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INTENSITY- AND TIME COURSE-BASED CLASSIFICATIONS OF OXIDATIVE STRESSES

VOLODYMYR LUSHCHAK

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).

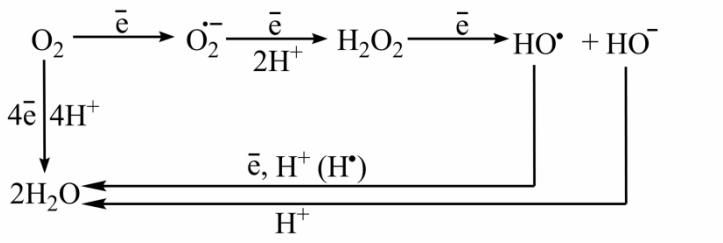

Fig. 1. Reduction of molecular oxygen via four– and one-electron schemes.

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.

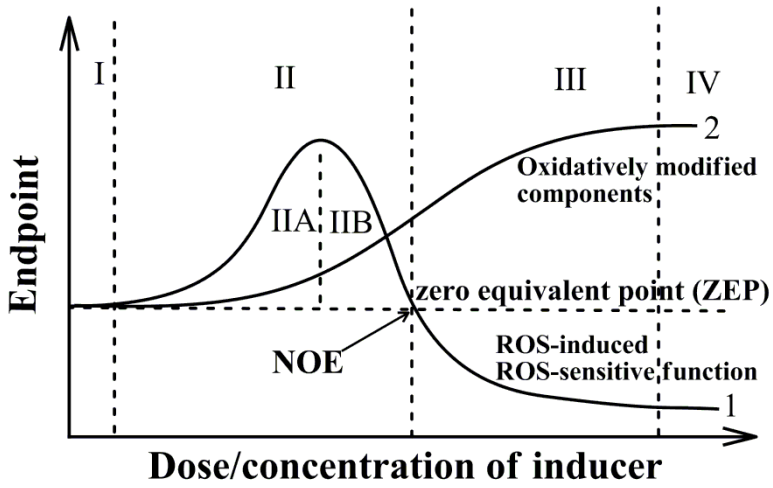

Fig. 2. Relationships between the dose of an inducer of oxidative stress and commonly used endpoints (end parameters) that may be measured. Zone I – no observable effects are registered due to very low (basal) intensity oxidative stress (basal intensity oxidative stress – BOS); zone II – low intensity (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.

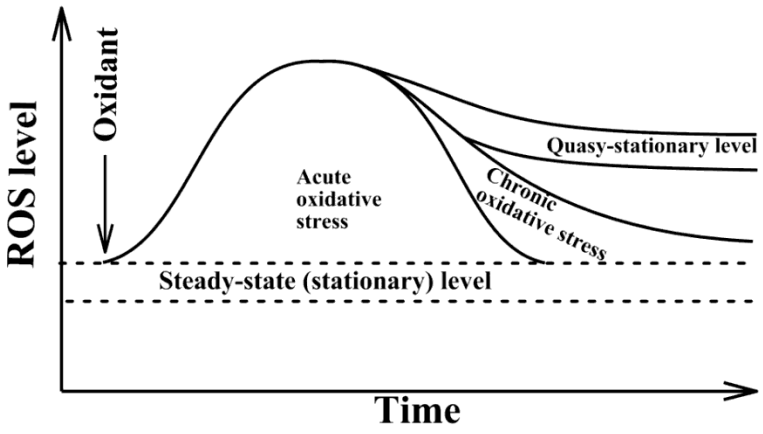

Fig. 3. The dynamics of levels of reactive oxygen species in biological systems. The basic steady-state (stationary) level of reactive oxygen species fluctuates in a certain range under normal conditions. However, under stress ROS levels may increase beyond the normal range resulting in either acute, 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.

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UNDERINVESTIGATED ROLES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

DMYTRO GOSPODARYOV

Abstract. The review examines certain aspects of the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) in living organisms of different taxa, from bacteria to humans. Particular emphasis is put to the role of G6PDH in iron homeostasis and lipogenesis. Prooxidant and antioxidant roles of G6PDH are also highlighted along with several currently known mechanisms of G6PDH regulation.

Keywords: NADPH, superoxide, thiol, iron-sulfur cluster, glutathione.

1. INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PDH) is an ancient enzyme, which is present in both, prokaryotes and eukaryotes. This dehydrogenase is the very first and regulatory step of pentose phosphate pathway (PPP). In turn, PPP interplays with many other important metabolic pathways in cells (Fig. 1). Indeed, PPP allows producing of ribose-5-phosphate, carbohydrate part of ribonucleotides as well as the metabolite of Calvin cycle in photosynthetic organisms. One of the products of the reaction catalyzed by G6PDH is reduced nicotinamide dinucleotide phosphate (NADPH). This compound is an essential reductant used in numerous biosynthetic processes [48]. Particularly, NADPH takes part in fatty acid and sterol synthesis, reduction of low and high molecular mass thiols [48], and consequently, in synthesis of heme and assembly iron-sulfur (Fe/S) clusters [14, 68]. These roles for NADPH, which is reduced mainly by G6PDH, characterize the latter as lipogenic and related to antioxidant defense enzyme. They underlie also specific regulation of G6PDH. It is known that the expression of the gene coding for G6PDH in multicellular organisms is regulated by transcriptional factors which are responsible for lipogenesis and antioxidant defense [3, 8, 42]. However, the lipogenic role of G6PDH is more pronounced in multicellular organisms which have lipid storages and insulin signaling. Indeed, NADPH does not react with either hydrogen peroxide or superoxide anion-radical directly, but instead reduces thiol-containing antioxidant proteins such as glutaredoxins and thioredoxins, and also tripeptide glutathione. Interestingly, all these antioxidant thiols are common for bacteria and humans. Using of NADPH as main thiol reductant adds a new layer of complexity in understanding of G6PDH role. Thus, glutaredoxins and thioredoxins, in addition to their antioxidant function, play also many metabolic roles. For instance, thioredoxin is used for the synthesis of deoxyribonucleotides from ribonucleotides [62]. This step integrates production of ribose-

5-phosphate in PPP and ribonucleotide biosynthesis with production of NADPH in PPP and deoxyribonucleotide biosynthesis. Reduced glutaredoxins are necessary for the assembly of Fe/S clusters and heme synthesis [14, 68] as it was mentioned above for NADPH. These roles of NADPH and reduced thiol-containing compounds may also partially explain development of hemolytic anemia in response to certain drugs for people with G6PDH deficiency that will be examined in more details in the review. The complexity and intertwining relationships between all processes which are related to G6PDH combined with huge amount of new data, demands scrupulous systematization. Despite the constant focus on G6PDH as an important enzyme, majority of past and recent reviews are devoted predominantly to G6PDH deficiency.

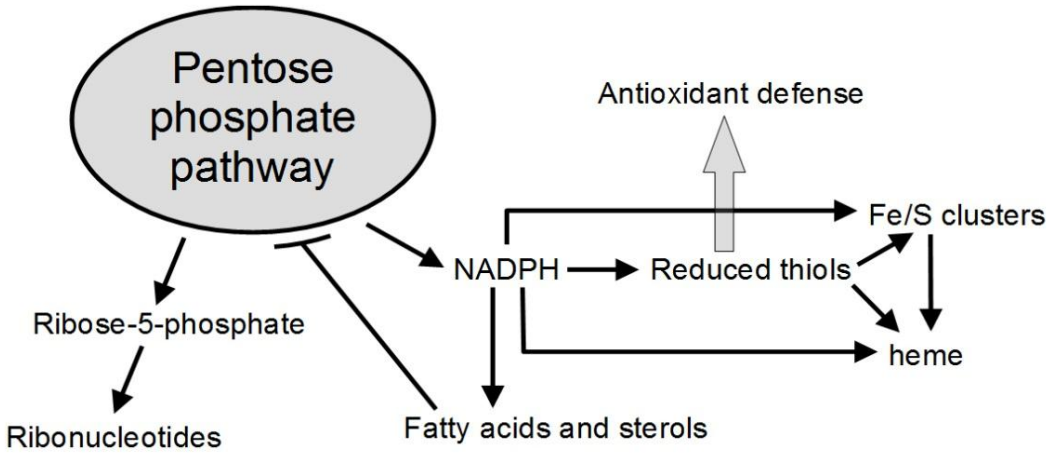

Fig. 1. Roles of pentose phosphate pathway in cells.

Thus, the goal of this review is to summarize and systematize the current knowledge on the roles of G6PDH in living organisms, from bacteria to humans. Particular emphasis is put on the points which were not emphasized previously, namely the role of G6PDH in Fe/S cluster biogenesis and the complex regulation of G6PDH activity, including responses to different environmental stimuli. This systematization is also required for better understanding of the integrated metabolic scheme for cells, tissues, organs and a whole organism. The crucial point here is that certain enzymes and metabolites of the organism can be set for distinct and specific roles. Particularly, despite so many important roles for G6PDH in cells, its deficiency is rarely characterized by a strong phenotype. The deficiency is exhibited as severe hemolytic anemia in response to redox-cycling compounds and may be accompanied by chronic granulomatous disease. However, there were no reports about disorders in lipid and sterol synthesis or nucleotide synthesis. The other example of such specific phenotype is fumarase deficiency which leads to predominantly renal cancer or leiomyomas [1], although fumarase is present in every cell and is an important enzyme in tricarboxylic acid cycle. The tissue-specificity of these and other similar diseases is poorly understood, and requires more profound understanding of metabolic integration, including signaling pathways which can be modulated by intermediates of key metabolic pathways.

2. G6PDH IN IRON-SULFUR CLUSTER HOMEOSTASIS

The gene *zwf* coding for G6PDH in enterobacteria is a member of the SoxRS regulon which coordinates defense systems against deleterious effects of superoxide anion-radical and hydrogen peroxide. Genes *sodA*, *acnA*, *fpr*, *fldA*, *fur*, *yggX*, and others which code for manganese-containing superoxide dismutase, oxidant-resistant aconitase, NADPH-ferredoxin oxidoreductase, flavodoxin A, ferric iron uptake regulator and putative Fe^{2+} transporter, respectively, are other members of this

regulon [21]. Superoxide dismutase (SOD) is the main enzyme detoxifying superoxide. This enzyme dismutates two molecules of superoxide yielding hydrogen peroxide and molecular oxygen. Importantly, superoxide is a very short living reactive oxygen species [21]. Thus, under intensive intracellular generation of superoxide or during attack of immune cells, catalase and peroxidases are a second line of the defense after SOD. Among peroxidases, glutathione peroxidase is one of the key enzymes for detoxification of hydrogen peroxide [65]. This enzyme uses tripeptide glutathione to reduce hydrogen peroxide as well as many other peroxides. Afterwards, the pool of reduced glutathione is replenished by *de novo* synthesis or in reaction catalyzed by glutathione reductase (GR) where NADPH is used as a reductant. This is a simple explanation of the regulation of *zwf* expression by superoxide. However, it is known that the gene coding for GR in *Escherichia coli*, *gorA*, is regulated by the OxyR transcriptional factor responding to hydrogen peroxide [40]. In contrast, G6PDH has so called "soxbox" and is regulated by the SoxS regulatory protein responding to superoxide anion-radical [11, 79]. Hydrogen peroxide may activate also SoxS target genes but there was no *zwf* among them [43, 81]. However, the increase in expression at the level of G6PDH activity in response to hydrogen peroxide treatment was described in other papers [60, 61]. Redox-cycling compounds may also drive OxyR-regulated genes [15]. Nevertheless, differential regulation of G6PDH and GR in *E. coli* suggests more specific role of G6PDH in response to superoxide attack. This specific role of G6PDH may supposedly be connected with repair of Fe/S clusters, the one of the main targets for superoxide. It is known that biogenesis of Fe/S clusters in bacteria and eukaryotes requires NADPH [12, 21, 49, 68] and is mediated by NADPH-dependent enzymes such as NADPH-ferredoxin oxidoreductase [12]. Remarkably, the latter enzyme is also under SoxS regulation [12, 21]. In addition, it was demonstrated that expression of SoxS targets can be triggered by changes of NADPH/$NADP^+$ ratio [26]. This demonstrates specific role for G6PDH in Fe/S cluster repair shared by bacteria and budding yeast *Saccharomyces cerevisiae*. Particularly, yeast strains deficient in G6PDH are auxotrophic for lysine and methionine. Metabolism of both these amino acids includes enzymes with Fe/S clusters and stages which require NADPH. Particularly, biosynthesis of lysine in yeast goes through the conversion of homocitrate to homoisocitrate [35]. This conversion is catalyzed by homoaconitase Lys4p, the enzyme containing Fe/S cluster and sensitive to superoxide treatment [34]. Additionally, lysine biosynthesis pathway includes two NADPH-consuming reactions, catalyzed by Lys2p and Lys5p, α-aminoadipate reductase and saccharopine reductase, respectively. Yeast methionine biosynthesis includes sulfite reductase Met5p, which also contains Fe/S clusters [72]. Sulfite reduction is simultaneously a NADPH-consuming step [66, 72]. In turn, the step catalyzed by phosphoadenylyl sulphate reductase requires thioredoxin as a reductant [72]. Thioredoxin is subsequently reduced by thioredoxin reductase, thus the thioredoxin reduction is also a NADPH-dependent step. Details of this regulation are shown on the scheme below (Fig. 2).

In humans, G6PDH deficiency causes severe erythrocyte hemolysis only after consumption of redox-cycling compounds contained in formulations of certain drugs and in foods like fava beans [6]. It means that despite importance for the whole organism, G6PDH is dispensable enzyme and its reduced activity does not result in strong phenotypes [6, 36]. It is believed that erythrocyte hemolysis under G6PDH deficiency is rather caused by superoxide and hydrogen peroxide than induced by redox-cycling agents directly [50, 78]. Thus, incapability of G6PDH-deficient cells to cope with superoxide toxicity results from glutathione depletion under NADPH deficit. Subsequently, superoxide and its derivatives, hydroperoxyl radical, hydrogen peroxide, and peroxinitrite oxidize many functional molecules in the cell, leading to its death. However, erythrocyte hemolysis may have also other causes, including those that anticipate the role of G6PDH in iron-sulfur cluster assembly. Particularly, ferrochelatase which catalyzes the final step of heme synthesis is a Fe/S-containing enzyme can be one of the potential candidates. Heme synthesis requires also involvement of other Fe/S-containing proteins such as ferredoxins [63]. Also, heme biosynthesis is a NADPH-consuming process [31]. Opposing to this concept, would be the fact that GR deficiency is characterized by similar symptoms that G6PDH deficiency [24]. However, it was recently found that glutathione plays an important role in Fe/S cluster biogenesis [27, 38].

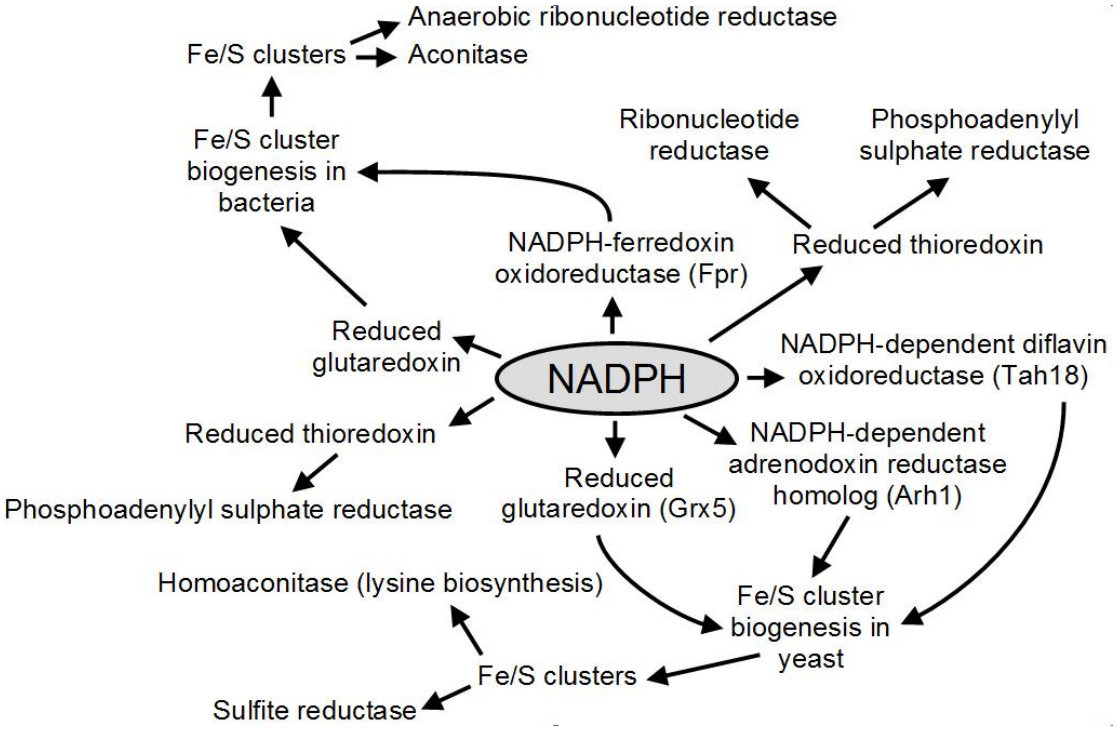

Fig. 2. Roles of NADPH in iron-sulfur cluster biogenesis.

Other reasons for hemolytic anemia at G6PDH deficiency can be related to involvement of NADPH in protection of erythrocyte membranes, particularly unsaturated fatty acids, against free radical attack resulting in lipid peroxidation, and/or its involvement in maintaining of correct redox state of Fe^{2+} in hemoglobin.

3. Antioxidant and Prooxidant Roles of G6PDH

As it was mentioned above, G6PDH is considered to be an enzyme closely related to antioxidant defense. The connection of G6PDH with antioxidant system is via NADPH which is further used for thiol reduction. However, it was highlighted above that thiols may equally be important for both, antioxidant defense and Fe/S repair. Thus, it is still difficult to distinguish between the antioxidant roles of G6PDH in either detoxification of peroxides, including hydrogen peroxide, by glutathione- or thioredoxin peroxidases, or in repair of cellular damage caused by reactive oxygen species (ROS). Indeed, impairment of Fe/S clusters may operate as prooxidants. Particularly, certain antioxidant enzymes, like catalase, contain heme and tightly bound NADPH (Fig. 3). As it was shown above, Fe/S-containing proteins, ferrochelatase and ferredoxins, are required for heme biosynthesis. Numerous Fe/S-containing proteins are involved in cellular energy metabolism. These proteins are in complexes I (NADH-ubiquinone oxidoreductase), II (succinate dehydrogenase), and III (ubiquinol-cytochrome c oxidoreductase) of mitochondrial respiratory chain as well as cytosolic and mitochondrial aconitases. Inhibition of mitochondrial respiratory complexes and aconitase may lead to increased intracellular ROS production by the respiratory chain. For instance, this increase in ROS production is obsered in case of mutations in gene *BCS1L*. This gene encodes an AAA family ATP-ase which is responsible for insertion of Rieske Fe/S center into the complex III [47]. Production of ROS can also be enhanced via disturbances in iron homeostasis since iron is a reactant in Fenton reaction, in which hydroxyl radical is formed from hydrogen peroxide [14, 39]. Interestingly, Fe/S clusters of the complex I themselves are

sites of ROS production [44]. Inhibition of aconitase may also prevent ROS-induced damage to a certain extent via blocking of citrate conversion and citrate accumulation. Citrate is itself an iron chelator and may show antioxidant properties [53], although citrate-iron complexes rather promote than hinder oxidative damage in many cases [73]. The complexity of the situation may be even higher if to take into account that NADPH is also used for the transport of iron and copper [20, 80], metals that present in active centers of antioxidant enzymes, catalase and SOD, respectively. The transport of these metals includes subsequent steps of reduction, oxidation, and permeation. The reduction step is mediated by NADPH-dependent enzymes ferric and copper reductases (Steap1-4 proteins in humans and Fre1-8p in budding yeast) [20, 80].

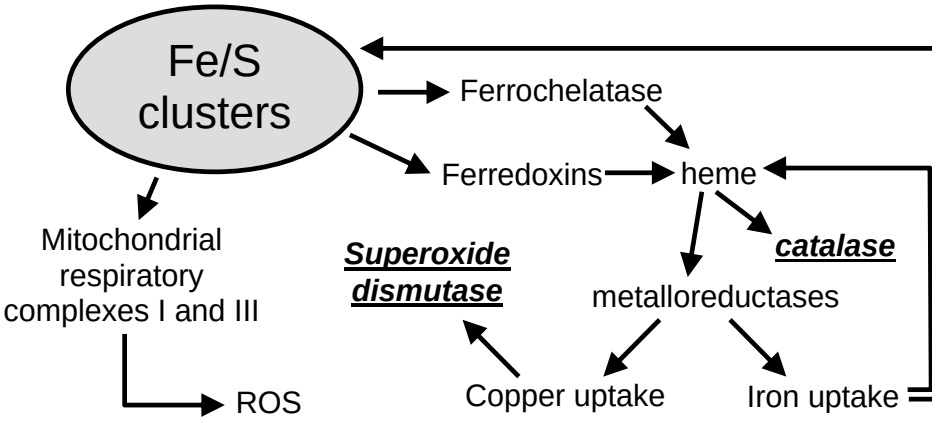

Fig. 3. Significance of iron-sulfur clusters for antioxidant defense. Antioxidant enzymes are underlined.

On the other hand, G6PDH may have also prooxidant role. In multicellular eukaryotes, NADPH is also used for superoxide production by NADPH oxidases [18]. Superoxide and its derivatives, including peroxynitrite, are used by immune cells to kill bacteria or eukaryotic parasites. Thus, G6PDH is an important enzyme for immune defense. Moreover, severe G6PDH deficiency was found to be accompanied by chronic granulomatous disease [2]. In some cases, deficiency of G6PDH and related decrease in superoxide production may confer certain advantages. For instance, apolipoprotein E-deficient mice with simultaneous lack of G6PDH showed less atherosclerotic lesions of aorta under high-fat feeding [45]. Interestingly, up-regulation of G6PDH accompanies cell proliferation, and consequently tumor formation [74]. Therefore, G6PDH activity should be strictly regulated in multicellular organisms.

4. Regulation of G6PDH Activity

The activity of G6PDH is mainly regulated at transcription level. Upstream region of the gene *zwf* that codes G6PDH in *E. coli* contains "soxbox", or SoxS-binding sequence AATCGACGGGTGGATAAGCGTT serving for regulation of *zwf* expression by superoxide via SoxRS system [79]. It is also known that G6PDH is regulated by the MarA transcription factor, which is responsible for bacterial resistance to multiple antibiotics [22]. In unicellular *S. cerevisiae*, expression of the *ZWF1* gene coding for G6PDH is shown to be regulated by the Yap1p transcription factor [32, 58]. It was also shown that G6PDH can be involved in high sugar stress tolerance by unknown mechanism [10]. In multicellular eukaryotes, expression of the gene coding for G6PDH is likely regulated in multiple ways because G6PDH is responsible for antioxidant defense and lipogenesis. The model of G6PDH regulation at the level of gene expression was proposed by Kletzien and colleagues [25]. It takes into account promoter regions via which the G6PDH expression can be controlled by insulin, polyunsaturated fatty acids, monasaccharides and glycerol. The analysis

performed by these authors revealed tissue-specific mode of G6PDH regulation by insulin and glucocorticoids, and epidermal growth factor via AP-1 (activator protein 1, heterodimer of protooncogens c-Jun and c-Fos) sites. The promoter of human G6PDH gene contains E-box (for binding regulatory proteins with basic helix-loop-helix motif), sterol regulatory element (SRE), and SRE-like sequence [3]. Additionally, Sp1 site was also found in the promoter of human gene encoding G6PDH [55]. The expression of G6PDH was found to be strongly up-regulated by sterol regulatory element binding protein 1a (SREBP-1a) and to the lesser extent – by SREBP-1c and SREBP-2 [3, 4]. The presence of E-box implies also regulation by carbohydrate response element binding protein (ChREBP). In turn, SREBPs and ChREBP are themselves activated via insulin signaling pathway [8]. Moreover, G6PDH demonstrated high activity in cancer cells due to upregulation via phosphatidylinositol-3-kinase (PI3K) along with other pro-oncogenic signaling proteins such as mTORC1 (mammalian target-of-rapamycin complex 1), K-ras (small GTPase), ATM (ataxia-telangiectasia mutated kinase), and protein kinase A. At the same time, anti-tumor proteins, transcription factor p53, protein phosphatase PTEN (phosphatase and tensin homolog), AMP-dependent protein kinase, and second messenger molecules, like cyclic AMP, regulate G6PDH activity negatively [23, 67]. Oxidative stress may induce G6PDH at transcriptional level via nuclear factor-erythroid 2-related factor-2 (Nrf2) [23, 67]. Indeed, antioxidant responsive element (ARE), the sequence ATGACACAGCA, was detected in the promoter of G6PDH gene [46]. This regulation has several more interesting links related to the noted above regulatory pathways. Particularly, Nrf2 is recognized as potentially pro-oncogenic regulatory protein [46, 64]. The activity of this transcription factor was revealed to be modulated by pro-oncogenic proteins like PI3K and downstream kinase Akt (protein kinase B) [46, 64]. On the other hand, Nrf2 could be involved in regulation of metal homeostasis as it binds to AREs of genes coding for ferritin L and metallothionein-1 as well as number of genes encoding antioxidant enzymes [7, 77].

Polyunsaturated fatty acids, such as arachidonic acid, can affect expression of G6PDH at posttranscriptional level via complex mechanism related to pre-mRNA splicing [55]. Arachidonic acid acted by multiple ways preventing induction of G6PDH expression by insulin and high carbohydrate diet. Particularly, arachidonic acid blocks insulin pathway by inhibition of PI3K, while activates kinase p38 [71]. Further, this polyunsaturated fatty acid was shown to decrease phosphorylation of serine-arginine-rich proteins, specifically SRSF3, which regulate pre-mRNA splicing by binding to exonic splicing enchancer sites [75]. At the same time, insulin increased phosphorylation of SRSF3 and resulted in correct splicing of G6PDH mRNA. Human gene coding for G6PDH consists of 13 exons. In case of incorrect splicing due to inhibition of SRSF3 binding, introns flanking the exon 12 are retained [75]. This posttranscriptional regulation comprises also splicing silencers, particularly, heterogeneous nuclear ribonucleoproteins (hnRNPs) K, L and A2/B1 which bind to the regulatory sites in G6PDH pre-mRNA near exon 12 in the starved state and dissociate during refeeding [17]. Recently, this mechanism was specified, and protein phoshatase PTEN, an anti-tumor protein which negatively regulates insulin signaling, was introduced to the scheme [19]. Protein phosphatase PTEN was shown to interact with hnRNP K, activating it, thus impeding correct splicing and lowering G6PDH activity. Simultaneously, PTEN activates glycogen synthase kinase 3β (GSK-3β) by dephosphorylation. This kinase phosphorylates, in turn, the co-activator of Akt kinase, the Tcl1 protein. This protein, known as an inducer of insulin signaling, interacts with hnRNP K, but inhibiting it, hence increasing G6PDH expression. The details of this regulation are shown on the figure Fig. 4. Such complex mechanism underlies hepatocancerogenesis [19] caused by disorders of insulin signaling pathway, and is also important in healthy tissue for relatively rapid response to refeeding after starvation [4].

There are many ways of G6PDH activity regulation at posttranslational level. It was found that G6PDH was allosterically regulated by NADPH/$NADP^+$ ratio [56, 67] and sterols [59, 67]. In cold-blooded animals, G6PDH was shown to be reversibly phosphorylated/dephosphorylated [70]. These posttranslational changes to G6PDH were observed in anoxia-tolerant animals like marine mollusk *Littorina littorea* and crayfish *Orconectes virilis* [29, 30], and animals with hypometabolic stages [9, 54]. In these animals, G6PDH is phosphorylated mainly by protein kinase G, and additionally by protein kinases A, calmodulin-dependent and AMP-activated protein kinases [9, 29, 30, 54]. The enzyme is

dephosphorylated by protein phosphatases 1 and 2C [29]. Here, G6PDH is likely a part of adaptive mechanism for prevention of oxidative damage caused by re-oxygenation after prolonged anoxia. Phosphorylated G6PDH was shown to be more active in comparison with dephosphorylated one. During anoxia and aestivation, G6PDH is phosphorylated, what results in changes in substrate affinities [9, 29, 30, 54] and enzyme activation.

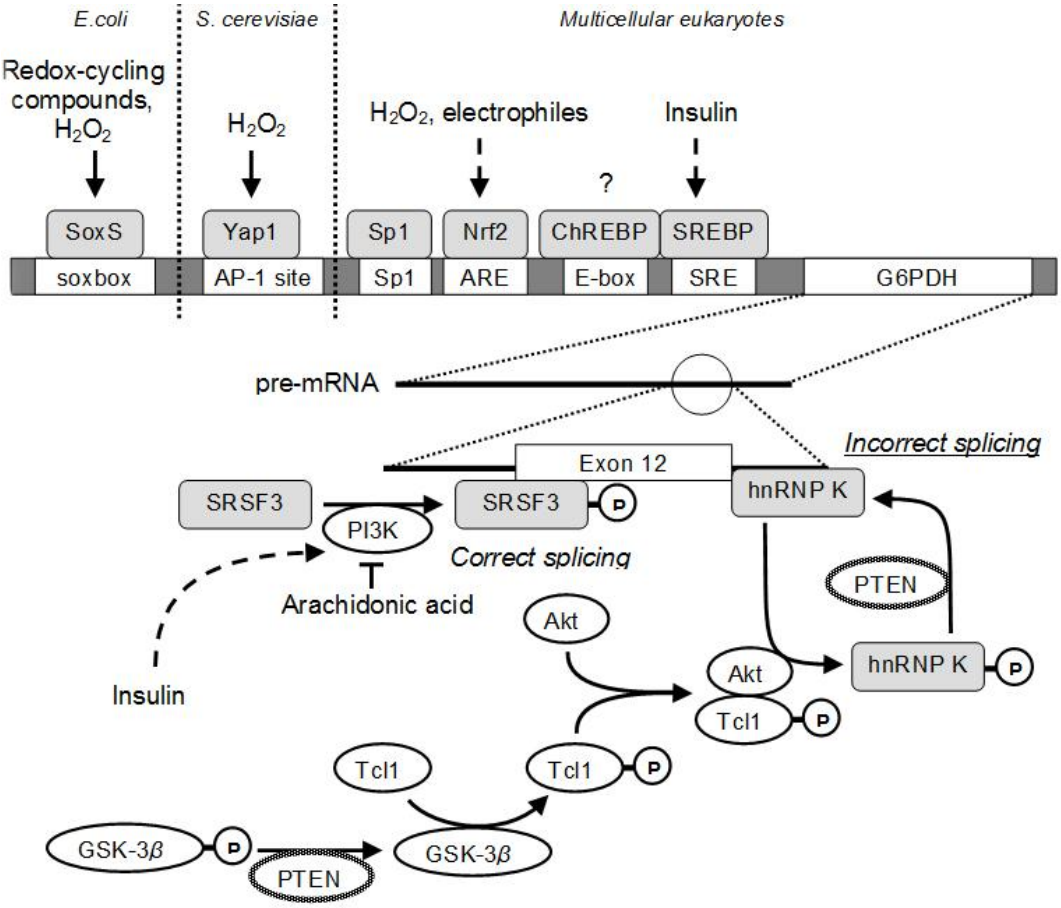

Fig. 4. Scheme of G6PDH expression regulation at transcription and posttranscription levels.

