Chapter 14

Differential pH Sensitivity of Tissue Superoxide Dismutases

Introduction

During mitochondrial electron transport there is considerable oxygen slippage which may amount to form 3-5% (1-3). This in turn results in the generation of superoxide radical, O_2^- (1-3). Additionally, significant amount of O_2^- is also produced by the microsomal electron transport chain and other enzyme systems e.g. xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase, and a group of flavoprotein dehydrogenases (1, 4). O_2^- is also produced by intact granulocytes during phagocytosis and by illuminated chloroplasts (4, 5). Within any given type of cell, quantitatively most significant sources of O_2^- and the absolute rates of O_2^- production are variable (4). However, most importantly, the fact remains that respiring cells produce significant amount of O_2^-.

Mc Cord and Fridovich reported for the first time presence of an enzyme superoxide dismutase (SOD) which eliminates the O_2^- by converting it to H_2O_2 (4, 5). The enzyme SOD is widely distributed in nature and is found to be present in bacteria, animal tissues and erythrocytes, and most higher plants (1, 4-6). In the animal tissues the cytosolic SOD is a Cu^{2+}- Zn^{2+} enzyme while the mitochondria contain a Mn^{2+}- dependent SOD. The erythrocyte SOD is a Cu^{2+}- Zn^{2+} enzyme (4, 5). The Cu^{2+}- Zn^{2+} SOD and Mn^{2+}- SOD are designated SOD 1 and SOD 2 respectively (4, 5). Additionally, presence of extra-cellular SOD (ec SOD) has also been reported which is designated as SOD 3 (7, 8). Mutations in SOD 1, 2 and 3 lead to polymorphism which has been shown to correlate with several pathological/clinical conditions (8).

In their original method McCord and Fridovich measured the activity of SOD by determining its ability to dismutate O_2^- radical. Xanthine/ xanthine oxidase (XO) system was used to produce O_2^- (9) and the rate of O_2^- generation was monitored in terms of reduction of cytochrome c (9). Unit of the enzyme activity was defined as the amount which will inhibit the reduction of cytochrome c by 50% under standard assay conditions (9). Since this time, several systems for generating O_2^- have been described in which generation of O_2^- is facilitated under alkaline conditions (4).

Using pyrogallol, Marklund and Marklund reported that the rate of O_2^- generation was optimum at pH 9.2 (10). Further, using the SOD purified from bovine erythrocytes, it was demonstrated that activity was stable over the pH range of 7.9 to 9.2 and that the sensitivity of the assay was significantly high at pH 9.2 (10). The results thus confirmed the notion that SOD is an unusually stable enzyme (4). The significantly high sensitivity of the assay at high alkaline pH becomes a tempting factor for determining SOD activity in different tissues/systems at pH 9.2.

However, the question as to whether the SODs from different tissues/systems would display similar stability over a wide range of pH and especially at high alkaline pH in the pyrogallol assay (10) has not been addressed to thus far (4). In the light of the above, we decided to measure and compare the SOD activities in human and rat erythrocytes as well as in mitochondria and post-mitochondrial fractions from various rat tissues at pH 8.0 and at pH 9.2. The results of these experiments depicting differential sensitivities of the tissue SODs are described here.

Materials and Methods

Chemicals

Bovine serum albumin fraction V (BSA) was purchased from SRL, India. 4-Morpholinopropanesulfonic acid (MOPS) was obtained from Sigma, USA. Pyrogallol (Loba Chem) was dissolved in 0.5 N HCl to obtain stock solution (200 mM) which was kept refrigerated in an amber colored stoppered tube. The stock solution was diluted in distilled water 1:50 prior to use. All other chemicals were of the highest purity grade and were purchased locally.

Animals

Adult male albino rats of Charles – Foster strain (8-10 week old, 220-225 g body weight) were used.

Isolation of RBC

Human blood samples were collected from normal healthy volunteers, both male and female (25- 30 Yr. old) in heparinized vials. The blood samples were centrifuged at 2000 rpm for 10 min to separate plasma from RBCs. The RBCs were then resuspended in saline and washed twice by centrifuging at 2000 rpm for 10 min. The washed RBCs were then resuspended in saline to the original volume of the blood sample.

