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Age-related Changes in Activity of Catalase in Selected Bovine Muscles

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ABSTRACT

Background: The unavoidable consequence of ageing process is oxidative stress which is harmful to cells. Muscles as a post-mitotic cells are especially susceptible for it. They are protected by antioxidants. One of the main antioxidant enzymes is catalase (CAT). Although muscles were extensively examined for antioxidant defense, there is a lack of comparative study regarding CAT activity in skeletal muscles, heart muscle and diaphragm in cows. The aim of present study was investigate the link between age related alterations and antioxidant defense system and confirmation that CAT activity depends on age and type of muscle tissue dependent on metabolic rate.

Materials, Methods & Results: Samples of skeletal muscles, heart and diaphragm were collected from healthy Holstein-Friesian cows aged between 14 and 27 months (n = 12; sexually mature) and female calves aged between 2 weeks and 2 months (n = 9; sexually immature) in slaughterhouse. The enzyme activity was measured by spectrophotometric method. Additionally the presence of CAT was confirmed by SDS-PAGE separation and detected by zymography. In current investigation CAT activity in heart and diaphragm showed significantly higher ($P < 0.05$) values than in skeletal muscles. Moreover, CAT activity was higher in cows than in calves in heart (0.28 ± 0.044 U /mg protein vs. 0.4 ± 0.035) as well as in diaphragm (0.27 ± 0.047 vs 0.37 ± 0.077), however in skeleton muscles there were no significant differences between examined animals (0.076 ± 0.016 vs 0.084 ± 0.0197). According to received results CAT activity increased during aging in heart and diaphragm while in skeleton muscles age-related changes were not observed.

Discussion: Skeletal muscles are particularly vulnerable to oxidative stress because they are composed by post-mitotic cells. Moreover, they have also some tendency to accumulate products of oxidative damage during consumption a large amount of oxygen. That is why highly active tissues like skeletal and cardiac muscles are more vulnerable to damage than other tissues. The metabolism of muscle tissues depends on the type of fibers which differ from each other with the concentration of myoglobin as well as resistance to fatigue. Other factors which have the influence on oxidative stress and activity of antioxidant enzymes are age and sex. The age-related changes in antioxidant system in muscles could be different from other tissues like liver, kidney or brain. Taking into consideration the fact that metabolic rate and demands for muscles are significantly different between each other, the examination of CAT activity in different type of bovine muscles (skeleton, diaphragm and heart) was undertaken. In current studies m. semitendinosus as a skeleton muscle was examined. There are some data describing differences between metabolism and contractile fibre type (MHC I proportion) in different parts (proximal, medium, distal) of that muscle which also change with age. Glycolytic metabolism dominated in proximal part while oxidative metabolism in distal part and in both cases the tendency decreased during ageing. The highest CAT activity was found in liver, kidneys, red blood cells and bone marrow, while connective tissue showed the lowest activity of the enzyme.

Present study confirms that CAT activity differs between type of examined muscles and may be related to metabolic characteristics of selected muscles. Age-related alterations that appeared may suggest the influence of sex steroids on antioxidative defense mechanisms.

Keywords: catalase, cow, ageing, skeletal muscles, diaphragm, heart.

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INTRODUCTION

During cellular processes the oxygen is involved in the generation of reactive oxygen species (ROS) which are dangerous for cell components because of high reactivity [1,6,20]. Production of free radicals is controlled by enzymatic and non-enzymatic antioxidant defense systems (ADS) [3,13]. The main antioxidant enzymes are glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) [4]. The product of the SOD-catalyzed reaction is hydrogen peroxide, while CAT decomposes hydrogen peroxide to water [9].

The efficiency of antioxidative protection decreases during ageing. The drop of CAT activity may lead to accumulation of hydrogen peroxide and consequently cause the oxidation of the most important components of cells [33]. Reduced number of mitochondria in older animals and dysfunction of these organelles caused by an excess of free radicals may lead to the reduction of oxidative capacity in muscles [8]. The activity of antioxidant enzymes is supposed to be controlled by sex steroids [17,23] which also change during ageing.

