Superoxide radical production by sponges *Sycon* sp.

Alexander V. Peskin[a,*], Yulii A. Labas[b], Alexander N. Tikhonov[c]

[a] *Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia*
[b] *Institute of Animal Morphology, Russian Academy of Sciences, Moscow, Russia*
[c] *Department of Biophysics, Faculty of Physics, M.V. Lomonosov Moscow State University, Moscow 119899, Russia*

Received 21 July 1998

Abstract Using the catechol Tiron as an $O_2^{-\bullet}$ scavenger, we showed that sea sponges (*Sycon* sp.) produce superoxide radicals in sea water at a high rate without any stimuli added. The rate of $O_2^{-\bullet}$ outflow from sponges to their water surroundings reaches a value of 0.5 nmol/min per sponge at pH 6.5. The generation of $O_2^{-\bullet}$ was inhibited by Cu,Zn-superoxide dismutase, and restored by the addition of KCN. We also confirmed the abiotic production of $O_2^{-\bullet}$ in sea water, detected earlier with a different method by Petasne and Zika [Nature 325 (1987) 516–518].

Key words: Free radical; Superoxide; Electron paramagnetic resonance; Sea water; Sea animal; Sponge

1. Introduction

The generation of reactive oxygen species is a common feature of aerobic organisms. Superoxide radicals ($O_2^{-\bullet}$) are known to be formed by oxygen metabolizing cells, and their efflux from the cells has been shown for several cell types [1–4]. However, high rates of $O_2^{-\bullet}$ generation outside the cell have been shown only for stimulated phagocytizing cells [5]. Stimulation of phagocytes causes the oxidative burst which results in the cyanide-resistant consumption of oxygen. This burst is triggered by the activation of NADPH-oxidase which is localized in the plasma membrane. Electrons from NADPH molecules localized in the cytosol are transferred by activated NADPH-oxidase across the plasma membrane to reduce the extracellular oxygen, thus providing the $O_2^{-\bullet}$ formation outside the cell [6,7]. The production of $O_2^{-\bullet}$ radicals by resting phagocytes is negligible.

In this study, we demonstrate for the first time that a whole organism without any added stimuli steadily releases $O_2^{-\bullet}$ radicals into the surroundings. Using the catechol Tiron as a $O_2^{-\bullet}$ scavenger, we showed that sea sponges, namely *Sycon* sp., produce superoxide radicals in the sea water at a high rate without any stimuli added.

2. Materials and methods

2.1. Animals and chemicals

Marine sponges, *Sycon* sp., were taken from the sea aquarium for tropical flora and fauna in the Moscow Zoo. Artificial sea water, whose composition was typical of natural sea water, was prepared in the Zoo by dissolving in distilled water the relevant amount of salt mixture purchased from Tropical Marine (Germany). Tiron (1,2-dihydrobenzene-3,5-disulfonate, Na_2 salt) and bovine Cu,Zn-superoxide dismutase (CuZnSOD) were purchased from Sigma or ICN.

*Corresponding author.
E-mail: peskin@ibrran.msk.su

2.2. EPR measurements of superoxide production

Depending on the experimental requirements, we used sea water that was equilibrated either with atmospheric air or by bubbling with air for 30 min. After dissolving Tiron, the pH of sea water was adjusted to the appropriate value by the addition of Tris. In a typical experiment, three sponges were usually placed on the bottom of an open hemisphere Japanese porcelain cup containing 3 ml of 10 mM Tiron solution in sea water. To avoid pH shifts, before placing the sponges into the cup they were soaked for 3 min in a Tiron solution of the same pH. The Tiron solution with sponges was steadily stirred by gently shaking the incubation vessel. 50 μl aliquots were picked up from the solution surrounding the sponges, and then transferred into the cell of an EPR spectrometer Varian E-4. EPR signals from the semiquinone form of Tiron were registered at room temperature (22°C), microwave power was 5 mW, modulation amplitude was 0.025 mT.

To determine the concentration of Tiron radicals, we referenced to a solution of spin label TEMPOamine (2,2,6,6-tetramethyl-1-piperidinyloxy-4-amine) as the standard.