Blood from rats was collected from orbital sinus in test tubes containing saline citrate and washed RBCs were collected as described above. Finally the RBCs were suspended in minimum volume of saline.

The RBC count was determined using a hemacytometer.

Isolation of mitochondria and post-mitochondrial fraction for liver, kidney and brain were by the procedure given in Chapter 11 and for heart the procedure was given in Chapter 12 of the Thesis.

Measurement of SOD activity

Measurement of SOD activity was carried out spectrophotometrically at 25 °C by the method of Marklund and Marklund (10) with some modifications i.e. the cacodylate based buffer system was changed to potassium phosphate buffer. Also, the measurements were carried out at two different pHs, i.e. pH 8.0 and pH 9.2. The assay medium in a total volume of 1.0 ml contained: 100 mM potassium phosphate buffer (pH 8.0 or 9.2) and 0.2 mM pyrogallol. Autoxidation of pyrogallol was monitored at 420 nm for 3 min in the absence and presence of added enzyme from different sources; at least three concentrations of the enzyme which produced between 30 to 60 % inhibition of pyrogallol autoxidation were used.

One unit of the enzyme activity is defined as, the amount which produced 50% inhibition of pyrogallol autoxidation under the standard assay conditions. Mitochondrial and cytosolic SOD activities are expressed as Units/ mg protein.

Total mitochondrial SOD activity/g tissue was computed based on the mitochondrial protein content in the various tissues (liver, kidney, brain and heart contain respectively

50.6, 64.0, 33.5 and 65.0 mg mitochondrial protein/g tissue) (12, 13). Total cytosolic SOD activity/g tissue was computed based on the volume of the post mitochondrial supernatant which was used as the source of the enzyme. The protein content of cytosolic fractions from the four tissues were 9.37 ± 0.23, 6.62 ± 0.20, 4.30 ± 0.21 and 2.91 ± 0.09 / ml, respectively

The SOD in the erythrocytes is expressed as units/10^6 RBCs.

Protein estimation was according to the method of Lowry et al. using bovine serum albumin as the standard (14).

The results are given as mean ± SEM, Statistical evaluation of the data was by Students' t-test.

Results and Discussion

In the initial experiments, the rates of pyrogallol autoxidation were determined at pH 8.0 and 9.2. As can be noted (Fig. 1) the autoxidation of pyrogallol was rapid at pH 9.2 but followed a non-linear pattern as reported by Marklund and Marklund (10). By contrast, at pH 8.0 the autoxidation of pyrogallol was slow but followed a linear kinetics.

SOD activities in various systems/sub-cellular fractions, are shown in Fig. 2 and 3. Thus at pH 8.0 the specific activity of SOD in rat erythrocytes was about 70% higher compared to human erythrocytes (Fig. 2). Higher specific activity of SOD in the rat erythrocytes

Figure legends

Figure 1 Typical curves depicting autoxidation of pyrogallolat pH 8.0 (—■—) and pH 9.2 (—o—). Autoxidation of pyrogallol was monitored by recording the increase in the absorbance at 420 nm in a Shimadzu model UV 160A.

Figure 2. Superoxide dismutase activity in human and rat erythrocytes. Measurements were carried out at pH 8.0 ☐ and pH 9.2 ■. The activity is expressed as mU/10^6 RBCs. Results are given as mean ± SEM of 12 independent samples in each group.
a, $p<0.05$ and b, $p<0.001$ compared with the corresponding values at pH 8.0.

Figure 3. Superoxide dismutase activity in liver, kidney, brain and heart mitochondrial and cytosolic fractions. The activities were measured at pH 8.0 ☐ and pH 9.2 ■ as described above in Fig. 2. A. Liver, B. Kidney, C. Brain and D. Heart.
Results are given as mean ± SEM of 12 independent samples in each group.
a, $p<0.001$ compared with the corresponding values at pH 8.0.

