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INTENSITY- AND TIME COURSE-BASED CLASSIFICATIONS OF OXIDATIVE STRESSES

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Abstract. In living organisms, production of reactive oxygen species (ROS) is counterbalanced by their elimination and/or prevention of formation which in concert can typically maintain a steady-state (stationary) ROS level. However, this balance may be disturbed and lead to elevated ROS levels and enhanced damage to biomolecules. Since 1985, when H. Sies first introduced the definition of oxidative stress, this area has become one of the hot topics in biology and, to date, many details related to ROS-induced damage to cellular components, ROS-based signaling, cellular responses and adaptation have been disclosed. However, some basal oxidative damage always occurs under unstressed conditions, and in many experimental studies it is difficult to show definitely that oxidative stress is indeed induced by the stressor. Therefore, usually researchers experience substantial difficulties in the correct interpretation of oxidative stress development. For example, in many cases an increase or decrease in the activity of antioxidant and related enzymes are interpreted as evidences of oxidative stress. Careful selection of specific biomarkers (ROS-modified targets) may be very helpful. To avoid these sorts of problems, I propose several classifications of oxidative stress based on its time-course and intensity. The time-course classification includes acute and chronic stresses. In the intensity based classification, I propose to discriminate four zones of function in the relationship between "Dose/concentration of inducer" and the measured "Endpoint": I – basal oxidative stress zone (BOS); II – low intensity oxidative stress (LOS); III – intermediate intensity oxidative stress (IOS); IV – high intensity oxidative stress (HOS). The proposed classifications may be helpful to describe experimental data where oxidative stress is induced and systematize it based on its time course and intensity. Perspective directions of investigations in the field include development of sophisticated classifications of oxidative stresses, accurate identification of cellular ROS targets and their arranged responses to ROS influence, real *in situ* functions and operation of so-called "antioxidants", intracellular spatiotemporal distribution and effects of ROS, deciphering of molecular mechanisms responsible for cellular response to ROS attacks, and ROS involvement in realization of normal cellular functions in cellular homeostasis.

Keywords: oxidative stress, time-course intensity, classification, free radicals, reactive oxygen species.

Abbreviations: BOS, basal oxidative stress; HOS, high intensity oxidative stress; IOS, intermediate intensity oxidative stress; LOS, low intensity oxidative stress; NOE, no observable effect point; RNS, reactive nitrogen species; ROS, reactive oxygen species; RS, reactive species; ROSISP, ROS-induced ROS-sensitive parameter; ZEP, zero equivalent point.

1. INTRODUCTION

Free radicals were discovered by Moses Gomberg (born in 1866, Yelizavetgrad, Russian Empire, now Kirovohrad, Ukraine) more than a century ago [17]. For a long time it was believed that they did not exist in biological systems due to their short life time resulting from high chemical activity. In the late 1930s, however, Leonor Michaelis proposed that all oxidation reactions involving organic molecules would be mediated by free radicals [43]. This actually incorrect prediction stimulated interest in the role of free radicals in oxidative biological processes. In the early 1950s, free radicals were detected in biological systems [13] and virtually immediately were applied to diverse phenomena including human pathologies [16], and aging [19]. Discovery of the presence of free radicals in biological systems was the first critically important finding in the field of free radical research in living organisms. Since that time, our knowledge on the involvement of free radicals in diverse processes in living organisms has increased enormously. In the 1970s, H. Sies and B. Chance used noninvasive spectrophometric method to evaluate the operation of catalase *in vivo*, which provided information on steady-state hydrogen peroxide levels in perfused rat liver [61]. This work was virtually the first attempt to characterize ROS homeostasis in animal tissues. In the 1980th, it became clear that generation and elimination of free radicals in living organisms are normally well-balanced and imbalances between these two processes underlie many pathologies.

At the beginning of free radical research in living organisms, serious debates took place, because it was supposed that if free radicals really did exist in biological systems, the latter should possess systems controlling the levels of reactive species (RS), particularly reactive oxygen species (ROS), *i.e.* some mechanisms for their elimination should exist. Therefore, the second principal discovery in free radical research in biological systems was extremely important. In 1969, J. McCord and I. Fridovich described a new function for an already well-known protein – erythrocuprein (hemocuprein); this enzyme was found to catalyze the dismutation of the superoxide anion radical and subsequently was renamed superoxide dismutase [42]. The third critically important discovery in the field showed that free radicals were not always deleterious but actually had beneficial biological functions as well. Their involvement in combating infection as part of the cellular immune response, where ROS, reactive nitrogen species (RNS), and reactive halogen species operate in concert with other RS to fight invading microorganisms was disclosed [2, 3, 9, 15, 50, 59]. Finally, identification of the signaling functions of ROS and RNS was the fourth principle discovery in free radical biology [23, 29, 31, 46, 51, 59, 65, 68, 71]. These four discoveries, along with the deciphered mechanisms of finely regulated RS production and their involvement in diverse homeostatic processes, were used to propose and develop Denham Harman's Free Radical Theory of Aging [19, 20]. It seems now that of all theories of aging, Harman's Free Radical Theory of Aging is the most consistent and, moreover, the most experimentally supported aging concept. However, it is also challenged by certain experimental data, and, therefore, needs further investigation.

Generally, the main problems in the investigation of free radical processes in living organisms are related to: (*i*) the high reactivity and low stability of free radicals; (*ii*) their low concentrations; (*iii*) absence of technical tools for reliable evaluation of absolute and sometimes even relative levels of free radicals *in vivo*; (*iv*) their low chemical specificity; (*v*) the huge diversity of reactions that radicals can take part in; (*vi*) complicated spatiotemporal distribution in the cell; (*vii*) for multicellular organisms, the heterogeneity of cells in organs and tissues; (*viii*) changes in free radical processes depending on organism's physiological state.