3. Results and discussion

3.1. Abiotic formation of superoxide radicals in sea water

Earlier, Petasne and Zika demonstrated that superoxide radicals are generated in coastal sea water at an average rate of 5×10^{-7} mol/(l·h) [8]. Measuring the concentration of H_2O_2 formed as the product of $O_2^{-\bullet}$ dismutation, they evaluated the steady-state concentration of $O_2^{-\bullet}$ as 10^{-8} M. In our work, we detected the formation of superoxide radicals in sea water using Tiron as the indicator of $O_2^{-\bullet}$ radicals. Tiron is known to be a rather specific scavenger of $O_2^{-\bullet}$ radicals [9–11]. Oxidation of Tiron by superoxide radicals results in the production of the paramagnetic semiquinone form of Tiron which gives the characteristic EPR signal. Fig. 1A (spectrum a) shows that the artificial generation of $O_2^{-\bullet}$ ions by the addition of small amounts of KO_2 to distilled water induces the appearance of the EPR signal from Tiron. This signal represents the quartet of similar EPR lines. In the case of sea water (Fig. 1A, spectrum b), the EPR spectrum from Tiron reveals the splitting of first, third and fourth components that could arise due to the interaction of sea water cations with Tiron molecules.

For Tiron dissolved in distilled water, there is no visible EPR signal (Fig. 1A, spectrum c). This result means that the rate of $O_2^{-\bullet}$ production in distilled water is negligibly small, while the rate of Tiron radical dismutation is rather high. On the other hand, being dissolved in sea water, Tiron spontaneously gives the EPR signal characteristic of its semireduced form (Fig. 1A, spectrum d). This signal is similar to the EPR spectrum induced by the addition of KO_2 to Tiron solution in sea water (compare spectra b and d in Fig. 1A). Aeration of the sea water after the Tiron was dissolved did not influence the EPR signal (data not shown). On the other hand, aeration of the sea water before the addition of Tiron substantially delayed signal appearance (Fig. 1B, compare

PII: S0014-5793(98)00981-8

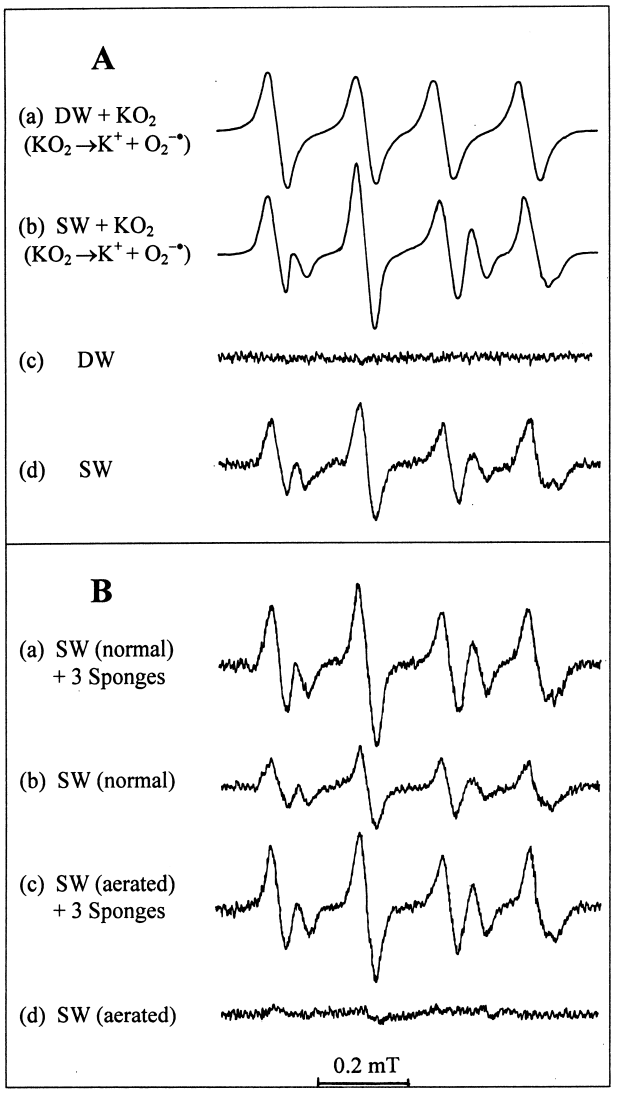

Fig. 1. EPR spectra of 10 mM Tiron. A: pH 7.4. a, c: Tiron in distilled water (DW); b, d: Tiron in sea water (SW); a, b: after addition of KO_2; c, d: without KO_2. B: EPR spectra of Tiron in sea water in the presence of three sponges (pH 6.5). a, c: EPR signals after 15 min incubation of three sponges; b, d: background EPR signals in control solutions (without sponges); a, b: Tiron was dissolved in sea water equilibrated with air; c, d: sea water was preliminarily bubbled for 30 min with air.

spectra b and d). The latter result indicates that sea water ions in the reduced state could be involved in the spontaneous abiotic formation of $O_2^{-\bullet}$ radicals and stabilization of Tiron radicals.

