Biochemical and Biophysical Research Communications 443 (2014) 957–961

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Formation of long-lived reactive species of blood serum proteins by the action of heat

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ARTICLE INFO

Article history:
Received 4 December 2013
Available online 19 December 2013

Keywords:
Protein oxidation
Reactive oxygen species
Long-lived reactive protein species
Hydrogen peroxide
Action of heat

ABSTRACT

It has been previously established that heat induces the formation of reactive oxygen species (ROS) in aqueous solutions. In biological systems, ROS cause oxidative damage predominantly to proteins due to their abundance and sensitivity to oxidation. Proteins oxidized by the action of X-rays represent long-lived reactive species, which trigger the secondary generation of ROS (Bruskov et al. (2012) [25]). Here we studied the possibility of formation of long-lived species of the blood serum proteins bovine serum albumin and bovine gamma-globulin in air-saturated solutions under the action of heat. It is shown that heat induces the generation of long-lived protein species, which in turn generate ROS ($^1O_2$, $\cdot O_2^-$, $\cdot OH$, and H_2O_2). The formation of the long-lived reactive species of BSA and BGG with a half-life of about 4 h induced by moderate hyperthermia was revealed using the chemiluminescence of protein solutions. It was found that long-lived reactive species of BSA and BGG cause prolonged generation of H_2O_2. The results obtained suggest that H_2O_2 produced by proteins after heating represents a messenger in signaling pathways and produces therapeutic effects in living organisms.

1. Introduction

Reactive oxygen species (ROS) emerge in living organisms both during the normal cell metabolism and in response to various physicochemical factors. If the level of ROS exceeds the neutralizing capacity of cellular antioxidant systems, they induce the oxidative stress, produce a damaging effect on biological molecules, and initiate various pathological processes. However, under normal physiological conditions, ROS in cells, first of all hydrogen peroxide, mediate diverse responses as a secondary messenger [1–3]. Recent data on the role of H_2O_2 in the intra- and intercellular signaling in mammalian cells, including its influence on cellular growth, death, and aging have been reported in reviews [1–5]. A change in the H_2O_2 content induced by various physical impacts may be an important factor of the therapeutic effect and the adaptation of the organism to unfavorable environmental conditions [2]. Moderate hyperthermia and a number of physiotherapeutic procedures leading to a local warming of tissues are widely used in medical practice. However, the biological mechanisms of their curative effect are poorly understood.

Previously we have shown that moderate heating of aqueous solutions in the presence of dissolved oxygen of air causes the formation of ROS (singlet oxygen, superoxide anion, hydroxyl radicals, and hydrogen peroxide) [6–13] as the result of complex coupled chain-radical reactions. There is good reason to believe that the formation of H_2O_2 is a cause of the medical action. Proteins, due to their abundance and high reactivity to oxidation, are the major and more vulnerable targets for ROS oxidants as compared with lipids, DNA and other constituents of biological systems [14–21]. Proteins account for about 15% of the cell mass (~70% of the dry weight of the cell). The oxidation by ROS leads to the loss of specific functions of proteins in normal and pathological processes and accelerate aging [4,5,16–21].

Deleterious effects of ROS were investigated in detail in biological systems exposed to ionizing radiation. Radiation-induced lesions in the cell are formed mainly by short-lived ROS due to water radiolysis. In the presence of oxygen, ROS produce long-lived reactive protein species (LRPS), which include long-lived protein radicals and protein hydroperoxides [16,17,19,22–25]. At present, the generation of long-lived protein radicals has been demonstrated for many proteins. These species are formed by the action of γ, X-ray, and UV radiations, as well as peroxynitrite and the products of decomposition of hydrogen peroxide by immobilized

Abbreviations: BGG, bovine gamma-globulin; BSA, bovine serum albumin; CCA, coumarin-3-carboxylic acid; LRPS, long-lived reactive protein species; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.bbrc.2013.12.073

peroxidase [26]. It was found that long-lived protein radicals induce mutations and cell transformation [22] and are formed in minor amounts in cells of animals and plants during their vital activity [23]. It was shown earlier that LRPS are the source of secondary free radicals, which induce further oxidation of proteins and other biomolecules, including DNA [17–19,24,25]. Radiation-induced LRPS can generate ROS for extended periods of time, thereby producing genotoxic effects on DNA [24,25], and may be the cause of prolonged oxidative stress after the action of ionizing radiation. The neutralization of LRPS-produced ROS by some natural antioxidants administered after irradiation can eliminate the oxidative stress [24–26].

