Generation of hydrogen peroxide in protein solutions under the influence of thermal and optical electromagnetic radiation

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Abstract. Using the method of induced chemiluminescence in solutions of bovine serum albumin (BSA) and bovine gamma globulin (BGG) under the influence of thermal and optical electromagnetic radiation, the formation of long-lived reactive protein species (LRPS) with a half-life of about 4-5 hours was registered. The oxygen effect was established, namely, the dependence of the chemiluminescence intensity of protein solutions on the concentration of dissolved oxygen. Using the enhanced chemiluminescence method in the luminol-p-iodophenol-peroxidase system, the ability of LRPS, induced by heat and laser radiation, to generate hydrogen peroxide in solution was studied. The dependence of the formation of H2O2 under the influence of LRPS at different times after exposure was established. Using the fluorescent probe coumarin-3-carboxylic acid, the formation of hydroxyl radicals in protein solutions after exposure to laser radiation and heat was detected. The obtained experimental data suggest that the generation of hydrogen peroxide by LRPS after exposure to thermal and optical electromagnetic radiation may be one of the mechanisms for activating protective cellular mechanisms that help overcome diseases, including those associated with oxidative stress.

1 Introduction

Reactive oxygen species (ROS) are constantly formed in aerobic cells as a result of normal metabolism and when exposed to external factors such as ionizing, ultraviolet, microwave radiation, xenobiotics, thermal effects, visible and infrared radiation with wavelengths corresponding to the dimolecular absorption lines oxygen, uranyl ions, etc. [1-4]. An increase in the intracellular concentration of ROS above the level of antioxidant protection causes "oxidative stress," which is accompanied by processes that are dangerous to cell activity, such as lipid peroxidation, oxidative modification of nucleic acids and proteins [5, 6]. It has been established that ROS not only damage cell molecules, but also performs a

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signaling role in the body, associated with the disruption of redox homeostasis [7]. Of all ROS in mammals, hydrogen peroxide is the main secondary messenger [8]. Changes in the content of H$_2$O$_2$ in the body, caused by various physical influences, can be an important factor in the therapeutic effect and adaptation of the organism to adverse environmental conditions as a result of signal-regulatory processes [9]. Currently, in medical practice a number of physiotherapeutic procedures are widely used, based on the use of moderate hyperthermia and sources of coherent and incoherent light exposure [10, 11]. However, the biological mechanisms of their therapeutic effects remain poorly understood [12, 13]. In this regard, it is important to study the possibility of long-term generation of H$_2$O$_2$ by long-lived reactive protein species (LRPS) after exposure to thermal and optical electromagnetic radiation. This may be one of the mechanisms of activation of protective cellular mechanisms that help overcome diseases, including those associated with oxidative stress.

2 Methods

Helium-neon laser LGN 208A (MedApparatura, Russia) with radiation at 632.8 nm (power 1.7 mW, flux density 0.7 mW/mm$^2$) was used as an artificial light source. Protein solutions in phosphate buffer (10 mM, pH 7.4) were irradiated in glass vials wrapped with a light-reflecting foil. Hyperthermia was modeled using a U-10 liquid ultrathermostat (Prüfgeräte-WerkMedigen, Germany) with an accuracy of ± 0.1 degrees. The study of LRPS was carried out by measuring the chemiluminescence of protein solutions induced by heat (2 hours at temperatures of 35-50 °C) and laser radiation using a highly sensitive chemiluminometer Biotoks-7 Ultra (Econ, Russia) [14]. The formation of H$_2$O$_2$, induced by LRPS in protein solutions under the influence of heat and laser radiation, was measured by a highly sensitive method of enhanced chemiluminescence in the luminol-p-iodophenol-horseradish peroxidase system. A counter for liquid scintillation Beta-1 (MedApparatura, Ukraine), operating in the mode for counting single photons (with one photomultiplier and the coincidence scheme disengaged), was used as a highly sensitive chemiluminometer [14]. The H$_2$O$_2$ content was determined using calibration graphs of the dependence of chemiluminescence on the known concentration of hydrogen peroxide in solution. The initial concentration of H$_2$O$_2$ used for calibration was determined spectrophotometrically at 240 nm using a molar absorption coefficient of 43.6 M$^{-1}$ × cm$^{-1}$ [15]. The concentration of hydroxyl radicals in the solution was determined using coumarin-3-carboxylic acid, a highly specific to OH-radicals fluorescent probe [16]. Protein solutions were additionally saturated with argon or oxygen by bubbling for 15 min [17]. Oxygen concentration in the solution was measured with an electrode DKTP 2,4 (Jonomer, Russia) using an oximeter Ekspert-001 (Ekoniks, Russia).