Due to the reasons listed above and many other ones, investigations of the processes involving RS and interpretation of experimental data are very complicated. For example, in many cases the same compounds at the same concentrations may increase or not affect the observable level of RS-modified molecules or increase/decrease activities of antioxidant enzymes, and yet all of these different states have been declared to represent the state of oxidative stress after introduction of this definition in 1985 [57]. In the present paper, using data from our laboratory as well as the literature ones, I propose explanations for the frequent contradictions in results found at analysis of RS-induced stresses. This

paper will focus only on primary oxidative stress induced by ROS, because it seems to be the simplest situation for description and analysis and the best-studied stress induced by ROS, the most commonly studied types of radicals. The state of secondary oxidative stress induced indirectly, for example, by heat shock, energy exhaustion, starvation, overfeeding and others will not be covered here in order to simplify presentation of the key ideas.

2. What are Free Radicals and Reactive Oxygen Species?

From the chemical point of view, a free radical is any atom, molecule or its part (particle) possessing unpaired excited electron(s) in external molecular or atomic orbitals. The negative electrical charge of electron(s) may be counterbalanced by the positive nuclear charge of protons resulting in a neutral particle, or if not counterbalanced results in anion or cation radicals. However, in biology there is another popular understanding of free radicals, less accurate, but widely used and, since we work in this field we will also use this broadly accepted understanding of free radicals. So, according to common biological understanding, a free radical is an unstable particle (atom or molecule or its part) possessing unpaired electron(s) in external atomic or molecular orbitals [18].

From the biological point of view, the dioxygen molecule (O_2) is a biradical, because it contains two electrons with the same spin in external antibonding molecular orbitals. Due to Hund's restriction rules, these should be located in different orbitals and, therefore, are not paired. They can be identified by electron paramagnetic resonance technique, because they interact with an electromagnetic field [40]. Molecular oxygen can be reduced via a four-electron mechanism with acceptance of four protons yielding two water molecules (Fig. 1). In this case, the free biradical is simply converted to a nonradical species due to acceptance of the four electrons and four protons. However, there is another way to reduce molecular oxygen – this is one-electron successive reduction (Fig. 1).

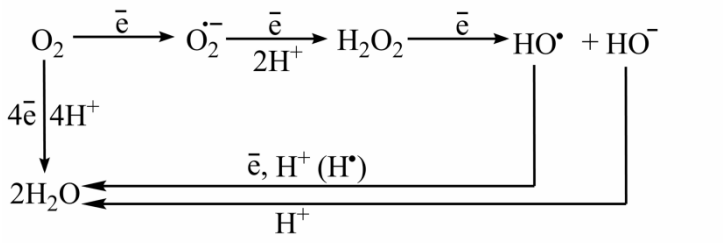

Fig. 1. Reduction of molecular oxygen via four– and one-electron schemes.

Receiving one electron, O_2 is converted to the superoxide anion radical ($O_2^{\bullet-}$), containing one unpaired electron in an external antibonding orbital. Accepting the second electron and two protons, the superoxide anion radical is converted into hydrogen peroxide (H_2O_2). The latter has a non-radical nature and is chemically more active than molecular oxygen, but less active than $O_2^{\bullet-}$. Formation of the most reactive of oxygen species, the hydroxyl radical ($HO^{\bullet}$), results from the further reduction of H_2O_2. Finally, acceptance of a fourth (final) electron and one more proton $HO^{\bullet}$ forms a water molecule. Usually, the chance directly and separately to bind an electron and proton is negligible, and this reaction generally occurs via the abstraction of a hydrogen atom from any substrate that may lead to free-radical chain reactions. Since $O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$ are chemically more reactive than molecular oxygen, they are collectively called ROS, but only $O_2^{\bullet-}$ and $HO^{\bullet}$ are actually free radicals, whereas H_2O_2 is not. Therefore, in biological research, the term "free radicals" is frequently replaced by "reactive oxygen species" (ROS), which is a more general term and includes both free radical and non-radical species. Singlet oxygen and various inorganic and organic peroxides as well as many other oxygen-containing compounds are also included in ROS group. It must be added that generally ROS are more

chemically active due to cancelling of restriction of the ground state (triplet) oxygen. Finally, it should be noted that in many cases the terms "oxygen free radicals" and "reactive oxygen species" are used interchangeably; in many cases this is not correct and authors should pay attention to the correct use of these terms.

3. Generation and Elimination of Reactive Oxygen Species

It is believed that in eukaryotic organisms more than 90% of ROS are produced by the mitochondrial electron-transport chain [59, 63]. Some amounts of ROS are also formed by electron transport chains located in plasmatic [38], nuclear [67] and endoplasmic reticulum [8] membranes. ROS generation takes place, because some active electrons "escape" electron transport carriers and reduce molecular oxygen to yield $O_2^{\bullet -}$. Superoxide is then spontaneously or enzymatically converted to H_2O_2. The latter accepting one more electron is converted to $HO^{\bullet}$ and OH^- in reaction that is frequently catalyzed by transition metal ions (Fe^{2+} or Cu^+). Finally, $HO^{\bullet}$ and HO^- receiving hydrogen atom or proton, respectively, are converted to water. Many oxidase enzymes, such as oxidases of xanthine, carbohydrates, aldehydes, monoamines and amino acids also form ROS.