We examined here whether the serum proteins bovine serum albumin (BSA) and bovine gamma-globulin (BGG) can form LRPS under moderate hyperthermia and showed that heat induces LRPS, which in turn continuously generate ROS, in particular H_2O_2.

2. Materials and methods

2.1. Materials

The following reagents and materials were used: *p*-iodophenol, coumarin-3-carboxylic acid (CCA), 7-OH-coumarin-3-carboxylic acid (7-OH-CCA), BSA, horseradish peroxidase, superoxide dismutase (SOD), tiron (Sigma–Aldrich, USA) and bovine gamma-globulin (Serva, Germany); sodium chloride (NaCl) (Solvey, France), sodium phosphate mono- and dibasic ($NaH_2PO_4 \times 2H_2O$ and $Na_2HPO_4 \times 12H_2O$) (Amresco, USA); luminol (AppliChem, Germany); hydrogen peroxide and sodium azide (NaN_3) (KhimMed, Russia); and 99.9% D_2O (Izotop, Russia). All reagents were used without additional purification. Freshly bidistilled water had a pH of 5.8 and a conductivity of 120 μSi/m. To change the oxygen concentration in solution, 99.5% oxygen and 99% argon (Institute for High Energy Physics, Russian Academy of Sciences, Russia) were used. The gas bubbling was carried out using a water trap to remove any extraneous particulate matter.

2.2. Long-lived reactive protein species

LRPS were studied by measuring the heat-induced chemiluminescence of protein solutions using a specially elaborated highly sensitive photon-counting chemiluminometer Biotoks-7 AM (Econ, Russia). After heating, all samples were held in the dark at room temperature for 30 min. Then measurements were carried out in the dark at room temperature in 20-ml plastic polypropylene vials for liquid scintillation counting (Beckman, USA) [24–26]. The measured values of chemiluminescence were background-corrected.

2.3. Determination of hydrogen peroxide

The concentration of H_2O_2 formed in heated solutions of BSA and BGG was measured by the method of enhanced chemiluminescence in the system luminol–*p*-iodophenol–peroxidase [6–12]. The liquid scintillation counter Beta-1 (MedApparatura, Ukraine) for measuring β-radiation, which operates in the regime of counting single photons (without the scheme of coincidence), served as a chemiluminometer. Due to the high sensitivity of the method, it was possible to detect H_2O_2 at concentrations of less than 1 nM. The H_2O_2 content was determined using the calibration plots of the dependence of chemiluminescence on the known concentration of hydrogen peroxide in solution. The initial H_2O_2 concentration used for the calibration was determined spectrophotometrically at 240 nm using the molar absorption coefficient of 43.6 M^{-1} cm^{-1}.

2.4. Determination of the concentration of hydroxyl radicals

The concentration of hydroxyl radicals in solution was determined using coumarin-3-carboxylic acid, a fluorescence probe specific to OH radicals [8,12,27]. The final concentration of CCA in working solutions was 0.5 mM. The fluorescence intensity was measured on a Cary Eclipse spectrofluorimeter (Varian, Australia) with λ_{ex} = 400 nm and λ_{em} = 450 nm. The calibration was carried out using commercial 7-OH-CCA.

2.5. Determination of the concentration of dissolved oxygen

Protein solutions were additionally saturated with argon or oxygen by bubbling for 15 min. The oxygen concentration in solution was measured with the aid of an oxymetric electrode DKTP 02.4 on an Ekspert-001 device (Ekoniks, Russia).

2.6. Statistical analysis

The results are expressed as the means and standard errors of means (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by the Fisher's post hoc test. Differences were considered significant at $P < 0.05$.

3. Results

Chemiluminescence is a suitable technique for the detection and investigation of long-lived protein radicals [24–26]. Fig. 1 shows the concentration dependence of BSA and BGG solutions heated at 40 °C for 2 h on luminescence intensity. The plot of the chemiluminescence intensity on the concentration of BSA and BGG had a biphasic bell shape. The chemiluminescence of the solutions was maximal at ~15 μM BSA and ~3 μM BGG. These concentrations were used for the determination of the half-life of LRPS. Considering the molecular weights of BSA and BGG (67 and 150 kDa, respectively; the ratio 1:2.24) and the concentrations of these proteins corresponding to the maximum values of chemiluminescence (0.44 g/l for BSA and 1 g/l for BGG; the ratio 1:2.27), one can conclude that the maximum amount of luminescent products formed by the action of heat per unit mass of these proteins is approximately equal.