3 Figures and tables

3.1 The formation of long-lived reactive protein species under the influence of thermal and optical electromagnetic radiation

Using the method of induced chemiluminescence, we studied the effect of exposure to optical radiation with a wavelength corresponding to a maximum (632.8 nm) in the absorption spectrum of molecular oxygen, and heat (40 °C, 2 h). Figure 1A shows the dependence of the chemiluminescence intensity of BSA and BGG solutions exposed to radiation with a wavelength of 632.8 nm from concentrations of proteins. The dependence of chemiluminescence intensity on the concentration of BSA and BGG had a two-phase
form. The luminescence of solutions was maximal at ~ 10 μM BSA and ~ 2 μM BGG. These concentrations were used to determine the half-life of LRPS. Considering the molecular masses of BSA and BGG (67 and 150 kDa, respectively, the ratio is 1: 2.24) and the concentration of these proteins corresponding to the maximum chemiluminescence values (0.67 g/l for BSA and 0.3 g/l for BGG; ratio 1: 2.23), it can be concluded that the maximum number of luminescent products formed by the action of optical radiation per unit mass of these proteins is approximately equal. Figure 1B shows the dependence of the chemiluminescence intensity of BSA and BGG solutions exposed to heat (40 °C, 2 h) on the protein concentration. The dependence of the intensity of chemiluminescence on the concentration of BSA and BGG had a two-phase bell-shaped form. At a concentration of 15 μM BSA and 3 μM BGG, the chemiluminescence of the solutions reached maximum values. These protein concentrations were used to determine the half-life of LRPS to reduce the chemiluminescence after heat exposure. Considering the molecular masses of BSA and BGG (67 kDa and 150 kDa, respectively; the ratio is 1: 2.24) and the concentrations of these proteins corresponding to the maximum chemiluminescence values (0.44 g/l for BSA and 1 g/l for BGG; ratio 1 : 2.27), it can be seen that the maximum number of luminescent products formed as a result of heat exposure is, on average, approximately the same, per unit mass of these proteins. Thus, it has been established that under the action of thermal and optical electromagnetic radiation, LRPS is generated in aqueous solutions of proteins, and this process depends on their concentration.

Fig. 1. The dependence of the intensity of chemiluminescence on the concentration of BSA and BGG solutions exposed to low-intensity laser radiation (632.8 nm, 5 min) and heat (40 °C, 2 h). The measurements were carried out 1 hour after exposure. Background chemiluminescence values were subtracted from the results. The mean values of three independent experiments and their standard errors are given.

Irradiation of He-Ne laser with a wavelength of 632.8 nm protein solutions for 10–30 min at BSA and BGG concentrations corresponding to maximum levels of chemiluminescence resulted in the formation of LRPS (Figure 2). An increase in the time of exposure of optical radiation to protein solutions of both BSA and BGG is accompanied by a proportional increase in the intensity of chemiluminescence (Figure 2 (A, B)). Reducing the intensity of chemiluminescence with time after irradiation allows us to determine the half-life of LRPS. The half-life of the BSA and BGG radicals was about 4–5 h after the action of optical radiation. Thus, it was shown that the half-life of BSA and BGG radicals is 4-5 h and the generation of LRPS linearly depends on the time of laser exposure (Figure 2C).
Fig. 2. Changes in the intensity of chemiluminescence of solutions (A) BSA (10 $\mu$M) and (B) BGG (2 $\mu$M) in phosphate-buffered saline during various time incubations after exposure to laser radiation (632.8 nm). The measurement was carried out 1 hour after physical impacts. Background chemiluminescence values were subtracted from the results. (C) - dependence of the chemiluminescence of BSA (10 $\mu$M) and BGG (2 $\mu$M) solutions on the exposure time of laser radiation (632.8 nm). The mean values of three independent experiments and their standard errors are given.