Figure 1 demonstrates relationships between molecular oxygen, water and ROS. $O_2^{\bullet -}$ can spontaneously interact with an electron donor and accepting two protons be converted to H_2O_2. This reaction is substantially accelerated by superoxide dismutase (SOD, EC 1.15.1.1):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \quad (1)$$

Reduction of H_2O_2 leads to the formation of $HO^{\bullet}$ and OH^-. Hydroxyl radical is the most reactive of all ROS and oxidants known up to now. There is no enzymatic system to defend living organisms against $HO^{\bullet}$, and, therefore, prevention of its formation is the most efficient way of protection against this highly reactive oxidant. There are several enzymatic systems dealing with H_2O_2. Catalase (EC 1.11.1.6) dismutates H_2O_2 to water and molecular oxygen:

$$2\ H_2O_2 \xrightarrow{catalase} 2\ H_2O + O_2 \quad (2)$$

There is also a large family of peroxidases that degrade other hydroperoxides as well as H_2O_2. For example, glutathione-dependent peroxidases (GPx, EC 1.11.1.9) can reduce H_2O_2 and lipid peroxides (LOOH) at the expense of reduced glutathione:

$$H_2O_2 + 2\ GSH \xrightarrow{GPx} 2\ H_2O + GSSG \quad (3)$$

$$LOOH + 2\ GSH \xrightarrow{GPx} LOH + GSSG + H_2O \quad (4)$$

The level of reduced glutathione is maintained/replenished by the reduction of glutathione disulfide by glutathione reductase (GR, EC 1.6.4.2):

$$GSSG + 2NADPH \xrightarrow{GR} 2GSH + 2\ NADP^+ \quad (5)$$

In some organisms, such as *Drosophila*, thioredoxin glutathione reductase (Dm TrxR-1, or TR, EC a1.8.1.B1) replaces GR for the replenishment of GSH (reaction 5).

Finally, the oxidized coenzyme $NADP^+$ is reduced to NADPH by several enzymes. This mainly involves pentose phosphate pathway (PPP) enzymes, namely glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.43):

$$NADP^+ + \text{glucose-6-phosphate} \xrightarrow{\text{G6PDH}} NADPH + \text{6-phosphoglucolactone} \quad (6)$$

$$NADP^+ + \text{6-phosphogluconate} \xrightarrow{\text{6PGDH}} NADPH + \text{ribuloso-5-phosphate} + CO_2 \quad (7)$$

In some tissues, particularly in the brain, malate dehydrogenase (oxaloacetate-decarboxylating) utilizing $NADP^+$ called also as NADP-malic enzyme (NADP-ME, EC 1.1.1.40) catalyzing reaction (8) may also be important producer of NADPH:

$$\text{(S)-malate} + NADP^+ \xleftrightarrow{\text{NADP-ME}} \text{pyruvate} + CO_2 + NADPH \quad (8)$$

$NADP^+$-isocitrate dehydrogenase (IDH, threo-DS-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) also provides substantial NADPH amounts in some cases:

$$\text{Isocitrate} + NADP^+ \xrightarrow{\text{IDH}} \text{2-oxoglutarate} + CO_2 + NADPH \quad (9)$$

The above enzymatic systems are responsible for elimination of $O_2^{\bullet-}$ and H_2O_2, and, therefore, prevent $HO^\bullet$ formation. Usually, these enzymes are grouped in two sets – the first set contains so-called primary antioxidant enzymes that directly deal with ROS (SOD, catalase, and other peroxidases), whereas the second set includes so-called associated or auxiliary antioxidant enzymes, assisting the first group. For example, these provide the reducing equivalents needed for ROS elimination (*e.g.* GR, TRR, G6PDH, 6PGDH, NADP-ME, IDH, *etc.*). The antioxidant enzymes and other proteins involved in antioxidant defense collectively form a group called high molecular weight (mass) antioxidants. Other antioxidants belong to a group of low molecular weight (mass) antioxidants. This includes compounds with molecular mass usually less than 1000 unified atomic mass (carbon) units or Daltons, (overall molecular mass < 1000) such as vitamins C and E, carotenoids, anthocyanins, glutathione (GSH), uric acid and many other natural or synthetic compounds. It should be noted that low molecular mass antioxidants may protect organisms against $HO^\bullet$. In concert, low and high molecular mass antioxidants form a unique and very efficient system to maintain ROS levels within in a certain range [60].

Under homeostatic conditions in organisms, the operation of two systems, generation and elimination of ROS, is well balanced due to which the steady state ROS, at least H_2O_2, level is maintained well below 10 nM [59]. However, even if the elimination systems work ideally, some ROS escape them resulting in basic level of modification of cellular components. Due to that, we always find some amount of ROS-modified biomolecules in unstressed organisms. This is so-called basic level of ROS-induced modification of cellular components.