Strong oxidative stress may lead to irreversible oxidation of G6PDH resulted in its inactivation. For instance, G6PDH was inactivated during growth of catalase-deficient yeast on ethanol, a non-fermentable carbon source [41]. It was shown in this and other cases that G6PDH activity positively correlated with catalase activity, suggesting potential G6PDH protection against oxidation by catalase [5, 37]. However, there could also be other explanation for the link between G6PDH and catalase. Particularly, G6PDH and catalase activities also positively correlated in *Drosophila melanogaster*, although in this instance organism was not subjected to a strong oxidative stress [37]. Thus, activities of both enzymes are higher in fruit fly males as compared with females, and they both are higher on high carbohydrate diets as compared with low carbohydrate ones [37]. Both enzymes are involved in metabolism of fatty acids: catalase operates in peroxisomes during breakdown of very-long-chain fatty acids to decompose H_2O_2 produced as side product, while the reaction catalyzed by G6PDH produces NADPH for fatty acid biosynthesis and reduction of oxidized glutathione. At the same time, G6PDH is inhibited by certain fatty acids (e.g., arachidonic acid) at posttranscriptional level [55]. Low carbohydrate diet results in a less active lipid biosynthesis, a lesser accumulation of lipids and lesser fatty acid oxidation in peroxisomes. It may subsequently lead to expression lowering

of both, catalase and G6PDH at transcriptional level via factors responsible for lipid biosynthesis and fatty acid oxidation, or at posttranscriptional level by free fatty acids.

5. Conclusions and Perspectives

It is now obvious that G6PDH is an important enzyme for supplying cells with NADPH. As a regulatory step of pentose phosphate pathway, G6PDH controls ribonucleotide biosynthesis (since ribose-5-phosphate is formed in this pathway). In bacteria, G6PDH and 6-phosphogluconate dehydrogenase reactions are the main NADPH sources, while eukaryotes possess a battery of other enzymes which may substitute for G6PDH: malic enzyme, NADP-dependent isocitrate and malate dehydrogenases, aldehyde dehydrogenases, transhydrogenase and others. The importance of these NADP-reducing enzymes, including G6PDH, is defined by NADPH roles in cells. These roles are thiol-reduction and direct, and indirect (thiol-mediated) participation in Fe/S cluster biogenesis and heme synthesis, fatty acid and sterol biosyntheses, metal transport and sulfur metabolism. Also, NADPH is involved in many other assimilatory pathways. These roles for NADPH, especially reduction of low and high molecular mass thiols, which detoxify ROS and mediate repair under oxidative stress, characterize G6PDH as an enzyme related to antioxidant defense. However, NADPH is also used by NADPH-oxidases, ROS-producing enzymes, which are essential for immune defense.

Mutations in G6PDH cause sensitivity of *E. coli* to oxidative and related stresses [18, 57]. In the yeast *S. cerevisiae,* such mutations result in lysine and methionine auxotrophies, and in disability to grow on non-fermentable carbon sources [66]. In humans, G6PDH is characterized by hemolytic anemia under certain conditions and can be accompanied by chronic granulomatous disease [2, 6]. Over-expression of G6PDH in *Drosophila* prolongs lifespan [33], while in humans such over-expression can be deleterious and favor carcinogenesis [28, 52, 76]. Abnormalities in regulation of G6PDH activity may lead to increased lipid biosynthesis and obesity [51, 52]. These peculiarities suggest very strict regulation of G6PDH activity. Indeed, G6PDH is an example of housekeeping enzyme which is regulated by multiple ways from transcription of corresponding gene to posttranscriptional and posttranslational levels. These multiple ways of regulation provide very precise response of G6PDH activity to different stimuli. This is also natural in view of simultaneously beneficial and adverse roles of G6PDH for cell and organism survival.

From the time of its discovery, G6PDH structure, functions, and regulation in different organisms were extensively investigated. Today, this enzyme is one of the most studied ones. The reaction catalyzed by G6PDH, its substrates, coenzymes and products, as well as regulation, serve as a model of complex metabolic integration, as we see how many other reactions it affects. Presence of G6PDH in all taxa, makes this enzyme also a good example for studying the evolution of metabolic integration and metabolic pathways [69]. These aspects are just starting to be investigated and require combined approach, involving contemporary methods of biochemistry, molecular and systems biology. The G6PDH reaction is also an example for tissue-specificity of metabolic pathways and their role in providing specific cell functions. In this context, current knowledge on G6PDH has gaps in understanding of complex picture for regulation of its activity and integration with multiple processes in cells. Further studies should be devoted to untangling intertwining between reaction catalyzed by G6PDH and other metabolic pathways, including multiple feedback regulation loops and complex signaling cascades.

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Господарьов Д.В. Мало досліджені ролі глюкозо-6-фосфатдегідрогенази. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 25–37.

Огляд розглядає певні аспекти реакції, яку каталізує глюкозо-6-фосфатдегідрогеназа (Г6ФДГ), у живих організмів усіх таксонів: від бактерій до людини. Особливий наголос зроблено на ролі Г6ФДГ у гомеостазі заліза та ліпогенезі. Також висвітлені прооксидантна та антиоксидантна ролі Г6ФДГ і декілька відомих на даний час механізмів регуляції Г6ФДГ.

Ключові слова: глюкозо-6-фосфатдегідрогеназа, НАДФН, супероксид, тіол, залізо-сірчані кластери, глутатіон.

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COPPER AND COPPER-CONTAINING PESTICIDES: METABOLISM, TOXICITY AND OXIDATIVE STRESS

VIKTOR HUSAK

Abstract. The purpose of this paper is to provide a brief review of the current knowledge regarding metabolism and toxicity of copper and copper-based pesticides in living organisms. Copper is an essential trace element in all living organisms (bacteria, fungi, plants, and animals), because it participates in different metabolic processes and maintain functions of organisms. The transport and metabolism of copper in living organisms is currently the subject of many studies. Copper is absorbed, transported, distributed, stored, and excreted in the body via the complex of homeostatic processes, which provide organisms with a needed constant level of this micronutrient and avoid excessive amounts. Many aspects of copper homeostasis were studied at the molecular level. Copper based-pesticides, in particularly fungicides, bacteriocides and herbicides, are widely used in agricultural practice throughout the world. Copper is an integral part of antioxidant enzymes, particularly copper-zinc superoxide dismutase (Cu,Zn-SOD), and plays prominent roles in iron homeostasis. On the other hand, excess of copper in organism has deleterious effect, because it stimulates free radical production in the cell, induces lipid peroxidation, and disturbs the total antioxidant capacity of the body. The mechanisms of copper toxicity are discussed in this review also.

Keywords: copper, copper-containing pesticides, fungicide, bactericide, oxidative stress.

1. INTRODUCTION

For centuries, pesticides have been used in agricultural practice to enhance food production by controlling unwanted pests [2]. Many of these pesticides used extensively worldwide are copper-based formulations, including copper sulfate, copper oxychloride and copper carbonate. Pesticides containing copper have a historical significance in that the fungicidal properties of "Bordeaux mixture", named after the Bordeaux region in France, were accidentally discovered. When Bordeaux mixture, a chemically undefined mixture of copper sulfate and hydrated lime, was applied to grapes to discourage local pilfering, it was observed that downy mildew disappeared from the treated plants. It was from this serendipitous event that commercialization of fungicides originated [19].

USEPA (2008) listed copper as a pesticide and copper compounds are extensively used in various agricultural settings. Millions tons of copper are applied annually, predominantly in crop protection [64]. Copper is relatively safe from a handling perspective, but there is some concern regarding its buildup in agricultural soils. After application on plants in the field, residual Cu typically accumulates in the upper 15 cm of soil, bound to the organic matter and fine clay fraction [29]. Most

importantly, the ecological risk assessments indicate that copper is relatively non toxic for use as a broad-spectrum fungicide on many food and ornamental crops, and for direct use in water applications as an algicide, aquatic herbicide, bactericide, and molluscicide. Copper compounds also are registered for antimicrobials [67].

Copper-based agrochemicals can affect human health, causing different types of cancer, degenerative diseases, and many immune, hematological, neurological and reproductive disorders [50].

With the increasing interest in improving "old" copper-containing pesticides and especially in developing new organic pesticides, it becomes of considerable practical importance to understand molecular mechanisms of copper action. This review article describes the metabolism, toxicity and several mechanisms of oxidative stress induction by copper and copper-containing pesticides in living organisms.

2. METABOLISM OF COPPER IN LIVING ORGANISM

Copper is absorbed, transported, distributed, stored, and excreted in the body according to complex homeostatic processes which ensure a constant and sufficient supply of the micronutrient while simultaneously avoiding excess levels [56]. It is mainly absorbed through the gastrointestinal tract of animals. From 20 to 60% of the dietary copper is absorbed, with the rest being excreted through the faeces. Various factors influence copper absorption. For example, copper absorption is enhanced by ingestion of animal protein, citrate, and phosphate. Copper ions are better adsorbed from salts, including copper gluconate, copper acetate, or copper sulfate, are more easily absorbed than from oxides [63]. Elevated levels of dietary zinc and cadmium, as well as high intakes of phytate and simple sugars (fructose, sucrose) inhibit dietary absorption of copper [12, 67, 68]. Following the metal passes through the basolateral membrane, it is transported to the liver bounded to the serum albumin. The transport of copper to the peripheral tissues is performed via the plasma attached to serum albumin, ceruloplasmin, or low-molecular-mass complexes [24]. In blood, copper is distributed into a non-exchangeable red cell pool, a plasma pool associated with proteins, and a labile pool of low molecular mass complexes. In humans, approximately 80-90% of the plasma copper is tightly bound with ceruloplasmin while the rest is bound to albumin and amino acids.

Bury et al. (2003) showed that in the fish gills, Cu^{2+} is probably reduced to Cu^{+} and enters body via either a putative epithelial sodium channel (EnaC) or copper transporter 1 (CTR1). Metallochaperones (MC) bind Cu^{+} and transport it to the Golgi network (GN), where copper enters into the Golgi lumen via a Menkes Cu^{+}-ATPase (MNK). Cu^{+} is incorporated into metal binding proteins (MBP) within the GN. The vesicles of GN then transfer copper to the basolateral membrane for release via exocytosis. Other ATPases exporting copper (i.e. Ag^{+}/Cu^{+}-ATPase) may also be presented in the basolateral membranes. Intestinal export of copper may be realised via a Cu^{-}/Cl^{-}-symporter, or via the MNK pathway. Excess of copper is bound to low molecular mass proteins, such as metallothioneins (MT) (Fig. 1) [8].

Interestingly, in mammals intestinal copper uptake primarily occurs in the small intestine [67], whereas in fish, copper uptake is found on the mid/posterior region [23].

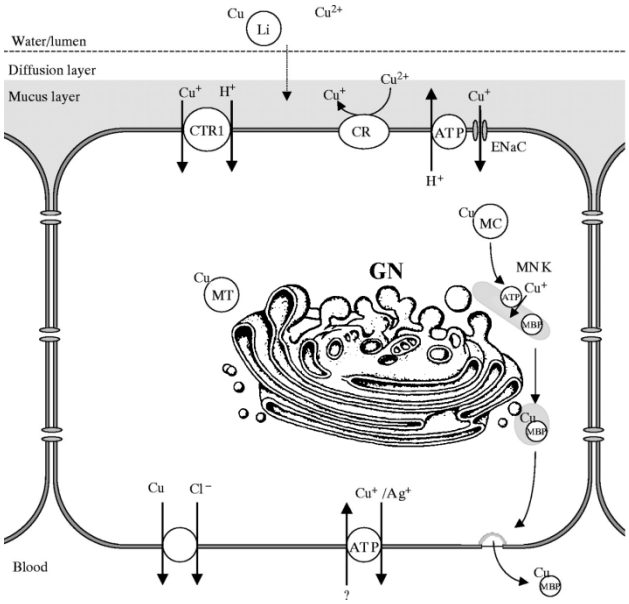

Fig. 1. Hypothetical representation of cellular copper uptake pathways in fish, combined data from gills and intestine (modified from [8]). EnaC, epithelial sodium channel; CTR1, copper transporter 1; MC, metallochaperones; GN, Golgi network, MNK, Menkes Cu⁺-ATPase; MBP, metal binding proteins; Li, aquatic ligand; CR, copper reductase.

Liver is the major organ involved in copper homeostasis [22, 31]. It accumulates a large proportion of the copper absorbed from the diet or water, and is the site for synthesis of the most abundant copper-containing protein in the body, ceruloplasmin. Ceruloplasmin is secreted into the blood and acts as a source of copper to extrahepatic organs [24]. Copper may also circulate in the body in complex with albumin and other low-molecular mass proteins [24]. The bile is the main site for secretion of excess copper in teleost fish [22]. In mammals, there are three possible secretory pathways: (1) a Cu-ATPase, identified in patients suffering from Wilson's disease (termed Wilson's protein or ATP7B) [24]; (2) a multiorganic cation transporter (cMoat) [16] and (3) lysosomal secretion [21]. Upon entering the cell through the copper transporter CTR1, copper is delivered to ATP7B in the Golgi apparatus by the copper chaperone ATOX1. In the Golgi apparatus copper is incorporated in various cuproenzymes including ceruloplasmin. When copper levels in the cell rise, ATP7B redistributes to a vesicular compartment. Upon relocalization of ATP7B, copper is excreted from the hepatocyte through the bile via an unknown mechanism that probably involves COMMD1 (Fig. 2) [13].

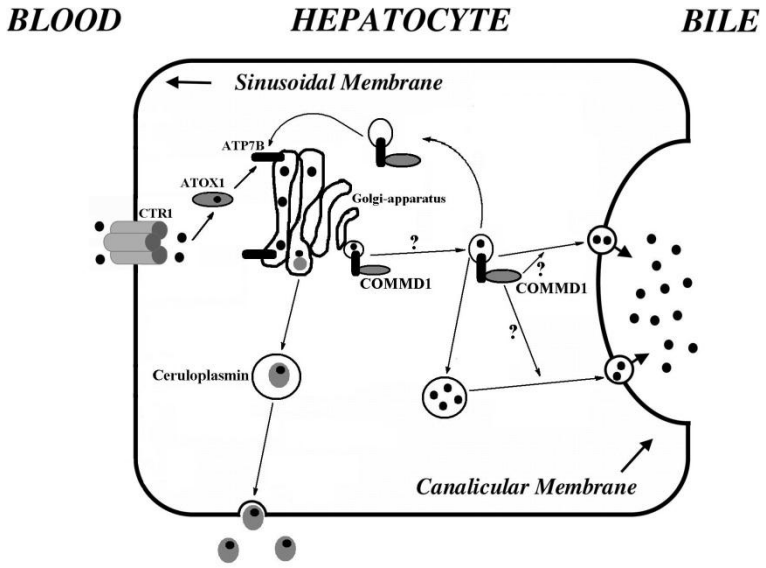

Fig. 2. The ATP7B-mediated copper export pathway in the hepatocytes (modified from [13]). CTR1, copper transporter 1; ATOX1, copper chaperone ATOX1; ATP7B, Wilson's protein; COMMD1, copper metabolism (Murr1) domain-containing protein 1.

In baker's yeast, copper is transported into the cell by two high-affinity copper transporters Ctr1 and Ctr3, or by a low-affinity Cu/Fe-transporter Fet4, depending on extracellular copper concentrations. Ctr2 could be involved in copper efflux from the vacuole. Copper is distributed via unknown mechanism to three different metallochaperones: Atx1, CCS and Cox17 [51]. Transport of copper to the secretory compartment involves the metallochaperone Atx1, that shuttles copper to Ccc2, a P-type ATPase located in the trans-Golgi network, and is responsible for Cu translocation. When Cu is transported into the lumen of the secretory pathway, it is loaded on Fet3, a multicopper-ferroxidase essential for high-affinity iron uptake that partners with the Ftr1 subunit. The delivery of copper to Sod1 is mediated by the copper chaperone for SOD, CCS. This protein contains three different domains: an Nt domain I similar to Atx1, a central domain II with homology to Sod1 and an essential Ct domain III. CCS directly interacts with Sod1 through the central domain and contacts from domain III to form a heterodimer. The metallochaperone Cox17 transports copper to Sco1, and other proteins, located in the inner mitochondrial membrane. Then copper is transferred to specific subunits of COX (Fig. 3) [51].

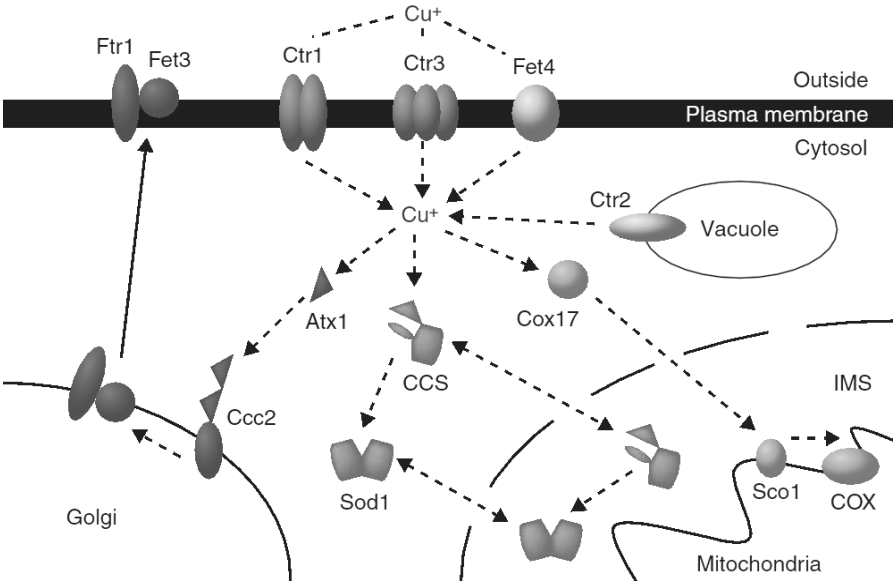

Fig. 3. Copper transport and distribution in baker's yeast (modified from [51]). Ctr1, Ctr2, Ctr3, Ctr4, copper transporters; Ftr1, Fet3, Fet4, Cu/Fe-transporters; Atx1, Cox17, CCS, metallochaperones; Ccc2, P-type ATPase; Sod1, Cu,Zn-SOD; Sco1, cytochrome C oxidase assembly protein; COX, cytochrome oxidase; IMS, intermembrane space.

3. COPPER AS A COMPONENT OF PESTICIDES

The first registration for a copper-containing pesticide, copper sulfate, was issued in 1956 [52]. Currently, 16 copper active ingredients have active food use registrations subject to tolerance reassessment and reregistration review [64].

Copper-based pesticides include aqueous solutions, wettable powders, dry flowables, dusts, flowables, water-dispersible granules, emulsifiable concentrates, and granules. There are two broad categories of copper-based products. Among them water soluble, such as copper sulphate, and insoluble in water, such the oxychlorides (Coppox®WG) and hydroxides (Hydrocop®WG). Water-soluble formulations are short lived, whereas the insoluble products release active copper ions over a period of time and are comparatively stable in nature. Concentrated water dispersible granules with additives, which ensure effective dispersion, also referred to as Dry Flowable Formulations seems are the most advanced user-friendly formulations [11].

Copper pesticides are formulated into various forms of copper, i.e., different salts and complexed forms, which ultimately dissociate into the cupric ion, the active component of concern. Among the copper-containing pesticides most common are the following: "Algimycin PWF" (copper in the form of

chelates of copper citrate and copper gluconate), a suspension concentrate formulation containing 5% metallic copper equivalent; "Macc 80" (Bordeaux mixture), a wettable powder formulation containing 200 g/kg of Cu; "Cuproxat SC" (tribasic copper sulfate), a suspension concentrate formulation containing 190 g/L of Cu; "Kocide 101" (copper hydroxide), a wettable powder formulation containing 500 g/kg of Cu; "Cuprocaffaro WP" (copper oxychloride), a wettable powder formulation containing 500 g/kg of Cu; "Nordox 75 WG" (copper (I) oxide), a wettable granule formulation containing 750 g/kg Cu; "MicroPro 200C-TS" (copper carbonate), a suspension concentrate formulation containing 28 % metallic copper equivalent; "Cutrine®-Plus" (copper ethanolamine complex), a suspension concentrate formulation containing 9 % metallic copper equivalent (Tab. 1) [11].

Copper-based pesticide	*Active ingredient*	*Use pattern(s)*
Copper (metallic)	Copper (metallic)	Algaecide, antifouling paint
Algimycin PWF	Copper (metallic in the form of chelates of copper citrate and copper gluconate)	Algaecide, fungicide, bactericide
Macc 80 (Bordeaux mixture), Cuproxat SC	Copper sulfates	Fungicide, algaecide, bactericide, herbicide, desiccant
Kocide 101, Copperhycide WP, Champion WP, Copperhycide WP, Hydrocop®WG, Zoom WP	Copper hydroxide	Bactericide, fungicide, plant growth regulator, wood preservative, antifouling paint
Cuprocaffaro WP, Rolex WP, Q-Copper WP, Curenox WP, Coppox®WG, Copral WP	Copper oxychloride	Algaecide, bactericide, fungicide
Nordox 75 WG, Caocobre, Fungi-Rap	Copper (I) oxide	Algaecide, wood preservative, antifouling paint
MicroPro 200C-TS	Copper carbonate	Algaecide, herbicide, wood preservative
Cutrine®-Plus	Copper ethanolamine complex	Algaecide, wood preservative

Tab. 1. Use patterns of copper-based pesticides.

4. TOXICITY OF COPPER AND COPPER-CONTAINING PESTICIDES TO TARGET ORGANISMS

Bacteria, fungi, and mollusks are generally the most sensitive to Cu compared with flowering plants and vertebrate animals. Common applications include controlling fungi in plants; controlling roots and other plant growth in sewers; controlling algae in swimming pools, ponds and lakes; controlling aquatic plant growth on boat hulls; serving as biocides in commercial products; and preventing rot and mildew on wood, roofing and other outdoor surfaces.

4.1. PLANTS AND ALGAE

The metal copper is a trace element essential as a micronutrient for cyanobacteria, algae and higher plants at low concentration because it is a reactant in biochemical functions of photosynthetic organisms, but at high concentrations it can be toxic [4]. In the later case, copper is very toxic for algae. It increases permeability of the cell membranes and leakage of the cellular constituents. However, the most important effect of copper on plants and algae is associated with the inhibition of photosynthesis. At high concentrations, it can be toxic by interrupting electron transport through photosystem II (PSII) [7]. The reaction center of PSII composes of a heterodimer of two integral membrane proteins, named D_1 and D_2 which bind electron transfer prosthetic groups such as P_{680}, pheophytin, and plastoquinon. PSII uses light energy to drive two chemical reactions – oxidation of water and reduction

of plastoquinone. The photosystem II complex is composed of more than fifteen polypeptides and at least nine different redox components (chlorophyll, pheophytin, plastoquinone, tyrosine, Mn, Fe, cytochrome b_{559}, carotenoids and histidine) [14, 42]. However, only five of these redox components are known to be involved in electron transport from H_2O to plastoquinone pool. There are also water oxidizing manganese cluster $(Mn)_4$, amino acid tyrosine, reaction center chlorophyll (P_{680}), pheophytin, and plastoquinone molecules, Q_A and Q_B.

Inhibition of oxygen evolution accompanied by quenching of variable fluorescence is the most apparent effect of the toxic action of copper on PSII [3, 26]. However, the precise location of the copper inhibitory binding site is still unknown. Most authors relate the target of the Cu-inhibition of PSII to its oxidizing side [7, 54, 55]. At higher copper concentrations, primary quinone acceptor Q_A [27], pheophytin-Q_A-Fe region [70], non-haem iron [28], and secondary quinone acceptor Q_B [44] were identifed as the target sites of Cu inhibitory action on the acceptor side of PSII (Fig. 4). Schroder et al. (1995) showed that Cu specifically inhibited the electron donation from Tyr_z to P_{680}, either by a modification of this amino acid in D_1 protein and/or its microenvironment [57]. Furthermore, it was demonstrated that the central magnesium atom of chlorophyll can be substituted by several metals (e.g., mercury, copper or cadmium), damaging the photosystem [34].

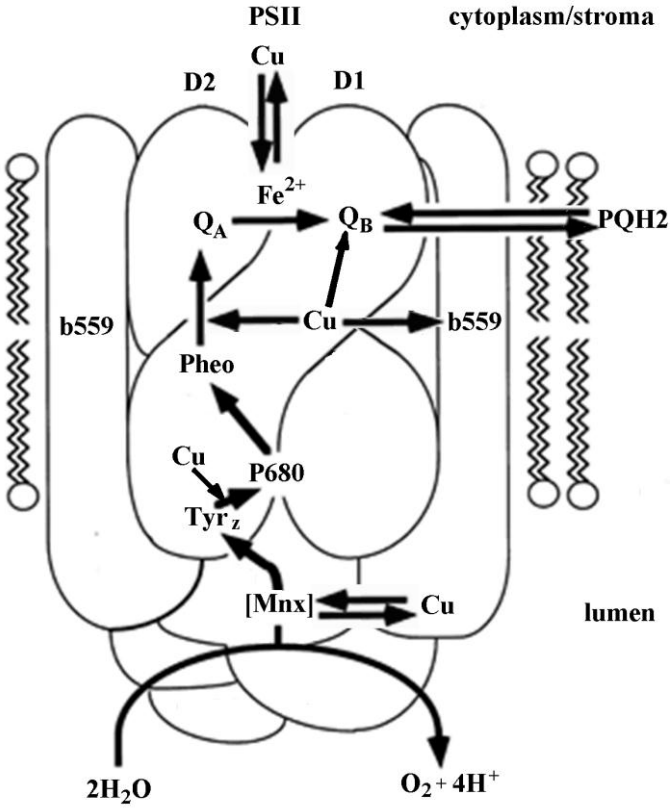

Fig. 4. Cu-inhibitory sites and action sites of different electron donors and acceptors in PSII-mediated electron transport. PSII, photosystem II; D1 and D2, bind the electron carriers involved in transfer of electrons from Tyr_z to plastoquinone; b559, cytochrome b_{559}; Tyr_z, tyrosine; Pheo, pheophytin; Q_A and Q_B, bound plastoquinone; P680, reaction center of chlorophyll (primary electron donor); PQ, reduced plastoquinone.

Cytochrome b_{559} is a heme protein and an essential component of all photosystem II reaction centers. If the membrane lacks cytochrome, a stable PS II reaction center cannot be formed [69]. It was also found that copper ions oxidized directly the low potential form cyt b_{559} at low concentrations (1-10 μM) and the high potential form at higher concentrations (10-100 μM), probably by deprotonation of this labile cyt b_{559} form [7].

Copper-induced inhibition of photosynthesis was found to be strongly related to the production of reactive oxygen species (ROS), since a number of studies reported activation of the antioxidant defense system, as well as an increase in the levels of ROS-modified lipids and proteins [15, 38]. Nevertheless,

all investigations on the specific Cu inhibitory binding site imply direct interference of the metal ion with the photosynthetic apparatus, resulting in a reduced electron flow.

4.2. Fungi, Bacterium and Mollusks

Copper is a relatively non-specific bactericide and fungicide and can kill naturally occurring microorganisms on leaves as well as those that have been applied as biocontrol including *Bacillus sp.*, *Trichoderma* and others [52].

Copper fungicides may be grouped into three general types: basic salts, normal salts, organic complexes. By far the greater part of all research on copper fungicides has been focused on Bordeaux mixture, including attempts to explain the nature of fungicidal action. Most copper fungicides are applied as foliar sprays.

Following absorption into the fungus or bacterium, the copper ions will link to various chemical groups (imidazoles, phosphates, sulfhydryls, hydroxyls) presented in many proteins and disrupt the function of these proteins and enzymes, resulting cell damage and membrane leakage [43, 52]. Thus, the mode-of-action of copper hydroxide (or any other copper fungicide) is the nonspecific denaturation (disruption) of cellular proteins.

The toxic copper ions are absorbed by the germinating fungal spores and thus for best results copper must be reapplied as plants grow to maintain coverage and prevent disease establishment [39, 40]. Up to now, little is known about mechanisms of copper induced killing before it permeation into the spore.

Copper containing compounds are among most effective for bacterial diseases including *Erwinia* soft rot (calla, orchid and poinsettia), *Pseudomonas* leaf spots (bedding plants) and *Xanthomonas* leaf spots (geranium, ranunculus and zinnia). Copper products can also react with other mode-of-action group, in the conditions of bacterial diseases or resistance development to copper. Additionally, in mollusks, copper sulfate disrupts surface epithelia function and peroxidase enzymes [52].

5. Toxicity of Copper and Copper-Containing Pesticides: Non-Target Organisms

5.1. Aquatic Organisms

Increased copper concentrations in the aquatic ecosystems can occur naturally but can also be related to different anthropogenic sources, e.g., agricultural fungicide and herbicide runoff [37]. Copper is highly toxic to most aquatic species. The main reason of copper toxicity to fish and aquatic invertebrates is rapid binding of copper to the gill membranes, causing damage and disturb osmoregulatory processes. The amount of cupric ion in the environment, and its toxicity to aquatic animals due to gill damage, depend on a number of water quality parameters including pH, alkalinity, and dissolved organic carbon [17].

Copper sulfate is toxic to aquatic invertebrates, such as crabs, shrimps, and oysters. It is used as a pesticide to control tadpole shrimp in rice production. The 96-hour LC_{50} of copper sulfate to pond snails is 0.39 mg/L at 20 °C. Higher concentrations of the material caused some behavioral changes, such as secretion of mucous, and discharge of eggs and embryos [17].

5.2. Terrestrial Organisms and Human Health

There is some uncertainty in the finding of risk to birds and mammals because, although copper is toxic at high concentrations, it is also an important essential trace element for terrestrial animals [17]. Many terrestrial animals have the ability to cope with some amount of excess copper exposure by storing it in the liver and bone marrow. Laboratory toxicity studies demonstrated that exposure to high levels of copper in the diet can overwhelm the ability of birds and mammals to maintain homeostasis. However, animals which are repeatedly exposed to levels of copper which do not cause permanent harm may undergo enzymatic adaptation which allows them to cope with greater levels of exposure [64].

Most agrochemicals, in particular copper-based formulations, seem to be responsible for several adverse effects on human health. These problems include different types of cancer, degenerative diseases, and many immune, hematological, neurological and reproductive disorders [50].

Toxic response in humans has been observed at concentration 11 mg/kg of copper. Ingestion of copper sulfate is often not toxic because vomiting is automatically triggered by its irritating effect on the gastrointestinal tract. However, the acute toxicity of copper-containing pesticides is not attributed to systemic toxicity, but to the efforts of the body to equilibrate copper concentrations [52]. Skin contact may result in itching or eczema. Copper is a skin sensitizer and can cause allergic reactions in some individuals. Eye contact with this material can cause conjunctivitis, inflammation of the eyelid lining, cornea tissue deterioration, and clouding of the cornea (Fig. 5) [17]. Ingestion of copper sulfate irritates the digestive system and may cause emesis and limiting toxicity. Copper hydroxide is less acutely toxic than copper sulfate, with an oral LD_{50} in rats of 833 mg/kg. It is also not readily absorbed through the skin, with a dermal LD_{50} of over 5000 mg/kg in rats [46]. Tissue corrosion, shock and death may occur after exposure to large doses of copper. Damage to blood cells, liver and kidney has also been reported [32].

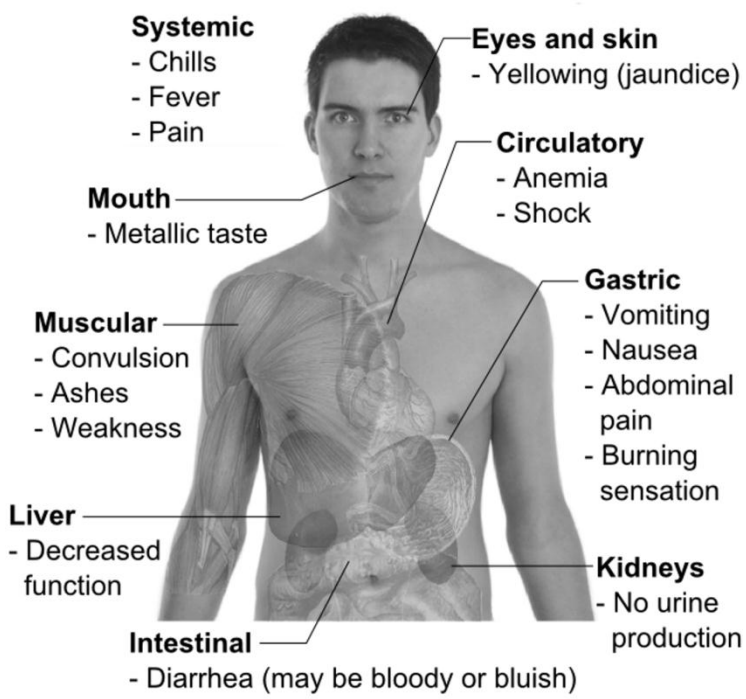

Fig. 5. Main symptoms of copper poisoning (by Mikael Häggström).