Heating of protein solutions (35–50 °C, 2 h) at BSA (14.7 $\mu$M) and BGG (3.3 $\mu$M) concentrations in phosphate-saline solution led to the formation of LRPS (Figure 3). An increase in temperature from 35 °C to 40 °C was accompanied by a twofold increase in the intensity of the chemiluminescence of proteins, and to 50 °C, a threefold increase. In addition, an oxygen effect was established, namely, the dependence of the chemiluminescence intensity of protein solutions on the concentration of dissolved oxygen (Figure 3C). Reducing the intensity of chemiluminescence over time after heating makes it possible to determine the half-life of LRPS. The half-life of the BSA and BGG radicals was about 4 hours when exposed to heat in the range of 35-50 °C. Thus, it is shown that with an increase in temperature, the intensity of LRPS generation increases. With an increase in the concentration of oxygen in the protein solution, the rate of formation of LRPS increases.

Fig. 3. Changes in the intensity of chemiluminescence of solutions (A) BSA (14.7 $\mu$M) and (B) BGG (3.3 $\mu$M) in phosphate-buffered saline (10 mM Na2HPO4 and 150 mM NaCl, pH 7.4) with different time incubations after heat exposure (35-50 °C, 2 h). The measurement was carried out 1 hour after heating. Background chemiluminescence values were subtracted from the results. (C) - dependence of the chemiluminescence of a solution (A) of BSA (14.7 $\mu$M) and (B) of a BGG solution (3.3 $\mu$M), measured 1 h after heating 40 °C for 2 h, on the oxygen concentration. The concentration of O2 in protein solutions was changed by bubbling with argon or oxygen before heating. The mean values of three independent experiments and their standard errors are given.
3.2 The formation of hydrogen peroxide under the action of long-lived reactive protein species induced by thermal and optical electromagnetic radiation

Fig. 4. Concentration dependence of $\text{H}_2\text{O}_2$ formation in BSA and BGG solutions induced by low-intensity laser radiation (632.8 nm) for 15 minutes (A) and heating at 45 °C for 2 hours (B). The background values of the concentration of $\text{H}_2\text{O}_2$ subtracted from the obtained results. The mean values of three independent experiments and their standard errors are given.

The formation of $\text{H}_2\text{O}_2$ was investigated under the action of He-Ne laser radiation on BSA and BGG protein solutions. The dependence of the generation of hydrogen peroxide after exposure to laser radiation for 30 min on the concentration of BSA and BGG is presented in Figure 4A. In the case of gamma globulin, the dependence is two-phase with a clearly defined maximum at a concentration of 2 μM. The dependence for BSA is also two-phase, but more flattened with a maximum of $\text{H}_2\text{O}_2$ generation at a concentration of about 10 μM. In both cases, the concentration of hydrogen peroxide after 1 hour of heating was about 4-6 nM. It was established that the formation of $\text{H}_2\text{O}_2$ under the action of laser radiation with a wavelength of 632.8 nm linearly depends on the exposure time in the range of 5-30 minutes (Figure 5). The rate of formation of hydrogen peroxide in the BSA solution is about 0.4 nM / min, and in the BBG solution - 0.3 nM / min.

Fig. 5. Dependence of hydrogen peroxide generation in BSA and BGG solutions on the time of exposure to laser radiation (632.8 nm). The background values of the concentration of hydrogen peroxide subtracted from the obtained results. The mean values of three independent experiments and their standard errors are given.