4. Routinely Used Markers of ROS-Induced Modification of Cellular Components

It seems that despite their high chemical reactivity most generated ROS do not lead to serious deleterious physiological consequences for organisms. That is mainly due to the action of highly efficient systems of ROS neutralization operating in concert with reparation and elimination of ROS-modified molecules. Thus, a certain level of ROS-modified molecules always exists, that may be called the basal steady-state (stationary) level [29, 31, 33, 35, 59]. Reactive oxygen species can modify most types of biomolecules including proteins, lipids, carbohydrates, nucleic acids, metabolic intermediates, etc. It is widely accepted that the use of only one type of modification to assess oxidative damage during oxidative stress is not sufficient. That is due to the different sensitivity, dynamics, and nature of ROS-promoted modifications. Instead, in order to evaluate the intensity of ROS-involving processes, several approaches for the evaluation of particular oxidatively modified molecules have been selected. They reflect the level of products of interaction between ROS and cellular components of different natures. "Classically", several essential markers are used. They are: (*i*) for lipids – formation of malonic dialdehyde, isopsoralens, and lipid peroxides; (*ii*) for proteins – protein carbonyl groups; and (*iii*) for DNA – 8-oxoguanine. Malonic dialdehyde is commonly measured via its reaction with thiobarbituric acid (TBA). However, this reaction is not specific and many other compounds react with TBA under the assay conditions (high temperature and low pH). The array of products formed is collectively called thiobarbituric acid reactive substances (TBARS) to reflect this low chemical specificity. Certain amino acids, carbohydrates, aldehydes and other compounds interfere with the reaction measurement and, therefore, this method should be used with precaution and discussed taking into account the highlighted issues [37]. In the last decades, an HPLC technique was applied to evaluate MDA levels and this method, along with immunochemical identification [12] can now be recommended as more reliable than the TBARS assay. There are also many other approaches to evaluate the intensity of ROS-induced lipid peroxidation and the measurement of lipid peroxides [12], 4-hydroxynonenal [73] or exhaled carbohydrogens [41] are some of them. Selection of methods depends on many things, particularly tools available [1, 18].

Probably the most popular method for detection of ROS-modified proteins is the one based on the formation of additional carbonyl groups with their visualization due to interaction with 2,4-dinitrophenylhydrazine [26, 32, 37]. The hydrazones formed are measured spectrophotometrically. Specific antibodies that interact with carbonyl groups of proteins [26, 70] have also been developed. In some cases, there is also the possibility to evaluate amount of dityrosines and other products of free radical-induced oxidation of proteins [4, 11].

Oxidation of nucleic acids also forms big array of products, but in this case there are some favorites that are relatively easy to quantify. These are mainly oxidatively modified guanine derivatives, of which 8-hydroxyguanine (8-OHG) is the most commonly used marker [27, 28], but 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanine (8-oxoGua) [45] are also measured.

Certainly, there are many more different markers of ROS-induced modification of cellular constituents, but those listed here are the most widely used and applied approaches.

5. Oxidative Stress: Description and Definitions

As described above, under normal conditions, living organisms maintain a basal steady-state (stationary) ROS level within a certain range. Homeostasis is provided due to the fact that systems of ROS generation are counterbalanced by prevention and elimination systems along with any other components interacting with ROS. However, under certain circumstances this balance may be shifted resulting in an enhanced ROS steady-state level even up to 100 nM [59]. Certainly, this has consequences due to enhanced oxidative modification of diverse macromolecular components of an organism. The state, when ROS levels exceed the basal values leading to functional disturbances has

been called "oxidative stress". It was first defined in 1985 by Prof. Helmut Sies [57]: Oxidative stress "*came to denote a disturbance in the prooxidant-antioxidant balance in favor of the former*". Next year he published a definitive review summarizing the accumulated knowledge at the time about ROS effects on nucleic acids, proteins, lipids, and carbohydrates, as well as relationships between ROS and inflammation, carcinogenesis, ageing, radiation damage, and photobiological effects [56]. Later H. Sies modified the mentioned above definition to "*An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed "oxidative stress*" in order to emphasize the damage to certain cellular components [58]. Finally, the definition was modified also to underline ROS-based signaling *"a disruption of redox signaling and control"* [62]. More recently I proposed one more definition: "*Oxidative stress is a situation where the steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents*" [31]. This reflects the steady-state level of products of ROS-promoted processes along with ROS effects on the functioning of living organisms. Clearly, these definitions are rather "theoretical" and practical questions remains: how to define oxidative stress over the basal state of ROS levels and operation of organisms?

6. Problems in Interpretation of Experimental Data on Induced Oxidative Stress

Oxidative stress induced by external factors, particularly primary oxidative stress, can be caused by the direct effects of ROS on living organisms. In the simplest case, unicellular organisms or cells in cultures are subjected to certain RS. Curve 1 in Fig. 2 shows schematically a typical response by a cellular endpoint to different concentrations of oxidants producing hydrogen peroxide or direct H_2O_2 application. Endpoint parameters of interest such as cell survival or activity of antioxidant enzymes are frequently used for evaluation of ROS effects on living organisms. At very low concentrations ROS do not affect these parameters (zone I). However, they can be altered by ROS addition in a concentration-dependent manner. Although at first glance may seem paradoxical, an increase in concentration of the inducer enhances cell survival and activity of antioxidant enzymes (zone II) [5, 54]. These effects are primarily related to the activation of many cellular processes, particularly directed to increase cell resistance to oxidative or general stresses. Up-regulation of antioxidant enzymes is a perfect example of this. So, at these levels the oxidant assists to develop the adaptive response in order to improve biological functions. This sort of relationship between toxicant/oxidant and the measured end parameter (endpoint) has been called "hormesis" [10, 24, 30, 47-49]. The cellular response to ROS is measurable up to a maximum level at certain ROS concentration/s followed by decrease in the endpoint parameter come back to control (basal) level. Increases in oxidant concentration may reduce the measured parameter to approximately zero or to some other horizontal asymptote. To underline the behavior of curve 1, zone II may be divided for zone IIa where the endpoint parameter increases and zone IIB where the parameter decreases to "no observable effect" (NOE) point. A further increase in inducer dose results in curve 1 passing through the NOE and decreased levels of the endpoint parameter in zones II and IV.