The pH of sea water influences the kinetics of Tiron radical formation and decay. The EPR signal intensity depends on the balance between Tiron semiquinone formation (Eq. 1) and decay (Eq. 2):

$$TH_2 + O_2^{-\bullet} + H^+ \leftrightarrow TH^\bullet + H_2O_2, \quad (1)$$

$$2TH^\bullet \leftrightarrow TH_2 + T, \quad (2)$$

$$TH^\bullet \leftrightarrow T^{-\bullet} + H^+. \quad (3)$$

The pH dependence of the signal amplitude reveals the bell-like shape with a maximum at pH 7.6 (Fig. 2A). At alkaline pH (pH > 7.6), the formation of Tiron semiquinone (Eq. 1) is restricted by the low activity of hydrogen ions. Depending on the pH, Tiron semiquinone can be either in protonated ($TH^\bullet$) or anionic ($T^{-\bullet}$) form (Eq. 3). The shift in Eq. 3 towards the protonated form of Tiron should accelerate the dismutation reaction (Eq. 2). Thus, one can explain the decrease in the EPR signal with the acidification of the reaction medium.

For an aliquot of Tiron solution placed in the closed cell of the EPR spectrometer, repetitive recording of the EPR signal for 10–20 min did not reveal a substantial change in the signal amplitude (Fig. 2B). Such a stability of the Tiron EPR signal allowed us to measure the kinetics of $O_2^{-\bullet}$ generation by taking aliquots from the samples of sea water.

3.2. Production of superoxide radicals by sponges

After the sponges were placed into Tiron solution in sea water, we observed that the amplitude of the EPR signal from Tiron semiquinone began to overcome the background level (compare the relevant EPR spectra in Fig. 1B). The kinetics of the signal rise is presented in Fig. 3. We did observe the signal increase in the range of pH 6–8.5. This interval includes the pH value of normal sea water (see the kinetics of the signal rise at pH 8.3, Fig. 3A). After 20 min of incubation, the EPR signal reached a steady-state level which corresponds to the equilibrium between the rates of signal generation and its decay due to Tiron radical dismutation (theoretical analysis of this kinetics will be published by us elsewhere). It should be noted that the stopping of the signal rise does not mean a ceasing of $O_2^{-\bullet}$ generation. Indeed, after transferring sponges into the cup with the Tiron solution used for the background measurements, we again observed a rise of the signal over the background level (Fig. 3A).

Routinely, we measured the kinetics of $O_2^{-\bullet}$ generation at acidic pH because of the low background signal and the higher affinity of Tiron for $O_2^{-\bullet}$ radicals. The time course of the second component of the Tiron EPR signal at pH 6.5 is shown in Fig. 3B. This component does not reveal splitting and its line shape does not depend on the pH. One can see that in the presence of sponges the EPR signal increases much faster than in the control solution (Tiron solution in sea water without sponges). The rather fast increase in signal amplitude was due to the intensive production of $O_2^{-\bullet}$ radicals by sponges, because the addition of CuZnSOD to the incubation medium inhibited the increase in the EPR signal (Fig. 3B). The immediate inhibition of the signal rise by CuZnSOD strongly suggests that Tiron scavenges the $O_2^{-\bullet}$ radicals from the bulk phase of the solution.

