDS at 42°C for 15 min. For the control experiment, partial cDNA of dog glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was cloned by the RT-PCR method with a set of human, mouse and rat primers (G3PDH 0.45 Kb control, Clontech Inc., USA). The membrane was rehybridized with G3PDH cDNA labeled with [α - 32 P]dCTP.

Results

Anti-oxidant enzyme activities in erythrocytes: Catalase, GPx and SOD activities in the erythrocytes of

normal and acatalasemic Beagle dogs are shown in Table 1. In the acatalasemic dog hemolysates, catalase activity was not detected at all, but GPx and SOD activities were detected at the same levels as those in the normal dog hemolysates.

Catalase activities in other tissue and organs: The tissue and organ distributions of catalase activity in normal and acatalasemic Beagle dogs are summarized in Table 2. The activity in the liver and kidneys of acatalasemic dogs, the organs having the highest catalase activity in mammalian species, was 0.71 and 0.41, respectively, of those in the normal dogs. The activity of acatalasemic dogs relative to normal dogs varied widely from 0.35 (spleen) to 1.00 (muscle). Activity in the kidneys, lung, spleen and brain of the acatalasemic dogs was significantly lower than in the normal dogs ($p < 0.05$).

Detection of catalase protein in erythrocytes, liver and kidneys by immunoblotting: The result of the immunoblotting analysis of the hemolysates of normal, hypocatalasemic and acatalasemic Beagle dogs with

Table 1. Catalase, GPx and SOD activities in erythrocytes of normal and acatalasemic Beagle dogs

	Catalase activity (units/g Hb)	GPx activity (units/g Hb)	SOD activity (U-525 units/ml)
Normal	8.9 ± 0.9	78.5 ± 2.1	33.2 ± 0.4
Acatalasemic	N.D.	83.4 ± 2.6	32.2 ± 0.9

The results are presented as mean values for three samples $\pm$ S.E. N.D.: not detected.

Table 2. The catalase activities in various tissues from normal and acatalasemic dogs

	Catalase activity (units/mg protein)		Ratio of catalase activity (AC/N) ^{a)}
	Normal dogs	Acatalasemic dogs	
Liver	286.4 ± 47.8	203.4 ± 56.0	0.71
Kidneys	154.3 ± 27.7	$63.0 \pm 7.3^*$	0.41
Heart	6.8 ± 1.9	6.1 ± 0.2	0.90
Lungs	9.6 ± 0.9	$5.4 \pm 0.2^*$	0.56
Spleen	4.3 ± 0.6	$1.5 \pm 0.3^*$	0.35
Pancreas	5.5 ± 1.2	4.2 ± 0.6	0.76
Intestines	16.7 ± 2.2	13.8 ± 2.0	0.83
Brain	1.9 ± 0.2	$1.0 \pm 0.1^*$	0.52
Muscle	1.3 ± 0.2	1.3 ± 0.2	1.00

The results are presented as mean values for three samples $\pm$ S.E. *: $p < 0.05$. a) AC/N means the ratio of catalase activity in acatalasemic dogs to normal dogs.

anti-dog erythrocyte catalase IgG is shown in Fig. 1. A single band corresponding to a monomer subunit of catalase was detected at 63 kDa in the normal dog hemolysates (Fig. 1, lane 1), but no catalase band was detected at all in the acatalasemic dog hemolysates (Fig. 1, lane 3), indicating that the catalase protein is not present in the acatalasemic dog erythrocytes. In the hypocatalasemic hemolysates, which had approximately 60% of the catalase activity of normal hemolysates, a catalase band with about one half the intensity of the catalase band in the normal dog hemolysate was detected at the same mobility on the gel (Fig. 1, lane 2). Quantitative immunoblotting analysis of the liver and kidney homogenates was carried out with purified bovine liver catalase as standard (Fig. 2). As shown in Fig. 3, the amounts of immunoreactive catalase protein in the liver and kidneys were in good correlation with the catalase activities ($R=0.971$), indicating that catalase is involved predominantly in the decomposition of hydrogen peroxide by those organs in both normal and acatalasemic dogs, and therefore the acatalasemic dogs have catalytically normal catalase.

RNA analysis: The result of Northern blotting analysis is shown in Fig. 4. The mRNA expression of catalase in the blood, which is quite likely in the eryth-

Fig. 1. Immunostain of catalase in erythrocytes from normal, acatalasemic and hypocatalasemic Beagle dogs with anti-dog catalase antibody. Lane 1: Normal dog hemolysate (100 μ g), which has a catalase activity of 7.4 units/mg protein. Lane 2: Hypocatalasemic dog hemolysate (100 μ g), which has a catalase activity of 4.2 units/mg protein. Lane 3: Acatalasemic dog hemolysate (100 μ g), which has no catalase activity. The arrow indicates the positions of the catalase bands.