The aim of the present study was to determine and to compare CAT activity in muscles differing from each other with their metabolic rate, such as skeletal muscles, heart muscle and respiratory muscles (diaphragm) in sexually mature cows and sexually immature female calves. CAT activity in cows depending on the age through the H_2O_2–induced oxidation *in vitro* was investigated. CAT may shift the equilibrium between antioxidant and oxidant to be pro-antioxidant and thus protects against oxidative stress in above-mentioned tissues during aging.

MATERIALS AND METHODS

Study design

The samples of heart, skeletal muscles and diaphragm were taken from cows aged between 14 and 27 months (n = 12) and female calves aged between 2 weeks and 2 months (n = 9). All animals were healthy and of Holstein-Friesian breed.

Tissues were collected immediately after slaughter of animals, washed in 0.9 % $NaCl$[2], portioned and frozen. Tissues were homogenized in phosphate buffer (0.05 mol/L, pH 7.2), centrifuged at 4ºC for 20 min at 6,000 x *g*. Obtained supernatants were subjected to further determinations.

CAT activity

CAT activity was measured according to the method of Cohen *et al.* [10] which is based on monitoring the rate of enzyme catalyzed decomposition of hydrogen peroxide (H_2O_2) using potassium tetraoxomanganate (VII) ($KMnO_4$)[2].

The 0.45 mL of supernatant was mixed with 4.5 µL of ethanol (final concentration 0.17 mol/L) and incubated on ice for 30 min to decompose the Complex II of Catalase – H_2O_2. After the incubation 50 µL of 10% TRITON[2] was added. Before measuring CAT activity the supernatant was 5-fold diluted with calcium phosphate buffer, 0.01mol/L, pH 7.0.

To 0.5 mL of diluted supernatant 5 mL of 6 mmol/L H_2O_2 was added and incubated on ice for 3 min. After that time the reaction was stopped by adding 1 mL 3 mol/L H_2SO_4.[2] Thereafter 7 mL of 2 mmol/L $KMnO_4$ was added and absorbance[1] was measured at 480 nm immediately against buffer. Spectrophotometric standard consisted of 5.5 mL of calium phosphate buffer (0.01 mol/L, pH 7.0) instead of sample and substrate.

The measured activities were recalculated per protein content of each sample and expressed in CAT units per protein content of homogenate.

Electrophoretic analysis

The supernatants were subjected to electrophoretic separation in 10% non-denaturing PAGE [15] by using Mini- Protean Tetra Cell[3]. The running buffer was 0.025 mol/L Tris/glycine (pH 8.3). The amount of protein which was added to each lane was 20µg. Electrophoresis was performed during 2 h at 100V, 15 mA per gel. After electrophoretic separation, proteins were silver stained according to Shevchenko *et al.* [26].

Obtained bands were compared with the molecular weight standards[3] (20,7; 28,8; 34,3; 50; 77; 103 kDa respectively).

Zymography

Zymography was performed according to Woodbury *et al.* [31]. After electrophoretic separation gels were incubated in 2.5% Triton X-100[2] solution for 15 min and then three times for 15 min in distilled water. The next step was the incubation of gels in 0.003% H_2O_2.[2] solution for 15 min and quick wash in distilled water. Thereafter the gels were incubated in solution composed by two equal parts of 2% $FeCl_3$.[2] and 2% $K_3Fe(CN)_6$.[2] for 10 min. The whole gel turned dark green due to the precipitation of ferric chloride and potassium ferric cya-

nide mediated by H_2O_2 while the place with active CAT was not stained and there appeared colourless enzyme bands against the greenish blue background of the gel.

The gels were scanned as a negative for better visualization and were evaluated densitometrically.