Irritant effects from occupational exposures to copper-based pesticides have been fairly frequent, including allergic reactions, itching, and eczema. Early signs and symptoms of copper poisoning include a metallic taste, nausea, vomiting, and abdominal pain. Chronic effects have been reported with vineyard workers who experienced liver disease after 3 to 15 years of exposure to Bordeaux mixture. The EPA does not require data on the teratogenic, mutagenic, carcinogenic, and reproductive effects on mammals for many of the copper-based pesticides [19].

Copper imbalances in humans lead to serious diseases such as Menkes syndrome or Wilson disease, characterized by the inability to appropriately distribute copper to all cells and tissues [41]. Additionally, copper has been strongly implicated in neurodegenerative diseases such as familial amyotropic lateral sclerosis, Alzheimer's disease, and prion diseases of neuronal spongiform encephalopathy [60].

6. Copper as Active Ingredient of Pesticides and Oxidative Stress

Copper as active ingredient of different pesticides found in all living organisms. It is absolutely necessary for survival and plays a crucial role as a catalytic cofactor in mammals in the active site of proteins, such as cytochrome c oxidase, tyrosinase, lysyloxidase, *p*-hydroxyphenyl pyruvate hydrolase, dopamine beta hydroxylase, and Cu,Zn-superoxidase dismutase (Cu,Zn-SOD). However, excessive amounts of Cu can oxidize important biomolecules, such as lipids, proteins, and DNA, mainly through the Fenton reaction [20]. Peturbations in copper concentrations in living organisms can cause oxidative stress.

Many researchers found adverse effects both *in vivo* and *in vitro* of Cu overload [5, 25, 33, 62]. Copper injection demonstrated the modification of activities of antioxidant enzymes. Enhanced concentrations of copper in living organisms caused increase in the activites of Cu,Zn-SOD and glutathione-S-transferase and decrease in the activities of catalase and selenium dependent glutathione peroxidase [35, 36].

Free Cu ions can be involved in ROS generation. Both cupric (Cu^{2+}) and cuprous (Cu^{1+}) ions can participate in oxidation and reduction reactions to form hydroxyl radicals via the Haber-Weiss reaction [6]:

$$O_2^{\cdot-} + Cu^{2+} \rightarrow O_2 + Cu^{+}$$
$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + HO^{\cdot}$$

Generation of hydroxyl radicals has been confirmed by analysis of the products of DNA damage [20, 30]. Copper binds readily to DNA to form adducts. The endogenous DNA-associated copper could promote local production of hydroxyl radicals and hence oxidative damage to DNA. The fact that copper accumulates within the nucleus at copper overload obviously enhances the likelihood of such reactions occurring [53]. In this case, formed complex Cu-DNA promotes hydroxyl radical-dependent DNA fragmentation.

Stimulation of lipid peroxidation is one of the main consequences of copper-induced production of ROS. This has been manifested as increased production of pentane and hepatic malondialdehyde when liver homogenates or hepatocytes are exposed to ionic copper. Moreover, dietary copper overload in rats resulted in *in vivo* peroxidation of mitochondrial membrane lipids demontstrated by increased concentrations of conjugated dienes and thiobarbituric acid-reacting substances (TBARS) [25, 58]. Copper-catalyzed lipid peroxidation also appears to underlie the alterations in hepatocyte lysosomes in copper-loaded rats [45]. Concentrations of TBARS in the isolated lysosomal membranes of these rats doubled, with an increase in their fragility and decrease in their fluidity. There were also changes in the membrane content of selected fatty acids, with an increase in polyunsaturated fatty acids. Lysosomal pH also increased and these membrane alterations might affect the function of the proton ATPase pump [6].

Cu-overloaded rats exhibited oxidative injury including decreased levels of hepatic GSH and α-tocopherol, increased levels of mitochondrial lipid peroxidation products, decreases in state 3 respiration of mitochondria and the respiratory control ratio in hepatic mitochondria, and decreased complex IV (cytochrome c oxidase) activity [58].

Similar to Cu overload, Cu deficiency also increased cellular susceptibility to oxidative damage, which might account for some of the pathological changes observed with low Cu status [47]. Predictably, the activities of Cu,Zn-SOD and ceruloplasmin are sensitive to tissue Cu as these enzymes require Cu as a catalytic cofactor. A Cu deficiency-induced decrease in the activities of these enzymes in humans and animals was found [48, 65]. Erythrocytes from Cu deficient rats reduced Cu, Zn-SOD activity and increased oxidative damage to several subunits of erythrocyte spectrin [61]. A deficiency of Cu also decreases the activities of certain non-Cu containing enzymes of the antioxidant defense system including catalase and selenium-dependent glutathione peroxidase [10, 59]. Cu-deficient rats exhibited increased liver lipid peroxidation, elevated hepatic Fe level, hepatic and blood glutathione and blood cholesterol concentrations [9, 18]. Furthermore, oxidative DNA damage was also detected in Cu-

deficient tissues and cells. For example, cytogenetic analysis of lymphocytes from cattle showed a significant negative association between plasma Cu concentrations and frequency of chromosomal aberrations [1].

8. CONCLUSIONS

Copper is an essential nutrient and a redox-active transition metal that may initiate oxidative damage. Virtually all organisms require copper as a catalytic cofactor for biological processes such as respiration, iron transport, oxidative stress protection, peptide hormone production, pigmentation, blood clotting and normal cell growth and development. Copper-containing pesticides primarily used as fungicides to control bacterial and fungal diseases of fruits, vegetables, nuts and field crops. Some of the diseases that are controlled by these fungicides include mildew, leaf spots, blights and apple scab. They are also used as an algaecide, an herbicide in irrigation and municipal water treatment systems, and as a molluscicide, a material used to repel and kill slugs and snails. Copper also participates in redox reactions that generate hydroxyl radical, which causes catastrophic damage to lipids, proteins and DNA. Additionally, Cu-induced oxidative damage has been implicated in disorders associated with abnormal Cu metabolism and neurodegenerative changes. Additionally, a deficiency in dietary Cu also increases cellular susceptibility to oxidative damage.

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Мета даної статті полягає в наданні короткого огляду сучасних знань про метаболізм та токсичність міді і мідь-вмісних пестицидів в живих організмах. Мідь є одним з важливих мікроелементів у всіх живих організмах (бактеріях, грибах, рослинах і тваринах), тому що вона бере участь у різних метаболічних процесах і підтримує важливі функції організмів. Транспорт і метаболізм міді в живих організмах в даний час є предметом багатьох досліджень. Мідь поглинається, транспортуються, поширюється, зберігається, і виводиться з організму через комплекс гомеостатичних процесів, які забезпечують підтримання постійного рівня цього мікроелементу в організмі і уникнення його надмірних кількостей. Багато аспектів гомеостазу міді вивчалися на молекулярному рівні. Пестициди, активним інгредієнтом яких є іони міді, зокрема, фунгіциди, бактерициди і гербіциди, широко використовуються в сільськогосподарській практиці в усьому світі, що збільшує ризик інтоксикації даним металом. Надлишок міді в організмі має шкідливий вплив, оскільки він стимулює виробництво вільних радикалів у клітині, індукує пероксидне окислення ліпідів і порушує загальну антиоксидантну здатність організму. В цьому огляді мова йтиме про механізми токсичності міді та мідь-вмісних пестицидів.

Ключові слова: мідь, мідь-вмісні пестициди, фунгіциди, бактеріоциди, оксидативний стрес.

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AMINO ACIDS: SENSING AND IMPLICATION INTO AGING

OLEH LUSHCHAK

Abstract. An ability to sense and respond to nutrient availability is an important requisite for life. Nutrient limitation is among main factors to influence the evolution of most cellular processes. Different pathways that sense intracellular and extracellular levels of carbohydtrates, amino acids, lipids, and intermediate metabolites are integrated and coordinated at the organismal level through neuronal and humoral signals. During food abundance, nutrient-sensing pathways engage anabolism and storage, whereas limitation triggers the mechanisms, such as the mobilization of internal stores including through autophagy. These processes are affected during aging and are themselves important regulators of longevity, stress resistance, and age-related complications.

Keywords: Aging, Amino acids, Macronutrients, Sensing.

1. INTRODUCTION

Macronutrients are organic compounds that undergo biochemical reactions to produce energy or plastic materials of the cell. Glucose and related carbohydrates, amino acids and lipids are main cellular nutrients, and distinct mechanisms to sense their availability operate in the cell. Essentiality is not necessarily a hallmark of nutrients; for certain amino acids essentiality is context dependent. In healthy organism, the *de novo* synthesis of these amino acids from different precursors meets organismal requirements. However, under particular metabolic needs, such as during rapid growth they must be obtained from the environment [37, 52]. Nutrient deficiency is a strong pressure for selecting efficient mechanisms of nutrient sensing in all species. Taking into account the importance of nutrient homeostasis for all living organisms it is surprising that we know relatively little about direct nutrient-sensing mechanisms. Direct binding of the molecules to their sensors or indirect mechanisms relying on the detection of intermediate molecule that reflects nutrient abundance are principal mechanisms of sensing of a particular nutrient. Regardless of the manner in which nutrient sensing occurs, for a protein to be considered as a sensor, its affinity must be within the range of physiological fluctuations of the nutrient concentration or its surrogate.

Unicellular organisms are directly exposed to environmental fluctuations of nutrients. They sense intracellular and environmental nutrient levels. In multicellular eukaryotes most cells are not directly exposed to changes in environmental nutrients, and homeostatic responses aim to control circulating nutrient levels within a narrow range. Nevertheless, internal nutrient levels fluctuate, and hence intracellular and extracellular nutrient-sensing mechanisms are also present in multicellular organisms.

In these organisms, nutrients also trigger release of hormones. They act as long-range signals with non-cell-autonomous effects, to coordinate proper responses in the organism.

In this Review, I discuss intracellular and extracellular sensing mechanisms of amino acids and their implication in aging, stress resistance and age-related disturbances.

2. SENSING OF AMINO ACIDS

Amino acids are the building blocks for proteins, critically most important molecules of living organisms. Protein biosynthesis is energetically expensive and complex. The cell senses extracellular and intracellular amino acid levels to couple their abundance and organism needs. When amino acids are limited, internal reserves may be used due to protein degradation by proteolylis for example via proteasome-mediated hydrolysis and autophagy. Amino acids are subsequently recycled and allocated for the biosynthesis of specific proteins required under nutrient limitation. Additionally, during periods of prolonged starvation and hypoglycaemia, amino acids are used for the production of other cellular constituents such as glucose and ketone bodies to fuel the particular needs of certain organs such as brain. Hence, the accurate sensing of amino acid levels is a key for proper homeostasis of proteins and amino acids, and in some cases their use as energy sources.

2.1. GENERAL CONTROL NONDEREPRESSIBLE 2 PROTEIN

In protein biosynthesis, no amino acid compensates for the absence of another. The cell must be able to efficiently detect the lack of any amino acid to prevent potential failures in peptide biosynthesis. Ribosome incorporates amino acids into a peptide with the help of specific transfer RNA in the form of aminoacyl-tRNA. The enzymes catalyzing formation of aminoacyl-tRNA, amino-acid-specific aminoacyl tRNA synthetases, execute the loading of amino acids to their cognate tRNAs [19] and uncharged tRNAs accumulate during low levels of free amino acids. Failure to complete a peptide chain under amino acid limitation is inefficient due to which cells recognize this situation and prevent translation initiation. Single protein is able to detect any uncharged tRNA independent of its amino acid specificity. This allows detection of low levels of any amino acid in the context of an abundance of the other ones. This protein is general control nonderepressible 2 (GCN2) protein that possesses high affinity to all uncharged tRNAs (Fig. 1a), and represents an elegant example of amino acid sensing by the indirect detection. Under low intracellular amino acid levels, the binding of GCN2 to a certain uncharged tRNA triggers a conformational change resulting in kinase activation and inhibition of phosphorylation of a key early activator of translation initiation – eukaryotic translation initiator factor 2α (eIF2α) [4]. Mouse models have proven the importance of GCN2 and eIF2α in mammalian responses to transient decrease in amino acids [43, 55]. This amino acid-sensing pathway seems to play a key role in the central nervous system for the detection of imbalances in amino-acid composition in food [16].

Inhibition of protein synthesis by GCN2 and eIF2α occurs in concert with other cellular responses to amino acid depletion such as the inhibition of the mechanistic target of rapamycin (mTOR) pathway. This restricts translation to those messenger RNAs that encode proteins required for cellular adaptation to nutrient starvation and suppressing biosynthesis of most other proteins [48].

2.2. MTORC1

The mTOR kinase as part of mTOR complex 1 (mTORC1) controls cellular energetic processes by inducing numerous anabolic processes including biosynthesis of proteins and lipids [22]. Growth factors activate mTORC1 through a well-studied signal transduction cascade initiated by the binding of a receptor at the membrane resulting in the activation of the Rheb GTPase. Rheb binds mTORC1 and activates its kinase in a growth-factor-dependent manner [13, 47, 55]. In addition to hormones, intracellular amino acids also activate mTORC1. In this way complex integrates information on both systemic and cellular nutrient levels. However, being highly responsive to changes in amino acid levels mTORC is not an amino acid sensor. Indeed, mTORC1 activation is an example of a key sensing signaling process, the actual nutrient sensor remain unidentified (Fig. 1b). mTORC1 is not equally

sensitive to all amino acids: for example, leucine is particularly important for its activation [17]. One can speculate on the selective importance of leucine levels for mTORC1 activation. It is one of the most reliable amino acid in proteins and probably is limiting during protein synthesis. Interestingly, GCN2-knockout mice fed a leucine-deficient diet have a more severe phenotype than the same animals fed diets lacking tryptophan or glycine [55]. Thus, leucine seems to be crucial for the organismal sensing of amino acid sufficiency and deprivation by different pathways. Molecular characterization of the amino acid-dependent activation of mTORC1 started with identification of the Rag GTPase family [39], which regulates mTORC1 through a mechanism distinct to that of growth factors. The growth factors regulate kinase activity of mTORC1 and the Rag GTPases recruit mTORC1 to the lysosomal surface to activate it [38].

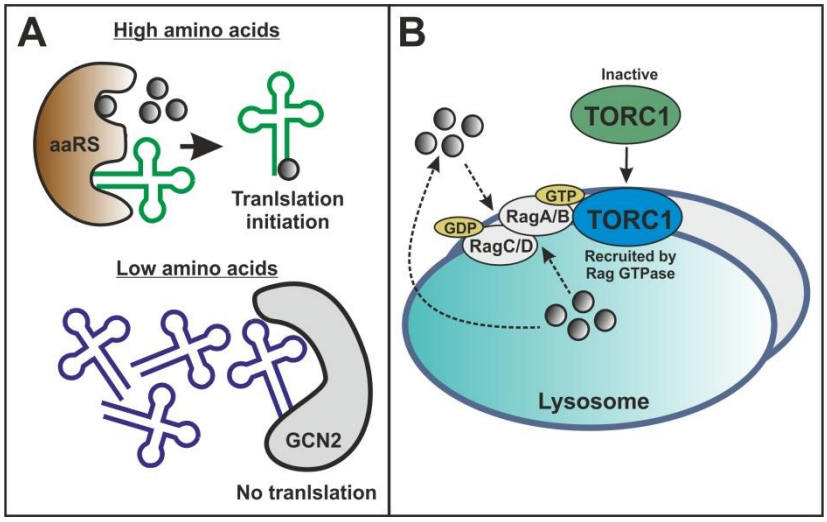

Fig. 1. Mechanisms of amino acid sensing by GCN2 protein (A) and TORC1 recruitment to the lysosomal membrane by Rag GTPases (B).

Because mTORC1 kinase activation by Rheb occurs at the outer lysosomal surface, it is only possible to follow recruitment of mTORC1 (Fig. 1b). Hence, amino acid abundance and the consequent recruitment of mTORC1 is a prerequisite for the activation of mTORC1 by growth factors. Cell-based biochemical studies have identified the proteins responsible for tethering the Rag proteins to the lysosomal surface [38] like guanine exchange factors and GTPase-activating proteins, as well as other regulatory proteins operating upstream of the Rag GTPases [3, 34, 35, 49].

The lysosomal-centered mechanism of mTORC1 activation is puzzling, but some pieces of evidence suggest that the lysosome has a key role in amino acid homeostasis. The yeast vacuole, an organelle similar to the mammalian lysosome, accumulates nutrients including amino acids (Kitamoto et al., 1988), and mTORC1 recruitment is conserved in yeast [5]. In addition, high intraluminal concentrations of certain amino acids have also been shown in lysosomes [18]. Protists such as Dictyostelium *discoideum* obtain energy through phagocytosis and lysosomal degradation [33] that is followed by a transient increase in intralysosomal nutrient levels. The lysosome and vacuole are the organelles in which amino acids and other nutrients are scavenged through the catabolic process of autophagy. Thus, high levels of amino acids within the lysosome or vacuole system seem to partially reflect cellular amino acid abundance and it is reasonable to couple its sensing with recruitment and activation of mTORC1. Germline and sporadic mutations in genes involved in the signal transduction of nutrient levels upstream of the Rag GTPases have been found in human syndromes with growth defects, neurological disorders, tumorogenesis and immunological problems [11, 49].

2.3. Amino Acid-Sensing Taste Receptors

Living organisms have to obtain energy and nutrients from external sources. Predicting the nutritional value of food before digestion allows for the accurate selection of food sources and for the

anticipation of increased nutrient abundance. Few mechanisms act synergistically, including experience and social interactions, but a fundamental nutrient-sensing event occurs at the level of the taste. Nutrient sensing by taste receptors is not only a means of sensing of extracellular nutrients, but also is a mechanism of extra-organismal sensing that allows the inquiry of prospective food sources. In humans, five test categories are present including sweet, umami, bitter, sour and salty. In taste-sensitive cells the taste receptors are exposed in the apical membrane oriented towards the environment [2].

Taste receptors T1R and T2R families belong to G-protein-coupled receptors with seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus. The T2R family is involved in the detection of bitter molecules as potentially toxic compounds. Two T1R family members are responsible to sense the presence of amino acids. Although other taste receptors also exist [2] and the genetic studies using heterologous expression experiments showed that the T1R1–T1R3 heterodimer senses amino acids. Human amino acid taste receptors have particularly high affinity to glutamate, but other L-amino acids also may be bound to them, whereas D-amino acids do not [32]. Amino acid binding to a taste receptor triggers signal transduction through the plasma membrane via activation of G-protein cascade to induce the release of neurotransmitter [8], which is then integrated with other neurotransmission events at the level of the central nervous system. Additionally, taste receptors also presented in endocrine cells of the gut [53]. Intestinal taste receptors operate through G-protein in a similar manner to that of the oral epithelium. This binding is followed by the cascade elicited by enteral taste receptors leading to release of incretins into the blood circulation. That serves as an anticipatory signal for the imminent digestion of nutrients.

Interestingly, extracellular amino-acid sensing by taste receptors can modulate mTORC1 activation without affecting intracellular amino acid levels [50], a meaningful cross-talk that evolves an anabolic machinery of the cell in anticipation of an elevation in intracellular amino acid levels.

3. Effects of Dietary Proteins and Amino Acids on Aging

Since proteins and their constituents the amino acids are major constituents of living organisms, many studies show that dietary proteins generally act as lifespan-regulating factors (Fig. 2). For example, deprivation of certain essential amino acids extends the lifespan of several model organisms, including Drosophila and mice.

3.1. Contribution of Dietary Proteins to Animal Aging

Studies with various model animals indicate a general negative correlation between the amounts of dietary proteins and lifespan. Many studies compared the effects dietary proteins and carbohydrates on aging by changing the dietary protein:carbohydrate (P:C) ratio. Investigation with Drosophila *melanogaster* show that a low-protein/high-carbohydrate diet is associated with longer lifespan [25, 28], whereas overall caloric intake had minimal effects on lifespan [29]. Similarly, low-protein/high-carbohydrate diets are linked to health and longevity in mice [44]. In fruit fly Drosophila, insulin-like peptides (dILPs) seem mediate the effects of the P:C ratio on lifespan regulating expression of target of brain insulin gene, which encodes an α-glucosidase [6]. Reduced protein intake also appears to extend lifespan by inhibiting the insulin/IGF-1 or TOR signaling pathways [20]. Additionally, it may reduce the levels of proteins with oxidative damage [6, 42].

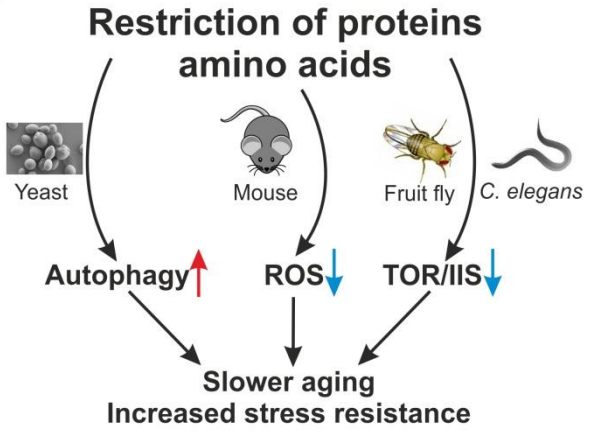

Fig. 2. Lifespan regulation by protein and amino acid restriction in varied organisms is mediated by TOR/IIS signaling, ROS and authophagy.

Despite a lack of direct evidences linking protein intake to longevity, the source of dietary protein may affect human health. A study performed on human, indicates that high animal-protein intake positively correlates with the risk of developing of urothelial cell carcinoma. Oppositely, high plant-protein intake negatively correlates with this risk [1]. This work also suggests that IGF-1 is a risk factor for the development of urothelial cell carcinoma in the setting of high animal-protein intake. A study of aged population (50-65 years) shows that subjects that consumed high amount of protein had a 75% increase in overall mortality and fourfold increase in cancer-related death risk [26]. Interestingly, plant-derived protein diet did not show this harmful effect. Further, a nutritional investigation study demonstrates that a plant-based, low-calorie diet significantly reduces total serum cholesterol and body fat percentage in obese people compared with those achieved with a traditional, low-calorie diet [27]. However, the underlying mechanisms remain undiscovered. These studies reveal potential health benefits from diet that is enriched with plant proteins. Comparably, plant proteins contain significantly lower methionine content than animal proteins [30] and this specific peculiarity may underlie the beneficial effects of dietary plant proteins.

3.2. Role of Specific Amino Acids in Longevity

Many studies have determined the effects of specific dietary amino acids on lifespan. Under low amino acid status, particularly methionine restriction, lifespan increases lifespan in Drosophila by down regulating TOR signaling [24]. Restriction in methionine extends lifespan in a variety of rat strains with different pathologies, suggesting that methionine deficiency alters the rate of aging rather than fixing a specific pathological defect [57]. Amino acid restriction significantly extends the mean and maximum lifespans of mice [31, 45]. Mice, restricted in methionine, displays reduced levels of insulin, IGF-1 and glucose, similar to calorie-restricted mice. Gene expression profiles of methionine-restricted and calorie-restricted mice were significantly different. Thus, these two dietary regimens may affect longevity through partly independent pathways. Methionine restriction also increases the lifespan of male Wistar rats and decreases the production of mitochondrial reactive oxygen species and DNA damage [40, 41].

Lifespan extension in *C. elegans* due to treatment with metformin, a well-known antidiabetic drug, and mutations in metr-1/methionine synthase are associated with decreased levels of internal methionine [7]. Several studies suggest that methionine has a positive impact on longevity [14]. Additionally, methionine supplementation does not shorten long lifespan in Drosophila with DRs [15]. Supplementation with all kinds of amino acids or essential amino acids suppresses DR-promoted longevity. Methionine restriction also causes a slight decrease in the average lifespan without affecting reproductive fitness in Drosophila [54]. Thus, other amino acids have a role in lifespan regulation. In agreement with this concept, restriction of tryptophan increases mouse lifespan [9]. Dietary amino acid composition affects lifespan by regulating various nutrient-sensing signaling pathways. In yeast, GCN2, which binds to uncharged cognate transfer RNAs [10, 51], eIF2α kinase and TOR pathway

components mediate longevity by acting as cellular amino acid sensors [12]. At reduced levels of amino acids, TOR signaling is inhibited, whereas GCN2 is activated. This inhibits overall protein biosynthesis and increases the translation of specific proteins involved in longevity. In addition, restriction of dietary tryptophan protects mice from renal and hepatic ischemic injury and reduces inflammation in a Gcn2-dependent manner in association with reduced serum IGF-1 [36]. In yeast, longevity extension due to methionine restriction is mediated by TOR [23] and autophagy [46]. The anti-aging effects of calorie restriction are largely conserved from yeast and nematodes to primates. However, it is worth to investigate whether the mechanisms through which amino acid restriction promotes life- and healthspan.

4. Conclusions and Perspectives

Despite intensive research, our understanding of nutrient-sensing mechanisms is far from complete. For instance, the amino acid sensor upstream mTORC1 has not been indentified yet. It is possible that this sensor in some way is associated with the lysosome. Since it is widely believed that mTORC1 may potentially integrate hormone and nutrient signals, its interplay with other related pathways coordinating organisms' response to change in nutrient supplementation have to be disclosed. General picture will have to be provided to clarify interplay between regulatory pathways involved in nutrient sensing, but also incorporate regulation by other signaling events. Nutrient abundance not only affects development of metabolic disturbances, but also to some extent is associated with cancer development and the ageing process.

There are many remaining challenges ahead in our understanding of the role of specific macronutrients in lifespan regulation. Alteration of one nutrient can affect lifespan, changing nutrient intake or processing of other nutrients in the mixture. Some studies on the reduction of a single nutritional component may have been misinterpreted. Recent studies indicate that dietary balance among nutrients has bigger effects on aging than individual components. Also many studies show that protein/nonprotein nutrient ratio rather than amount of proteins or calories plays key roles in the regulation of lifespan. The genetic factors that mediate effects of nutritional components on aging are mostly focused on insulin/IGF-1 signaling, TOR and sirtuins, but it does not necessarily mean that these factors are the most important ones. Therefore, identification of genetic factors using unbiased methods and systems biology approaches may lead to better mechanistic insights. Additionally the studies on human subjects uncover the effects of dietary nutritional components on health and aging. It will be exciting to combine all available knowledge to translate discoveries from those done in model organisms into therapeutic applications.

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Здатність відчувати і адекватно реагувати на доступність поживних речовин є важливим фактором для життя. Обмеження доступності поживних речовон є одним з основних факторів, що впливає на еволюцію більшості клітинних процесів. Різні сигнальні механізми, що задіяні у детекції внутрішньо- і зовнішньоклітинного рівня вуглеводів, амінокислот, ліпідів, а також проміжних метаболітів, взаємодіють на рівні організму через нервову і гуморальну системи. При доступності поживних речовин сигнальні шляхи активують анаболічні процеси, а при їх обмеженні актвуються механізми мобілізації з внутрішніх резервів включаючи аутофагію. Протікання цих процесів змінюється з віком проте вони самі є важливими регуляторами тривалості життя, чутливості до стресів, а також розвитку порушень повязаних з віком.

Ключові слова: старіння, амінокислоти, поживні речовини, чутливість.

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OXIDATIVE STRESS IN MUSCLE GROWTH AND ADAPTATION TO PHYSICAL EXERCISE

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Abstract. In a few last decades oxidative stress detected in a variety of physiological processes where reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a central role. They are directly involved in oxidation of proteins, lipids and nucleic acids. In certain concentrations they are necessary for cell division, proliferation and apoptosis. Contractile muscle tissue at aerobic conditions form high ROS flow that may modulate a variety of cell functions, for example proliferation. However, slight increase in ROS level provide hormetic effect which may participate in adaptation to heavy weight training resulted in hypertrophy and proliferation of skeletal muscle fibers. This review will discuss ROS types, sites of generation, strategies to increase force production and achieve skeletal muscle hypertrophy.

Keywords: oxidative stress, reactive oxygen species, muscle hypertrophy.

1. INTRODUCTION

In modern society, healthy people are often associate with sports lifestyle. Regular training provides a lot of benefits from enhancing immune system, metabolism and muscle force to normalizing blood pressure, body weight etc. No matter in what type of sport people are engaged their muscles always produce reactive oxygen species (ROS) in a greater or lesser extent. The reason for this lies in the mechanism of energy production. Mitochondria produce ATP during oxidative phosphorylation and therefore the generation of ROS occurs. ROS are chemically reactive molecules that formed as a natural byproduct of the normal metabolism of oxygen. In certain circumstances their levels may increase and significantly damage cell components. These ROS molecules act as oxidants leading to myoblast apoptosis or stimulators of proliferation to increase force and endurance of muscle fiber. High ROS formation occurs during intense muscle contractions and myogenesis inhibition [19, 26]. Low-to-moderate ROS levels lead to activation of growth factors, receptors, signal transduction cascades, and transcriptionally regulated genes linked to proliferation. Character of action depends on ROS type and place of its formation. Hormonal stimulation is also an important part in increasing strength and endurance of skeletal muscle.

2. ROS CLASSIFICATION AND SITES OF ITS FORMATION

The most common types of ROS are: superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$) [37]. Formation of these ROS types begins with superoxide anion. Further, hydroperoxyl radical, hydrogen peroxide and hydroxyl radical forms in below presented scheme:

$$O_2 \xrightarrow{e^-} O_2^{\bullet -} \xrightarrow[H^+]{} HO_2^{\bullet} \xrightarrow[H^+]{e^-} H_2O_2 \xrightarrow{e^-} {}^{\bullet}OH + OH^-$$

The main source of superoxide anion in the cell is the mitochondrial electron transport chain [34]. It is considered that the place of superoxide anion formation in mitochondrial electron transport chain is ubiquinone Q-cycle [27]. In this cycle ubiquinone accepts electrons from cytochrome b on the matrix side of the inner mitochondrial membrane, becoming a free radical compound – ubisemihinon. While accepting protons and electrons, ubisemihinon is reduced to ubiquinol, which translocates to the cytoplasmic side of the mitochondrial membrane. Here ubiquinol again oxidized to ubisemihinon by transferring one electron to cytochrome c1 through an intermediary Fe-S-containing protein [9]. At the last step reaction ubisemihinon reduce cytochrome b, thus transforming to ubiquinone. Some electrons can escape from ETC, especially from complexes I and III [31] and its leakage is greater during basal respiration state compared to maximal ADP-stimulated respiration [16]. Other sites of superoxide generation represented in transverse tubules, sarcolemma and sarcoplasmic reticulum by skeletal muscle NADP(H) oxidases [38, 50]. To provide NADP(H) oxidase activity after contraction, regulatory subunit $p40^{phox}$ translocates from cytoplasm to sarcolemma [42]. Following muscle contraction, superoxide anion has been detected in the extracellular space [51]. NADP(H) oxidase activity can be stimulated by calcium-dependent and independent forms of phospholipase A_2 in both cytosol and mitochondria [32]. The last notable superoxide-producing enzyme in skeletal muscle is xanthine oxidase [21]. This enzyme catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. Xanthine oxidase can also act on certain other aldehydes, purines and pterins. For instance, it efficiently converts 1-methylxanthine, a metabolite of caffeine. Caffeine is widely used before training as mental stimulator and its action may demand xanthine oxidase activity. Xanthine oxidation provides uric acid when oxygen atom from molybdenum transferred to xanthine. The reformation of the active molybdenum center occurs by the addition of water [29].

Hydrogen peroxide is one of the most persistent form of ROS with a comparably long half-life and it freely diffuses at a relatively large distance from cite of its formation [49]. In response to specific signals mitochondria produced H_2O_2 by activation of $p66^{Shc}$ protein [30]. It functions as a redox enzyme that generates mitochondrial H_2O_2 and causes swelling of mitochondria and apoptosis. $p66^{Shc}$ uses mitochondrial reduction equivalents from ETC by direct oxidation of cytochrome *c*. These data suggest that H_2O_2 generation in mitochondria is not just a by-product of respiration, but also the product of specific enzymes, such as $p66^{Shc}$. H_2O_2 interaction with copper or iron ions leads to the formation of hydroxyl radical which is highly reactive with a strong oxidizing potential and can damage carbohydrates, lipids, aromatic amino acid residues on proteins. Skeletal muscles produce $HO^{\bullet}$ during intermittent static contraction.