Then, the formation of $\text{H}_2\text{O}_2$ induced by LRPS in BSA and BGG solutions under the influence of heat was investigated. The dependence of the generation of hydrogen peroxide after heat exposure (45 °C, 2 h) on the concentration of BSA and BGG is shown in Figure
In the case of BGG, there is a two-phase dependence of H$_2$O$_2$ formation after heat exposure with a clearly defined maximum at 1-2 $\mu$M protein. For BSA, the two-phase dependence is more than a canopy with the highest H$_2$O$_2$ value at 10 $\mu$M protein. In both cases, after 1 h after warming up, the concentration of H$_2$O$_2$ was about 40 nM.

Concentrations of BSA and BGG, which correspond to the maximum generation of H$_2$O$_2$, were additionally used to measure the content of hydrogen peroxide within 6 hours after irradiation (Figure 6A). In control solutions without proteins, the concentration of H$_2$O$_2$ was maximum immediately after irradiation and decreased after 6 hours to background values. In a solution of BSA, a gradual increase in the concentration of hydrogen peroxide was observed for 3 hours, followed by a slow drop. In the case of BGG, the H$_2$O$_2$ concentration increased during the first hour, and then decreased gradually.

Concentrations of BGG (2 $\mu$M) and BSA (10 $\mu$M) at which the “maximum effect” was observed were later used to measure the content of hydrogen peroxide within 6 hours after heat exposure (Figure 6B). In the control solution without protein, the concentration of H$_2$O$_2$ was about 4-5 nM and decreased to 6 nM by 6 h. In the BSA solution, a gradual increase in the H$_2$O$_2$ concentration was observed for 4 h, and then its rapid decrease by 6 h. In the case of BGG, its concentration increased to 1 h, followed by a decrease to 6 nM by 6 h. It can be assumed that during the half-life of LRPS, which is about 4 hours, intense generation of H$_2$O$_2$ occurs, leading to an increase in its concentration for BSA for 4 hours and 1 hour in the case of BGG, followed by decay. Laser exposure (Figure 6A) in a BSA solution showed a gradual increase in the concentration of hydrogen peroxide for 3 hours, followed by a slow drop. In the case of BGG, the H$_2$O$_2$ concentration increased during the first hour, and then decreased gradually.

Using fluorescent probe coumarin-3-carboxylic acid (CCA), whose hydroxylation product, 7-OH-coumarin-3-carboxylic acid (7-OH- CCA), intensively fluoresces, measured the formation of hydroxyl radicals in solutions BSA and BGG after exposure they have laser radiation with a wavelength of 632.8 nm for 30 min and after 2 h of heating at 45 °C. After heating, the content of 7-OH- CCA was 9.2 ± 1.0 nM for BSA and 15.2 ± 1.9 nm for BGG (n = 3), after exposure to laser radiation - 3.5 ± 0.5 nM for BSA and 5.2 ± 0.7 nM for BGG (n = 3).

To clarify the mechanism of formation of H$_2$O$_2$ in a BSA solution under the action of laser radiation and heat, the influence of various factors on this process has been studied (Table 1). The oxygen effect is established - the effect of the concentration of dissolved oxygen on the formation of H$_2$O$_2$ under the action of LRPS. The concentration of hydrogen
peroxide generated by LRPS in a solution additionally saturated with oxygen for 15 minutes before exposure is 1.5-1.6 times higher. When the BSA solution is saturated with argon under the action of laser radiation and heat, 40% less H$_2$O$_2$ is formed. It is known that in the presence of D$_2$O, the lifetime of singlet oxygen increases. In a protein solution containing 25% D$_2$O, the concentration of hydrogen peroxide under these effects increases by 1.2-1.3 times. Sodium azide, a singlet oxygen quencher, reduces the LRPS-induced H$_2$O$_2$ generation by 40% for laser radiation and by 30% for heat. These data indicate that singlet oxygen is involved in the formation of hydrogen peroxide. The use of superoxide dismutase (SOD) has shown that superoxide anion radicals are involved in the formation of H$_2$O$_2$ in solution under the action of LRPS. SOD increased the concentration of hydrogen peroxide by 30% due to the additional dismutation of these radicals. Addition of tiron, a spin trap of superoxide anion radicals, reduced the concentration of H$_2$O$_2$ by 30%.