It is critically important to note that the whole dose dependence of curve 1 in Fig. 2 is connected with the interaction between ROS and certain cellular components. This interaction leads to oxidative modification of cellular components which is reflected by curve 2 in Fig. 2. These characteristics of cellular response to different concentrations of oxidants are frequently found in experiments. Interestingly, presence of these complicated relationships can frequently be misleading and result in discrepancies in the interpretation of experimental data, especially if only a single dose of oxidant is evaluated (as compared with analysis of multiple points on a dose-response curve). Complicated behavior of the system is explained by the many components involved and different sensitivity of cellular components to ROS-induced modification, their localization, and target accessibility to ROS, subject to repair, reduction and degradation pathways.

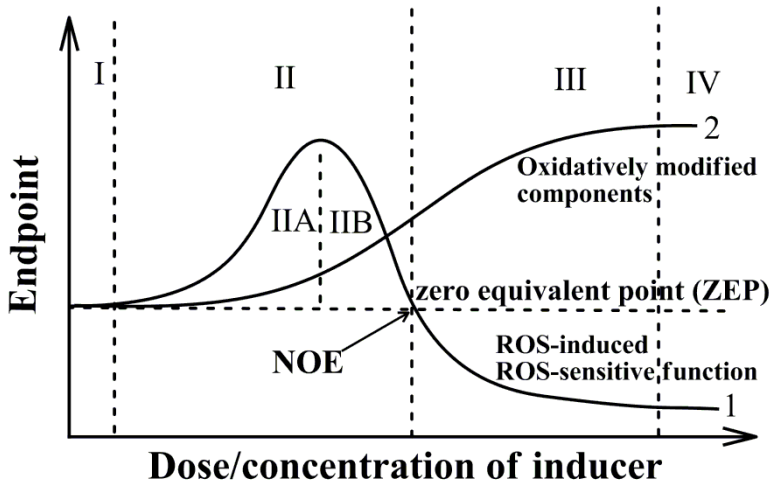

Fig. 2. Relationships between the dose of an inducer of oxidative stress and commonly used endpoints (end parameters) that may be measured. Zone I – no observable effects are registered due to very low (basal) intensity oxidative stress (basal intensity oxidative stress – BOS); zone II – low intensity (mild) oxidative stress (LOS) with a slightly enhanced level of oxidatively modified molecules and enhanced activity of antioxidant enzymes in response to oxidative stress; zone III – intermediate intensity oxidative stress (IOS); and zone IV – high intensity (strong) oxidative stress (HOS). Curve 1 – ROS-induced ROS-sensitive function (ROSISP), curve 2 – level of oxidatively modified components. Abbreviations: NOE – no observable effect point; ZEP – zero equivalent point – the level of components of interest correspond to the initial (basic) level in the absence of inducers of oxidative stress.

Analysis of thousands of reliable publications with the term "oxidative stress" in them lets us categorize given interpretations. Induction of oxidative stress is usually evidenced by: (*i*) enhanced levels of oxidized cellular constituents; (*ii*) increased levels or activities of antioxidant and associated enzymes; (*iii*) decreased levels or activities of antioxidant and associated enzymes, and, finally, by a combination of the above mentioned responses. In certain cases, the levels of ROS-modified molecules may also be decreased due to their elimination by specific systems (this case is a relatively rare event and complicates the description; therefore it will not be covered here). Why do such different responses, sometimes opposite, all lead to a conclusion of the induction of oxidative stress? The first case from the above list (enhanced levels of oxidized cellular constituents) usually does not raise serious questions if evaluated correctly and if several markers are measured simultaneously. The most common practice includes evaluation of level of oxidized lipids (*e.g.* lipid hydroperoxides) and oxidized proteins (*e.g.* protein carbonyls). Other parameters like glutathione disulphide levels or the ratio of oxidized to total or reduced glutathione are also measured as well as oxidized nucleic acids or various complexes formed at interaction between carbohydrates, proteins and/or nucleic acids. The situation with respect to the levels or the activities of antioxidant enzymes is even much more complicated issue. As mentioned above, under oxidative insults, enzyme activity may demonstrate some or all of the potential responses: decrease, increase, or no changes. Decreased activities of the enzymes are usually discussed from the point of view of enzyme inactivation by ROS. Indeed, many antioxidant and associated enzymes have been shown to be inactivated by ROS [7, 22, 25, 36, 54, 55, 72]. Specific mechanisms may differ substantially, but a decrease in the activity is a common event. Increases in the activities of antioxidant and associated enzymes under oxidative stress are usually connected with their *de novo* synthesis [29, 35] or activation of preexisting inactive molecules [5, 6, 52]. Although activation of inactive enzyme molecules is still debatable issue, up-regulation of their biosynthesis is well-established. The process of up-regulation may involve enhanced gene transcription, protein translation and posttranslational modification or maturation [29, 59, 65]. Several regulatory systems responsible for up-regulation of antioxidant and associated enzymes have been described in different organisms. These systems are regulated by transcription factors, the best-known ones being SoxR and OxyR in bacteria [14, 34], Yap1 in budding yeasts [35], Rap2.4a and Npr1 in plants

[29, 64], and Nrf2/Keap1 and NF-kB in animals [29, 69]. The molecular mechanisms involved in redox signaling by the listed above and other transcription factors are based on the reversible oxidation of cysteine residues of sensor proteins, and in bacteria also [4Fe-4S] cluster of SoxR [29, 59, 65]. These have been shown to be responsible for realization of adaptive responses to the introduction of inducers of oxidative stress at low or intermediate concentrations.

In summary, we can say that oxidative stress is clearly presented when: (*i*) a steady-state level of ROS-modified cellular components is enhanced; (*ii*) ROS-regulated transcription factors are activated and antioxidant and associated enzymes are up-regulated; and finally, (*iii*) real evidence of ROS-induced inactivation of antioxidants or their consumption is demonstrated.