Figure 1

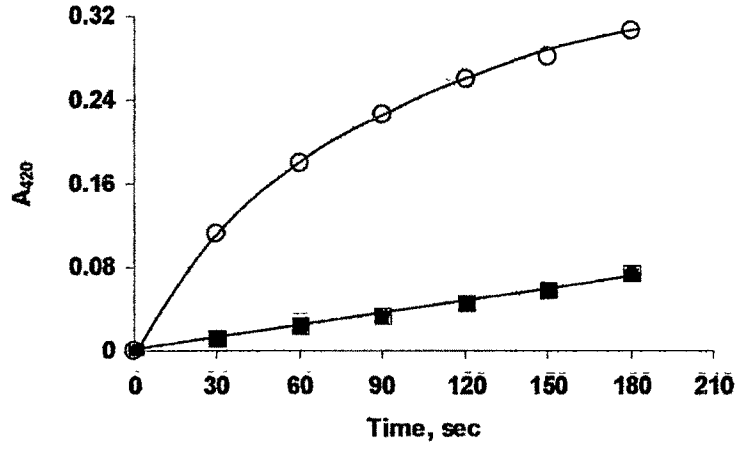


Figure 2

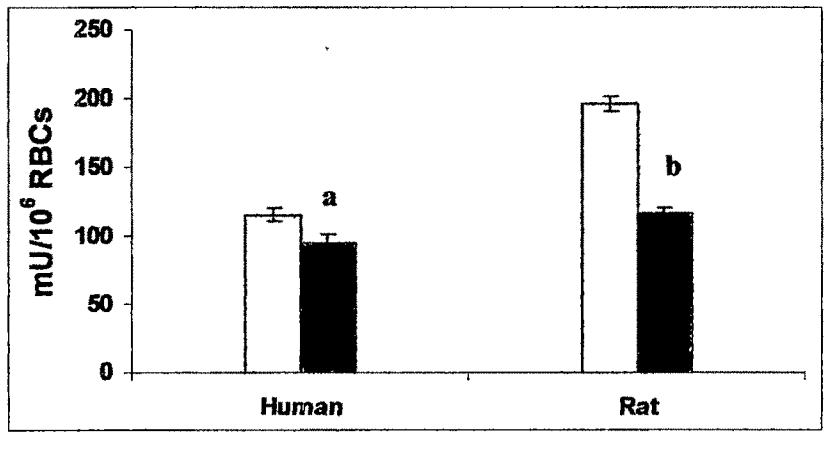
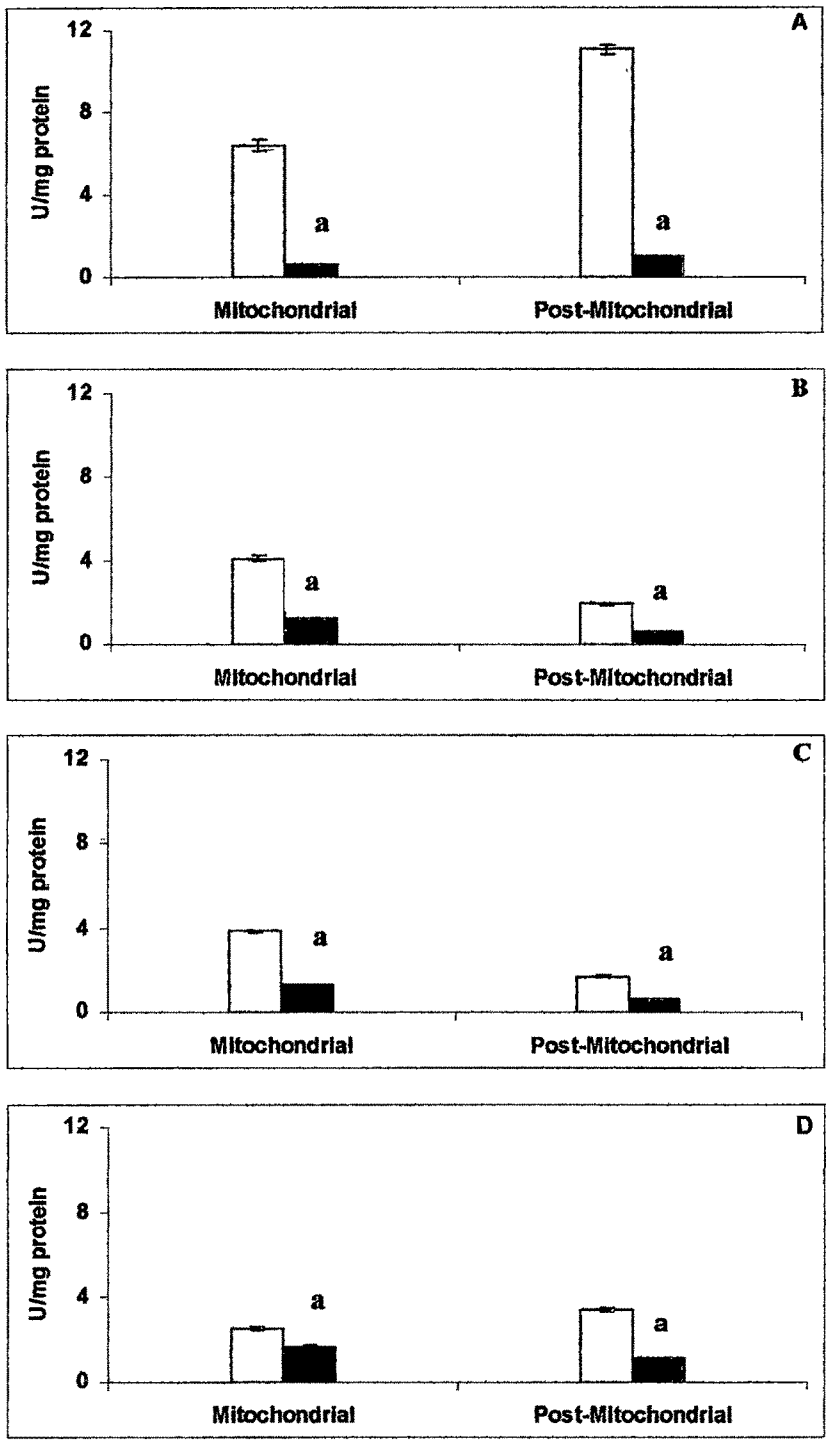