Determination of protein concentration

The concentration of total protein in the samples was measured by the biuret method using a kit of reagents Cormay[4] according to the attached leaflet from the manufacturer.

Statistical analysis

The effects of age and the type of tissue on enzyme activity was examined by two-way analysis of variance (ANOVA).

To investigate the influence of age and type of tissue on CAT activity the Tukey's multiple comparison tests were used.

All calculations were done with SAS 8.0 system[5]. P values < 0.05 was considered as significant.

RESULTS

The results of CAT activity in examined tissues are shown in Figure 1.

In the group of cows the highest activity ($P < 0.05$) was noticed in heart (0.4 ± 0.035 U/mg protein) however in diaphragm (0.37 ± 0.077) the level was similar. In skeletal muscles the activity of enzyme was significantly lower (0.076 ± 0.016).

In the group of calves the CAT activity in heart and diaphragm was similar (0.28 ± 0.044 and 0.27 ± 0.047 respectively). In skeletal muscles enzyme activity was significantly lower (0.084 ± 0.0197).

In heart and diaphragm CAT activity was higher in cows than in calves but without statistical significance and that tissues created homogenous group.

CAT activity in muscles was significantly lower than in other tissues in cows as well as in calves.

The zymography of CAT (Figure 2) is a method of negative staining where enzyme is detected as colourless bands on a greenish blue coloured background due to potassium ferricyanide ferrichloride complex.

Liver (lane h) was used as reference tissue and strong bands at 120 kDa - dimer (optical density 48.2) and 180 kDa - trimer (optical density 3.5) were clearly visible. Bands in muscle tissues were less visible but densitometric analysis detected bands at 180 kDa with optical density of 10.4; 8.8; 12.5; 8.6; 5.4 and 6.2, respectively (lanes b-g).

The reason why examined tissues exhibited weak positive reaction could be too low level of enzymatic protein in examined tissue sample and lower activity as compared to liver tissue. At 240 kDa no bands were observed.

The gel was scanned as a negative for better visualization of bands. Vertical lines show densitometric analysis and peaks at enzyme bands in gel.

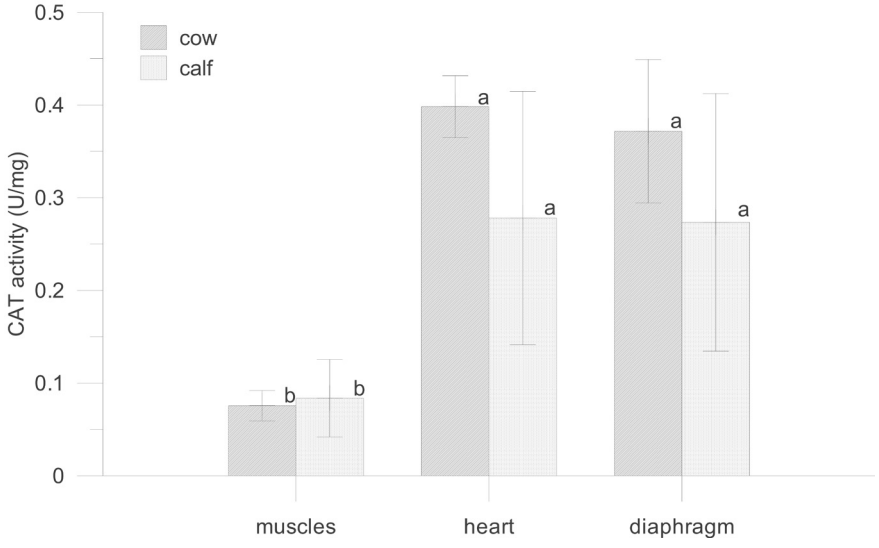

Figure 1. Catalase activity in bovine muscles. Different superscripts depict significant differences between bovine muscles.