Now the question is: how can all accumulated information available in the literature be categorized? In the following section I am going to propose a system which may provide interpretation for virtually all experimental results with induction of oxidative stress if they meet criteria described above. The key idea used to systematically categorize the effects of oxidative stress is based on its different intensity due to the application of different doses/concentrations of inducers in different studies.

7. CLASSIFICATION OF INTENSITY OF OXIDATIVE STRESS: MILD, MODERATE OR STRONG?

Investigation of different modes of oxidative stress induction in all groups of organisms (*e.g.* bacteria, fungi, plants and animals) has always been complicated [29]. For example, a "classic" inducer of oxidative stress, hydrogen peroxide (H_2O_2), affects the levels of oxidized lipids and proteins in bacteria [53] and yeasts [5, 6, 52] often increasing the levels in one case and decreasing in another (due to the operation of specific defense and detoxification systems), but mainly showing enhanced levels. The activities of antioxidant and associated enzymes were similarly increased, decreased or not changed in different cases. In most of these cases, we were talking about induction of oxidative stress with the need to explain obvious differences. This experience and discussion with many colleagues made it clear that there was a desperate need to sort the accumulated wealth of experimental data and determine why responses were so variable between different studies.

Curve 1 in Fig. 2 shows the relationship between the dose of oxidant effector and the endpoint parameter measured. The latter parameter may vary in different studies, *e.g.* cell survival, activation of ROS-sensitive regulatory proteins, activity of antioxidant enzymes, *etc*. For analysis, we will use those which are ROS-sensitive and at the same time are induced/enhanced by ROS exposure at low concentrations. Curve 2 in Fig. 2 shows the relationship between concentration of the oxidant and the level of oxidatively modified components. Those may be different products of oxidation of proteins, lipids, nucleic acids, carbohydrates, *etc*. These provide an integral marker of ROS-induced modification of cellular constituents. Next, we will analyze the behavior of curves 1 and 2 at different concentrations of inducers of oxidative stress.

At very low concentrations (zone I) no observable effects are seen – oxidant effects are near negligible and significant responses cannot be discerned. In living systems, ROS are always present and the introduction of additional small amounts of oxidant (*e.g.* levels similar to basal amounts *in vivo* or even slightly higher) does not disturb the cellular processes to an extent that may be detected using conventional assay approaches. However, a further increase in the concentration of the inducer (zone II) enhances the observable level of oxidatively modified components and, at the same time, increases the endpoint parameter measured – *i.e.* the ROS-induced ROS-sensitive parameter (ROSISP). The mechanisms responsible for this induction were discussed briefly above. In this zone, elevation of the inducer concentration results in the development of either full response (zone IIA) or reduction in the ROSISP level despite a concomitant increase in the levels of ROS-modified components (zone IIB). In other words, in zone II, we can see that the expression of the ROSISP rises to a maximum but then decreases again to the point when no observable effect (NOE) is seen. The levels of oxidatively modified components at the NOE point are substantially increased, but after that point, the ROSISP

further decreases (zone III). Finally, in zone IV both measured functions converge to some plateau – *i.e.* virtually all available potential substrates are oxidized in this situation which results in the development of a near maximum response. Fig. 2 represents an "idealized" relationship between the concentrations of the inducer, the levels of oxidatively modified components, and ROSISP, but it can be seen that these relationships account for many different dose dependency relationships that have been reported in the literature.

To our best knowledge, there have been no serious attempts to date to categorize oxidative stress depending on its intensity. Therefore, based on the information provided above, the following attempts to provide such an exercise using Figure 2. Zone I where no observable effects of added ROS are seen can be called "*no stress at all*" or "*no stress*". Zones II, III and IV where the stress can be observed are labeled "mild", "moderate" and "severe (strong)" oxidative stress, respectively. Under *mild oxidative stress* (zone II), an elevated level of ROS-modified molecules is observed, and the ROSISP situates above zero equivalent point (ZEP), which means that ROSISP is increased. For convenience, zone II may be subdivided for zone IIA where ROSISP is increasing from ZEP to its maximum level, and zone IIB where ROSISP decreases from the maximum to ZEP and crosses at the NOE point. Under *moderate oxidative stress* (zone III), the level of ROS-modified molecules is higher than that under mild oxidative stress and the ROSISP situates below the ZEP, which means that it is decreased. Finally, under *strong oxidative stress* conditions (zone IV), the level of ROS-modified molecules reaches the maximum, and the ROSISP also situates below the ZEP and reaches minimum values. The entire concept is mainly related to simplified *in vivo* systems. Reactive oxygen species affect targets more or less nonspecifically, but induce defense systems specifically. The specificity of the pair is provided by properties of the affected target and the ROS that interacted with it.

It is also possible to propose more convenient classification from a semantic point of view. Using again Fig. 2, the four zones of for "Endpoint" *vs* "Dose of inducer" may be called: I – basal intensity oxidative stress zone (BOS); II – low intensity oxidative stress (LOS); III – intermediate intensity oxidative stress (IOS); and IV – high intensity oxidative stress (HOS). The proposed classification may be helpful to describe experimental data where oxidative stress is induced and systematize it basing on its intensity. Interested readers may propose their own vision of the problem or discuss the issues proposed here in order to choose the most adequate and convenient classification system.