Figure 3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Post-Mitochondrial

may relate to the higher rate of metabolism and rapid turnover of the rat erythrocytes (15). Interestingly, at pH 9.2 the SOD activity of the two RBCs was differentially inactivated; the activity in the human erythrocytes decreased by 17% while that in rat erythrocytes showed a greater decrease of 41% (Fig. 2). Thus the results point out that unlike the ox erythrocyte SOD (10), the human and rat erythrocyte SODs are unstable and are differentially susceptible to alkaline pH.

The specific activities of SOD in mitochondria from different rat tissues measured at pH 8.0 and 9.2 are shown in Fig.3. At pH 8.0, the specific activity was highest in the liver followed by kidney and brain. The activity in the heart mitochondria was the lowest. The latter observation seems rather surprising in view of the reported observations that the respiration rates are the highest in the heart mitochondria (12, 13, 16). It may hence be suggested that oxygen slippage may be tightly regulated and minimum in the electron transport chain of heart mitochondria compared to that in the mitochondria from other tissues. At pH 9.2 the mitochondrial SOD from the four tissues showed differential susceptibility. The liver mitochondrial enzyme was most susceptible and 90% of the activity was lost at pH 9.2, while the losses were 60-70% in kidney and brain and only 34% in heart respectively.

For the post-mitochondrial fractions once again at pH 8.0 the liver displayed the highest specific activity whereas kidney, brain and heart showed only about 15-31% activity compared to the liver value (Fig. 3). Exposure to pH 9.2 resulted in 91% inactivation of liver enzyme which was similar to that noted above the liver mitochondrial enzyme (Fig.

3). The inactivation of the enzyme in the other tissues was almost comparable but of lesser magnitude and ranged from 66-68%.

It may be anticipated that the total content of the SOD activity in a given tissue would be important for the overall organ functions towards O_2^- dismutation. Hence it was of interest to find out the total SOD content - both mitochondrial and cytosolic - in all the tissues. The mitochondrial total SOD content was computed based on the reported values of mitochondrial protein contents in the different tissues (12, 13), while the cytosolic SOD content was computed from the volume of the 12000 X g supernatants. These results are given in Table 1.