Table 1. The effect of various factors on the formation of H$_2$O$_2$ in a BSA solution (10 $\mu$M) under the action of a He-Ne laser (15 min) and in a solution of BSA (14.7 $\mu$M) under the action of heat (45 $^\circ$C, 2 h). The background values of the concentration of H$_2$O$_2$ subtracted from the obtained results. The mean values of three independent experiments and their standard errors are given.

| Treatment                  | [O$_2$], $\mu$M | Irradiation | Heating |
|----------------------------|----------------|-------------|---------|
| Control                    | 270            | 6.5 ± 0.6$^a$ | 1       | 39.2 ± 3.0 | 1 |
| Saturated with O$_2$$^b$   | 270            | 9.8 ± 0.8$^a$ | 1.5     | 61.7 ± 3.7$^a$ | 1.6 |
| Saturated with Ar$^b$      | 130            | 3.8 ± 0.3$^a$ | 0.6     | 23.8 ± 1.6$^a$ | 0.6 |
| D$_2$O (25% v/v)           | 270            | 8.4 ± 0.6$^a$ | 1.3     | 47.6 ± 0.8$^a$ | 1.2 |
| NaN$_3$ (0.1 $\mu$M)$^c$  | 270            | 3.8 ± 0.4$^a$ | 0.6     | 28.0 ± 2.6$^a$ | 0.7 |
| Tiron (100 nM)             | 270            | 4.3 ± 0.2$^a$ | 0.7     | 25.5 ± 3.6$^a$ | 0.7 |
| SOD (10$^{-3}$ U/ml)       | 270            | 8.7 ± 0.6$^a$ | 1.3     | 49.3 ± 2.4$^a$ | 1.3 |

Measurement was performed after 1 hour. K - change in the concentration of H$_2$O$_2$ under the influence of the studied agent relative to the control.

$^a$ – P < 0.05 vs control.

$^b$ - BSA solution was saturated for 15 min with gas before exposure to thermal and optical electromagnetic radiation by bubbling.

$^c$ - Sodium azide at this concentration did not cause a marked inhibition of peroxidase activity.

Thus, it has been established that long-lived reactive protein species, induced by heat and optical radiation, can lead to the long-term generation of hydrogen peroxide and the formation of a hydroxyl radical. Both under the action of optical radiation in the absorption bands of dimoles of molecular oxygen, and under the action of heat, a decrease in the rate of formation of H$_2$O$_2$ is observed with a decrease in the concentration of protein solutions. Using inhibitory analysis, it was shown that the superoxide anion radical and singlet oxygen are involved in the process of LRPS-induced generation of hydrogen peroxide in aqueous solutions of BSA proteins and BGG.

This work shows that the dependences of the intensity of chemiluminescence of protein solutions (Figure 1) and the formation of hydrogen peroxide (Figure 4) under the influence of thermal and optical electromagnetic radiation on the protein concentration are biphase with a maximum at a certain protein concentration. This complex two-phase nature of dependencies indicates the existence of two competing processes. Such processes can be both the generation of ROS and LRPS, and their neutralization, depending on the concentration of protein in the solution. Initially, with increasing protein concentration when exposed to radiation, the process of ROS generation dominates due to the formation of LRPS. However, when a certain protein concentration is exceeded, the processes of neutralization of ROS, in particular H$_2$O$_2$, begin to predominate. In the case of
chemiluminescence, a decrease in intensity may be caused by additional scattering or absorption of optical radiation.