Heating the protein solutions at 35–50 °C for 2 h at a concentration of BSA and BGG of 6.7 μM [22] in 10 mM Na_2HPO_4 and

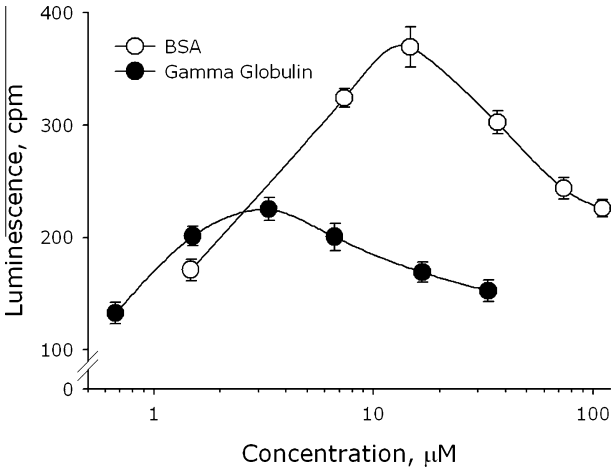

Fig. 1. Effect of different concentrations of BSA heated at 40 °C for 2 h on luminescence intensity. Measurements were carried out 1 h after the exposure. The data are given as the means ± SEM of three separate experiments. The background luminescence values were subtracted from the results.

150 mM NaCl, pH 7.4, led to the formation of long-lived protein radicals (Fig. 2). The elevation of temperature from 35 to 40 °C was accompanied by a twofold increase in the intensity of protein chemiluminescence, whereas if it was elevated from 35 to 50 °C, the increase in chemiluminescence was threefold. In addition, the oxygen effect was revealed, namely, the dependence of protein chemiluminescence on the concentration of dissolved oxygen in solutions (Fig. 2, inset).

A decrease in chemiluminescence with time after thermal treatment enables one to determine the half-life of LRPS. The half-life of BSA and BGG radicals was about 4 h after the heating in the range of 35–50 °C. Thus, it was shown that moderate heating leads to the formation of long-lived protein radicals due to protein oxidation.

Further, we examined the formation of H_2O_2 upon the heating of protein solutions. The dependence of H_2O_2 production after a 2-h heating at 45 °C on the concentration of BSA and BGG is shown in Fig. 3. In the case of BGG, the dependence is biphasic with a clearly defined maximum at a concentration of 1–2 μM. The dependence for BSA is also biphasic but more flattened with the maximum of H_2O_2 generation at a concentration of 10 μM. In both cases, the H_2O_2 concentration a 1-h after heating was about 40 nM.

To determine the mechanism of hydrogen peroxide formation in a BSA solution by the action of heat, the effect of different factors on this process was studied (Table 1). It was found that the concentration of dissolved oxygen affects the LRPS-induced generation of hydrogen peroxide (the oxygen effect). The LRPS-induced generation of hydrogen peroxide in a solution additionally saturated with oxygen and argon for 15 min before the treatment was 1.6 times higher and by 40% lower than in control, respectively. In the presence of 25% D_2O, which increases the life-time of $^1O_2$, the concentration of H_2O_2 rose 1.2 times. Sodium azide, a quencher of $^1O_2$, decreased the LRPS-induced H_2O_2 generation by 30%. These data indicate that singlet oxygen is involved in the formation of H_2O_2. Using SOD, it was shown that superoxide anion radicals participate in the formation of hydrogen peroxide in aqueous solution by the action of LRPS. SOD increased the H_2O_2 concentration by 30% due to the additional dismutation of these radicals. The addition of Tiron, a spin scavenger of superoxide radicals, decreased the H_2O_2 concentration by 30%. The concentrations of BGG and BSA