A. Bovine liver catalase

C. Kidney

B. Liver

Fig. 2. Immunostain of catalase in hepatic and renal homogenates from normal and acatalasemic Beagle dogs. A: Purified bovine liver catalase (Sigma) in the range 40–250 ng as the calibration standard. B: 2 μ g of hepatic homogenates from three normal dogs (N) and three acatalasemic dogs (AC). C: 5 μ g of renal homogenates from three normal dogs (N) and 10 μ g of renal homogenates from three acatalasemic dogs (AC).

Fig. 3. Catalase activities and expression of catalase protein in liver and kidneys from normal and acatalasemic Beagle dogs. (●): Normal dog liver. (■): Acatalasemic dog liver. (○): Normal dog kidney. (□): Acatalasemic dog kidney. Each bar represents the mean $\pm$ S.E. The amount of catalase was normalized as a ratio in relation to the amount of bovine liver catalase.

rocytes [14], as well as in the liver and kidneys from acatalasemic Beagle dogs was at almost the same level as that of the normal Beagle dogs. No difference was found between the acatalasemic and normal Beagle dogs in the size of mRNA.

Discussion

To investigate whether the erythrocytes of acatalasemic dogs are associated with abnormal catalase protein having no catalytic activity or with the complete absence of this enzyme protein, we performed the detection of catalase in erythrocytes with a specific antibody raised against catalase purified from normal Beagle dog erythrocytes [9]. No catalase protein was detectable in acatalasemic dog erythrocytes and the enzyme protein expression in erythrocytes of normal, hypocatalasemic and acatalasemic dogs correlated well with the catalase activity (Fig. 1). These results clearly demonstrate that the deficiency of the catalase activity in the acatalasemic Beagle dogs is due to a complete lack of the catalase protein in the erythrocytes (Table 1), and therefore the possibility of the occurrence of a catalytically inactive enzyme was excluded.

Regarding the residual catalase in the liver and kid-

Fig. 4. Northern blot analysis of liver, kidney and blood RNA from normal and acatalasemic Beagle dogs. Total RNA (each 30 μ g) from the liver, kidneys and blood of the normal (N) or acatalasemic (AC) Beagle dogs were subjected to formaldehyde containing 1.2% agarose gel electrophoresis. The samples were blotted onto Hybond-N membrane and hybridized with 32 P-labeled dog catalase cDNA probe (1×10^6 cpm/ml) at 42°C for 16 hr (panel A), followed by rehybridization with 32 P-labeled dog G3PDH cDNA probe (1×10^6 cpm/ml) at 42°C for 16 hr (panel B). The positions of the 18S and 28S ribosomal RNA bands are shown in panel A.

neys from acatalasemic dogs, there was no difference between the normal and acatalasemic dogs in the mobility of immunodetectable catalase in SDS-polyacrylamide gel electrophoresis (Fig. 2). This result and that shown in Fig. 3 indicated that the catalase protein expressed in the acatalasemic Beagle dog is catalytically normal.

In the case of Japanese-type acatalasemic patients, unlike acatalasemic Beagle dogs, almost no catalase activity was detected in the tissues or erythrocytes examined [12, 15]. The complete lack of catalase activity is explained by an error in the splicing of premature mRNA to mature mRNA leading to entirely no expression of the catalase protein [20]. Therefore the dog acatalasemia seems to differ biologically from Japanese-type acatalasemia reported so far, although acatalasemic dogs occasionally exhibit signs of an oral gangrenous disease very similar to Takahara's disease [17].

Interestingly, the acatalasemic mouse strain shows

some similarities to the acatalasemic dog strain. The residual catalase activity in the erythrocytes of the acatalasemic mouse was only about 2% that of normal activity [19] but much higher levels of catalase activity were detected in the liver, kidneys and lungs [19]. As in acatalasemic dogs, hypocatalasemic mice exhibited only about 50% of the normal catalase activity in the erythrocytes [5].

As described above, the catalase activities in the tissue and organ of acatalasemic dogs were lower than those in normal dogs, and the difference was statistically significant in the kidneys, lungs, spleen and brain (Table 2). Catalase mRNA was expressed in the tissues and organs of acatalasemic dogs at normal levels including the blood, suggesting that the catalase protein is translated normally. These facts give rise to the conjecture that this enzyme protein is disposed of by an unknown mechanism in the erythrocytes, which are well-differentiated mature cells, and have no machinery for protein synthesis. The mechanism for the complete lack of the catalase protein in the erythrocytes is not yet clear and awaits further investigation.

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