Thus the computed content of mitochondrial SOD at pH 8.0 was the highest in the liver followed by the kidney (80 % compared to the liver value). The contents in the brain and the heart were to about 40-50% compared to liver. Consistent with the earlier observations (Fig. 3), the liver, kidney and brain mitochondrial enzymes were more susceptible to alkaline pH in that order whereas the heart enzyme was relatively more resistant. Even in the post-mitochondrial fraction the SOD content measured at pH 8.0 was the highest in the liver. Compared to this, cytosolic SOD content in the kidneys, brain and heart was only 6-11%. Measurements at alkaline pH resulted in 90% inactivation of the liver cytosolic enzyme which is similar to that noted for the mitochondrial SOD. In case of the other tissues, there was an overall 65-68 % inactivation. Thus even the heart cytosolic enzyme seemed to be more susceptible to alkaline conditions. One interesting point to note here is that in the liver the content of the

Table 1. Comparison of SOD content in mitochondrial and post-mitochondrial fractions from rat tissues.

Tissue	Mitochondria (Units/g tissue)			Post-Mitochondrial (Units/g tissue)		
	pH 8.0	pH 9.2	% Change	pH 8.0	pH 9.2	% Change
Liver	325.5 ± 13.9	31.2 ± 1.0[a]	- 90.4	1057.2 ± 20.4	93.5 ± 1.9[a]	- 91.2
Kidney	264.4 ± 9.2	83.2 ± 3.0[a]	- 68.5	116.4 ± 3.6	37.6 ± 1.2[a]	- 67.7
Brain	129.0 ± 2.1	43.2 ± 2.5[a]	- 66.5	62.5 ± 2.0	21.6 ± 1.0[a]	- 65.4
Heart	165.1 ± 4.9	108.6 ± 5.2[a]	- 34.2	77.6 ± 1.2	25.2 ± 1.2[a]	- 67.5

The experimental conditions are as described in the text. The results are given as mean ± SEM of 12 independent observations in each group.

a. $p<0.001$ Compared with the corresponding values at pH 8.0.

cytosolic enzyme was 3.25 times higher compared to the mitochondrial counterpart. Similar trend has also been reported by other researchers (!7). By contrast in the other three tissues the cytosolic enzyme content was only about half the mitochondrial contents. The higher cytosolic enzyme content in the liver may possibly relate to the higher rate of metabolic/overall synthetic activities whereby the flux of O_2^- in the cytosolic compartment may be much higher.

From the foregoing results it is clear that the SODs from the different systems had differential pH sensitivity which is evident from the differential inactivation of mitochondrial and post-mitochondrial SOD activities from different tissues. Besides, our results suggest that measurements around physiological pH can give more realistic estimations of the SOD activities. Additionally, our studies have also highlighted that the relative levels of the mitochondrial and cytosolic SOD in various rat tissues are different. It may also be pointed out here that bovine erythrocyte SOD may represents an unusual form with respect to its pH stability (10).

Since involvement of oxygen - derived free radicals in pathological/clinical conditions has been implicated (2, 3, 7), it would be of interest to find out if the levels of the mitochondrial and cytosolic SODs would change in a pathological/clinical condition.

Summary

Superoxide dismutase (SOD) activities in the human and rat RBCs and rat liver, kidney, brain and heart mitochondria as well as cytosolic fractions were determined by the pyrogallol assay procedure with slight modifications. Measurements were carried out in 0.1 M potassium phosphate buffer pH 8.0 and 9.2 to assess the pH stability of the SODs from various systems. Under these conditions the SODs from different systems including RBCs exhibited differential pH stability i.e. they displayed differential susceptibility at pH 9.2. Even in a given tissue, the mitochondrial and cytosolic SODs were inactivated differentially at pH 9.2. We also observed that the total mitochondrial and cytosolic SOD contents show a tissue-specific pattern. Our results also suggest that measurements carried out at pH 8.0 may give more realistic estimates of SOD activities.

References

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