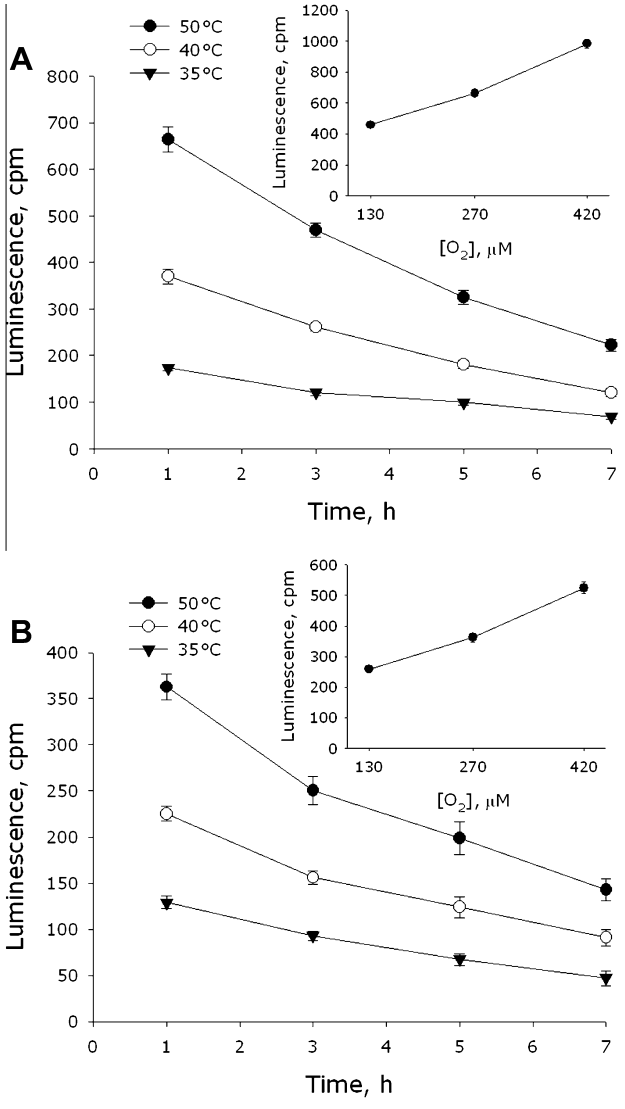

Fig. 2. Time dependence of the luminescence intensity decay of solutions of (A) BSA (14.7 μM) and (B) BGG (3.3 μM) in phosphate-buffered saline (10 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4) after heating for 2 h. The background luminescence values were subtracted from the results. The data are the means ± SEM of three separate experiments. Inset: Dependence of chemiluminescence of (A) BSA solution (14.7 μM) and (B) BGG solution (3.3 μM) measured 1 h after its heating at 40 °C for 2 h on the concentration of oxygen. The O_2 concentration was changed by bubbling argon or oxygen through the solutions. The data are the means ± SEM of three separate experiments.

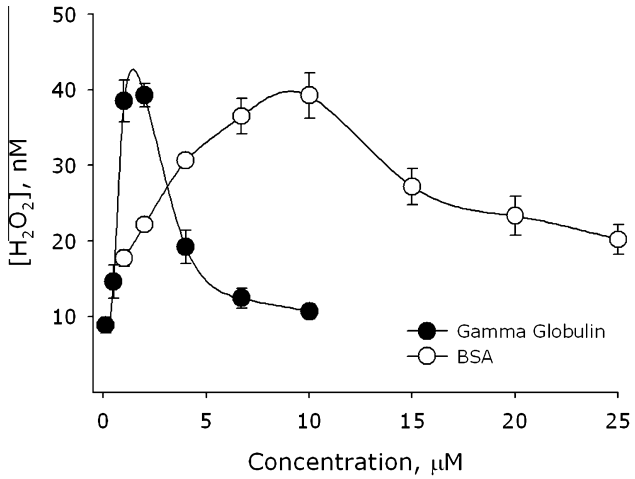

Fig. 3. Concentration dependence of hydrogen peroxide formation in BSA and BGG solutions, induced by heating at 45 °C for 2 h. The background values of hydrogen peroxide concentration were subtracted from the results. The data are the means ± SEM of three separate experiments.

Table 1
The effect of different factors on H_2O_2 formation by the action of heat (45 °C, 2 h) in aqueous solutions of BSA (1 g/l). The background values of hydrogen peroxide concentration were subtracted from the results. The data are the means ± SEM of three separate experiments.