Nitric oxide (NO) is synthesized by nitric oxide synthases from *L*-arginine with formation of *L*-citruline. This process requires reducing equivalents of NADPH. Both the neuronal NOS (nNOS) and the endothelial NOS (eNOS) isoforms are expressed in skeletal muscle [23]. nNOS is thought to be localized in the subsarcolemmal region of skeletal muscle and at the neuromuscular junction. eNOS is uniformly distributed in muscle fibers and vessel wall [24]. *L*-Arginine is considered a dietary supplement that improves arterial vessels tone, which depends on blood pressure and nutrition of tissues, including muscle. A blood pressure decrease was observed with both *L*-arginine enriched by natural foods and oral *L*-arginine supplementation [46]. Serum total cholesterol, triglyceride decreased

and HDL cholesterol increased only in case with oral *L*-arginine supplementation. Lack of arginine and insufficient activity of NO-synthase manifested in increased blood pressure.

3. Skeletal Muscle Force Production and Hypertrophy

One of the most common effect of weight training is increase in force production. Skeletal muscle generates a number of reactive oxygen species that are increased during contraction. ROS and NO are well established compounds involved in contractile force production. Both of them demonstrate a positive impact at specific cellular levels. For example, low-to-moderate ROS levels promote full and even increased force production in unfatigued muscle [39], while antioxidant supplementation decreases this parameter [7]. NO also modulates force production at submaximal contraction intense [36], but does not involved in maximal force production. Role of ROS and RNS in fatigue postponing is controversial. Some studies report positive effect of ROS and RNS scavenging in promotion of longer contractile activity. For example, NAC (*N*-acetylcystein) administration increases duration of contractile activity and delay onset of muscle fatigue [45, 28]. Other study conclude that under physiologically relevant conditions, the recovery of force after fatigue could not be improved with antioxidants or with a nitric oxide synthase inhibitor [6]. Neither vitamin C nor vitamin E supplementation improves exercise performance in humans. Further, no beneficial effects have been observed with the combination of vitamins C and E [1, 3]. However, one report indicate improved recovery from diaphragm fatigue [10].

Progressive overload of skeletal muscle leads to adaptation in the way of increased size and amount of contractile proteins. Strength training resulted in muscle injury. Satellite cells found in outside of the muscle fibers between the basal lamina (basement membrane) and the plasma membrane (sarcolemma) of muscle fibers proliferate to these sites resulted in fusion between themselves or with muscle fibers. This process depends on nitric oxide and lies in the increasing of cross-sectional muscle area or hypertrophy. Muscle cells increase in thickness and number by division and differentiation [47]. Satellite cells fusion with muscle fibers resulted in providing additional nuclei to increase the myofilaments number. This satellite cell activation and proliferation period lasts up to 48 hours after muscle injury [15] and maintained at higher levels after several weeks of training [22]. Satellite cells express Pax7 and inactivation of Pax7 results in severe depletion of these muscle stem cells [43]. They are classified as myogenic stem cells because of their ability to maintain their own population by self-renewal. Muscle growth and regeneration highly dependent on satellite cells, because their number is not restored after ablation.

Insulin-like growth factor 1 (IGF-1) is a downstream effector of growth hormone (GH). IGF-1 closely linked to protein synthesis and repair. Mechano growth factor, a splice variant of IGF-1, suggested to play crucial role in muscle growth in response to strength training. Mechano growth factor causes muscle hypertrophy in response to a mechanical stimulus by activating muscle satellite cells and increasing the upregulation of protein synthesis through IGF-1 receptor. ROS has a critical function in muscle hypertrophy via MAPK signaling and action of IGF-I. A recent study found that the ingestion of a leucine-enriched essential amino acid-carbohydrate mixture resulted in significant increase in muscle protein synthesis, reduced AMPK phosphorylation, increased Akt/PKB and mTOR phosphorylation, increased mTOR signaling to its downstream effectors increasing both S6K 1 and 4E-BPI phosphorylation, and reduced eEF2 phosphorylation [13].

Many athletes face with a reduction in the protective function of the immune system. This results in decreased physical performance or even risk to fall ill. Some researchers have demonstrated that the leukocytes and lymphocytes have been increased due to stress of exercise. Muscle injury stimulate macrophages activity, which are necessary to provide adequate response by secreting cytokines and growth factors [35]. Interleukin-1, Interleukin-6 and tumor necrosis factor alpha are responsible for protein breakdown, an increased production of prostaglandins and removal of damaged muscle cells. It has been reported that caffeine ingestion prior to exercise enhances the activation of both the

hypothalamic-pituitary-adrenal axis and the autonomic nervous system, which in turn, may affect the immune response to exercise. Nonessential amino acid *L*-glutamine is highly utilized by cells of the immune system for proliferation, antigen presentation, phagocytosis, cytokine, nitric oxide and superoxide production [33]. Thus, *L*-glutamine supplementation may provide additional protection and boost the efficiency of the immune system.

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone and combined with exercise, increase fat-free mass, muscle size and strength in normal men with previous weight-lifting experience who were on standardized diets [2]. Using AAS provide higher resistance to muscle fatigue during training. Authors speculate, that it can be the effect of blocking cortisol action, since cortisol level increases in response to steroid administration [17]. Cortisol is a steroid hormone, which is produced in the adrenal cortex of the kidney in response to stress. Increase in cortisol is related to an increased rate of protein catabolism, in a way of breaking down muscle proteins, inhibiting skeletal muscle hypertrophy [18]. Receptor binding and susceptibility to biotransformation govern androgenic anabolic steroids efficacy.

AAS and GH are widely administrated by athletes and bodybuilders to increase muscle hypertrophy and strength [11]. Extremely high doses or long period of use may cause tumorigenesis, water retention, hyperglycemia, testicular atrophy, gynecomastia, myocardial infarction, heart failure and many other side effects. Some AAS are directly involved in oxidative stress appearance. For example, repeated low level administration of turinabol and methanabol had no significant effects on oxidative stress markers, while the high dose created oxidative stress and myocardial dysfunction in young rabbits [14]. Authors emphasize high hazard from high doses of these AAS, especially in young human subjects. In addition, nandrolone decanoate administration to male rats induces oxidative stress, seminiferous tubules dysfunction, and sperm DNA fragmentation [48] and disrupts redox homeostasis in liver, heart and kidney of male Wistar rats [12]. For instance, acute effects of nandrolone decanoate do not promote the production of ROS and even possess some anti-oxidative potential [20]. Anabolic steroid stanozolol decreases mitochondrial ROS generation during acute exercise [41]. High doses of GH also involved in detrimental effects related to energy metabolism and oxidative stress [44], whereas close-to-physiological levels prevents memory deficits in early stages of the neurodegenerative process and slow down adverse effects of immobilisation in old rats muscle tissue [5, 40].

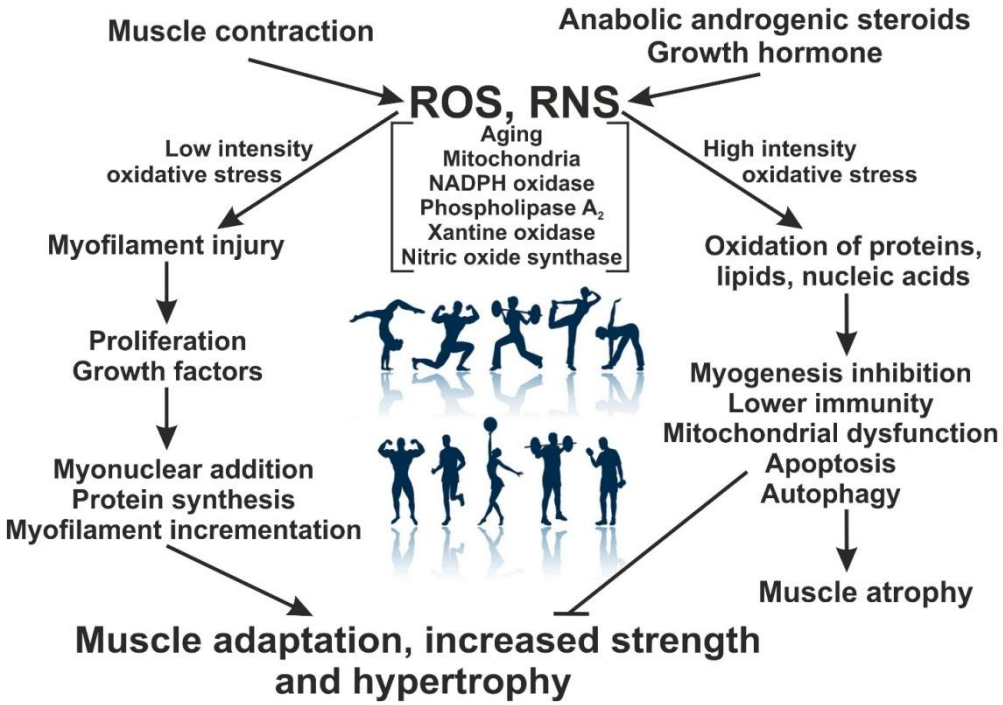

Fig 1. Possible role of ROS/RNS in muscle growth and adaptation to physical exercise. Muscle contraction, anabolic androgenic steroids and growth hormone cause induce changes in ROS and RNS levels. Low intensity oxidative stress promotes hypertrophy and adaptation of myofibers through stimulation of proliferation and action of growth factors. This provides additional nuclei and increases the myofilaments number, enhances protein synthesis and as a result myofilament incrementation. Oxidative stress of high intensity causes unfavorable oxidation of proteins, lipids and nucleic acids. Oxidation of this macromolecules decreases myogenesis, immunity, mitochondrial functions and stimulate apoptosis and autophagy. These degenerative processes results in muscle atrophy.

4. CONCLUSIONS

Oxidative stress is directly involved in contractile muscle force production, proliferation and hypertrophy. Many muscle processes need ROS and RNS for its functions. To achieve muscle hypertrophy hormone therapy does not required. Yes, it would take more time and will require close attention to details, such as training methodology, nutrition and recovery, but keeps healthspan or even improve it. Nowadays allot of non-hormonal supplements available in the markets of sport nutrition. Some of them work, some of them is just a waste of money. Nonetheless, well established creatine monohydrate, BCAAs, *L*-glutamine, *L*-carnitine, *L*-arginine-α-ketoglutarate and weight training combined with of course protein whey enhance muscle hypertrophy and force production [8, 25, 4].

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Юркевич І.С. Оксидативний стрес при м'язовому рості та адаптації до фізичних навантажень. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 61–69.

За останні десятиліття було встановлено роль вільних радикалів в багатьох фізіологічних процесах. Активовані форми кисню (АФК) та активовані форми нітрогену (АФН) безпосередньо задіяні в цих процесах. Вони беруть участь в окисленні білків, ліпідів та ДНК. В певних концентраціях вони необхідні для клітинного поділу, проліферації та індукції апоптозу. М'язова тканина в аеробних умовах генерує велику кількість АФК, які можуть модулювати різні клітинні функції. Проте, невелике збільшення рівня АФК створює горметичний ефект, який може забезпечити адаптацію до силового

тренування, яке призводить до гіпертрофії і проліферації скелетних м'язових волокон. У цьому огляді ми зупинимося на класифікації АФК, місцях їх утворення, стратегіях для збільшення сили і гіпертрофії скелетних м'язів.

Ключові слова: оксидативний стрес, активні форми кисню, гіпертрофія м’язів.

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PESTICIDES: BENEFITS AND HAZARDS

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Abstract. Pesticides are an integral part of modern life used to prevent growth of unwanted living organisms. Despite the fact that scientific statements coming from many toxicological works provide indication on the low risk of the pesticides and their residues, the community especially last years is deeply concerned about massive application of pesticides in diverse fields. Therefore evaluation of hazard risks particularly in long term perspective is very important. In the fact there are at least two clearly different approaches for evaluation of pesticide using: the first one is defined as an objective or probabilistic risk assessment, while the second one is the potential economic and agriculture benefits. Therefore, in this review the author has considered scientifically based assessment of positive and negative effects of pesticide application and discusses possible approaches to find balance between them.

Keywords: pesticides, xenobiotics, benefits, hazards, toxicity.

Abbreviations: DDT, dichlorodiphenyltrichloroethane; BHC, benzene hexachloride; 2,4-D, 2,4-dichlorophenoxyacetic acid; FAO, Food and Agriculture Organization of the United Nations.

1. INTRODUCTION

Modern agribusiness includes extensive use of pesticides in their activity and it is absolutely clear that in the near future the use of pesticides will be increased. This is due to growing consumption of food at global scale. But, food production faces different problems. For example, new approaches to cultivate and increase agricultural production from the areas are developed. Mechanization and technological advances and the emergence of new pesticides allow farmers to grow and manage bigger areas of crops with the reduced labor force [7, 29].

Ideal pesticides must act selectively against certain pest organisms without adverse effects to to non-target organisms. However, is difficult to achieve absolute selectively and most pesticides are a toxic also to humans and other non-target organisms. Pesticides are the most important reason of self-poisoning in the developing world. Three million cases of pesticide poisonings, nearly 220,000 fatal, occur world-wide every year [7, 12]. Certain pesticides can be used safely and effectively. But if proper care is not taken, pesticides can harm the environment by contaminating soil, surface and ground water, and ultimately kill wildlife.

Increase in food consumption resulted in enhanced agriculture productivity to big extent relied on extensive use of pesticides. Extensive application of pesticides allowed to prevent or to reduce

agricultural losses to pests and promoted great availability of food at a reasonable price and at any season [12].

However, modern agricultural development has led to great increase in production of agrochemicals. Therefore, pesticides are an integral part of modern life used to prevent growth of unwated species [7]. That is why, in this review the author considers objective assessment of positive and negative effects of pesticides used. In other words, this question can be formulated rhetorically: use or not use the pesticides? Certainly, the question is too radical, and obviously pesticides will be used, but how to minimise their negative effects – that is really matter of life.

2. WHAT ARE PESTICIDES?

The term "*pesticide*" indicates any substance or mixture of substances used to kill, repel, or otherwise control a "*pest*", including insects, snails, rodents, fungi, bacteria, and weeds [7]. Pesticides are used for a long time. Documented history of pesticides began in ancient Rome, but their extensive began after World War II with the introduction of dichlorodiphenyltrichloroethane (DDT), beta-hexachlorocyclohexane (BHC), aldrin, dieldrin, endrin, and 2,4-dichlorophenoxyacetic acid (2,4-D). Food and Agriculture Organization of the United Nations (FAO) defines, pesticide as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances [12].

Pesticides can be classified by target organism (e.g., insecticides, herbicides, fungicides), chemical structure (organochlorines, organo-phosphates, carbamates, phenoxy acids), and physical state (solid, liquid, aerosol) [6]. So, depending on the chemical structure the most popular pesticides may be divided into the following groups [7, 15, 20]:

1. Organochlorines (endosulfan, hexachlorobenzene);
2. Organophosphates (diazinon, omethoate, glyphosate);
3. Carbamic and thiocarbamic derivatives;
4. Carboxylic acids and their derivatives;
5. Urea derivatives;
6. Heterocyclic compounds (benzimidazole, triazole derivatives etc.);
7. Phenol and nitrophenol derivatives;
8. Hydrocarbons, ketones, aldehydes and their derivatives;
9. Fluorine-containing compounds;
10. Copper-containing compounds;
11. Metal organic and inorganic compounds;
12. Natural and synthetic pyrethroids and others.

Any rational approach to pesticide use should include a risk-benefit comparison. Many people want to know more about pesticides, their benefits and risks for them.

3. THE BENEFITS OF PESTICIDES

The primary benefits are known as consequences of the direct pesticides' effects such as protection of people, animal and crop health and protection of recreational turf. The secondary benefits arise from primary and these are the less immediate, less intuitively obvious, or longer term consequences. Table 1 summarizes effects, primary and secondary benefits, and their interactions. The interplay between negative effects and benefits is complex and not easy to follow always.

Primary benefits	*Secondary benefits*
1. Controlling pests and plant disease vectors	**Community benefits**
Improved crop/livestock quality Reduced fuel use for weeding Reduced soil disturbance Invasive species controlled	Nutrition and health improved Food safety/security Life expectancy increased Reduced maintenance costs
2. Controlling disease vectors and nuisances organisms	**National benefits**
Human lives saved Human disturbance reduced Animal suffering reduced Increased livestock quality	National agricultural economy Increased export revenues Reduced soil erosion/moisture loss
3. Prevent or control of organisms that harm other human activities and structures	**Global benefits**
Tree/bush/leaf hazards prevented Recreational turf protected Wooden structures protected	Less pressure on uncropped land Fewer pest introductions elsewhere International tourism revenue

Tab.1. The complexity of the effects, primary and secondary benefits of pesticides [12].

Over last 60 years, farmers achieved significant progress in production of foodstuff by using pesticides. They have done this principally to prevent or reduce agricultural losses due to activity of pests which resulted in improved yield and greater availability of food, at a reasonable price and over all seasons. By the use of pesticides in agriculture, the productivity has increased dramatically in most countries. For example, wheat yields in the United Kingdom [4], corn yields in the USA [23], and total yields in the Russia and other countries were enhanced enormously [11, 21, 32].

It has been long believed that diets containing fresh fruits and vegetables far outweigh potential risks from eating very low residues of pesticides in crops [9]. Improved nutrition and reduced drudgery both improve the quality of life and longevity [16]. Improved medical care and drug treatments along with hygiene have played a significant role in extending lives, but the value of nutritious, safe and affordable food should not be underestimated as a health promoter that increases life expectancy [3, 31].

Control of wide range of human and livestock disease vectors thus reducing the number of infected individuals and deaths accompanied by prevention of international disease spread is among critical obvious benefits of broad pesticide use. Killing of vectors is the most effective method to struggle them. According to the World Health Organization [2] without access to chemical control methods life will be unacceptably dangerous for a large proportion of mankind.

Pesticides play an important role in destruction of various organisms which have a negative impact on human activities, infrastructure and the materials of everyday life. In many specific sectors of human activity, pesticides are used to control unwanted organisms, such as prevention of accelerated corrosion of metal constructions, maintain the turf on sport pitches, cricket grounds and golf courses, helping to facilitate a hugely popular pastime that provides fresh air and exercise for millions of people around the world in domestic and ornamental gardening etc.

4. HAZARDS OF PESTICIDES

Pesticide use raises a number of environmental concerns, including human and animal health hazards. Food contaminated with toxic pesticides is associated with severe effects on the human health

because it is the basic necessity of life. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water and soil [28].

Pesticide toxicity can result from ingestion, inhalation or dermal absorption. Continued exposure to these chemicals for a long period may result in various diseases some of which are listed below:

- Neurological, psychological and behavioral dysfunctions;
- Hormonal imbalances, leading to infertility, breast pain;
- Immune system dysfunction;
- Reproductive system defects;
- Cancers;
- Genotoxicity;
- Blood disorders.

Pesticides can contaminate soil, water, turf, and other vegetation. In addition to killing insects or weeds, pesticides can be toxic to a host of other organisms including birds, fish, beneficial insects, and non-target plants.

Recent articles and reports review toxicological and epidemiological evidences for various health effects associated with pesticides [22, 25, 26]. Extensive toxicological studies in animals demonstrate that a number of pesticides to which the general population may be chronically exposed are potential carcinogens, neurotoxins, reproductive toxins, and immunotoxins [5, 8]. González with colleagues [18] showed DNA damages under 2,4-D exposure in Chinese Hamster ovary cells (CHO). There are evidences on involvement of pesticides in development of neurodegenerative diseases [15, 19]. Many scientists reported impact of pesticides on biochemical parameters, in particular on protein metabolism [24], endocrine [13, 17, 30], and reproductive systems [1].

Pesticide contamination of both surface and ground waters can affect aquatic fauna and flora, as well as human health when water is used for public consumption [10]. Aquatic organisms are directly exposed to chemicals resulting from agricultural production via surface run-off or indirectly through trophic chains.

5. PESTICIDE ALTERNATIVES

In fact, modern agricultural business leads to environmental crisis. Excessive use of pesticides and to increase the food production may result in serious environmental "diseases". They may be grouped into two sets [29]:

1. Diseases of ecotopes (erosion, loss of soil fertility, depletion of nutrient reserves, salinization and alkalinization, pollution of water systems);
2. Diseases of biocoenosis (loss of crop, wild plant, and animal genetic resources, elimination of natural enemies, pest resurgence and genetic resistance to pesticides, chemical contamination, and destruction of natural control mechanisms).

Due to this and many other reasons, search for "safest" analogue pesticides of natural origin is for one of the most important problems of our civilization. Potential alternatives to pesticides are available and include specific methods of plant cultivation, use of biological pest controls (such as pheromones and microbial pesticides), plant genetic engineering, and methods of interfering with insect breeding [28]. Application of composted yard waste has also been used as a way of controlling pests [27].

The major alternatives to traditional chemical pesticides are listed below:

1. Natural pesticides;
2. Biological pest control;
3. Plant genetic engineering;
4. Interfering with insect breeding;
5. Application of composted yard waste;
6. Cultivation practices;

7. Release of organisms that fight the pests;
8. Interfering with insects' reproduction;
9. Soil steaming.

These methods become increasingly popular and usually are safer than traditional chemical pesticides. The polyculture (growing multiple types of plants together), crop rotation, planting crops in areas where the pests that damage them do not live and use of trap crops that attract pests away from the real crop are elements of cultivation practices [29]. Another example of an alternative to pesticide is using of other organisms that fight the pest. These organisms can include natural predators or parasites of the pests [28].

There is no enough information on the effectiveness of discussed above alternative methods, but further research in this area may help to replace chemical pesticides by ecologically friendly technologies.

6. CONCLUSIONS

Due to growing consumption of food, modern agribusiness involves extensive use of pesticides in their activity. This has led to significant progress in food production, but many problems in the field of environmental protection and health arised. The data, described in this review, on benefits and environmental-health risk assessment studies may be regarded as an aid towards a better understanding of the problems related to global using of pesticides.

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Максимів І.В. Пестициди: переваги та ризики. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 70–76.

Пестициди є невід'ємною частиною сучасного життя і використовуються для запобігання росту небажаних живих організмів. Незважаючи на те, що результати багатьох наукових досліджень свідчать про низький ризик використання пестицидів та продуктів їх перетворень, суспільство, особливо в останні роки, серйозно стурбоване масовим застосуванням пестицидів у різних сферах. Тому так важлива оцінка загроз, зокрема, в довгостроковій перспективі. Насправді, принаймні, є два різні підходи для оцінки пестицидів: перший – це об'єктивна або ймовірнісна оцінка ризиків, а другий представляє потенційні економічні та сільськогосподарські переваги. Тому в даному огляді автор розглянув науково обґрунтовану оцінку позитивних і негативних наслідків використання пестицидів і обговорює можливі підходи для знаходження балансу між ними.

Ключові слова: пестициди, ксенобіотики, переваги, небезпеки, токсичність.

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REGULATION OF *DROSOPHILA* INTESTINAL STEM CELL MAINTENANCE AND PROLIFERATION

OLHA STRILBYTSKA

Abstract. To maintain gut homeostasis intestinal stem cells (ISCs) constantly replace damaged ones. This process is conservative from *Drosophila* to human. Proliferation and differentiation of ISCs in adult *Drosophila* midgut are regulated by growth factors which are secreted in the surrounding cells collectively forming ISCs niche. Here I discuss an interaction between ISCs with its niche through conservative signaling pathways. Several evidences on significance of cooperation between multiple signaling pathways including Notch, Wingless, JAK/STAT, EGFR, Hippo, and insulin signaling for regulation of stem cell maintenance and activity are provided. Further investigation in this area will allow us to understand how proper regulation of ISCs maintenance and differentiation can assist to ensure intestinal integrity.

Keywords: intestinal stem cells, *Drosophila*, niche, signaling pathway, midgut.

Abbreviations: ISCs, intestinal stem cells; EBs, enteroblasts; ECs, enterocytes; EEs, enteroendorcine cells.

1. INTRODUCTION

The digestive tract of *Drosophila melanogaster* plays a key role in digestion, absorbtion, transit, and excretion. *Drosophila* gut is one of the biggest organs in its body. It consists of foregut, midgut and hindgut. The midgut is composed of an epithelium covered by visceral muscle. The epithelium consists of intestinal stem cells (ISCs), enterocytes (ECs), and enteroendocrines (EEs). All these cells form the niche which controls the ISCs proliferation and differentiation of enteroblasts (EBs). Undergoing asymmetric division, ISC can produce two types of cells. One such type, stem cells, to keep the amount of ISCs constant and the second counterpart, enteroblasts, which further differentiate into enterocyte or enteroendocrine cell [22].

There are several signaling pathways like Notch, Wingless (Wg)/Wnt, and JAK-STAT pathways, which regulate maintenance, proliferation, and differentiation of ISCs [5, 13, 19]. Understanding of molecular mechanisms will allow us to uncover developmental origin of adult stem cells, the role of their niches and how the destiny of stem cells and their progenies are regulated.

In this review, I describe how intestinal stem cell niche creates an environment, which regulates self-renewal and differentiation of ISCs.

2. INTESTINAL STEM CELL NICHES

The adult *Drosophila* midgut has many similarities to the mammalian intestine and is well characterized. It is an attractive and useful model for investigation the ways of regulation stem cells maintenance, differentiation, and proliferation [7]. The *Drosophila* midgut consists of ISCs which are located on the basement membrane of the epithelium (Fig. 1). ISCs undergo frequent asymetric divisions. In average, midgut cells are renewed approximately four times during the eight week lifespan of the adult female fly [13]. After division, one dauther cell maintain its stemness and the other one become enteroblast, which subsequently differentiates into enterocyte or enteroendorcine. Approximately ninety percents of the EBs will become EC cells, and the rest will become EE cells [25]. These four cell types are characterized by specific array of differently expressed genes.

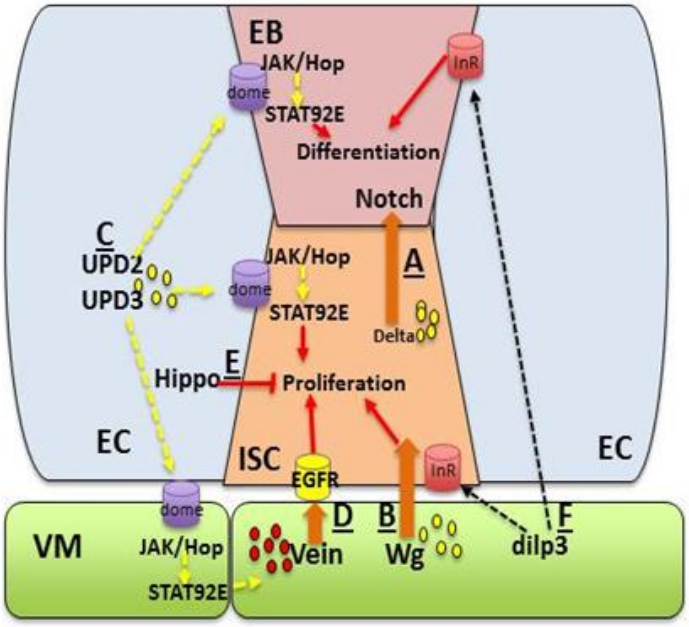

Fig. 1. Input signals from surrounding niche cells that regulate ISCs maintaining, differentiation and proliferation. The diagram represents the genetic interactions between ISCs and the cells of their niche. VM – visceral muscle; ISC – intestinal stem cell; EB – enteroblast; EC – enterocyte; Wg – Wingless; Upds – unpaireds; JAK – receptor-associated Janus Kinase; Hop – Hopscotch; STAT – Signal Transducer and Activator of Transcription; EGFR – Epidermal growth factor receptor; Dome – Domeless; InR – insulin receptor; dilp – Drosophila insulin like peptide.

ISCs are diploid, have a small nucleus and express ligand Delta, which is specific for the receptor of Notch-signaling pathways. EBs are diploid with a small nucleus and express the transcriptional reporter for Notch – Supressor of hairless (SuH) [31]. ECs are polyploid with a large nucleus and express transcriptional factor Pdm1. EEs are diploid cells with a small nucleus and express transcriptional factor Prospero.

ISCs contact with each other via visceral muscle integrins. The muscle express several potential niche factors that are capable to promote ISCs dividing, including wingless (Wg), vein (an Egfr ligand), and dilp3 (an insulin-like peptide) [19, 35]. That is why visceral muscle serves as the stem cell niche. On the other hand, ECs and EEs produce growth factors that regulate stemness and differentiation. These factors are important during regeneration after damage. During midgut development ISCs proliferate intensively and form clusters. Some cells in these clusters become peripheral cells and serve as a transient niche for the progenitors [21]. Cells in this niche produce Dpp (Decapentaplegic) that inhibit differentiation and epidermal growth factor receptor (EGFR) ligands.

3. NOTCH SIGNALING

There is a variety of evidences on Notch as a major regulator of ISCs self-renewal. Notch signaling is evolutionary conserved mechanism important for development. ISCs control differentiation of daughter cell by modulating Notch signaling [25], and Notch is specifically activated in EBs (Fig. 1) [36]. The Notch proteins (single-pass receptor) are activated by Delta ligand. They are transported to the plasma membrane and induce dissociation of Notch intracellular domain (NICD) from the plasma membrane. In the absence of NICD, the DNA-binding protein CLS associates with ubiquitous corepressor (Co-R) proteins and histone deacetylases (HDACs). These factors inhibit the expression of Notch target genes [12]. NICD binds to the CSL and in this way triggers the splitting of transcriptional repressors. Binding of NICD-CLS complex to the components of activation complex MAML1 and HDACs facilitates transcription activation of Notch target genes (Fig. 2) [12]. Numerous of findings indicate that proliferating cells and ECs require the activity of Notch. The downstream transcriptional repressor Hairless prevents transcription of Notch target genes and is sufficient for ISCs self-renewal [3].

Notch signaling has dual role: it controls balance between stem cells and their differentiating progeny and determines the type of progeny [32]. Notch pathway activates Hes-1, the bHLH transcription factor, which is expressed by ECs. The activity of Notch signaling is low in EEs and ECs, which express Math1 and Neurogenin 3, the bHLH proteins [17, 29]. Interestingly, these proteins belong to the same bHLH family and this may show common evolutionary origin of ECs and EEs [10]. Notch is a central signal mechanism for stemness maintaining.

4. WINGLESS (WG)/WNT SIGNALING

Investigation of Lin et al. [19] demonstrated the role of Wnt/Wg signaling in gut homeostesis and the role of ISC niche in the communication between intestinal epithelium and neighboring tissues (Fig. 1). Visceral muscle were found to produce a Wnt ligand, wingless (wg) (Fig. 2) which is very important for ISC maintenance [35]. It was observed that wg is expressed in the visceral muscle and Wg protein is located between visceral muscle and basement membrane [16]. According to this observation, visceral muscle secret Wg through basement membrane. Loss of wg significantly reduces the ISC number [19]. The proliferating effect of Wnt/Wg signaling is mild and the cooperation of Wnt/Wg with EGFR and JAK/STAT regulates a balance between ISC proliferation and maintenance [35].

Wingless activates proliferation that is why loss of this signal results in a loss of dividing cells, and also causes apoptotic cell death [33]. But ISCs proliferation induced by Wnt signaling is much weaker than induced by JAK/STAT or EGFR [14, 22]. Hence one can suggest that other signaling pathways play more important role in ISCs proliferation.

5. JAK/STAT

The JAK/STAT system consists of a receptor, Janus kinase (JAK), and signal transducer and activator of transcription (STAT) [1]. The JAK/STAT signaling pathway is highly conserved from flies to mammals, and plays essential roles during development. It also serves as a regulator of stem cells and their niches. *Drosophila* possesses relatively not complex JAK/STAT pathway. Signaling ligands for this pathway Upd2 and 3 (Unpaireds) both contribute to ISC mitosis, and both of them trigger synthesis of the antimicrobial peptide drosomycin-3. Upds are sufficient to activate JAK/STAT pathway activity in ISCs [4].