Being suppressed by CuZnSOD, the rise of the EPR signal was restored after the addition of potassium cyanide, an inhibitor of CuZnSOD (Fig. 3B). However, the effect of potassium cyanide on the EPR signal kinetics was complex. Immediately after KCN addition to the Tiron solution, we registered initially a sharp drop of the EPR signal followed by a rather fast increase in signal amplitude. The initial drop can be explained by the non-specific influence of CN^- ions, because the decrease in the intensity of the EPR signal was also observed in sea water solution without sponges in a concentration-dependent manner (Fig. 2C). Nevertheless, in the presence of sponges the KCN-induced initial drop after the lag phase was followed by a fast rise of the EPR signal. The latter implies that the generation of $O_2^{-\bullet}$ by sponges is resist-

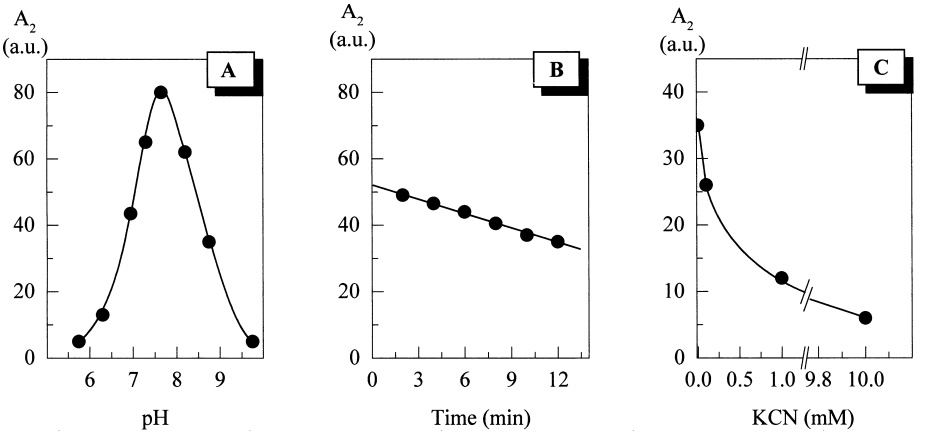

Fig. 2. A: The amplitude of the second component, A_2 (arbitrary units), of the EPR signal from Tiron versus the pH of sea water. B: The amplitude of component A_2 versus the time the sea water aliquot was kept in the closed EPR cell, pH 7.4. C: The effect of KCN on the A_2 value, pH 6.5.

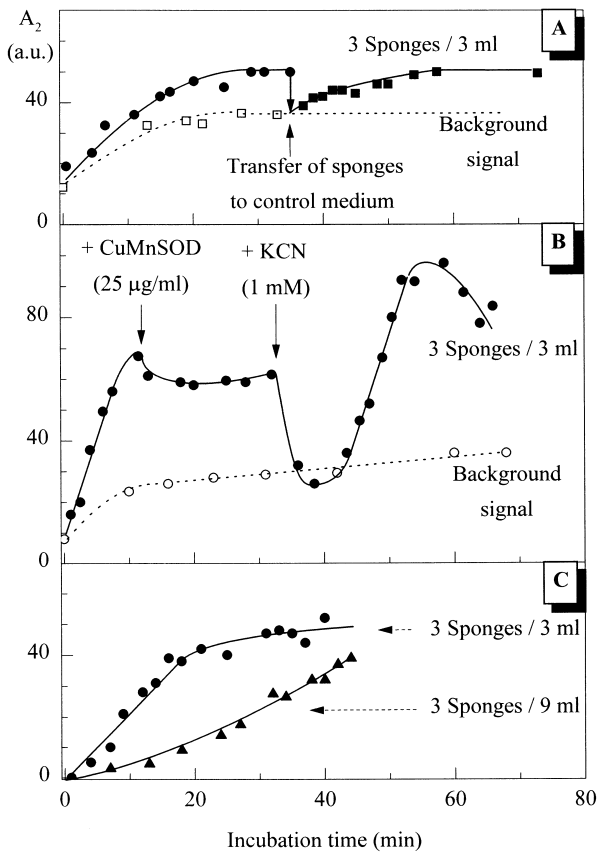

Fig. 3. The time courses of the amplitude of the second component, A_2, of the EPR signal from Tiron in sea water. Solid symbols correspond to the EPR signal generated in the presence of sponges; open symbols: control solutions (without sponges). A: pH 8.3, vertical arrow indicates the moment of transferring sponges from the incubation medium to the control solution. B: pH 6.5; the arrows indicate the additions of CuMnSOD and 1 mM KCN to the incubation medium. C: pH 6.5; three sponges were placed in 3 or 9 ml solution of Tiron as indicated. A, B: Tiron was dissolved in sea water equilibrated with air; C: sea water was preliminarily bubbled for 30 min with air.

ant to cyanide. The study of the mechanism of cyanide-insensitive superoxide generation by sponges is in progress.