Treatment	$[O_2]$, μM	$[H_2O_2]$, nM	K[a]
Control	270	39.2 ± 3.0	1
Saturated with O_2[b]	420	61.7 ± 3.7*	1.6
Saturated with Ar[b]	130	23.8 ± 1.6*	0.6
D_2O (25% v/v)	270	47.6 ± 0.8*	1.2
NaN_3 (0.1 μM)[c]	270	28.0 ± 2.6*	0.7
Tiron (100 nM)	270	25.5 ± 3.6*	0.7
SOD (10^{-3} U/ml)	270	49.3 ± 2.4*	1.3

[a] K, changes in H_2O_2 concentration by the action of the agent examined relative to the control.
[b] BSA solution was saturated for 15 min by bubbling with gas prior heating.
[c] Sodium azide at this concentration did not markedly inhibit the activity of peroxidase.
* $P < 0.05$ vs control.

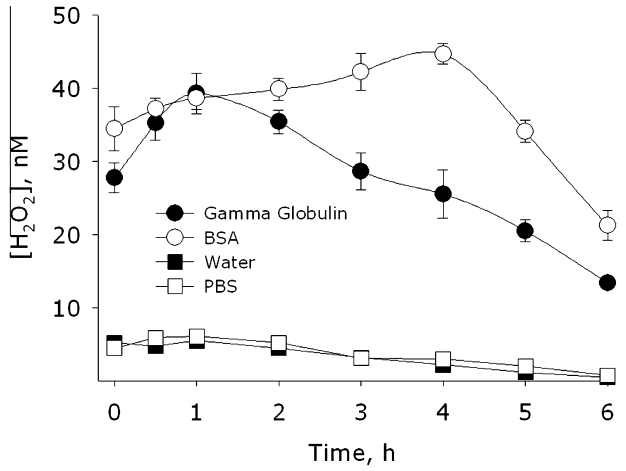

Fig. 4. Formation of hydrogen peroxide induced in BSA (10 μM) and BGG (2 μM) solutions at different time intervals after their heating at 45 °C for 2 h and cooling to 25 °C. The background values of hydrogen peroxide concentration were subtracted from the results. The data are the means ± SEM of three separate experiments.

that correspond to the maximal H_2O_2 generation (2 and 10 μM, respectively) were used further for measuring the H_2O_2 content within 6 h after the thermal treatment (Fig. 4). In control solutions without the proteins, the H_2O_2 concentration was about 4–5 nM and decreased by 6 h to 1 nM. In the BSA solution, a smooth increase in H_2O_2 concentration over a period of 4 h followed by its rapid fall by 6 h was observed. In the case of BGG, the H_2O_2 concentration increased within the first hour and then decreased to about 13 nM by 6 h. Using the fluorescent probe CCA whose hydroxylation product 7-OH-coumarin-3-carboxylic acid intensively fluoresces, we measured the formation of hydroxyl radicals in BSA and BGG solutions after heating for 2 h at 45 °C. The content of 7-OH-CCA was 9.2 ± 1.0 nM for BSA and 15.2 ± 1.9 nM for BGG ($n = 3$).

4. Discussion

It was found previously that long-term irradiation of immunoglobulins with ultraviolet low-intensity rays at 312 nm leads to the generation of H_2O_2 from dissolved air oxygen through its excitation into the singlet state [28]. In this case, the H_2O_2 production was predominantly observed in BGG solutions and significantly exceeded that in the case of albumins and other proteins at equimolar concentrations. In our experiments, we activated proteins by heating and observed no pronounced capacity of BGG to generate H_2O_2 as compared with BSA. It was shown that the UV-irradiation-induced activation of proteins, accompanied by the formation of singlet oxygen with subsequent H_2O_2 production, results from the excitation of aromatic amino acids [29]. However, no higher specificity toward BGG compared with BSA was observed [29].

It should be noted that, in the case of BSA, the kinetics of H_2O_2 production during the LRPS generation induced by heat significantly differs from that induced by X-rays [24,25]. In the case of radiation-induced LRPS, an almost exponential decrease in the H_2O_2 concentration takes place within 30 min after the irradiation. In the case of the heat-induced LRPS, the H_2O_2 production first increases within 4 h for BSA and 1 h for BGG and then decreases. This difference may be due to the different ratios of individual ROS species generated in water radiolysis and heat exposure. As a result, the contributions of two possible ways of H_2O_2 production, owing to the recombination of OH radicals and dismutation of hydroperoxide radicals, are different [6,11,12].