A ligand binding to the receptor Dome (Domeless) activates the associated JAK Hopscotch (Hop), which further recruits and phosphorylates the transcription factor STAT92E and finally these events

result in signaling triggering (Fig. 1) [4]. Dimerization of STAT92E allows its translocation into the nucleus where it activates expression of target genes (Fig. 2).

During normal tissue homeostasis in the *Drosophila* midgut, JAK/STAT signaling, does not act on itself, but is a part of a greater regulatory network. It coordinates collective operation of EGFR and Wg signaling pathways. This coordination is necessary for ISC maintenance, and promotes differentiation of EBs. It is suggested, that stresses or infections upregulate JAK/STAT signaling. In these conditions, the signals back from EC and EB to ISC and promote ISC proliferation [4].

6. EGFR

The study of Buchon et al. [6] revealed that a canonical EGFR pathway is required in ISCs and promotes their proliferation. EGFR pathway is activated in the adult intestine by three EGFs, namely Vein (Vn), Spitz (Spi), and Keren (Krn) [6, 35]. All three ligands have overlapping functions in activating of EGFR signaling [13]. Vn is expressed in visceral muscle (Fig. 1) whether Spi and Krn are expressed in the midgut epithelium [6, 35]. It was shown that flies with reduced EGFR activity in ISCs fail to repair their intestine after infection [6]. While Vn is partially regulated in visceral muscle by JAK/STAT, it was hypothesized that visceral muscles serve as a connective tissue between damaged ECs and ISCs [6].

EGFR and JAK/STAT are activated in response to regenerating inflammatory signaling. The interplay between these pathways is realized at the level of ligand induction. Interesting, the inhibition of EGFR completely blocs proliferation, that triggered by Upd3 or Dome in ISCs, while JAK/STAT inhibition only partially depresses proliferation which was induced by EGFR [6]. This fact confirms idea that EGFR plays more important role in ISCs proliferation. Besides EGFR and JAK/STAT control ISC proliferation they have very distinct functions while JAK/STAT controls EBs differentiation, EGFR is required for proper gut morphogenesis [6, 14]. It was shown that both EGFR and JAK/STAT are induced by lack of Hippo signaling in the ECs [28].

7. HIPPO SIGNALING

Hippo signaling was first discovered in *D. melanogaster* as a key regulator of body size. The *Drosophila* Hippo signaling pathway is highly conserved with all main components which have its homologues in mammals [27]. Signal transduction between the mammalian Hippo components is also analogous to that in flies. Hippo signaling negatively regulates stem cell proliferation through limiting cytokine and mitogen biosynthesis that activate JAK/STAT and EGFR pathways (Fig. 1) [28, 30]. It was shown that Hippo signaling is a damage sensor in the intestinal epithelium and other signaling pathways and it plays important role in the control of gut homeostasis.

The Hippo pathway includes Expanded (Ex), the serine-threonine kinases, Hippo (Hpo) and Warts (Wts), scaffolding protein Salvador (Sav), the Mats (Mob and tumor suppressor) and the transcriptional enhancer Yorkie (Yki) [11]. This pathway controls expression of cyclin E and DIAP by modulation Yki activity. There are several conserved components in mammals including *sav* (*hWW45*) and *mats* (*MATS1*). MST1 and MST2 (kinases) and their regulatory protein WW45. For activation, WW45 forms a core complex of the signaling pathway. RASSF family proteins activate MST1/2 by binding and recruiting it to the cell membrane [24]. Activated MST1/2 phosphorylates LATS1 and LATS2 (tumor suppressor homolog kinases), which are regulated by MOBKL1A/B. MST1/2 phosphorylates MOBKL1A/B promoting binding it to the LATS1/2 and they form a complex MOB1-LATS1/2 [26]. Lats1/2 phosphorylates YAP/TAZ, which interacts with 14-3-3 protein and modulates the cytoplasmic retention and trigger protein degradation.

It was demonstrated that Hippo signaling inhibits ISCs proliferation in the adult midgut and was described on-cell-autonomous mechanism of negative regulation of ISCs proliferation. This regulation

is realized through the restriction of cytokines and mitogen production, which can activate JAK/STAT and EGFR cascades [28].

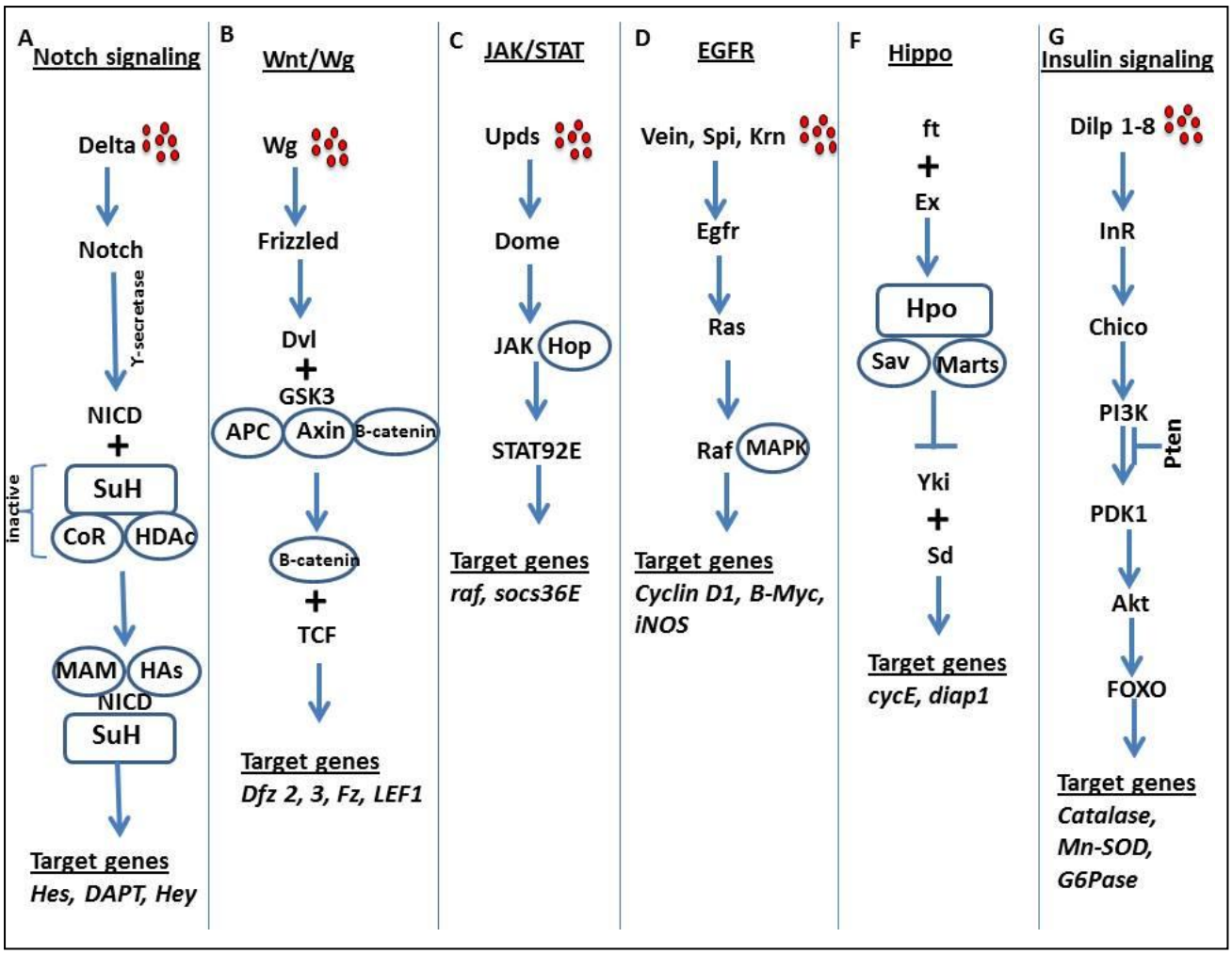

Fig. 2. Signaling pathways involved in interactions between ISCs and the niche cells.
(A) Ligand Delta activates Notch proteins in the EBs. Dissociation of Notch intracellular domain (NICD) from cell membrane is catalyzed be gamma-secretase. Than NICD forms a complex with Suppressor of Hairless (Su(H)) protein to replace a histone deacetylase (HDAc)/corepressor (CoR) from the Su(H). Active complex contains Mastermind (MAM) protein and histone acetyltransferases (HAc) which are recruited to the NICD/Su(H).
(B) Visceral muscle produces Wingless which activates the frizzled receptor (Fzd). This causes an activation of Dishevelled (Dvl), which binds to glycogen synthase kinase-3 (GSK-3) and inhibits it. As a result, ß-catenin dissociates from a complex which is composed of scaffold proteins Axin and APC (Adenomatous polyposis coli). β-catenin triggers the transcription of target genes.
(C) Unpaired 2 and 3 (Upd 2 and 3) activate a receptor-associated Janus Kinase (JAK) termed Hopscotch (Hop) which in turn triggers a Signal Transducer and Activator of Transcription (STAT).
(D) Epidermal growth factor receptor (EGFR) is activated by ligands Vein, Spitz and Kern which stimulate EGFR and activate RAS-RAF-MAPK signaling.
(E) Fat (ft) interacts with Expanded (Ex) and activates the core kinase cascade which includes kinase Hippo (Hpo), adaptor proteins Marts and Salvador (Sav). The main function is to inhibit phosphorylation of transcription co-activator Yorkie (Yki) causing its translocation to the nucleus and binds to transcription activator Scalloped (Sd).
(F) Dilp 3 acts directly through insulin receptor (InR) to activate Protein kinase B (Akt), which phosphorylates and inactivates FOXO.

8. The Role of Diet in ISC Proliferation

It was found that starvation reduces insulin signaling allowing gut to decrease in size during starvation [23]. The insulin signaling positively regulates ISCs proliferation and differentiation during aging and regeneration [2, 8]. Nutrient deprivation leads to inhibition of ISCs proliferation in

Drosophila [8]. Protein deprivation and reduced insulin signaling lead to increased number of lower ploidy enterocyte daughter cells per midgut. These observations indicate that endoreduplication in the midgut is regulated by nutrition [8].

Drosophila intestine expresses two insulin-like peptides Dilp3 and Dilp7 [9, 34]. Dilp7 is expressed in the neurons and in this way regulates intestinal physiology [9]. Dilp3 is expressed in the midgut and foregut muscles (Fig. 1) [34] and acts directly through *Drosophila* insulin receptor (InR) to induce proliferation and midgut growth via asymmetric and symmetric division (Fig. 2) [23]. Interestingly, blocking of brain neurons which produce Dilps leads to inhibition of bleomycin-induced midgut regeneration [2].

9. CONCLUSIONS

This review described the main signaling pathways that control maintenance and differentiation of *Drosophila* ISCs. Several conserved signaling pathways including Notch, Wnt/Wg, JAK/STAT, EGFR, Hippo, and insulin signaling are known to be involved here. Operation of these pathways depends on ISC niche and the ligands they produce. The intestinal regeneration is not controlled by individual signaling pathways, but mainly complex interplay between them. Regulatory signals exchange between epithelium and surrounding tissues to control gut homeostasis. Moreover, tissue damage induces ISC proliferation to replace damaged cells. Under these conditions JAK/STAT and insulin signaling act as important mediators of ISCs proliferation [2, 6, 13]. Little is known about the mechanisms of ISC decision between self-renewal and initiation of differentiation. It worth to underline high conservative level of all these signaling pathways. That is why fruit fly *Drosophila* plays very important role in our understanding many conserved characteristics of stem cells in animals.

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Стрільбицька О.М. Регуляція диференціації стовбурових клітин кишківника у *Drosophila. Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 77–84.

Функціонування стовбурових клітини кишківника (СКК) необхідне для підтримання цілісності шлунково-кишкового тракту за нормальних умов та при пошкодженнях. Задіяні регуляторні процеси характеризуються значною подібністю у *Drosophila* і людини. Проліферація та диференціація СКК кишківника дорослої дрозофіли регулюються факторами росту, що секретуються навколишніми клітинами, які формують нішу СКК. В даному огляді обговорюються механізми регуляції функціонування СКК клітинами ніші через консервативні сигнальні шляхи. Також представлені докази значимості взаємодії сигнальних шляхів Notch, Wingless, JAK/STAT, EGFR, Hippo та інсулінового шляху для забезпечення системності і функціонування СКК. Подальші дослідження в даній області допоможуть зрозуміти як належна регуляція диференціації СКК може сприяти забезпеченню цілісності кишківника.

Ключові слова: стовбурові клітини кишківника, *Drosophila*, ніша, сигнальний шлях, кишківник.

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OLFACTORY SYSTEM IN *DROSOPHILA*

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Abstract. Smell is an ancient sensory system presented virtually in organisms from bacteria to humans. In *Drosophila* odors elicit a variety of behavioral responses in relatively simple but sensitive olfactory system. An increasing number of mutants have been found to be defective in olfactory function. Genetic and molecular analysis of the olfactory system of the fruit fly have identified many molecular components, and have revealed some principles of its function and organization.

Keywords: olfaction, *Drosophila*, olfactory receptors, olfactory sensory neurons.

1. INTRODUCTION

Insect olfactory systems vary enormously. Some species possess highly specialized olfactory systems, possibly limited to few odorants with innate significance, such as the carrot psyllid with only 50 receptor cells of possibly only four types [22]. Oligolectic pollinators (some moth or bee species that live on nectar of very few flower species) find their preferred flower based on a few key compounds. Some jewel beetles (e.g., *Melanophila acuminata)* fly long distances to find burning forests, where they deposit their eggs in burnt trunks. The highest sensitivity described so far is that of male moths following the sexual pheromone trail of female conspecifics, often at a distance of several miles. At the other end of the spectrum, some insect species have widely specialized olfactory systems. Honeybees visit whatever flower will deliver nectar: their olfactory system can learn hundreds of odors and distinguish them [11, 13]. Fruit flies are sensitive to an extraordinary variety of odorants. Airborne molecules drive a number of behavior reactions, including attraction and repulsion [5].

Drosophila melanogaster offers several advantages as an organism which may help to address some of these problems [33]. The first advantage is that its olfactory system is relatively simple, containing only 103 neurons. The second advantage is a significant number of genetic and molecular tools that can be conveniently applied to explore the system. The third, and perhaps most compelling, the olfactory response can be easily quantified *in vivo* using either physiological, or behavioral measures. Recent progress and difficulties in the study of vertebrate olfaction have stimulated new interest to *Drosophila* as a model system to study olfaction [5].

2. Olfactory Receptors and Receptor Cells

In vertebrates, olfactory receptors (ORs) were first identified in 1991 as a very large family of related genes encoding members of the G protein-coupled receptor (GPCR) superfamily, which couples ligand binding to production of cAMP to induce downstream signaling [4]. During the 1990s, efforts by multiple investigators to find homologues of vertebrate ORs in insect genomes failed. In 1999, three groups used a combination of difference cloning [37] and mining of genome databases for multi-transmembrane domain proteins [7, 14, 37] to identify candidate *Drosophila* ORs. There are 62 ORs, encoded by a family of 60 genes through alternative splicing [28]. The fly OR genes encode a highly divergent family of membrane-associated proteins that are selectively expressed in *Drosophila* olfactory sensory neurons (OSNs) [7, 14, 37].

The carbon dioxide receptor (CO_2) of *Drosophila* is a special case: the heteromer consists of dGR21a and dGR63a, with no need for Orco. While CO_2 is clearly an odor for a fly, and not a taste, the receptors are within the gustatory clade of the gene tree. The CO_2 receptor is more related to other gustatory receptors than to the olfactory receptors [12].

A second family of olfactory receptor genes has been called IRs (ionotropic receptors). These receptors are similar in structure to ionotropic glutamate receptors (iGluRs), but have lost the binding site for glutamate. *Drosophila* has 11 neuron types expressing IRs in four coeloconic sensillum types. Each receptor cell expresses at least a pair of IRs, generally one specific IR and either one or both of IR8a and IR25a, suggesting that they work as receptor complexes, most likely as odorant-activated ion channels [2]. ORs and IRs are not coexpressed. IR response profiles to odorants are similarly broad as ORs, and some odorants elicit activity both in IRs and in ORs, though for other odorants responses have only been found in one or other family [12].

In addition to IRs and ORs, some other protein families play important roles in olfaction. These are olfactory binding proteins (OBPs) and sensory neuron membrane proteins (SNMP). OBPs are produced by the accessory cells in the sensillum and released into the lymph at high concentrations. They perhaps serve several functions which are still debated, but helping the transport of lipophilic odorants across the aqueous lymph is uncontroversial [21]. A special family of OBPs is represented by the PBPs (pheromone binding proteins). SNMPs are located in the membrane of receptor cells, and might belong to the receptor complex, participating in transduction of olfactory signal. SNMPs belong to the CD36 protein family, which have two transmembrane domains, and large extracellular binding domains. These proteins typically bind and transport cholesterol, fatty acids, and other hydrophobic molecules [12].

3. The Olfactory System

Drosophila detects odors through the two olfactory sensory organs on the head, the antenna and maxillary palp (Fig. 1.). They are covered with a large number of sensory hairs, called sensilla, which protect the underlying OSNs that are specialized to detect odors. Olfactory sensilla can be distinguished morphologically from thermo- and hygro-sensitive sensilla by the presence of a large number of small pores that perforate the shaft of the sensillum and which are believed to allow access to odors. A total number of about 410 olfactory sensilla cover the antenna, the maxillary palp has about 60 olfactory sensilla. These hairs can be divided into three distinct morphological and functional classes: club-shaped basiconic sensilla, long and pointed trichoid sensilla and short, peg-shaped coeloconic sensilla [23].

Morphological and functional distinctions subdivide both basiconic and trichoid sensilla into additional subclasses. They differ by the size and density of odor pores, the number of neurons housed in each sensillum and their distribution on the antenna [6, 8, 31, 32, 34]. The different sensilla types are distributed in a highly stereotyped fashion over the surface of the antenna. Large basiconic sensilla are

clustered at the medial face of the antenna, while the three types of trichoid sensilla are arranged in diagonal bands across the lateral face of the antenna [23].

Coeloconic sensilla are interspersed with other sensilla types, but are concentrated at the central face of the antenna. The relative position of these sensilla is well conserved as are the number of neurons innervating a given sensillum. Trichoid sensilla (T1, T2 and T3) contain one, two, or three OSNs, respectively [23]. Basiconic sensilla house contain two neurons, there are several cases of four neurons per basiconic sensillum [32, 34], coeloconic sensilla – two or three neurons. The third segment of the antenna is marked by a reproducibly ordered array of olfactory sensilla that house defined and stereotyped numbers of OSNs [16, 17, 26, 29].

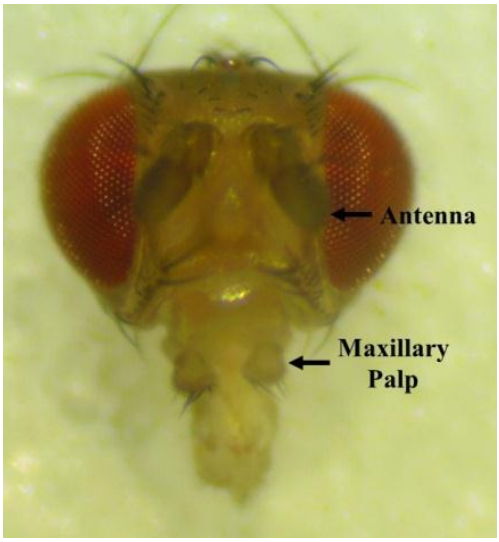

Fig. 1. Olfactory organs — the antennae and maxillary palps.

The maxillary palp is relatively simple olfactory organ, containing few OSNs housed in a small number of basiconic sensilla. About sixty basiconic sensilla each housing two OSNs can be found in this organ. Shanbhag et al. [32] used electron microscopic analysis of OSN terminal dendrite branching in the maxillary palp to further subdivide palp sensilla into three subtypes, PB-I, PB-II, and PB-III [31]. PB-I OSNs contain highly branched terminal dendrites, while PB-II OSNs are characterized by ribbon-shaped dendrites. PB-III OSNs are rarer on the palp and have an unusual thick, hollow dendritic segment. [12].

The antennal lobe (AL) is the deutocerebral neuropil of insects. AL receives the input from the olfactory sensory neurons on the antenna. Functionally, AL shares some similarities with the olfactory bulb in vertebrates.

In insects, the olfactory pathway starts at the antennae (Fig. 2.) (though in some insects like *Drosophila* there are olfactory sensory neurons in other parts of the body) from where the sensory neurons carry the information about the odorant molecules impinging on the antenna to the antennal lobe [18].

The antennal lobe is consist of tightly packed neuropils, called glomeruli, where the sensory neurons interact with the two other views of neurons, the projection neurons and the local neurons. In the *Drosophila* antennal lobe there are 43 glomeruli; in *Aedes aegypti* there are 32; locusts and social wasps may have over 1000 [18]. The projection neurons project to higher brain centers such as the mushroom body and the lateral horn [35]. The local neurons, have their neurites restricted to the antennal lobe. In fruit fly, each olfactory sensory neuron generally expresses a single olfactory receptor gene [38] and the neurons expressing a given gene all transmit information to one or two spatially invariant glomeruli in the antennal lobe [20]. Each projection neuron receives information from a single glomerulus. The interaction among the olfactory receptor neurons, local neurons and projection

neurons reformats the information received from the sensory neurons into a spatiotemporal code before it is sent to higher brain centers [23].

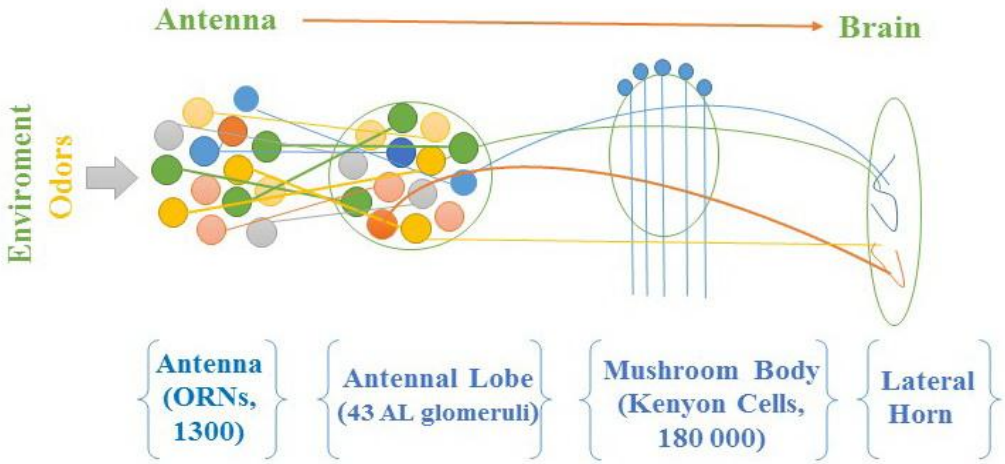

Fig. 2. Scheme Drosophila olfactory system. The olfactory receptor neurons (ORNs) are located in the antenna (1200 ORNs) and maxillary palp (120 ORNs). Each ORN that expresses the same odorant receptor targets its axons to a defined locus in the antennal lobe (olfactory bulb) called a glomerulus. In Drosophila, there are about 54 glomeruli. The antennal lobe is the first processing center for olfactory information. Olfactory information is then relayed by the projection neurons (PNs), which send their axons to synapse with the mushroom bodies and also the lateral horn (redrawn from [12, 23]).

The axons of antennal lobe projection neurons project to the protocerebrum, and here to the mushroom bodies and the lateral protocerebrum. The mushroom bodies have been studied in details, for two reasons. The first reason is that they were shown to be important for learning and memory. The second, they have a highly structured and geometrically regular architecture: their intrinsic cells, the Kenyon cells, form dense bundles of very fine axons that bifurcate to form the mushroom body peduncle and lobes. The cell bodies are arranged in a circular manner, forming a cuplike structure, and the axon bundles are like stalks of a mushroom. In most insects, Kenyon cells are the largest cell population in the brain: 180 000 in each hemilobe of the bee correspond to more than a third of all bee brain cells. In most insect species, mushroom bodies get olfactory and visual input, allowing multimodal processing and memory [12].

The lateral protocerebrum it is an important part of the brain olfactory circuitry: premotor neurons receive input in the lateral protocerebrum, thus input from the antennal lobe to premotor neurons in the lateral protocerebrum is the fastest connection from a stimulus to a behavioral response. [12].

4. Odorant Receptor Gene Expression

Drosophila OR genes encode a highly divergent family of membrane-associated proteins that are selectively expressed in *Drosophila* OSNs [7, 14, 37]. These proteins contain seven transmembrane domains, but do not possess obvious homology to vertebrate ORs or the GPCR superfamily [1, 37, 39]. Different members of the fly OR family show considerably less homology to each other than most vertebrate ORs [28]. Detailed information about the expression of each *Drosophila* OR gene is now available. Recent studies have shown that there is a segregation of gene expression between the two major appendages: ORs expressed in the antenna are not expressed in the maxillary palp and vice versa. Examination of a group of 57 fly ORs confirmed this initial impression of segregation in OR repertoire between antenna and palp. These appendages express non-overlapping subsets of 32 and 7 OR genes, respectively (Table 1) [38]. OR gene expression with transgenic reporter techniques bring the total number of antennal-specific genes to 40 and maxillary palp-specific genes to seven [8, 10].

Each OR gene is expressed in a small subset of the OSNs in either olfactory organ, which varies from two to 50 OSNs per OR. The relative position and number of OR-expressing OSNs is bilaterally symmetric in the two appendages and highly stereotyped between individual flies. Early reports discussed the existence of "zones" of OR gene expression, reminiscent of the zones of OR gene expression on the olfactory turbinates of the rodent [27, 36]. Careful examination of the relationship between OR gene expression and sensilla type has revealed that there is a nearly perfect correlation between the expression of OR genes and subsets of morphologically distinct basiconic, trichoid and coeloconic sensilla [3, 8]. Thus, the same developmental pathways that specify the morphology of the sensilla must also dictate the numbers and functional properties of the OSNs and the specific ORs they express [23].

Antena					Maxillary palp
Basiconic sensilla		Trichoid sensilla	Coeloconic Sensilla		Basiconic sensilla
Food odors	CO_2	Pheromones	Food odors	Water vapor	Food odors
OrX/GR83b	Gr21a/Gr63a	OrX/Gr83b	Or35a/Gr83b	Unknown recoptor	OrX/Gr83b
Or7a Or56a Or9a Or59b Or10a Or67a Gr10a Or67b Or13a Or67c Or22a Or69aA Or22b Or69aB Or33a Or82a Or33b Or83c Or42b Or85a Or43b Or85b Or47a Or85f Or49a Or92a Or49b Or98a Or98b	Gr21a Gr63a	Or2a Or19a Or19b Or23a Or43a Or47b Or65a Or65c Or67d Or88a	Or35a	?	Or33c Or42a Or46aA Or59c Or71a Or85d Or85e

Tab. 1. Molecular organization of the Drosophila olfactory system. Gene expression of chemosensory receptors responding to different classes of ligands is indicated. Red – co-receptors (redrawn from [9, 10, 19, 24, 40]).

There are two unusual features of OR gene expression in *Drosophila* that installed this system, in which each OSN expresses only a single OR gene [30]. First, each *Drosophila* OSN expresses a broadly expressed member of the OR gene family called Or83b, which associates with ORs and is necessary for the proper ciliary targeting and function of all OR genes [1, 24, 25]. Second, a given OSN can co-express up to three conventional ORs mediating ligand selectivity along with the Or83b co-receptor [8, 10, 15].

5. CONCLUSIONS

The use of *Drosophila* to study olfaction has been growing rapidly. Expanded state of knowledge concerning gene expression and synaptic organization of the early olfactory system of the fly makes this a compelling system to address questions in odor coding. For instance, it is not yet clear in any species how and where odor concentration is encoded; how the brain solves odor mixture problems. Little is known about how the olfactory system processes odors to produce stereotyped behavioral outputs. The small size, genetic manipulability and availability of robust olfactory behavior paradigms for *Drosophila* olfaction strengthen the role of this small insect as a powerful genetic model system for the foreseeable future. It seems clear that genetics will continue to play an important role in following the path from odor to behavior in the fly.

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Нюхова система виявлена практично у всіх організмів від бактерій до людини. У представників роду *Drosophila* запахи викликають різні поведінкові реакції за участі відносно простої, але чутливої нюхової системи. Генетичні та молекулярні дослідження нюхової системи *Drosophila* дозволили ідентифікували деякі компоненти, і виявили основні принципи її функціонування та організації.

Ключові слова: нюх, *Drosophila*, нюхові рецептори, нюхові сенсорні нейрони.

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2,4-DICHLORPHENOXICACETIC ACID AT LOW CONCENTRATIONS ENHANCES REPRODUCTIVE ABILITY AND OXIDATIVE STRESS RESISTANCE OF YEAST SACCHAROMYCES CEREVISIAE

MARIA BAYLIAK, NADIA BURDYLYUK

Abstract. 2,4-dichlorphenoxicacetic acid (2,4-D) is one of the most widely used herbicides with well documented toxic effects on non-target organisms. In this study, the effect of low concentrations of 2,4-D on reproductive activity and resistance of yeast *S. cerevisiae* to oxidative stress was evaluated. Supplementation of the cultivation medium with 0.1-100 μM 2,4-D did not affect the rate of yeast growth. In early stationary phase, yeast cultures grown with 0.1 and 1 μM 2,4-D had higher number of reproductively active cells than control ones (without 2,4-D). In exponential phase, *S. cerevisiae* cells grown in the presence of 1-100 μM 2,4-D were more resistant to hydrogen peroxide comparing to control ones. Thus, the herbicide increased reproductive potential and cross-resistance to oxidative stress in yeast but the effective concentrations of 2,4-D were different for these phenomena. In summary, the results suggest possible involvement of certain hormetic mechanisms in the influence of 2,4-D at low concentrations on yeast.

Keywords: colony forming unit, herbicide, hormesis, hydrogen peroxide, survival.

Abbreviations: CFU, colony forming unit; 2,4-D, dichlorphenoxicacetic acid; ROS, reactive oxygen species.

1. INTRODUCTION

Herbicides are agrochemicals that control the growth of undesired weeds, bringing about a significant overall increase in crop productivity. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most broadly used herbicides due to its relatively moderate toxicity to non-target organisms, when in concentrations resulting from adequate use in agriculture, and to its biodegradability in the soil [23]. Nevertheless, numerous scientific papers provide evidences of the significant toxicity of 2,4-D to non-target organisms in relevance to cancer risks, neurologic diseases, reproductive risks, hepatotoxicity and immunotoxicity [4, 10, 11, 15, 24]. Yeast *Saccharomyces cerevisiae* has been actively used as an eukaryotic experimental model to assess 2,4-D toxicity under different environmental and physiological conditions [5, 25, 29, 31], and to elucidate the mechanisms underlying the resistance to 2,4-D [9, 21, 25, 26, 28, 30]. It is supposed that the mechanisms underlying basic cellular processes and chemical stress resistance are apparently conserved among phylogenetically distant organisms, making it possible to extrapolate the knowledge gathered in yeast to higher eukaryotes. 2,4-D is a highly

lipophilic weak acid and it exists at low pH in its undissociated lipophilic toxic form (RCOOH), which can readily cross the plasmal membrane by passive diffusion. In the neutral cytosol, the molecular form of 2,4-D dissociates, leading to internal acidification [9, 21] and accumulation of the toxic anion (RCOO-), which cannot easily cross the plasmal membrane lipid bilayer due to electrically charged nature [5]. However, the acid ions may be actively exported through a specific inducible transporters [19, 27]. The induction of specific transporters such as PDR5 and TPO1, which are plasmal membrane multidrug resistance transporters of the ATP-binding cassette and major facilitator superfamilies [27], is postulated as effective adaptive defensive mechanisms against 2,4-D toxicity in yeast [25, 28]. On the other hand, oxidative stress development was demonstrated to accompany 2,4-D toxicity that being confirmed by increased production of reactive oxygen species (ROS) and enhanced levels of oxidative stress markers [1, 15, 23, 29]. The response to 2,4-D includes the upregulation of genes involved in peroxisomal beta-oxidation and mitochondrial oxidative phosphorylation, two metabolic processes leading to the endogenous generation of ROS [25]. Electron leakage from the mitochondrial respiratory chain might further increase production of ROS as a result of cell exposure to 2,4-D [23, 32]. Moreover, the transient increase in free radical generation and lipid peroxidation in the yeast cell exposed to 2,4-D correlated with stimulation of the activity of antioxidant enzymes, which were shown to be determinants of yeast resistance to 2,4-D [29]. While at high concentrations 2,4-D is lethal or possesses severe toxic effects, at low concentrations it can show beneficial effects on plants acting as an auxin analogue to promote plant growth [16]. This biphasic concentration-response can be a reflection of common hormesis phenomenon when generally favorable biological responses result from the action of low doses/concentrations of toxicants or other stressors, while exposure to high doses results in an inhibitory/detrimental outcome [6, 14]. ROS are considered as key mediators of low-dose beneficial events of different stressors [17]. Taking into account that toxicity of 2,4-D is connected with prooxidant mode of action and ROS generation, it can be hypothesized that low doses/concentrations of this herbicide can produce low levels of ROS which may function as signaling molecules that improve systemic defense mechanisms by inducing an adaptive response. To test this assumption, this work aimed to study the effect of 2,4-D low concentrations on S. *cerevisiae* reproductive ability and resistance to oxidative stress induced by hydrogen peroxide.