8. TIME-COURSE OF OXIDATIVE STRESS

Certainly, all processes in living organisms are dynamic, and, therefore, there is a reason to characterize development of oxidative stress in time [29]. Under normal conditions ROS level fluctuates in certain corridor which is defined by the balance between their generation and elimination providing in this manner certain steady-state ROS level. After induction of oxidative stress, for example by direct addition of hydrogen peroxide to cell suspension, steady-state ROS level may enhance (Fig. 3). Further, two different scenarios resulting from perturbations of ROS-related processes may take place. If the capacity of antioxidant system is not overwhelmed, the stress-enhanced ROS level can return to its initial range. In many cases, induction of ROS-regulated genes may be needed to cope with the enhanced ROS levels. Generally, if ROS steady-state levels return to the initial value within minutes/hours after stress induction, when organisms are capable and have enough resources for the corresponding response, the stress is called "acute oxidative stress" [31]. However, sometimes ROS levels do not return into initial range, but stabilize at a somewhat higher level or just extend the steady-state ROS range existing under normal conditions and in this case the stress does not last for prolonged time period. This scenario is called "chronic oxidative stress" and frequently occurs under diverse pathological conditions or substantial changes of physiological state. Finally, after some perturbations, particularly as a consequence of substantial physiological or pathological shifts or chronic intoxication, the steady-state ROS level does not return to its initial range but stabilizes at a higher level, called a quasi-stationary or quasi-steady-state one.

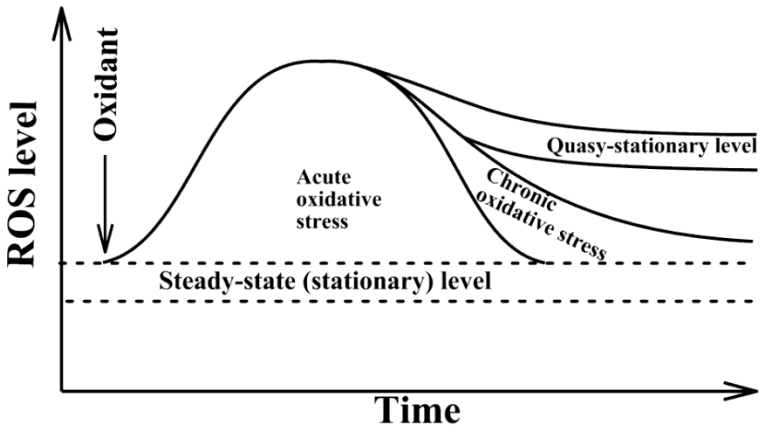

Fig. 3. The dynamics of levels of reactive oxygen species in biological systems. The basic steady-state (stationary) level of reactive oxygen species fluctuates in a certain range under normal conditions. However, under stress ROS levels may increase beyond the normal range resulting in either acute, or chronic oxidative stress. Under some conditions, ROS levels may not return to their initial range and stabilize at a new quasi-stationary level.

9. EXPERIMENTAL COMPLICATIONS

In Section 7, I represented the "idealized" cellular response to exposure to inducer of oxidative stress – a two-dimensional system with variable levels of inducer and cellular response. In reality, this ideal system is complicated by at least four factors. These are: (*i*) the time course of the response, (*ii*) tissue/cell specificity, (*iii*) accessibility of targets to the inducer especially when dealing with multicellular organisms, and (*iv*) the physiological state of the organism.

It is clear that in order to develop a response to any oxidizing effector, some time is required. Moreover, time courses of various processes are usually different. Therefore, in addition to concentration dependency, the investigator has to study the development of the response over time.

Some additional points should be highlighted here. (1) If we measure several parameters to characterize oxidative stress as it is the usual practice in most cases, the results from some of these parameters may classify the stress as strong, whereas others may indicate intermediate or even mild stress. In these cases, researchers should choose which intensity of the stress they deal with. Perhaps, some clues for selection can be provided by weighing all parameters evaluated and choosing the zone in which most of them are located. (2) How can we differentiate zones III and IV in Fig. 2, *i.e.* the zones of intermediate (moderate) intensity oxidative stress (IOS) and high intensity oxidative stress (HOS)? Here, I propose to use Hill equation. The relationship between oxidant dose and the level of ROS-oxidized products usually follows a sigmoid or S-shaped curve converging to a horizontal asymptote (although the asymptote is virtually never reached experimentally). In biochemistry such relationship is usually described by the Hill equation and the mathematical apparatus used to calculate the maximum parameter (saturation of the binding centers or maximum rate of the enzyme) may be applied. Since the maximum parameter is complicated to measure, the calculated one is used for these purposes. Actually, I propose to use the point where 90% of the calculated maximum level is reached as a border between zones III and IV (IOS and HOS). (3) The intensity of oxidative stress changes with increasing time of the stressor application comparing to zero-time point. For example, at the beginning the stress may be classified as HOS or IOS, but over time it might change to IOS or even LOS reflecting organisms' response or adaptation. For this reason, the researcher should be very accurate defining the time course of the stress effects. (4) In some cases, acute oxidative stress may be a mild stress, whereas chronic oxidative stress would correspond with intermediate or high intensity oxidative stress.

Probably, there is a need here to summarize once more biologically most relevant biomarkers to characterize oxidative stress. They are: (*i*) presence of ROS-modified molecules and products of ROS-promoted reactions (for lipids – malonic dialdehyde, isopsoralens, and lipid peroxides, for proteins – carbonyl proteins, and for nucleic acids – 8-oxoguanine, or their complexes); (*ii*) induction of defense systems (SoxR and OxyR in bacteria; Yap1 in yeasts; Npr1 and Rap2.4a in plants; and Nrf2 in animals). These events if not counterbalanced may lead to cell death via apoptosis or necrosis.