The time course of the EPR signal rise depends on the ratio of the number of sponges to the volume of the incubation medium. For three sponges placed in 3 ml of sea water, the rate of the signal rise was almost three times higher than in the case of the same sponges transferred into 9 ml of incubation medium (Fig. 3C). This means that the rate of the signal rise is proportional to the average concentration of $O_2^{-\bullet}$ radicals emerging in the solution outside the sponges.

The efflux of $O_2^{-\bullet}$ from cells was observed for several cell types [1–4]. The rate of $O_2^{-\bullet}$ production usually falls in the range of 1–10 nmol/h per 10^6 cells, which is about two orders of magnitude less than that of activated phagocytes [3,5]. The time course of the Tiron signal allowed us to evaluate the rate of $TH^{\bullet}$ formation, J_T. In the presence of sponges, the J_T value is not less than 0.5 mM/min. To numerate the rate of $O_2^{-\bullet}$ production, one has to take into account the yield of Eq. 1 and the rate constant k_d of $TH^{\bullet}$ dismutation (Eq. 2). From the kinetics of Tiron radical decay (Fig. 2A), we can estimate the k_d value. However, due to the lack of information about the rate constants of Eq. 1, one cannot calculate precisely the real rate of $O_2^{-\bullet}$ generation, J_o. Nevertheless, for the initial stage of Eq. 1, we can find the lowest limit of the value of J_o by taking the relationship $J_o = J_T$. We evaluate this value as $J_o \approx 0.5$ nmol/min per sponge at pH 6.5. Taking into account the Tiron radical dismutation (Eq. 2), the real rate should be definitely higher. With the alkalization of the incubation medium (pH > 7.5), the apparent rate of $O_2^{-\bullet}$ generation determined by the Tiron method decreases (compare Fig. 3A and B).

Thus, our results demonstrate that the sea water organism, *Sycon* sp., secretes into its surroundings a significant amount of $O_2^{-\bullet}$ in the absence of any added stimuli (about 0.5 nmol/min per sponge at pH 6.5). Such a high productivity of sponges can be compared with that of beef heart submitochondrial particles (2 nmol/min per 1 mg of protein [11]). For one sponge, the total content of protein is about 20 μg. Notably, the extremely high rate of production of $O_2^{-\bullet}$ was observed at acid pH. For nutrition purposes sponges are known to consume bacteria. Therefore, the $O_2^{-\bullet}$ produced by sponges could be used as an antibacterial agent. Also, one can

suggest that *Sycon* sp. can use oxygen radicals for signal transduction.

Acknowledgements: We thank all the members of the sea aquarium department of the Moscow Zoo headed by Vladimir Emanov for the generous supply of sponges. This work was supported in part by grants from the Russian Foundation for Basic Research.

References

[1] Cross, A.R. and Jones, O.T.G. (1991) Biochim. Biophys. Acta 1057, 281–298.
[2] Kelner, M.J., Bagnell, R., Montoya, M., Estes, L., Uglik, S.F. and Cerutti, P. (1995) Free Radical Biol. Med. 18, 497–506.
[3] Turner, C.P., Toye, A.M. and Jones, O.T.G. (1998) Free Radical Biol. Med. 24, 401–407.
[4] Caraceni, P., Ryu, H.S., van Thiel, D.H. and Borle, A.B. (1995) Biochim. Biophys. Acta 1268, 249–254.
[5] Shult, P.A., Graciano, F.M., Wallow, I.H. and Busse, W.W. (1985) J. Lab. Clin. Med. 106, 638–645.
[6] Segal, A.W. and Abo, A. (1993) Trends Biochem. Sci. 18, 43–47.
[7] Chanock, S.J., el Benna, J., Smith, R.M. and Babior, B.M. (1994) J. Biol. Chem. 269, 24519–24522.
[8] Petasne, R.G. and Zika, R.G. (1987) Nature 325, 516–518.
[9] Greenstock, C.L. and Miller, R.W. (1975) Biochim. Biophys. Acta 396, 11–16.
[10] Ksenzenko, M.Yu., Konstantinov, A.A., Khomutov, G.B., Tikhonov, A.N. and Ruuge, E.K. (1983) FEBS Lett. 155, 19–24.
[11] Konstantinov, A.A., Peskin, A.V., Popova, E.Yu., Khomutov, G.B. and Ruuge, E.K. (1987) Biochim. Biophys. Acta 894, 1–10.