2. METHODS AND MATERIALS

2.1. STRAIN AND GROWTH CONDITIONS

The *S. cerevisiae* strain YPH250 (*MAT***a** *trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*) used in this study was kindly provided by Dr. Youshiharu Inoue (Kyoto University, Japan). Yeast cells were grown at 28 °C with shaking at 175 rpm in YPD liquid medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, and 2,4-D in a range of concentrations from 0.1 μM to 100 μM. The addition of 2,4-D at indicated concentrations did not change pH of medium which was adjusted to 5.5 Although this pH does provide easy penetration of 2,4-D into yeast cells, it is used to study toxic effects of this herbicide [5]. Control cells were cultivated under the same conditions but without 2,4-D. The initial concentration of cells in the medium was about 0.3×10^6 cells ml^{-1}. Growth curves were followed by measuring culture optical density at OD_{620}. The parameter was monitored during 3 days of yeast cultivation.

2.2. EVALUATION OF REPRODUCTIVE CAPABILITY

Reproductive ability of yeast cells was analyzed by plating in triplicate on YPD agar after proper dilution of aliquots of the experimental cultures after 24 h growth (early stationary phase) under the conditions mentioned above. The plates were incubated at 28 °C for 72 h and the colony forming units (CFUs) counted [18]. Yeast colony growth was expressed as percentage of total amount of respective control cells plating on YPD agar.

2.3. Pre-Treatment with 2,4-D and Stress Induction

Yeast cells were harvested by centrifugation (5 min, 8000 g) after 16 h cultivation (exponential phase), re-suspended in fresh YPD medium, and incubated with different concentrations of 2,4-D (pH 5.5) at 28 °C for 2 h. Then the cells were harvested as described above, washed and re-suspended in equal volume of 50 mM potassium phosphate buffer (pH 7.0). Aliquots of the experimental cultures were exposed to 10 mM H_2O_2 at 28 °C for 1 h. Control cells were incubated under the same conditions but without hydrogen peroxide and 2,4-D. Cell survival after stress exposure was monitored by counting a number of CFUs on YPD agar plates as described above.

2.4. Statistical Analysis

Experimental data are expressed as the mean value of six independent experiments ± the standard error of the mean (SEM), and statistical analysis used Dunnett's t-test.

3. Results and Discussion

3.1. 2,4-D at Low Concentrations Does Not Affect Yeast Growth

Previously it was established that herbicide 2,4-D showed the toxicity on yeast *S. cerevisiae* in concentration-dependent and pH-dependent manner [5, 29, 30, 31]. Low values of pH (from 3.0 and lower) exacerbate toxic effects of the herbicide, in particular 100 μM 2,4-D being non-toxic at pH 4.5 becomes toxic for yeast grown at pH 3.5 [9]. In our experiments, YPD medium was supplemented with water solutions of 2,4-D in a range of concentrations from 0.1 to 100 μM. Adding of 2,4-D at indicated concentrations did not change pH of medium which was about 5.5. It can be conditioned by buffering properties of YPD medium [19]. Medium supplementation with 0.1-100 μM 2,4-D did not affect the growth pattern of *S. cerevisiae* YPH250 culture (Fig. 1). Thus, 2,4-D demonstrates no toxicity and does not stimulate yeast reproduction under conditions used in this study.

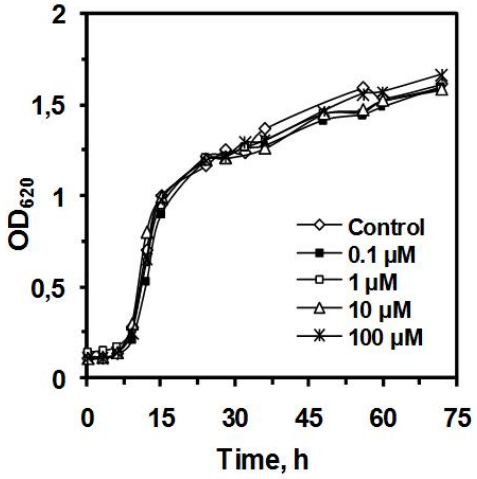

Fig. 1. Growth curves of S. cerevisiae in YPD medium without or in the presence of 2,4-D at different concentrations. The growth curves presented are representative of at least three independent experiments.

3.2. Growth with 2,4-D Increases Reproductive Capability of Stationary Phase Yeast Cells

It is known that any yeast population is heterogeneous and includes cells of different ages [22]. The ability of yeast cells to reproduce themselves reduces with age, and as a result in a population there are some alive cells unable to form colonies [3, 20]. Therefore next we studied the effect of growth with 2,4-D at low concentrations on reproductive potential of yeast cells entering early stationary phase. The reproductive activity of yeast was evaluated by monitoring cell ability to form colonies (CFUs) on complete medium. Fig. 2 demonstrates the colony-forming ability of YPH250 cells grown in the presence of 2,4-D at different concentrations. The percentage of cells capable to form colonies was

by ~19% higher in cultures cultivated with 0.1 and 1 μM 2,4-D compared to control cultures. At the same time, the number of reproductively active cells grown at 2,4-D higher concentrations, 10 and 100 μM, did not differ from that in control. Hence, 2,4-D can clearly affect a number of yeast cells in stationary-phase cultures capable to form colonies in dose-dependent manner. At very low concentrations 2,4-D increased a potential to reproduce and form colonies in stationary phase cells. In different models, the biphasic dose-response relationship for many toxic agents has also been demonstrated showing a stimulatory effect at low doses but inhibitory effect at high doses [2, 11, 18]. This phenomenon is well known as hormesis. In yeast, beneficial effects of low doses of toxicants are often connected with stimulation of colony growth which is experimentally evaluated as CFUs [7, 18]. Thus, one can conclude that hormesis might be responsible for enhanced CFUs.

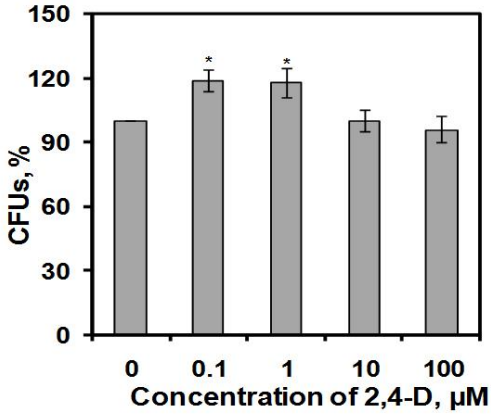

*Fig. 2. Colony forming units of S. cerevisiae after 24 h growth (early stationary phase) in YPD medium supplemented with different concentrations of 2,4-D. The number of reproductively active cells in control cultures is referred as 100%. *Significantly different from respective values in control cultures with $P < 0.05$. Results are shown as means ± SEM ($n = 6$).*

3.3. 2,4-D at Low Concentrations Increases Yeast Resistance to Hydrogen Peroxide

Hormetic action of many compounds was proposed to be connected with their direct or indirect pro-oxidant activity [17]. In this case, toxicants or/and ROS as byproducts of their metabolism are considered as mild stressors activating various defensive mechanisms resulting in the acquisition of resistance to further lethal stress [17, 18].

Since 2,4-D at low concentrations was found to enhance number of formed yeast colonies, next we evaluated the ability of this herbicide to induce cross-adaptation against hydrogen peroxide exposure. Growth with 0.1 μM 2,4-D did not influence yeast survival upon treatment with 10 mM H_2O_2 (Fig. 3). At the same time, yeast cells grown with higher concentrations of 2,4-D were more resistant to 10 mM H_2O_2 than control cells. Resistance to hydrogen peroxide enhances gradually with increasing of 2,4-D concentration accounting for 35%, 53% and 71% of survived cells after of H_2O_2 treatment in control cultures and cultures grown with 1 and 100 μM 2,4-D, respectively. Hence, 2,4-D at very low concentrations causes yeast cross-resistance to oxidative stress. Based on previous reports, it can be supposed that protective effects of 2,4-D may be attributed to its ability to stimulate antioxidant defense and enhance yeast survival under lethal oxidative stress. Analyzing the results, one can see that the concentrations of 2,4-D displaying growth stimulating and stress-protective effects are different. That is not surprising, since it is known that stimulating doses of stressors are usually lower than stress-protective ones, because under stimulating mild stress all resources are directed to ensure cell growth but not cellular protection. More severe stress may lead to redirecting resources, induction of defensive mechanisms and blocking of cell division [8]. In line with this, it was proposed that oxidative stress dependently on its intensity induces different signaling pathways providing an increase in adaptive potential or cell death [13].

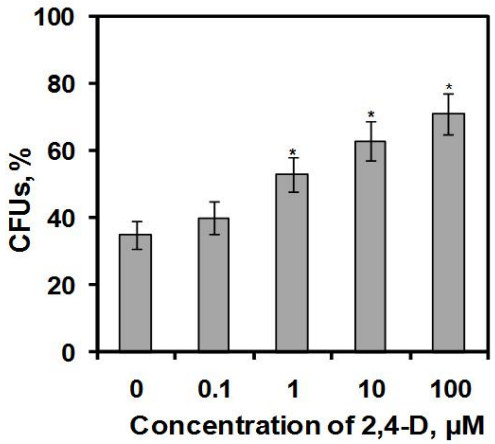

*Fig. 3. Effect of treatment with hydrogen peroxide on survival of exponentially growing S. cerevisiae after pre-incubation with 2,4-D at its indicated concentrations. Cells grown for 16 h in YPD medium were pre-incubated with 2,4-D at different concentrations for 2 h at 28 ºC were collected and re-suspended in 50 mM potassium phosphate buffer (pH 7.0), and then exposed to 10 mM H_2O_2 for 1 h at 28 ºC. *Significantly different from respective values without 2,4-D with $P < 0.05$. As 100% the cell survival in control cultures (without 2,4-D and H_2O_2) was accepted. Results are shown as means ± SEM (n = 6).*

4. CONCLUSIONS

Our results demonstrate that herbicide 2,4-D at low concentrations enhances reproductive potential of *S. cerevisiae* cells and yeast resistance to oxidative stress. It suggests that action of 2,4-D on yeast involves hormetic mechanism having beneficial effects at low concentrations and deleterious at high one. The effective concentrations of 2,4-D for yeast reproduction and cross-resistance to stress were different suggesting the dose-dependent induction of different signaling pathways in yeast cells.

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Байляк М.М., Бурдилюк Н.І. 2,4-дихлорфеноксиоцтова кислота за низьких концентрацій підвищує репродуктивну здатність і стійкість дріжджів *Saccharomyces cerevisiae* до оксидативного стресу. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 93–99.

2,4-дихлорфеноксиоцтова кислота (2,4-*Д*) є одним з найбільш широко використовуваних гербіцидів, який проявляє водночас значний токсичний вплив на нецільові організми. У цьому дослідженні вивчали вплив низьких концентрацій 2,4-*Д* на репродуктивну активність та резистентність дріжджів *S. cerevisiae* до оксидативного стресу. Додавання до середовища культивування 2,4-*Д* у концентраціях 0,1-100 мкМ не впливало на швидкість росту культур дріжджів. На початку стаціонарної фази росту культури дріжджів, які вирощували у присутності 0,1 і 1 мкМ 2,4-*Д*, мали більшу кількість репродуктивно активних клітин, ніж контрольні культури (без 2,4-*Д*). Клітини *S. cerevisiae*, вирощені до середини експоненційної фази в присутності 1-100 мкМ 2,4-*Д*, були стійкішими до дії пероксиду водню, ніж контрольні клітини. Таким чином, гербіцид збільшував репродуктивний потенціал і перехресну стійкість до оксидативного стресу в дріжджів, проте ефективні концентрації 2,4-*Д* були різними для цих двох феноменів. Загалом, отримані результати свідчать про можливе залучення горметичного механізму до реалізації ефектів низьких концентрацій 2,4-*Д* на дріжджі.

Ключові слова: колоніє-утворююча одиниця, гербіцид, гормезис, пероксид водню, виживання.

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INFLUENCE OF AMYLOSE STARCH ON DEVELOPMENT AND LIFESPAN OF FRUIT FLY DROSOPHILA MELANOGASTER

OLEKSANDRA ABRAT

Abstract. Last years, the concept of resistant starch (RS) has evoked a new interest in researchers in the context of bioavailability of starch and its use as a source of dietary fiber. Based on clinical and animal research, RS has been proposed to be the most potentially beneficial starch fraction for human health. In this study, the effects of amylose starch as a fraction of RS on development and lifespan of fruit fly *Drosophila melanogaster* were investigated. In both Canton S and w^{1118} strains, the diet with 20% amylose RS delayed fly development, increased triacylglyceride level in the body of adult insects and reduced their lifespan compared to the diet with 4% amylose starch. Thus, our data clearly demonstrate that amylose starch at high concentrations may negatively affect fruit fly.

Keywords: *Drosophila melanogaster*, resistant starch, pupation, triacylglycerides, lifespan.

Abbreviations: RS, resistant starch; TAG, triacylglycerides.

1. INTRODUCTION

In recent years, there has been an increasing interest in metabolic syndrome, a phenomenon describing clinical profiles of some of the world's major health problems today: obesity, heart diseases and diabetes [7, 19]. It is known that an excessive intake of all macronutrients, particularly carbohydrates, contributes to the development of obesity [2, 4, 6]. The rising prevalence of obesity in both, adults and children, is one of the most important public health concerns in developed and developing countries [1]. Therefore, slowly digestible nutrients has been proposed as a possible intervention to decrease the risk and complications related to metabolic disorders such as obesity and metabolic syndrome [1, 5, 15, 19]. Regarding starch, it is known that this component contains different fractions which are digested and absorbed at different rates in the human small intestine, resulting in varied glycemic responses [15]. Based on speed of digestion, starch is divided into such categories as rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) [1, 3, 15]. Rapidly digestible starch is rather quickly digested and hydrolysed products are absorbed in the duodenum and proximal regions of the small intestine leading to a rapid elevation of blood glucose and usually a subsequent episode of hypoglycemia, hungry feeling and overconsumption. Fast and substantial increase in blood glucose level can further lead to cellular, tissue and organ damages [1]. Resistant starch is digested partly and very slowly in the upper part of gastrointestinal tract, but it is also fermented by the colonic microflora producing short chain fatty acids that provide additional energy to the body. Slowly digestible starch is hydrolysed within the small intestine providing

sustained glucose release with low initial glycemia, leading to prolonged energy availability, compared to more rapidly digestible starch [1]. It is considered, that RS in the diet may assist to prevent and manage conditions associated with the metabolic syndrome via its potential effects on delaying of glucose delivery with subsequent fat utilisation and appetite control benefits [17, 19]. However, studies related to effects of resistant or slowly digestible starches on parameters of the metabolic syndrome have been relatively scanty.

The fruit fly *Drosophila melanogaster* is one of models that has become important for use in the investigation of metabolism. It is well established that central metabolic and regulatory pathways, including metabolism of carbohydrates, fat, proteins, and insulin signaling are conserved throughout evolution [2]. This creates a solid basis for the use of fly model to reveal certain metabolic disturbances and transfer gained knowledge to human. The current study aimed to examine the beneficial and deleterious effects of the diet with amylose starch as one of fractions of resistant starch [15, 19] on fat metabolism, development and lifespan of D. melanogaster.

2. Materials and Methods

2.1. Drosophila Melanogaster Stock and Media

The *D. melanogaster* strains w^{1118} and Canton S were obtained from Bloomington Stock Center (Bloomington, Indiana, USA). Stock flies and larvae were reared on yeast-corn-molasses (regular) food with 12 hour illumination at 25 ± 1°C and relative humindity of 55-60%. Nipagin (methyl-p-hydroxybenzoate) at concentration of 0.18% was added to the medium to inhibit mold growth [9]. Experimental media contained 4% yeast extract, 0.18% nipagin, 1% agar and starch in a range of concentrations from 0.25 to 20%.

2.2. Pupation

After egg laying for 3-4 h, the eggs were transferred into bottle containing food with different concentrations of starch (about 150 eggs per bottle containing 15 mL of food). In these vials eggs hatched and larvae developed until pupation. The number of formed pupae was recorded every day for 5 days. Mean larval development time was calculated accordingly to Olcott [12] as time at which 50% of larvae had pupated.

2.3. Triacylglyceride Assay

Pre-weighed flies were homogenized in chilled 10 mM phosphate buffered saline with tween (PBST buffer) (pH 7.4) in a ratio 1:50 (milligram flies per buffer microliter) at 4 °C. Homogenates were heated at 70 °C for 10 min to denature proteins followed by cooling to 4 °C. To precipitate denatured proteins, supernatants were centrifuged (16,000 g, 15 min, 21 °C). Final supernatants were used for assay of body triacylglycerides (TAG) which were measured using a diagnostic kit Liquic Cor-TG (PZ Cormay S.A., Poland) following kit guidelines. Standard TAG solutions in concentration range from 3 to 30 μg/mL were used for determination of TAG content in flies. Ttriacylglyceride levels in fly bodies were expressed as micrograms per fly (μg/fly).

2.4. Lifespan Assay

Experimental flies were raised at standard densities of 150 eggs per bottle with 15 mL of yeast–molasses medium. Newborn flies were transferred without anesthesia to fresh medium and maintained for 2 days. After separation by sexes, 100 two-day-old flies were placed into containers with 5 mL of the experimental food. Food was changed every second day, and died flies were counted. To minimize any density effects on mortality, two vials within cohorts were merged when the density of flies reached 50% of initial one [10]. Two or three independent trials with about 300 flies were performed.

2.5. Statistics

The values are presented as means ± S.E.M. Statistical analysis of all data was performed using one-way ANOVA followed by the Dunnett's test to compare multiple experimental treatments to the single

control value with the use of the Mynova program. The difference in survival between cohorts was calculated with log-rank test with a use of JMP 9.0 statistical software (SAS Institute) [14].

3. Results and Discussion

The effect of carbohydrate diets on physiological parameters is generally studied in relatively simple model organisms like nematodes, mice and rats. Recently, fruit fly *Drosophila melanogaster* has been started to be intensively used in nutritional studies [2, 4, 10, 16, 18].

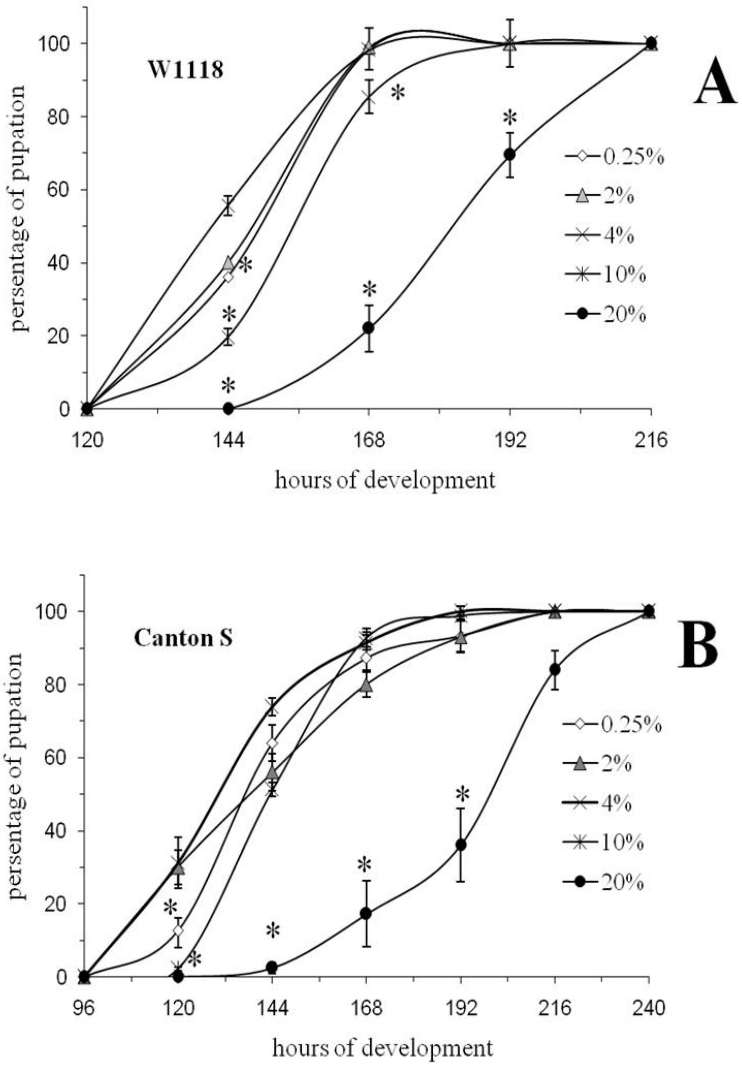

*Fig. 1. Developmental curves of D. melanogaster w^{1118} (A) and Canton S (B) grown on media containing amylose starch in different concentrations. Data are presented as means ± S.E.M.; cohorts with 1500 flies were used (n = 4). Diet with 4% starch was taken as a control group. *Significantly different from the control group with P < 0.05.*

Many *Drosophila* organs that regulate food intake and energy metabolism have analogs in humans, in part those that are potential targets of diabetic complications: heart, brain, kidney (nephrocytes, Malpighian tubules), liver and adipose (fat body), gastrointestinal tract, and blood (hemolymph) [2, 11]. In the current study, we used *D. melanogaster* as a model to investigate biological effects of starch with high amylose content. This type of starch belongs to so-called resistant starch and up to present time there is a too little information about mechanisms of action of this fraction.

First we evaluated the developmental patterns of fly larvae after *per os* uptake of amylose starch in different concentrations (Fig 1). Since previous studies demonstrated that effects of diets could be strain-specific [8], all experiments on the effects of dietary amylose were carried out with two *D.*

melanogaster strains, Canton S (wide type) and its derivative w^{1118}. Experimental medium supplemented with 4% starch was selected as the control and all parameters further were compared with flies fed under these conditions.

Strain	Starch concentration, %				
	0.25	2	4	10	20
w^{1118}	146±4	146±7	144±3	154±6*	175±6*
Canton S	137±3♣	139±2♣	130±6♣	144±5*,♣	202±7*,♣

Data are presented as the means ± S.E.M. (n = 3).
**Significantly different from the control group (4% carbohydrate) and ♣from w^{1118}.*

Tab. 1. Fly half-pupation time (time at which 50% of larvae pupated) on different starch-containing diets.

Consumption of food with 0.25-2% starch showed the tendency to retard normal development of insects relatively to the control (Fig. 1A-1B). This might be due to deficiency of energy nutrients required for normal fly development. High-caloric starch diet (10-20%) significantly prolonged development time in both strains.

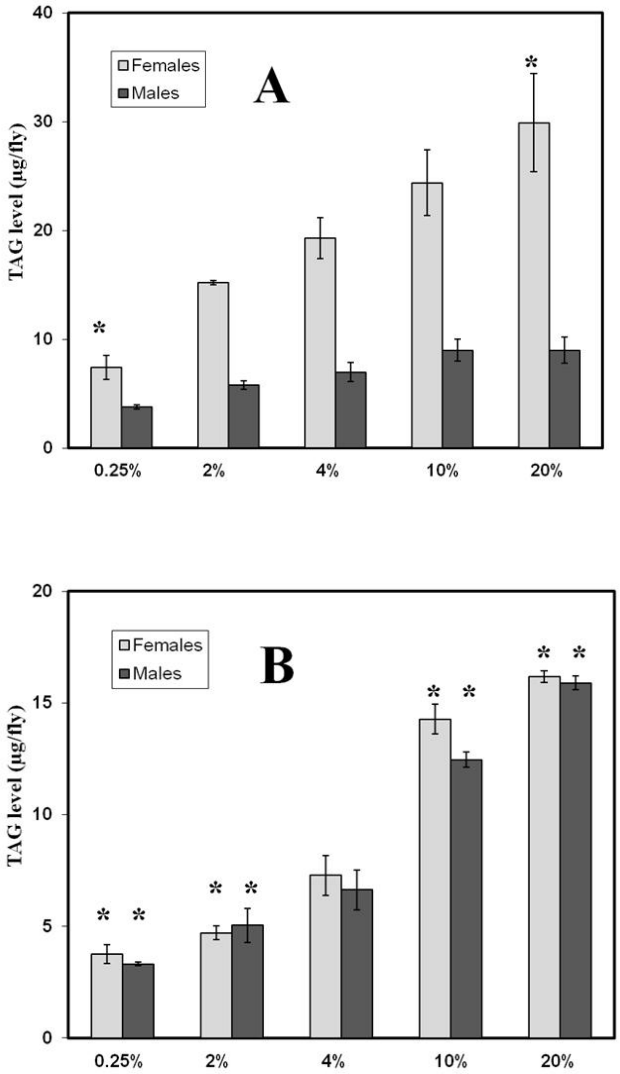

Fig. 2. The levels of triacylglicerides in the bodies of eight-day old w^{1118} (A) and Canton S (B) flies, kept on media containing amylose starch in different concentrations. Data are presented as means ± S.E.M. (n = 4). Significantly different from the control group (4% starch diet) with P < 0.05.

To determine the length of developmental delay, we calculated the time at which 50% of larvae had pupated (Tab. 1). For 20% starch groups in w^{1118} and Canton S strains, this parameter increased by 1.2- and 1.6-fold, respectively, compared the control diet with 4% starch. At the same time, pupae survival and pupation height on low (0.25%) and high (20%) starch diets were significantly lower in comparison with 2-10% starch diet (data not shown). Our results demonstrate that adverse effects of resistant starch at high concentrations on fly development were not related to the strains. We suppose that toxic effects of high concentrations of the starch could be connected either with excessive solidification of the culture medium making the food very hardly accessible for larvae, or with metabolic rearrangements in larvae bodies delaying their development. It can not be excluded that both mechanisms could be involved.

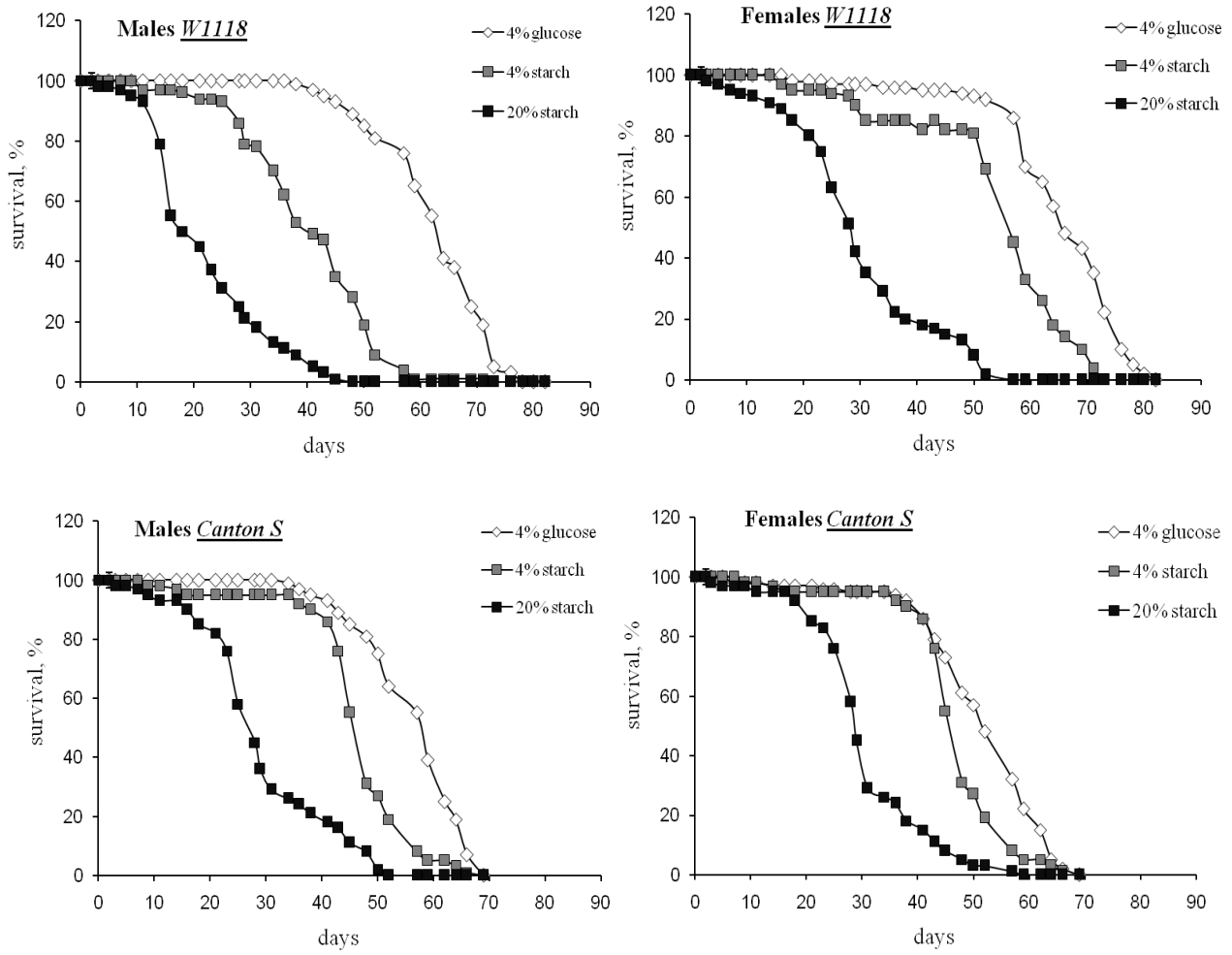

Fig. 3. Survival curves of D. melanogaster w^{1118} and Canton S flies, maintained on glucose or starch diet. Each curve represents survivorship about 300 flies.

Previously it has been shown, that high-sugar feeding enhances triglyceride (TAG) level in insect bodies [11, 16]. In this study, we examined the level of TAG in adult fruit flies consumed different starch diets (Fig. 2). As it could be expected, with increase in starch concentration the content of TAG proportionally increased in w^{1118} females (Fig. 2A) and both sexes of Canton S flies (Fig. 2B). Thus, our results regarding effects of starch diet are in a very good agreement with previous studies in *Drosophila* on other carbohydrates [11, 16]. Interestingly, under consumption of the high-starch diet the wet mass of fly bodies did not differ from other groups (data not shown). This is consistent with previous study which revealed that a diet high in RS reduced rat adipose tissue with no changes in body mass [13]. This work indicated that despite similar mean body mass, rats given high-starch food had more body fat and less lean body mass than those given low-starch food [13].

Since diet with different starch concentrations modified fly development and level of TAG in adult flies, next we investigated the long-term effects of amylose starch consumption on *D. melanogaster* lifespan. Again, the medium with 4% glucose was used as the control. The presence of starch in the food reduced the survival of male and female flies of both strains in dose-dependent manner compared to one on glucose medium (Fig. 3). For w^{1118} strain, median survival time on 4% glucose, 4 and 20% starch, was 65, 45 and 23 days for males, and 68, 58 and 30 days for females, respectively. For Canton S flies, this parameter was 63, 48 and 28 days for females, and 56, 47 and 28 days for males, respectively. Thus, dietary amylose starch does not appear to offer any benefits for lifespan of fruit fly and is actually toxic at higher concentrations. It seems that amylose starch induced such metabolic perturbations in fly bodies which lead to shortening of lifespan, but this statement needs the further investigations.