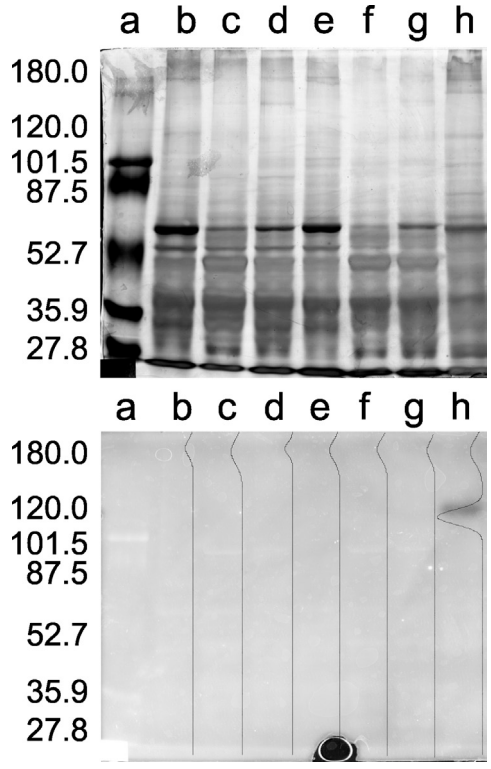

Figure 2. The eletrophoresis and zymography of bovine muscles. Lanes: a - mol weight standards; b - calf heart; c - calf skeletal muscles; d - calf diaphragm; e - cow heart; f - cow skeletal muscles; g - cow diaphragm; h - cow liver. Vertical lines represent densitometric analysis.

DISCUSSION

Current investigation provided with data on age-related changes in CAT activity in muscles of pre- and postpubertal cows. Moreover, the activity was examined in muscles, diaphragm and heart and the results showed that it differed between samples taken from muscles with different metabolic rate and demands.

Catalase (CAT; EC 1.11.1.6) for the first time has been isolated from bovine liver and these days it is an enzyme with the best known chemical structure [29]. CAT present in eukaryotic cells is a common bifunctional hemoprotein and can be used as both catalase and peroxidase [27]. The enzyme consists of four identical subunits including the heme system with iron atom centrally located and molecular weight approximately 60 kDa. CAT is located in peroxisomes, mitochondria, endoplasmic reticulum and cytosol of cells such as erythrocytes, liver and kidney cells and bone marrow [21].The enzyme is one of the main protein involved in antioxidant defense of cells.

Peroxidative processes are an inherent part of aerobic metabolism but their intensity depend on the specificity of organs. The balance between production and neutralization of ROS is important to maintain correct permeability of cell membranes and to protect against the damage to macromolecules.

Skeletal muscles are constituted with post-mitotic cells that is why they are especially susceptible to oxidative stress. Because of a large amount of oxygen consumption they have tendency to accumulate products of oxidative damage. The age-related changes in antioxidant system in muscles could be different from other tissues like liver, kidney or brain [18].

The metabolism of skeletal muscles is tightly correlated with the type of fibers which differ from each other with the concentration of myoglobin and resistance to fatigue. During studies about oxidative stress in skeletal muscles two type of muscles were taken into consideration 1) rich in slow-twitch fibers (soleus muscle) which are characterized by large amount of mitochondria, myoglobin and more resistant to fatigue and 2) rich in fast-twitch fibers (digitorum-longus muscle) which have less myoglobin and are more susceptible to fatigue.

In our studies m. semitendinosus was examined. However, the metabolism and the contractile fibre type (MHC I proportion) in muscle varied between its parts (proximal, medium, distal) and also changed during ageing in cattle [5]. In proximal part glycolytic metabolism dominated but decreased during ageing while in distal part oxidative metabolism dominated and also decreased during ageing. The MHC I proportions were higher in distal parts but increased slightly faster in proximal and median parts during ageing. In older animals the metabolism and MHC I proportion was similar in all parts of the muscle. In calves glycolytic fibre type dominated in m. semitendinosus but during ageing they changed to oxidative type I fibre especially after puberty [12].