It follows from Table 1 and the data on the generation of OH-radicals that, in the formation of H_2O_2 by LRPS, $^1O_2$, $HO_2^{\cdot}$ and $^{\cdot}OH$ are involved. Taking into account the generation of ROS in water by the action of heat [6–13], the following sequence of conjugated reactions leading to the formation of LRPS and H_2O_2 in protein solutions can be proposed:

$$O_2 + h\nu \rightarrow {}^1O_2 \quad (1)$$

where $h\nu$ is the quantum energy of thermal electromagnetic radiation corresponding to the transition of oxygen into the singlet state [6–13].

$$OH^- \rightarrow {}^{\cdot}OH + e^- + {}^1O_2 + H^+ \rightarrow HO_2^{\cdot} \quad (2)$$

where e^- is a hydrated electron.

A reaction of $^{\cdot}OH$ with the hydrogen atom at the α-carbon atom of the polypeptide chain will result in its abstraction and the formation of an α-carbon-centered radical [30,31]:

$$-NH-RC_{\alpha}H-CO- + {}^{\cdot}OH \rightarrow -NH-RC_{\alpha}^{\cdot}-CO- + H_2O \quad (3)$$

The reaction with oxygen results in the formation of peroxyl radical in proteins [31]:

$$-NH-RC_{\alpha}^{\cdot}-CO- + O_2 \rightarrow -NH-RC_{\alpha}OO^{\cdot}-CO- \quad (4)$$

Then the elimination of hydroperoxide

$$-NH-RC_{\alpha}OO^{\cdot}-CO- \rightarrow -N=RC_{\alpha}-CO- + HO_2^{\cdot} \quad (5)$$

and the formation of H_2O_2 due to dismutation of hydroperoxide radicals take place:

$$HO_2^{\cdot} + HO_2^{\cdot} \rightarrow H_2O_2 + {}^1O_2 \quad (6)$$

To verify the validity of this mechanism, further investigations are obviously required. The α-carbon radical can be formed as a result of the detachment of the proton from the side residues of β-, γ- and δ-groups of amino acids and the transfer of the damage to α-carbon in proteins due to the stability of the delocalized α-carbon radical [31].

Thus, we found a new fundamental property of serum proteins: by the action of moderate heating in the presence of dissolved air oxygen, they are able to transform into LRPS that produce H_2O_2 over a long period of time, apparently as a result of coupled electron radical chain autocatalytic reactions [25]. Seemingly, the formation of heat-induced LRPS is not restricted only to blood serum proteins but must be inherent in at least the majority of proteins, as it occurs in the case of X-radiation [25].

The impression may form that the concentration of H_2O_2 produced by reactive protein species of serum in our experiments in vitro is too low to have a marked biological effect. But H_2O_2 acts as a secondary messenger at concentrations from a few to hundreds of nanomoles per liter [1]. It should be emphasized that we register the average concentration of H_2O_2 in strongly diluted solutions, and its local concentration near the protein surface can be substantially higher. Because the concentration of serum proteins in the organism of mammals and humans is by more than two orders of magnitude higher than protein concentrations used in our experiments, the extrapolation-derived estimates show that long-term generation of H_2O_2 by heat-activated serum proteins *in vivo* may reach micromolar concentrations. The concentration of H_2O_2 in human blood plasma is no higher than 250 nM [32]. Therefore, it cannot be excluded that the heat treatment during physiotherapeutical procedures in medicinal practice is accompanied by local heating and the formation of LRPS. Hydrogen peroxide generated by these species may participate in signaling pathways and induce adaptive response in humans [1–4]. Moreover, it may be assumed that the temperature elevation in warm-blooded organisms in diseases is accompanied by the

formation of LRPS and long-term generation of H_2O_2. Probably, this is a trigger to activate protecting cellular mechanisms that contribute to the overcoming of the disease. The oxidation of proteins necessitates their turnover by means of degradation of damaged species and biosynthesis of new ones. It is also necessary to take into account that warming is associated with the synthesis of heat-shock proteins in cells, which are involved into signal transduction [33], possibly due to H_2O_2 formation.

It is believed that proteins are the oxidative sensors preventing cells from oxidative insults of OH-radicals and singlet oxygen. They are linked with various cellular signaling pathways, which are engaged in regulating pro- and antioxidant genes expression, and mediate cells oxidative injury and antioxidant defense system [34–37].

However, it should be taken into consideration that the heat-induced formation of ROS above the capacity of antioxidant protection systems of cells to inactivate them may lead to similar consequences as the action of ionizing radiation.

Acknowledgment

This work was supported in part by the Russian Foundation for Basic Research (Project Nos. 10-04-008000-a, 10-04-00949-a, and 13-04-00730-a).

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