In summary, our results demonstrate that dietary amylose starch at moderate and high concentrations demonstrates deleterious effects on fly development and lifespan, and induces metabolic reorganization accompanied by increased triacylglycerol levels. The results point out the need for further research to review the concept of resistant starch as beneficial food component.

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Абрат О.Б. Вплив крохмалю з високим вмістом амілози на розвиток та тривалість життя плодової мушки. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 100–106.

В останні роки використання харчових продуктів на основі резистентного крохмалю (РК) викликало новий інтерес у дослідників з позиції біодоступності його волокон. На підставі клінічних та експериментальних досліджень РК було запропоновано як потенційно найбільш корисну для здоров'я людини фракцію крохмалистих продуктів. Метою даної роботи було вивчення впливу крохмалю з високим вмістом амілози, що належить до фракції резистентного крохмалю, на розвиток та тривалість життя плодової мушки *Drosophila melanogaster*. Показано, що споживання резистентного крохмалю у високих дозах (20%) призводило до затримки розвитку двох ліній мух (Canton S and w^{1118}), збільшення рівня триацилгліцеридів у їх тілі та зменшення тривалості життя комах, порівняно з дієтою на 4% крохмалі. Таким чином, наші результати наштовхують на думку, що крохмаль на основі амілози у високих концентраціях може негативно впливати на плодову мушку.

Ключові слова: *Drosophila melanogaster*, резистентний крохмаль, лялькування, триацилгліцериди, тривалість життя.

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INVOLVEMENT OF CATALASE IN SACCHAROMYCES CEREVISIAE HORMETIC RESPONSE TO HYDROGEN PEROXIDE

RUSLANA VASYLKOVSKA, NADIA BURDYLYUK, HALYNA SEMCHYSHYN

Abstract. In this study, we investigated the relationship between catalase activity and H_2O_2-induced hormetic response in budding yeast *S. cerevisiae*. In general, our data suggest that: (*i*) hydrogen peroxide induces hormesis in a concentration- and time-dependent manner; and (*ii*) the effect of hydrogen peroxide on yeast colony growth positively correlates with the activity of catalase that suggests the enzyme involvement in overall H_2O_2-induced stress response and hormetic response in yeast.

Keywords: yeast, hormesis, hydrogen peroxide, reproductive ability, catalase.

1. INTRODUCTION

Hormesis is a phenomenon of particular interest in biology, medicine, pharmacology and toxicology. It has been observed in a variety of organisms: from bacteria to humans, responding to a wide range of chemical, physical, and biological stressors, including ionizing radiation, chemotherapeutic agents, metal ions, pesticides, antibiotics, ethanol, aldehydes, chloroform, pro-oxidants, hypergravity, and so forth [10, 20, 25, 26]. The specificity of stress response is determined by the nature of stressor, intensity/duration of its action, and physiological state of an organism. According to the hormesis theory, low doses of stress-inducing factors lead to stimulatory hormesis response and improvement of cellular and organism functions, whereas at high doses the deleterious effects prevail [19, 21]. Interestingly, hormesis may activate defense pathways ensure protection against higher doses of the same agent ("pre-adaptation") as well as other specific stressors ("cross-protection") [5, 28, 30]. Therefore, hormetic response suggests the existence of complex mechanisms, which sense and respond to different kinds of stress.

Recent studies strongly support the notion that hydrogen peroxide plays a dual role in biological systems [1, 6, 17, 27]. Its effect can be considered as either beneficial or harmful, because at high concentrations H_2O_2 causes oxidative damage to cell structures, whereas at low concentrations it is a part of many cellular signaling systems. At low concentrations hydrogen peroxide also plays a crucial role in the induction of hormesis [1, 17, 24, 28].

Manipulation of reproductive potential through hormesis-stimulating compounds, like hydrogen peroxide, appears to be an effective approach to improve yeast survival and cross-adaptation to different kinds of stress. In our recent study, it has been demonstrated that hormetic concentrations of hydrogen peroxide caused yeast cross-resistance to severe stress induced by high concentrations of

ethanol, acetic and propionic acids [28]. The global-stress transcription factors Msn2/4p and Yap1 have been found to play an important role in the hormetic effects by low concentrations of hydrogen peroxide and involved in yeast cross-adaptation by low concentrations of hydrogen peroxide [2, 28]. Since catalase is a member of H_2O_2-stimulon regulated by the Msn2/4p and Yap1 transcriptional factors in *S. cerevisiae* [14, 18, 22, 29], the enzyme could be a key element of either the hormetic stress response to hydrogen peroxide.

Here, we used model organism *S. cerevisiae* to study the effect of different concentrations of hydrogen peroxide on cellular reproductive ability and potential role of catalase in H_2O_2-induced hormetic response.

2. METHODS AND MATERIALS

The *Saccharomyces cerevisiae* strains used in this study were YPH250 (wild type *MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*) and its isogenic derivative YWT1 (YPH250 *ctt1Δ::URA3 cta1Δ::TRP1*) described earlier [16]. The strains were kindly provided by Prof. Yoshiharu Inoue (Kyoto University, Japan). Chemicals were obtained from Sigma-Aldrich Chemical Co. (USA) and Fluka (Germany). All chemicals were of analytical grade.

Yeast cells were grown with shaking at 175 r.p.m. and 28 °C in Erlenmeyer flasks containing YPD liquid medium (1% yeast extract, 2% peptone, and 2% glucose) in a volume that respected the ratio 1 : 5 regarding media volume to flask volume. For experiments, overnight cultures were diluted to about 10^6 cells/mL in YPD. Cells from experimental cultures after 24 h growth were split into two portions: one used for the reproductive ability evaluation and another used for cell-free extract preparation and catalase activity measurement.

Yeast reproductive ability was determined in yeast collected by centrifugation (5 min, 8000 g), washed with 50 mM potassium phosphate (K-phosphate) buffer (pH 7.0), resuspended in the same buffer. Aliquots of the suspension (10^8 cells/mL) were exposed to different concentrations of hydrogen peroxide followed by their incubation at 28 °C for different time periods as described earlier [4]. Control cells were incubated under the same conditions but without hydrogen peroxide. Yeast reproductive ability was analyzed by plating in triplicate on YPD agar after proper dilution. The plates were incubated at 28 °C for 3 days and the colony forming units (CFU) counted [11]. Reproductive ability was expressed as percentage of total amount of cells plating on YPD agar.

The activity of catalase was measured in cell-free extracts prepared from yeast treated with different concentrations of hydrogen peroxide for different periods of time in YPD medium at 28 °C as described earlier [3, 31]. Control cells were incubated under the same conditions but without hydrogen peroxide. Then yeast were collected by centrifugation (5 min, 8000 g), washed with 50 mM K-phosphate buffer (pH 7.0). The yeast pellets were resuspended in lysis buffer (50 mM K-phosphate buffer (pH 7.0), 1 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA). Cell extracts were prepared by vortexing yeast suspensions with glass beads (0.5 mm) as described earlier [3, 31] and kept on ice for immediate use. Catalase activity was measured spectrophotometrically with a SF-46 spectrophotometer (LOMO, USSR). The enzyme activity was determined by monitoring the disappearance of hydrogen peroxide at 240 nm using the extinction coefficient for hydrogen peroxide of 39.4 $M^{-1}cm^{-1}$ [3, 31]. One unit of catalase activity was defined as the amount of supernatant protein that utilized 1 μmol of substrate per minute. The activity was measured at 25 °C and expressed per milligram of soluble protein in supernatant.

Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method [7] with bovine serum albumin as the standard. Experimental data are expressed as the mean value of four to eight independent experiments ± the standard error of the mean (SEM), and statistical testing was carried out used Student's t-test.

3. Results and Discussion

Oxidative stress, depending on its intensity, can be considered as either harmful or beneficial [10, 19, 20, 21, 23]. Mild oxidative stress has been found to stimulate microorganisms' biological functions and resulted in an acquisition of their resistance to high doses of the same as well as other stressors [17, 23, 28]. An improvement of cellular and organismic functions by moderate stress is known as the phenomenon of hormesis, that may be graphically represented by the biphasic dose-response dependence, characterized by the stimulation in the low dose zone, followed by an inhibitory response at higher doses [10, 20]. Hormetic stimulatory response is usually limited to the 30–60% increase in a biological function under mild stress conditions [8, 9, 10]. Among many stress-induced agents, hydrogen peroxide has been found to play a crucial role in the induction of hormesis [17].

The findings of the present study also demonstrate typical biphasic concentration-response curve, exhibiting hormetic effect of hydrogen peroxide (Fig. 1). The reproductive ability of yeast cells, that was determined as colony forming units (CFU), was assessed after the 60-min incubation period with different H_2O_2 concentrations. The concentrations lower than 0.15 mM H_2O_2 did not affect reproductive ability. The peak hormetic response of yeast cells was observed at 0.15 mM H_2O_2. At the hormetic concentration of hydrogen peroxide, yeast showed about 160% of the control (without H_2O_2) colony growth of yeast. At higher concentrations (0.2-2.5 mM H_2O_2), reproductive activity decreased to almost control value, and the parameter dropped to 73-55% of the initial reproductive ability during yeast incubation with as high as 10-100 mM H_2O_2.

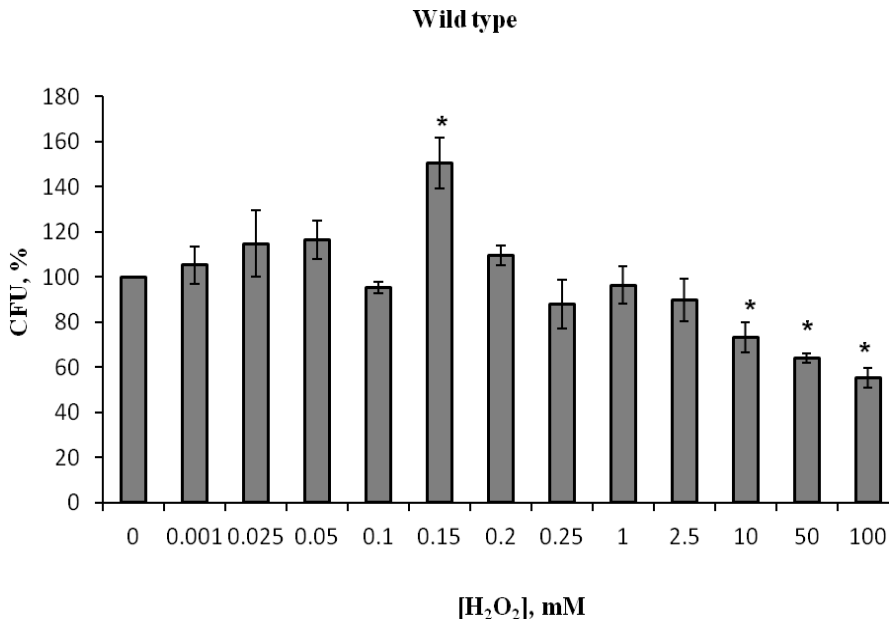

*Fig. 1. Effect of hydrogen peroxide on reproductive ability of S. cerevisiae YPH250 (wild type). Results are shown as the mean ± SEM (n = 4-10). *Significantly different from control (without H_2O_2) with P < 0.05.*

The data presented in Fig. 1 are consistent with previous studies reported stimulatory effect of low concentrations of hydrogen peroxide (≤0.4 mM H_2O_2) and its toxicity at the concentrations higher than 0.5 mM H_2O_2 in *S. cerevisiae* [13, 28]. Stimulation of the reproductive potential through hormesis-induced compounds, like H_2O_2, appears to be an effective approach to improve yeast survival and cross-adaptation to different stresses. Earlier we demonstrated that, unlike ethanol, hormetic concentrations of hydrogen peroxide caused yeast cross-resistance to different kinds of severe stress [28].

Molecular mechanisms of hormesis due to H_2O_2-induced stress are yet to be elucidated, but there are some, which can be suggested to involve catalase, a member of the H_2O_2-stimulon in *S. cerevisiae* [14, 22, 29]. Fig. 2 demonstrates the effect of different concentrations of hydrogen peroxide on the activity of catalase in yeast. The activity increased significantly (by 1.6-fold) during incubation with as

little as 25 μM H_2O_2 and reached a maximum content (about 2-fold higher than control) in the presence of 50-100 μM H_2O_2. In previous studies, the activation of catalase by low concentrations of hydrogen peroxide has been associated with a cytosolic CTT1 isoenzyme [3, 14]. Fig. 2 shows also that at higher H_2O_2 concentrations (0.15-2.5 mM H_2O_2), catalase activity decreased to control value, and dropped significantly by 2.1-4.8-fold comparing to control during yeast incubation with the highest concentrations used (10-100 mM H_2O_2, respectively). Lower catalase activity in cells exposed to 10-100 mM H_2O_2 could be explained by inactivation of catalase *in vivo* by high concentrations of the substrate. Our previous experiments also demonstrated that catalase activity did not depend linearly on hydrogen peroxide concentration, and results were interpreted from the point of view of enzyme inactivation by high peroxide concentrations [3, 31].

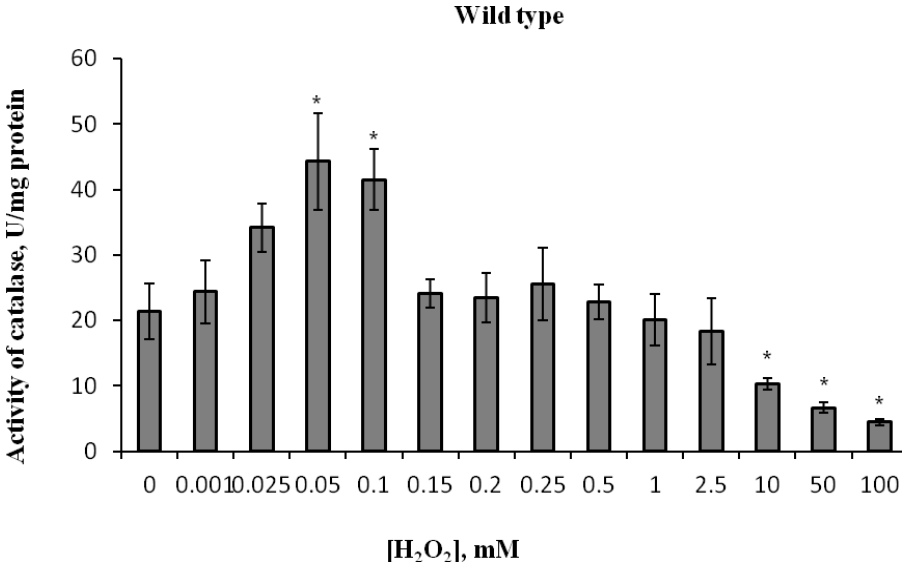

*Fig. 2. Effect of hydrogen peroxide on the activity of catalase in S. cerevisiae YPH250 (wild type). Results are shown as the mean ± SEM (n = 5-8). *Significantly different from control (without H_2O_2) with P < 0.05.*

Figure 3 shows the time course of the reproductive ability of yeast cells stressed by exposure to 0.15 (the hormetic concentration) and 100 mM H_2O_2 (the highest concentration used). As seen from the Figure the parameter did not change in the control cells over the 120-min incubation period without hydrogen peroxide. In the case of yeast treatment with 0.15 mM H_2O_2, the parameter gradually rose reaching the highest values on 40-120 min (~2.5-fold higher than zero time control). Incubation of yeast with 100 mM H_2O_2 over the 60-120-min incubation period caused 2.5-fold reduction of the reproductive ability as compared with zero time control. The activity of catalase rose over time in cells treated with 0.1 mM H_2O_2 (Fig. 4). A significant increase in the enzyme activity of 1.7-fold higher than zero time control was noted after 60 min incubation, but it significantly reduced over the 120-min incubation period and became virtually the same as that in control. At the same time yeast exposure to 1 mM H_2O_2 caused decrease in catalase activity over time (2-fold lower after 120 min than zero time control). Control experiments (without H_2O_2) showed that the activity did not change over the 120-min incubation period. Thus from the presented above data an important role of catalase in H_2O_2-induced hormetic response of yeast can be supposed. This suggestion is confirmed by the results demonstrated in Fig. 5. As seen in the Figure, hydrogen peroxide did not affect the reproductive ability of mutant yeast cells lacking catalase activity.

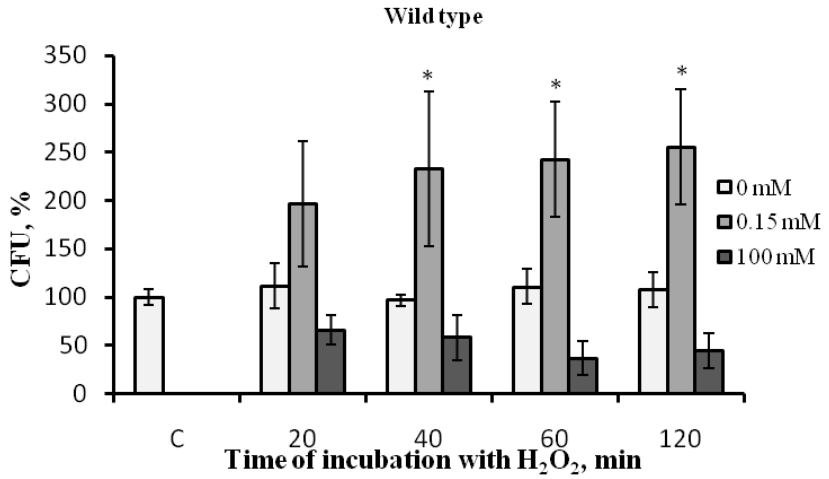

*Fig. 3. The time course of changes in reproductive ability of S. cerevisiae YPH250 (wild type). Results are shown as the mean ± SEM (n = 4-8). *Significantly different from control (without H_2O_2) with P < 0.05.*

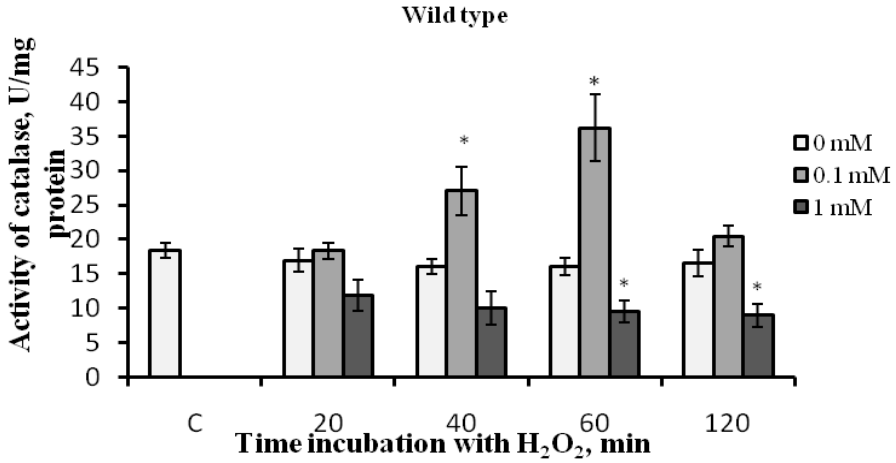

*Fig. 4. The time course of changes in catalase activity in S. cerevisiae YPH250 (wild type). Results are shown as the mean ± SEM (n = 4-8). *Significantly different from control (without H_2O_2) with P < 0.05.*

In general, yeast cells exposed to hydrogen peroxide respond by the reorganization of gene expression at different levels of the flow of the genetic information [18, 22, 29]. The oxidative stress response in yeast has been analyzed in detail at the genome, transcriptome, proteome and post-proteome level. However, many studies reported a lack of correlation between the expression of certain genes at different levels of cellular organization [12, 15, 29]. The weak elevation of catalase activity in yeast treated with sublethal concentrations of H_2O_2 reported earlier [3, 4, 16] as well as found in this study (2-3-fold higher than control), does not correspond to high level of the respective enzyme molecules (15-fold higher than control) [14]. It is well known that exposure of microorganisms to low sublethal concentrations of hydrogen peroxide may lead to the acquisition of cellular resistance to a subsequent lethal stress [13, 28, 30]. Earlier we supposed that yeast cells exposed to low concentrations of hydrogen peroxide respond accumulating stress-protectant molecules [4, 31]. Since a sudden increase in antioxidant activities could dramatically disturb the intracellular redox homeostasis, it seems most of the respective protein molecules synthesized *de novo* remain non-active. This provides the cells with the capability to respond quickly and survive consequent lethal challenge via rapid post-translational activation of these proteins, and catalase in particular.

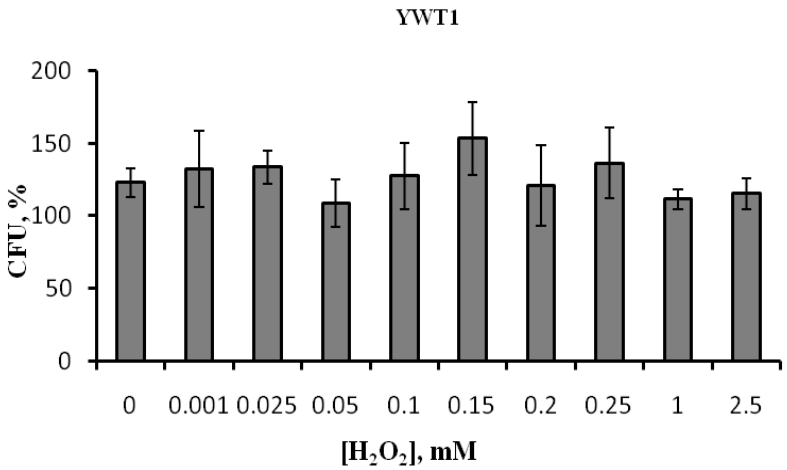

Fig. 5. Effect of hydrogen peroxide on reproductive ability of S. cerevisiae YWT1 (lacking catalase). Results are shown as the mean ± SEM (n = 3-4).

4. CONCLUSIONS

Therefore, considering the literature and present data it can be supposed that moderate stress induced by low concentrations of hydrogen peroxide results in catalase-dependent hormesis, the effect that provides organism with a potential to survive consequent lethal stress (Fig. 6). On the other hand, at high concentrations of hydrogen peroxide oxidative processes seem to predominate, therefore cellular proteins and even catalase may be inactivated. In turn, this leads to cell/organism death under further severe stress.

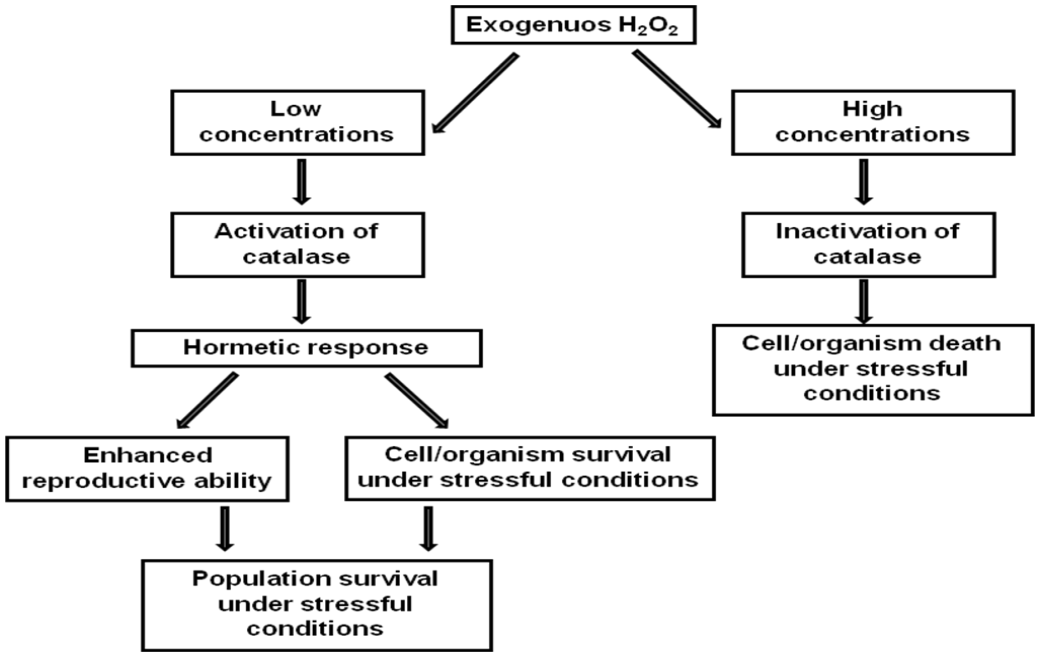

Fig. 6. Concentration-dependent dual effect of hydrogen peroxide in vivo.

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Васильковська Р.А., Бурдилюк Н.І., Семчишин Г.М. Участь каталази в горметичній чутливості *Saccharomyces cerevisiae* до пероксиду водню. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 107–114.

Досліджено взаємозв'язок між активністю каталази і горметичною відповіддю *S. cerevisiae* на дію пероксиду водню. Загалом, отримані дані свідчать про те, що: 1) горметичний ефект залежить від концентрації та тривалості інкубації клітин з H_2O_2; та 2) ріст колоній дріжджів за дії пероксиду водню позитивно корелює з активністю каталази, що свідчить про участь ферменту в загальній відповіді на стрес, викликаний H_2O_2, а також в горметичній відповіді дріжджів.

Ключові слова: дріжджі, гормезис, пероксид водню, репродуктивна здатність, каталаза.

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ALPHA-KETOGLUTARATE PARTIALLY PROTECTS FRUIT FLY *DROSOPHILA MELANOGASTER* FROM ETHANOL TOXICITY

HALYNA SHMIHEL

Abstract. Alpha-ketoglutarate (AKG) is an important intermediate in Krebs cycle and in metabolism of amino acids. Recently, it was proposed to apply as a dietary supplement to improve overall functional state of living organisms. In particular, AKG was supposed to use under exposure of animals and cell cultures to many toxic agents. In this context, this study aimed to elucidate the ability of dietary AKG to reduce toxic effects of ethanol on development of fruit fly *Drosophila melanogaster*, which is a popular model subject to research many aspects of biology of higher eukaryotes. For this aim, the effect of sodium salt of AKG on pupation speed of *D. melanogaster* w^{1118} on the medium supplemented with different concentrations of ethanol was studied. Ethanol at a low concentration (2%) did not affect the rate of larval pupation, whereas at higher concentrations it significantly delayed fly pupation and showed developmental toxicity reducing a number of total pupae formed. The most toxic developmental effects of ethanol were observed at its highest concentration (15%). The potential mechanisms of protective effects of AKG are discussed.

Keywords: rate of pupation; larvae; embryonic toxicity, dietary supplement.

Abbreviations: AKG, alpha-ketoglutarate; EthOH, ethanol.

1. INTRODUCTION

Currently, the substances which can improve adaptation of living organisms to various types of stresses are actively investigated. In this context, there is a growing interest in the study of alpha-ketoglutarate (AKG) as a dietary supplement to improve the overall functional state of organisms and increase their resistance to a number of stressors [3, 11]. Alpha-ketoglutarate is an organic ketoacid that is important for the transfer of cellular energy in the citric acid cycle and the proper metabolism of essential amino acids. Being a precursor for such amino acids as glutamate, glutamine and proline, AKG is involved in protein biosynthesis [11, 13]. It is considered also as one of the most important nitrogen transporters in metabolic pathways. The amino groups of amino acids are attached to it (by transamination) and carried to the liver where the urea cycle takes place to remove excessive ammonia from the body. One of the most important functions of AKG is to detoxify ammonia in tissues, especially in central nervous system. Alpha-ketoglutarate also scavenges ammonia released at catabolism of amino acids, thereby balancing the body's nitrogen homeostasis and preventing nitrogen overload in body tissues and fluids. In addition, it was proposed that AKG can display antioxidant

activity, in particular iron-chelating activity [22]. In contrast to oxaloacetate which cancels iron redox activity by forming inactive complexes, AKG can form active complexes with iron with potential pro-oxidant activity [22]. It was also shown that AKG prevented oxidative damages to lipids under ethanol administration in rats [26].

Ethanol is an important larval food resource and toxin for fruit fly *D. melanogaster* simultaneously [8]. The latter encounters ethanol in its natural habitat and possesses many adaptations that allow it to survive and thrive in ethanol-rich environments. Several assays to study ethanol-related behaviour in flies, ranging to have been developed in the past 20 years. These assays have provided the basis for studying the physiological and behavioural effects of ethanol and for identification of genes mediating these effects. In mammalian and insect models of ethanol intoxication, ethanol at low doses stimulated locomotor activity whereas at high doses was a sedative [5, 6]. *Drosophila* species which breed in fermenting fruits can encounter ethanol at concentrations up to 4–5% [9]. Some *Drosophila* species breed in wineries and breweries, where ethanol concentrations may be even higher [9, 17]. Ethanol can serve as a food resource at low concentrations, but at high concentrations it is toxic [20]. That ethanol has been an important selective agent for *Drosophila* which is supported by the results of interspecific comparisons: species which normally breed in fruit are more resistant to the toxic effects of ethanol, and have higher activity of the enzyme alcohol dehydrogenase [18]. Alcohol addiction is a common affliction with a strong genetic component [7]. Although mammalian studies have provided significant insight into the molecular mechanisms underlying ethanol consumption [4], other organisms like *D. melanogaster* are better suited for unbiased, forward genetic approaches to identify novel genes related. Behavioural responses to ethanol, such as hyperactivity, sedation, and tolerance, are conserved between flies and mammals [23, 27], as are the underlying molecular pathways [19].

Humans and flies share a large number of homologous genes: vertebrates have about four homologues for every gene found in *D. melannogaster*. Both, imago and larvae of *D. melanogaster*, have been used as classical tools for neuroscience and biology in general for over a century [2, 10]. Larvae have been workhorses for many aspects of behavioural neuroscience, including sensory research [12, 14], learning and memory studies [1, 21]. Recently, larvae have also been employed for drug discovery [24, 25]. In this study, we used *D. melanogaster* larvae as a model to study possible protective effects of alpha-ketoglutarate sodium salt (AKG) under exposure to high concentrations of ethanol. For this purpose, the effects of dietary AKG and ethanol at different concentrations either alone and in the mixture (AKG and ethanol) on pupation rate of fruit fly *D. melanogaster* were studied.

2. Materials and Methods

2.1. Drosophila Melanogaster Stocks and Media

The *D. melanogaster* strain w^{1118} flies were used in all experiments. Stock flies were reared on the standard yeast-corn-molasses food with 12:12 photoperiod at 25±1ºC. The experimental media consisted of 10% sucrose, 10% pressed yeast, 1% agar, and 0.2% nipagin (methyl-p-hydroxybenzoate, used to inhibit mold growth) (SY diet) were supplemented also with AKG and ethanol at different combinations. Fly pupation on different media was monitored.

2.2. Analysis of Pupation Rate

After egg laying for 5-6 h, eggs were transferred to vials containing SY diet either control or supplemented with different combinations of AKG and ethanol. About 100-150 eggs per vial containing 20 ml of food were placed. In these vials, eggs hatched and larvae developed until pupation. The number of pupated larvae was counted every day [15].