Age-related changes in antioxidant enzymes activity were usually examined in muscles of rats [22]. Taking into consideration that oxidative stress is different depending on the type of muscle and fibres two type of muscles were examined: soleus muscle (SO, rich in slow-twitch fibers) and extensor digitorum longus muscle (EDL, rich in fast-twitch fibers). The higher

activity of CAT was observed in SO than in EDL and increased during ageing but in the case of EDL there were no age-related changes [22,16]

Soleus muscles are more resistant to age-related changes, they indicate higher activity of antioxidant enzyme and are well protected from oxidative stress compared to muscles with a large percentage of type II fibers [25]. Moreover, rat soleus muscle has a very homogenous fiber composition (about 97% of myosin heavy chain I (MHC I) and only 3% of MHC IIa) [2]

The release of H_2O_2 also change during ageing and is different between type of muscles [7]. H_2O_2 release was lower in oxidative (soleus) than in glycolytic (tibialis anterior) muscles in young rats but increased during ageing while H_2O_2 release in tibialis anterior did not change during ageing.

Catalase activity increased significantly (+ 84%) in tibialis anterior during ageing but in soleus was not measured. However, glycolytic (tibialis anterior) muscles are more affected by the sarcopenia compared to oxidative (soleus) muscles. [7].

The significant increase of CAT activity in skeleton muscles was also observed in old mice [30]. Another parameter which is useful to describe age-related in antioxidant enzymes is mRNA expression. Activity and mRNA expression of CAT increased during ageing in muscles of pigs [32] as well as in rats [16]

In current investigation CAT activity did not change during ageing in skeleton muscles which is in disagreement with data from rats mentioned above. Similar results were received in people [24]. However, in some cases CAT activity decreased during ageing [11] with increased release of H_2O_2 [28]. The insufficient antioxidant protection during ageing may cause H_2O_2 accumulation and the onset of sarcopenia.

Cardiac muscle is characterised by dynamic metabolism that is why is especially vulnerable to oxidant injury. Moreover it is well known that mitochondria in heart produce more ROS with aging. CAT activity in heart was significantly higher in old animals while in other tissues (kidneys and liver) decreased during ageing [19]. This is in agreement with our results.

The level of glutathione (GSH) significantly decreased but GSSG level increased during ageing in heart of rats [14]. The increase of CAT activity could play compensatory role when GSH-Px which is also responsible for decomposition of H_2O_2, is insufficient because of glutathione depletion.

The diaphragm is constantly active and is oxidative muscle that is why is especially vulnerable to oxidative stress. Moreover the diaphragm metabolism is different between species and is higher in small animals like rats compared to large animals like cows.

Unfortunately there is lack of information about age-related changes in antioxidant enzymes in diaphragm.

CONCLUSIONS

In conclusion, current investigation provided with data that CAT activity increased during ageing in heart and diaphragm however in skeleton muscle age- related changes were not observed. The reason why CAT activity in heart and diaphragm is higher in older animals could be elevated level of H_2O_2 which is released during ageing. It is highly probable, taking into consideration the fact that heart and diaphragm as an oxidative muscles are especially rich in mitochondria. Additional reason for age-related alterations can be the influence of sex steroids which are supposed to control and regulate the activity and the expression of antioxidative enzymes. Our samples were collected from pre- and postpubertal animals where the influence of sex steroids is different.

Skeletal muscle tissue specific discrepancies between results of other studies and ours could be related also to differences in metabolic activity of examined muscles. Differences between laboratory animals and cows could be related to different metabolic rate of these animals as well as different workload of skeletal muscles of cows and different time frame of experiments. Additionally different muscles and even its parts were examined.

MANUFACTURERS

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Ethical approval. The experimental protocol was approved by appropriate Ethic Committee appointed at University of Life Sciences in Lublin (decision no 60/2011).

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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