It should be noted that the kinetics of the generation of hydrogen peroxide under the action of laser radiation, hyperthermia, and ionizing radiation is significantly different [14, 18-20]. Radiation-induced LRPS exhibits an almost exponential decrease in the rate of hydrogen peroxide generation after exposure. In the case of LRPS induced by laser radiation and heat, the rate of hydrogen peroxide generation after the end of exposure does not significantly decrease for several hours and even in some cases increases slightly (Figure 6). This is probably due to the different ratios of certain types of reactive oxygen forms generated on one side during radiolysis of water [1], and on the other when exposed to optical radiation with wavelengths corresponding to absorption lines of molecular oxygen dimoles [2] and hyperthermia [3]. As a result, the contributions of two possible methods for the production of H$_2$O$_2$, due to recombination of OH radicals and dismutation of hydroperoxide radicals, may be different [21].

As follows from Table 1 and data on the generation of OH radicals, LRPS -induced generation of hydrogen peroxide involves $^1$O$_2$, HO$_2^*$ and 'OH. Taking into account the formation of reactive oxygen species in water under the action of hyperthermia and optical radiation with wavelengths corresponding to the absorption lines of molecular oxygen dimoles [2,21], the following sequence of conjugate reactions can be proposed, resulting in the formation of LRPS and H$_2$O$_2$ in protein solutions:

$$\text{O}_2 + h\nu \rightarrow ^1\text{O}_2 / \text{O}_2 + kT \rightarrow ^1\text{O}_2,$$

(1)

where $h\nu$ is the quantum of energy of electromagnetic radiation, corresponding to the transition of oxygen to the singlet state [22], and $kT$ is the thermal energy [3].

$$\text{OH} \rightarrow \text{OH} + e + ^1\text{O}_2 + \text{H}^+ \rightarrow \text{HO}_2^*,$$

(2)

where $e$ is a hydrated electron. The reaction of 'OH with a hydrogen atom at the $\alpha$-carbon atom of the polypeptide chain will lead to its separation and the formation of the $\alpha$-carbon radical [23,24]:

$$\text{–NH} = \text{RC}_\alpha \text{H} = \text{CO} = + \text{‘OH} \rightarrow \text{–NH} = \text{RC}_\alpha^* + \text{CO} – + \text{H}_2\text{O}$$

(3)

The reaction with oxygen leads to the formation of a peroxyl radical in proteins [23,24]:

$$\text{–NH} = \text{RC}_\alpha^* + \text{CO} – + \text{O}_2 \rightarrow \text{–NH} = \text{RC}_\alpha \text{OO}^* –$$

(4)

Next is the elimination of hydroperoxide

$$\text{–NH} = \text{RC}_\alpha \text{OO}^* – \rightarrow \text{–N} = \text{RC}_\alpha \text{CO} = + \text{HO}_2^*$$

(5)

and the formation of hydrogen peroxide due to dismutation of hydroperoxide radicals:

$$\text{HO}_2^* + \text{HO}_2^* \rightarrow \text{H}_2\text{O}_2 + ^1\text{O}_2$$

(6)

To confirm the correctness of this mechanism, further research is needed. In this work, the participation of singlet oxygen, hydroxyl and superoxide (hydroperoxide) radicals in the formation of hydrogen peroxide was experimentally confirmed. It has been established that BSA and BGG under the action of laser radiation and heat in the presence of dissolved oxygen of the air can turn into LRPS, which subsequently generate H$_2$O$_2$ for a long period of time, as a result of conjugated electron-radical autocatalytic chain reactions [14]. It is believed that proteins are sensors that alert cells about the onset of oxidative stress. They are associated with various intracellular signaling cascades involved in the regulation of the
expression of pro- and antioxidant genes, and serve as a link between oxidative damage to cells and antioxidant defense systems [25]. Thus, a change in the H$_2$O$_2$ content and the formation of LRPS under the influence of heat and low-intensity laser radiation can be an important factor in their therapeutic effect, which contributes to the adaptation of the organism to adverse environmental factors through signal regulatory processes that are caused by the formation of hydrogen peroxide. The results are in good agreement with the results of studies by other scientists [26-37].

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