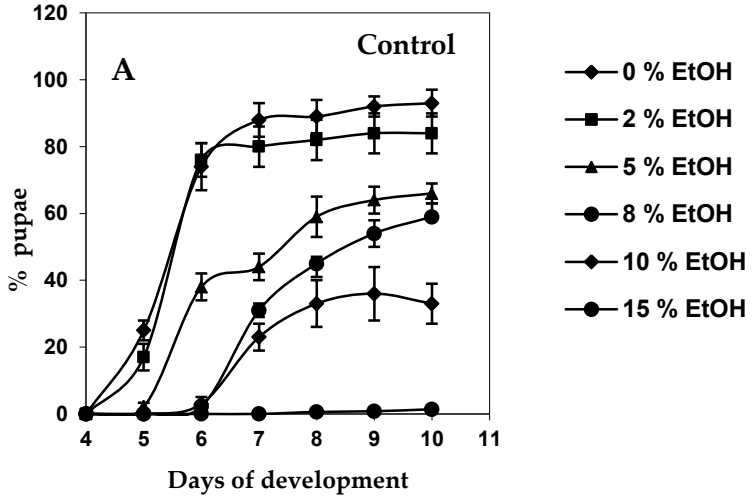
2.3. Statistical Analysis

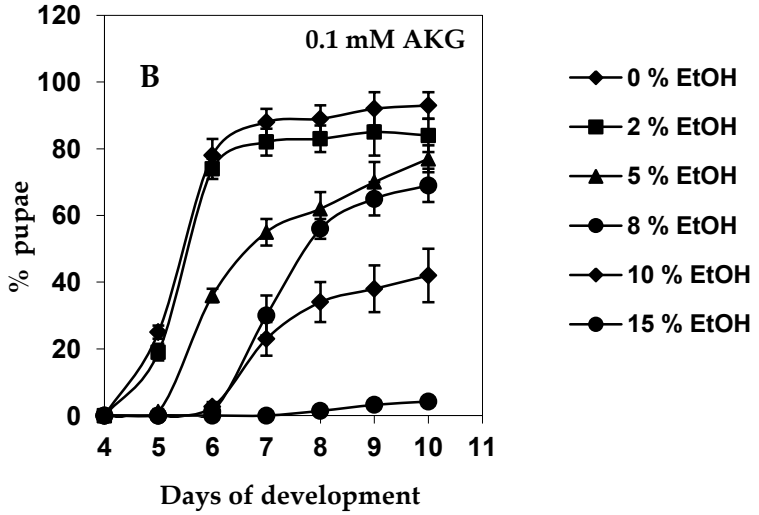
Experimental data are expressed as the mean value of 5 independent experiments ± the standard error of the mean (SEM), and statistical analysis was carried out using Dunnett's t-test.

3. Results and Discussion

Drosophila larvae stops feeding and initiate pupation at a very specific time after hatching and this can be used as a developmental transition point to assay growth pattern alterations [28]. In this study, the experiments regarding potential protective effects of AKG against ethanol toxicity were carried out with the w^{1118} fly strain. In each independent experiment, 100 eggs were added to sucrose-yeast medium containing different combinations of the used compounds and larvae were allowed to feed and develop to pupation. Percentage of pupae was calculated as the ratio between the numbers of pupae formed to the total number of eggs placed into the vial.

Fig. 1. (A) demonstrates the effect of ethanol at different concentrations on developmental pattern of w1118 flies rearing at control media. It is clear, ethanol delayed fly pupation in dose-dependent manner. At a concentration of 2%, ethanol virtually did not affect larvae pupation process. It is can be explained by evolutionary fitness of fruit fly to survive and live in fermented fruits where they can be exposed to ethanol at certain concentrations [9]. However, at higher concentrations ethanol delayed fly pupation. Finally, it demonstrated highest toxicity at highest concentration used (15%). These results are in good agreement with earlier data which demonstrated developmental toxicity of ethanol [16]. It was proposed that the developmental defects in *Drosophila* could be largely due to ethanol effects on insulin signaling. Supplementation of the medium with AKG at concentrations of 0.1 and 10 mM partly alleviated ethanol-induced developmental fly pattern (Fig. 1. (B)-(C)). Certainly, these effects were the most pronounced when we studied effects of 10 mM AKG under fly exposure to 15% ethanol.





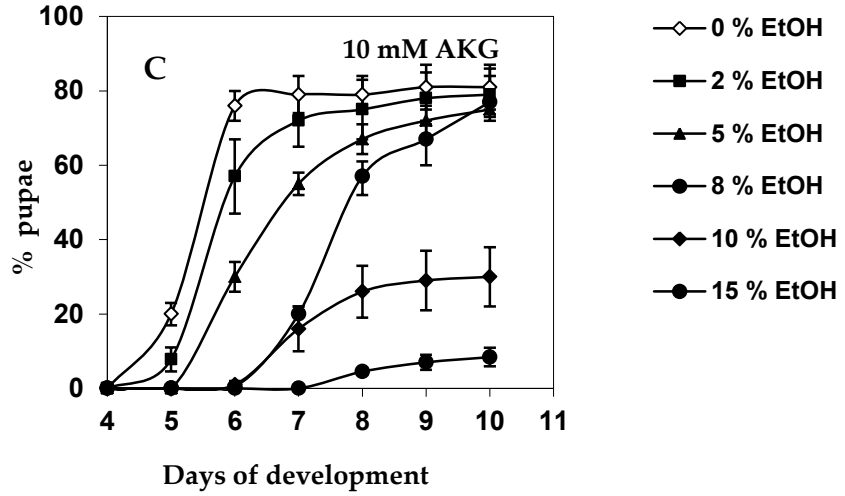


Fig. 1. Effects of different concentrations of ethanol on pupation pattern of D. melanogaster w^{1118} fed control SY food (A) or SY food supplemented with 0.1 mM (B) or 10 mM (C) AKG, n=5.

Fig. 2 shows total number of pupae formed when the larvae were reared on media supplemented with ethanol and AKG at different concentrations. Although in two groups, control and with ethanol at concentration 2%, virtually did not affect the number of pupated larvae, at concentrations 5, 8, and 15% it decreased the pupae number by 29, 40, and 98%, respectively. Supplementation of food with AKG did not affect the parameter in control and 2% ethanol fed groups, it influenced it in the rest ones, but to different extent. In the fly group maintained with 5% ethanol, AKG in both used concentrations, 0.1 and 10 mM, provided by 17 and 14% higher number of pupated larvae. Obviously, at highest concentrations used, 8 and 15%, negative ethanol effects were greatly prevented y AKG in concentration dependent manner, but the only at 10 mM AG the difference was statistically significant in 1.2 and 2.5-fold effects. This means that at high levels of ethanol, AKG partly diminished larval toxicity of ethanol, whereas at low ethanol levels AKG was not efficient.

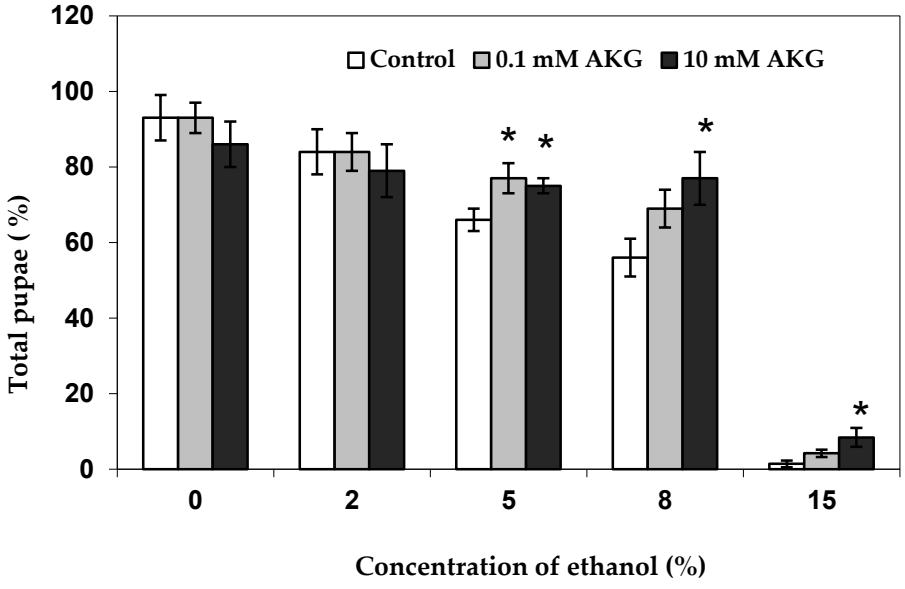


*Fig. 2. Total number of D. melanogaster w^{1118} pupae formed on the media supplemented with ethanol and AKG in different combinations. The number of eggs used was given as 100%. *Significantly different from respective control values with P < 0.05 using Dunnett's test, n=5.*

Thus, dietary AKG can partly alleviate developmental toxicity of ethanol at high concentrations on fruit fly *D. melanogaster*. It is possible that earlier found fact that AKG could prevent lipid peroxidation

in rats under chronic ethanol administration [26] may be one of the potential explanations of AKG effects. Therefore, it may be supposed that antioxidant AKG action can be potential mechanism in fruit fly against ethanol toxicity. The detailed mechanisms of AKG action need to be investigated further.

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Шмігель Г.В. Альфа-кетоглутарат частково захищає плодову мушку *Drosophila melanogaster* від токсичної дії етанолу. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 115–121.

Альфа-кетоглутарат (АКГ) – це важливий проміжний продукт циклу Кребса і метаболізму амінокислот. Нещодавно АКГ почали використовувати як харчову добавку для покращення загального функціонального стану живих організмів. Зокрема, було запропоновано використовувати АКГ при дії на тварин і клітинні культури токсичних речовин. У цьому контексті, метою даної роботи було з'ясувати здатність екзогенного АКГ зменшувати токсичну дію етанолу на розвиток плодової мушки *D. melanogaster*, яка є популярною моделлю для вивчення багатьох аспектів біології вищих

організмів. Нами досліджено вплив натрієвої солі АКГ на заляльковування плодової мушки *D. melanogaster*, в присутності етанолу за різних його концентрацій. Етанол за низької концентрації (2%) не впливав на швидкість лялькування плодової мушки, але за вищих концентрацій він сповільнював заляльковування. Найбільш токсичний вплив етанолу на розвиток мушок був знайдений при використанні його найвищої концентрації – 15 %. Обговорюються потенційні механізми захисної дії АКГ.

Ключові слова: швидкість заляльковування; лялечки; ембріональна токсичність, харчова добавка.

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EFFECTS OF SODIUM NITROPRUSSIDE ON SALT STRESS TOLERANCE OF TOCOPHEROL-DEFICIENT *ARABIDOPSIS THALIANA* PLANTS

NADIIA MOSIICHUK

Abstract. In the present study, effects of exogenous sodium nitroprusside (SNP), a nitric oxide (•NO) donor, on lipid peroxidation and antioxidant enzyme activities in wild type and tocopherol-deficient lines *vte1* and *vte4* of *Arabidopsis thaliana* subjected to 200 mM NaCl were studied. In wild type plants, pretreatment with SNP did not change level of thiobarbituric acid reactive substances (TBARS), but decreased the activities of dehydroascorbate reductase and guaiacol peroxidase under salt stress. In mutant line *vte1*, which lacks all forms of tocopherols, pretreatment with SNP reduced TBARS level and increases the activities of glutathione reductase and guaiacol peroxidase under salt stress. Ascorbate peroxidase activity decreased under salt stress conditions in both mutant lines, pretreated with SNP. It can be concluded, that pretreatment with SNP could attenuate salt-induced injuries in *A. thaliana* plants via up-regulation of activity of antioxidant enzymes and attenuate lipid peroxidation.

Keywords: antioxidant enzymes, lipid peroxidation, nitric oxide, oxidative stress, tocopherols.

Abbreviations: APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GuPx, guaiacol peroxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside; TBARS, thiobarbituric acid reactive substances.

1. INTRODUCTION

Salt stress is one of the most significant abiotic stresses and affects many aspects of plant physiology and homeostasis [2, 23]. The effects of high salinity on plants can be mainly classified from two different points: osmotic stress induced by high salt concentration in the environment and the toxic effect of sodium accumulated in the cell [23]. Along with these primary effects, secondary stress, such as oxidative one, occurs because high concentrations of ions disrupt cellular homeostasis and increase generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) [2]. The enhanced ROS production during stress induced high salinity can enhance oxidative modification of lipids, nucleic acids, and proteins. Plants possess several mechanisms to detoxify ROS which include non-enzymatic antioxidants as well as antioxidant enzymes [12]. Among non-enzymatic antioxidants, tocopherols (α-, β-, γ- and δ) play a key role because they eliminate singlet oxygen and prevent propagation of lipid peroxidation in

membranes by scavenging lipid peroxy radicals. Two major tocopherols possessed by plants are α-tocopherol in green tissues and γ-tocopherol in seeds [21].

In view of a number of studies, salt tolerance often correlates with a more efficient antioxidant system [23, 30]. Therefore, enhancing antioxidant potential in plants may improve plant tolerance to salt stress. Numerous studies reported important role of nitric oxide (•NO) in stress response of plants. Nitric oxide (•NO) is highly reactive free radical with diverse biological functions in plants – either cytotoxic or cytoprotective [18]. The cytoprotection is partly based on its ability to regulate ROS level and toxicity. However, various derivatives of •NO, collectively referred as reactive nitrogen species (RNS), can be toxic [18, 42]. In plants •NO also acts as an important inter- and intracellular signaling molecule involved in many physiological processes, as well as responses to biotic and abiotic stresses [18]. Many reports have shown that exogenous •NO exhibited an antioxidant role during pathogen infection [25], osmotic stress and salinity [39, 44, 47], heavy metal and herbicide toxicity [10, 28, 37, 41, 43, 45]. The possible mechanisms of such protective action include up-regulation of the activity of antioxidant enzymes, including superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR) that reduces oxidative damage and provides protection against ROS-promoted injury. An important role for •NO in regulating salt stress response in plant has already been suggested by several researches [3, 19, 44]. Uchida et al. [39] reported that exogenous •NO enhances antioxidant enzyme activities in rice under salt stress. Same investigators proved that preatreatment with NO-donors elevated seeds germination, root growth and dry weigh accumulation [17, 46]. Based on the above observations, this work aimed to investigate whether exogenous sodium nitroprusside (SNP), •NO donor, may alleviate oxidative damage induced by salt stress in wild type and tocopherol deficient lines *vte1* and *vte4* of *Arabidopsis thaliana* and to elucidate possible involvement of exogenous •NO in improvement tolerance of wild and tocopherol-deficient plants to salt stress. The *vte1* mutant lacks all four tocopherols [27], whereas *vte4* mutant lacks α-tocopherol, but instead posseses γ-tocopherol in leaves [5].

2. MATERIALS AND METHODS

Seeds of *Arabidopsis thaliana* wild type (Columbia) and mutant lines *vte4* (SALK_03676) and *vte1* (GABI_11D07), defective in *VTE4* and *VTE1* genes, respectively, were obtained from the Salk Institute [1] and GABI-Kat [29] and selected homozygote plants from the seeds at the Institute of Botany of Kiel University (Germany) were used in the present investigation. The plants were grown in hydroponic system using Rockwool supports as described earlier [38] at 28°C and naturally illuminated environmental conditions. The Gibeau nutrient solution [11] was used and changed every two weeks. Ten-week-old plants were used for experiment. Initially, roots of plants were placed for 24 h in solutions 0.1 mM SNP, $K_4[Fe(CN)]_6$ (additional control to SNP) or distilled water (control). Afterwards, plants from the three groups were exposed to nutrient solution containing 0 or 200 mM NaCl for 24 h. Therefore, the plants of each line were submitted to five treatments: control (pre-treated with H_2O and not NaCl stressed), NaCl (pre-treated with H_2O and NaCl stressed), SNP (pre-treated with SNP and not NaCl stressed), SNP-NaCl (pre-treated with SNP and NaCl stressed), and $K_4[Fe(CN)]_6$ -NaCl (pre-treated with $K_4[Fe(CN)]_6$ and NaCl stressed). After 24 h fully expanded leaves of plants were harvested and frozen with liquid nitrogen.

To measure the level of products of lipid peroxidation and activity of antioxidant enzymes the frozen leaves were powdered in liquid nitrogen with morta and pestle and mixed (1:5, w:v) with 50 mM potassium-phosphate buffer (pH 7.0) that contained 1 mM ethylenediamine-tetraacetic acid (EDTA) and 1 mM phenylmethylsulfonylfluoride (PMSF). Ascorbic acid (1 mM) was added to potassium-phosphate buffer in the case of ascorbate peroxidase (APX) assay. The homogenates were centrifuged at 13,000 *g* for 20 min at 4°C in Eppendorf 5415R (USA) centrifuge. The supernatants obtained from each sample was collected and used for further assay.

Supernatants were mixed with an equal aliquot of 40% (w/v) trichloroacetic acid (TCA) and then centrifuged for 10 min at 5000 *g*. The supernatants were used for determination of level of thiobarbituric acid reactive substances (TBARS) as described by Heath and Packer [14].

The activity of ascorbate peroxidase (APX) was measured spectrophotometrically following the decrease of absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) due the oxidation of ascorbic acid to dehydroascorbate [7]. Guaiacol peroxidase (GuPx) activity was assayed spectrophotometrically following the increase in absorbance at 470 nm due to guaiacol oxidation (ε = 26.6 $mM^{-1}cm^{-1}$) [31]. Dehydroascorbate reductase (DHAR) activity was determined by measuring increase in absorbance at 265 nm due the formation of ascorbic acid (ε = 14 mM^{-1} cm^{-1}) [35]. Glutathione reductase (GR) activity was determined as the decrease in absorbance at 340 nm (ε =6.22 mM^{-1} cm^{-1}) due to the oxidation of reduced NADPH [20].

One milliunit of APX, GuPx, DHAR and GR activities is defined as the amount of the enzyme consuming 1 nmol of substrate or generating 1 nmol of product per minute; activities were expressed as international milliunits per milligram of protein.

Protein concentration was determined with Coomassie brilliant blue G-250 according to Bradford's method [6] with bovine serum albumin as a standard.

All values were expressed as means ± S.E.M. of three independent experiments. For statistical analysis, the Student's *t*-test was used to compare values at stress conditions with their corresponding controls values, and to compare *vte4* and *vte1* mutant lines with the wild type.

3. Results and Discussion

The involvement of •NO in salinity tolerance has been studied intensively in the past few years. For instance, under salt stress conditions, the exogenous •NO can enhance salt tolerance by alleviating oxidative damage, enhancing activities of proton-pump and Na^+/H^+ antiport in the tonoplast, and K^+/Na^+ ratio (reviewed in [22]). In many cases, the protective role of •NO under salt stress conditions was related with its effects on the ROS elimination. It has been shown that ROS production, particularly $O_2^{\bullet -}$ and H_2O_2, is stimulated under salt stress conditions [15]. Free radical-induced peroxidation of lipids is one of commonly used markers of stress-induced damage [33]. A protective role of •NO against lipid peroxidation was previously reported by many researchers [32, 34, 39, 40]. Nitric oxide can affect lipid peroxidation due to interaction with lipid alcoxyl (LO•) and peroxyl (LOO•) radicals [18]. Decomposition of lipid hydroperoxides results in formation of diverse products including malondialdehyde (MDA). In this work the product of MDA condensation with thiobarbituric acid (TBA) was measured as thiobarbituric acid reactive substances (TBARS). The level of TBARS in leaves of wild type Arabidopsis plants was significantly higher up to 1.5 fold after treatment by salt stress (Fig. 1).

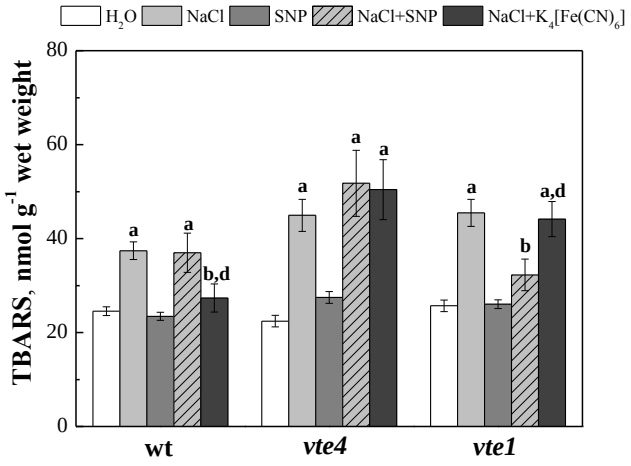

Fig. 1. Effect of SNP pretreatment on TBARS content in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M (n = 3). [a]Significantly different from respective control group (H_2O), [b]NaCl and [d]NaCl+SNP groups ($P < 0.05$).

Exogenous SNP did not changed TBARS content under both normal and salt stress conditions. Salt treatment with addition of potassium ferrocyanide resulted in 1.4-fold decrease of TBARS level in wild type plants. In leaves of mutant line *vte4*, salt stress increased by 2-fold TBARS level (Fig. 1). Under salt stress, the treatment with both potassium ferrocyanide and SNP did not change TBARS level in *vte4* plants. Similarly, in mutant line *vte1* the level of TBARS increased by 1.7-fold under salt stress conditions (Fig. 1). Application of SNP did not change this parameter under normal conditions, but significantly decreased it by 29% under the stress. The treatment with potassium ferrocyanide had no effect on TBARS level under salt stress as compared to plants exposed to 200 mM NaCl only. The *vte1* mutant plants do not synthesize tocopherols, therefore •NO can play the key role in a cell as a limiting factor of the chain reaction of lipid peroxidation and thus limit oxidative damage. Two mechanisms which may explain protective •NO action against oxidative damage have been widely reported. Firstly, •NO may detoxify ROS directly, such as superoxide radicals, to form peroxyntrite, which is less toxic and thus decrease cellular damage [42]. Secondly, •NO could function as a signaling molecule, which upregulating cellular antioxidant system [18, 22].

Wu and colleagues [40] showed that application of SNP slowed down the increase in MDA production in tomato leaves under NaCl treatment. Application of exogenous •NO dramatically decreased TBARS level in cucumber root mitochondria under salt stress, whereas sodium ferrocyanide did not affect TBARS level in salt-treated plants [34]. SNP treatment slightly reduced the increase in MDA contents in shoots of *Kosteletzkya virginica* seedlings exposed to 200 mM NaCl [13]. Pretreatment with SNP also decreased levels of lipid peroxidation products in tomato seedlings under osmotic stress induced by drought [24].

Induction of the antioxidant defense system is one of the mechanisms actively employed by plants to survive at high salinity [4, 16]. It was found that •NO induced activity of various ROS-scavenging enzymes [17].

An ascorbate-glutathione (AsA-GSH) cycle is the most important H_2O_2-detoxifying system in plant chloroplasts, which operates also in cytosol, peroxisomes, and mitochondria [26]. The enzymes of the ascorbate-glutathione cycle APX, DHAR and GR play an essential role in plant tolerance to the action of various biotic and abiotic stresses by eliminating of H_2O_2, as well as sustaining of reduced status of ascorbate and glutathione [12].

APX which uses ascorbate as a reductant in the first step of the AsA-GSH cycle is the most important plant peroxidase in H_2O_2 detoxification [12, 26]. Salt stress did not affect APX activity in wild type and both mutant lines, *vte4* and *vte1* (Fig. 2).

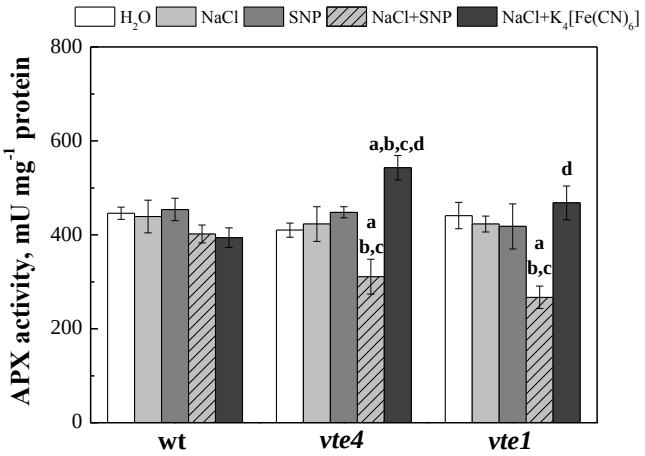

Fig. 2. Effect of SNP pretreatment on the activity of ascorbate peroxidase (APX) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M.(n = 3). [a]Significantly different from respective control group (H_2O), [b]NaCl and [c]SNP groups (P < 0.05).

Pretreatment with SNP resulted in a remarkable decrease by 26-36 % in the activity of APX in both mutant lines under salt stress. Pretreatment with potassium ferrocyanide did not significantly influence APX activity in *vte1* mutant plants, and slightly increased by 32 % APX activity in mutant plants *vte4* under salt stress conditions (Fig. 2).

Regeneration of ascorbate via AsA-GSH cycle requires the activity of DHAR and GR [12, 26]. In our study, DHAR activity significantly increased by 47 % in wild type plant exposed to 200 mM NaCl (Fig. 3).

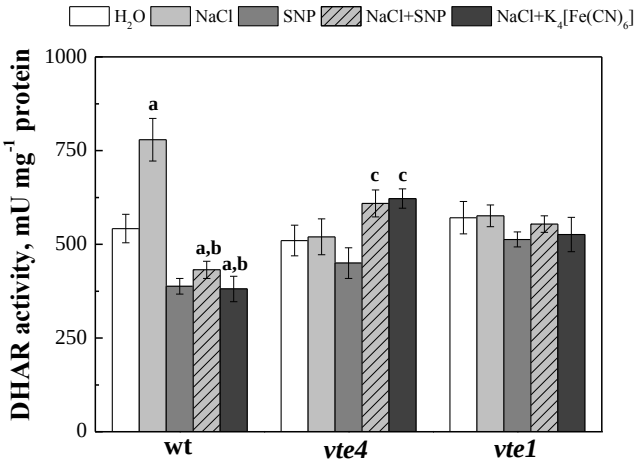

Fig. 3. Effect of SNP pretreatment on the activity of dehydroascorbate reductase (DHAR) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M. (n = 3). [a]Significantly different from respective control group (H_2O) and [b]NaCl groups (P < 0.05).

Under normal conditions, application of NO-donor had no significant effects on DHAR activity. Under salt stress conditions, the treatment with both potassium ferrocyanide and SNP decreased DHAR activity by 44% as compared with NaCl-treated plants. In leaves of *vte4* mutant line, neither NaCl nor SNP changed DHAR after treatment. However, pretreatment with SNP or potassium ferrocyanide increased DHAR activity by 19% under NaCl stress as compare with corresponding controls. None of the treatments had effects on DHAR activity in *vte1* mutant line (Fig. 3).

The activity of GR did not change in wild type plants after all treatments (Fig. 4). Exposure to 200 mM NaCl enhanced GR activity by 27-39 % in leaves of both mutant lines. In mutant line *vte4*,

pretreatment with SNP or potassium ferrocyanide slightly attenuated increase in GR activity under salt stress condition. At the same time, pretreatment with both potassium ferrocyanide and SNP did not change GR activity under salt stress conditions in *vte1* mutant line (Fig. 4).

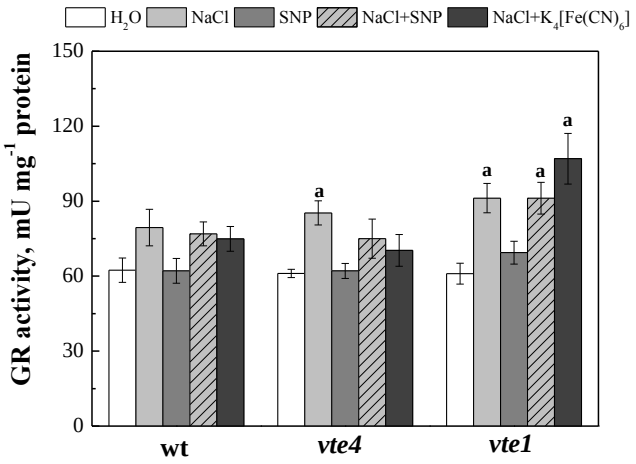

Fig. 4. Effect of SNP pretreatment on the activity of glutathione reductase (GR) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M. (n = 3). [a]Significantly different from respective control group (H_2O) ($P < 0.05$).

It is known that in plants guaiacol peroxidases (GuPx) also may participate in H_2O_2 detoxification. Similarly to APx, GuPx scavenge H_2O_2 using plant phenolic compounds, in particular guaiacol (*o*-methoxyphenol) as an electron donor [12]. In our experiments, salt stress strongly increased GuPx activity in all three plant lines (Fig. 5).

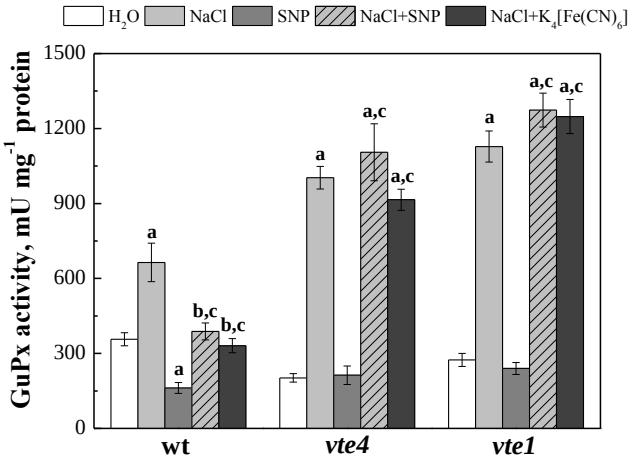

Fig. 5. Effect of SNP pretreatment on the activity of guaiacol peroxidase (GuPx) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M. (n = 3). [a]Significantly different from respective control group (H_2O), [b]NaCl and [c]SNP groups ($P < 0.05$).

Under normal conditions, application of SNP significantly decreased GuPx activity in wild type plants. Pretreatment with SNP and potassium ferrocyanide significantly reduced the increase in GuPx activity in wild type plants under salt stress conditions. In mutant lines *vte4* and *vte1*, pretreatment with both SNP and sodium ferrocyanide did not change GuPx activity under NaCl stress.

Some authors supposed that •NO could increase the activity of antioxidant enzymes by stimulation of H_2O_2 producing system(s) [8]. Guo and colleagues [13] proposed that exogenously applied SNP indirectly enhance activities of antioxidant enzymes under salt stress by the increasing proline content.

Previously Shi et al. [34] reported that application of SNP greatly induced the H_2O_2-scavenging enzymes CAT, APX and GuPx under salt stress. In the experiment, application of SNP also promoted DHAR and GR activities under salt stress, and such promotion was important for the efficient H_2O_2-scavenging by APX in cucumber mitochondria [34]. Tanou et al. [36] showed that exogenously introduced $^{\bullet}$NO (as SNP) effectively induced antioxidant enzyme activities, particularly ones of APX and GR, promoted the maintenance of the cellular redox homeostasis and mitigated the oxidative damage caused by $HO^{\bullet}$ under high salinity. The study of Uchida et al. [39] also revealed that pretreatment with SNP enhanced not only ROS scavenging enzymes activities, but also expression of transcripts for stress-related genes under salt stress conditions. SNP improved the activity of antioxidant enzymes of cucumber seedling leaves under NaCl-induced stress to different extent, and reduced the rate of $O_2^{\bullet-}$ production, membrane permeability, H_2O_2 and MDA contents simultaneously [9].

4. Conclusion

It can be concluded that pretreatment with SNP attenuated salt stress induced injuries in *Arabidopsis thaliana* plants via up-regulation of the activities of antioxidant enzymes and prevention of lipid peroxidation. The most pronounced SNP effects were observed in tocopherol-deficient *vte1* mutant plants, in which pretreatment with SNP reduced TBARS level and increased activities of GR and GuPx.

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Мосійчук Н. М. Вплив нітропрусиду натрію на стійкість до сольового стресу дефектних за токоферолом рослин *Arabidopsis thaliana*. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 122–131.

У даній роботі досліджено влив екзогенного донора оксиду азоту (•NO) нітропрусиду натрію (НПН) на пероксидне окислення ліпідів та активність антиоксидантних ферментів у дикого типу і дефектних за біосинтезом токоферолу ліній *vte1* та *vte4 Arabidopsis thaliana* за дії 200 мМ NaCl. У рослин дикого типу, попередня обробка НПН не впливала на рівень ТБК-активних продуктів, але знижувала активність дегідроаскорбатредуктази та гваяколпероксидази в умовах сольового стресу. У безтокоферольної лінії *vte1* попередня обробка НПН знижувала вміст ТБК-активних продуктів та підвищувала активність глютатіонредуктази та гваяколпероксидази за дії сольового стресу. Активність аскорбатпероксидази знижувалася за дії сольового стресу у рослин двох мутантних ліній, попередньо експонованих з НПН. Можна дійти висновку, що попередня обробка НПН може послаблювати дію сольового стресу у рослин *Arabidopsis thaliana* шляхом збільшення активності антиоксидантних ферментів та ослаблення пероксидного окислення ліпідів.

Ключові слова: антиоксидантні ферменти, пероксидне окислення ліпідів, оксид азоту, оксидативний стрес, токофероли.

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