Studies of multicellular organisms add complications for oxidative stress researchers. The delivery of inducers and tissue/cell specificity in the response to inducers are the main problems here. The routes for inducer delivery can vary substantially and include uptake through routes including the alimentary system, skin, gills, and lungs, *etc*. Chemical properties of inducers, specificity of the absorption system, as well as inducer metabolism and excretion from the body all combine to define the dose of inducer promoting specific response of each tissue type, and, therefore, the tissue-specific (as well as whole organism) response(s) that occur. It should also be noted, that it is not always the original oxidant compounds that may affect the target organism or its tissues, but also the products of their chemical modification or biological catabolism that may actively determine the overall response. Another important aspect of the induction of oxidative stress by exogenous ROS is the issue of tissue specificity or even cell specificity in those tissues with multiple cell types. Each cell/tissue type may respond differently to oxidative stress inducers including experiencing different local doses, showing different thresholds for damage, possibly undergoing different types of damage, and being differentially important in determining the overall whole organism response to the stress.

The listed above and many other experimental complications clearly demonstrate that the proposed classification systems rely on many parameters, depend on specific conditions, physiological state of the organisms, and parameters measured. Obviously, the models will not always work out and this leads to the conclusion that it should be used in a prognostic manner. I suggest that the proposed systems should be used not as "ideal" classifications, but rather as working model to develop a reliable system of classification of oxidative stress with predictive strength and which can be used for quantitative evaluation.

10. CONCLUSIONS AND PERSPECTIVES

Oxidative stress has been extensively studied for about four decades. Substantial progress has been achieved to date – from descriptive characterization of this process to delineation of molecular mechanisms underlining adaptive responses and targeted manipulations of expected responses. Up to now, descriptive works still prevail, but more and more frequently studies assessing the molecular mechanisms involved appear [21, 29, 31, 39, 44, 59, 65, 66, 72]. In the light of this article, it is still important to characterize internal processes induced by ROS. Which specific targets are important for survival and for adequate responses to oxidative insults? Again, this depends on many circumstances. For example, the loss of transmembrane ion gradients as a result of high levels of lipid peroxides may be responsible in some cases, whereas in other situation, irreversible changes can be triggered by oxidative damage to mitochondrial or nuclear DNA. In many instances, ROS-triggered damage to cellular components may direct the cell to apoptosis or necrosis.

Future progress in the field needs identification of the most crucial cellular targets for ROS action as well as further discovery of the underlying mechanisms and consequences of the interaction between ROS and cellular components. The mechanisms responsible for ROS combating and regulation of the systems involved would be the second hot topic for ongoing studies of ROS metabolism. Last years, it was discovered that ROS and ROS-regulated pathways are actively involved in modification of diverse cellular processes starting from core metabolism and hormonal signaling to complicated processes such as fertilization, development, *etc*. The latter along with some biotechnological avenues would also extend ROS-related studies in practical directions. Therefore, much remains to be learned about the

effects of ROS on biological systems, the adaptive strategies that overcome ROS attack, and the natural use of ROS in the signaling and regulation of metabolism.

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У живих організмах, між генерацією активованих форм кисню (АФК) та їх знешкодженням та/або попередженням утворення існує рівновага, яка може підтримувати сталий (стаціонарний) рівень АФК. Проте цей баланс може бути порушений, що призводить до підвищенння стаціонарного рівня АФК та посилення процесів пошкодження біомолекул. З 1985 року, коли Х. Сайс вперше застосував визначення оксидативного стресу, ця сфера досліджень стала однією з найгарячіших тем в біології і до сьогодні багато деталей стосовно клітинних пошкоджень, спричинених АФК, сигнальних процесів, клітинної відповіді та адаптації були розкриті. Проте, деякі базальні окисні пошкодження завжди відбуваються за нормальних умов і в багатьох експериментальних дослідженнях важко показати, що оксидативний стрес дійсно викликаний стресором. Тому, як правило, дослідникам важко правильно інтерпретувати розвиток оксидативного стресу. Наприклад, у багатьох випадках, підвищення або зниження активності антиоксидантних і асоційованих з ними ферментів інтерпретується як свідчення оксидативного стресу. Допомогти у вирішенні цієї проблеми може ретельний вибір специфічних біомаркерів (модифікованих АФК мішеней). Щоб уникнути такого роду проблем, я пропоную кілька класифікацій оксидативного стресу, базуючись на його тривалості в часі та інтенсивності. Класифікація за тривалістю в часі включає в себе гострі та хронічні стреси. В класифікації, що базується на інтенсивності, я пропоную вирізнити чотири зони функцій у залежності між «Дозою/концентрацією стресора» та визначенням «Кінцевої точки»: I – зона базального оксидативного стресу (BOS); II – оксидативний стрес низької інтенсивності (LOS); III – оксидативний стрес проміжної інтенсивності (IOS); IV – оксидативний стрес високої інтенсивності (HOS). Запропоновані класифікації можуть бути корисні для опису експериментальних даних, де виникає оксидативний стрес, а також його систематизації, базуючись на тривалості в часі та інтенсивності. Перспективні напрями досліджень в даній галузі включають розробку складних класифікацій оксидативного стресу, точну ідентифікацію клітинних мішеней АФК та їхньої відповіді на дію АФК, реальні функції та дію так званих «антиоксидантів", внутрішньоклітинний просторово-часовий розподіл та наслідки дії АФК, розшифровка молекулярних механізмів, відповідальних за клітинну відповідь на дію АФК та участі АФК в реалізації нормальних функцій клітинного гомеостазу.

Ключові слова: оксидативний стрес, динаміка інтенсивності, класифікація, вільні радикали, активовані форми кисню.