UNIVERSITY OF PÉCS
Biological Doctoral School
Molecular analysis of microorganisms' life processes

EPR studies in yeast cells: effect of carotenoids on plasma membrane dynamic and C(VI) reduction

Ph.D. Thesis

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PÉCS, 2010

Introduction

The yeast cells, like all living creatures, in their lives respond to a number of environmental impacts (drought, temperature changes, environmental pollution, etc.). These effects change the optimal living conditions, thereby forcing them to adapt. Too high oxidative stress load, in combination with not very effective reaction to the changes lead to a degree of cell damage, which ultimately lead to cell death.

Extracellular cell defence

The first line of defense from the environmental effect in the cell is the cell membrane. In the biological membrane, within the normal "operating temperature" of biological system, there is a high degree of motility between the lipids in the lipids double layer due to lack of covalent bond. The lipid mobility depends on the temperature and the membrane composition in the membranes.

Generally the cell membranes are in fluid state at body temperature. If the temperature falls below the phase transition temperature, the membrane becomes rigid known as gel state, hence the lipid motility is reduced. The membrane fluidity growth leads to an increase in permeability. In the fatty acid region above a given temperature shows a significant structural transformation and decreases their ordering. The fatty acid orientation decreases, its freedom of movement increases and the distance between the double-layer surfaces also increases, leading to volume expansion as well as the rate of lateral diffusion of phospholipids. In the phospholipids the presence of unsaturated fatty acids increase the fluidity, while the trans double bond did not cause significant change in the fluidity compared to the saturated bonds. On the other hand the cis double bonds, expresses significant difference in the fluidity state due to the flexibility of fatty acid. These bonds reduce the actual chain length, increasing the width of the molecule, preventing the establishment of too

close side chains. By contrast, a large amount of saturated fatty acids in the membranes reduces the fluidity and consequently the permeability.

Lipid soluble carotenoids incorporated in *Xanthophyllomyces dendrorhous* plasma membrane, their localization determine the membrane dynamics. The molecular structures of carotenoids generally affect and modify the biological membranes and play a role in various physiological processes. The carotenoids have antioxidant effect in the plasma membrane and effectively protect the cells against phospholipid peroxidation induced by radicals.

The intracellular cell defense against oxidative damage caused by chromium

Nowadays due to the industrial activity a large amounts of chromium accumulated in the environment and our organism. The mutagenic and the carcinogenicity of chromium have been well documented. Cr(VI) and Cr(III) are the only chromium ions that are stable under environmental condition. The reduction process may contribute to the adverse effects of Cr(VI) by producing hydroxyl radicals ($^{\bullet}OH$).

The Cr(VI) undergo the following processes upon entering the cell: (a) reduction of Cr(VI), (b) reaction with reactive oxygen species (ROS) resulting in production of harmful $^{\bullet}OH$, (c) neutralization by the cellular components including detoxifying enzymes and antioxidants. In a Fenton reaction between H_2O_2 and Cr(V)/Cr(IV)/Cr(III), the cyto and genotoxic effects of Cr(VI) is attributed to the formation of $^{\bullet}OH$. However the Haber-Weiss reaction between $O_2^{\bullet-}$ + Cr(VI) is unlikely to play a significant role in the formation of Cr(V) and hence the generation of $^{\bullet}OH$.

The yeast Cr(VI) tolerance is proportional to their ability of sulphate uptake from the environment. The Cr(VI) gradient between the two sides of the cell membrane is maintained by the metabolically active cells, which continuously reduce the accumulated Cr(VI) to lower oxidation states in both enzymatic processes (involving flavoenzymes) and non enzymatic processes (glutathione (GSH), NADPH and ascorbate). The low molecular mass reductant GSH which is widespread in yeast is thought to

be the most important agent participating in the reduction of Cr(VI). *In vitro* Cr(VI) is reduced by GSH producing glutathione derived thiol radical ($GS^{\bullet}$) and Cr(V). Among the GSH-dependent enzymes, using NADPH as co-substrate, *in vitro* experiments the GR reduces Cr(VI) directly to Cr(V) producing superoxide.

Cr(V) and Cr(IV) variant are able to react with H_2O_2 to produce $^{\bullet}OH$ in Fenton-type reaction. In this harmful process cyto and genotoxic radicals may also be generated in Cr(VI)-mediated Haber-Weiss type reaction, however the direct $O_2^{\bullet-}$ participation in the generation of $^{\bullet}OH$ is questionable. The severe cellular damage caused by $^{\bullet}OH$ is well known as there is no enzymatic defense against it. *S. pombe* does not produce non enzymatic $^{\bullet}OH$ scavengers such as ascorbate, α-tocopherol and carotenoids, it uses GSH and phytochelatins. The GSH and free radical metabolisms of fungi are rather complex and are influenced by numerous endogenous and exogenous factors.

The first line of defense against $O_2^{\bullet-}$ and H_2O_2 mediated injuries are well functioning antioxidant enzymes, including superoxide dismutases (SODs), peroxidases and catalase, which are also key elements in the cross-protection and adaptation to against various oxidative stressors. The GSH-dependent detoxification activity depends on the glutathione-S-transferase (GST) enzyme, which is crucial during superfluous glutathione disulfide (GSSG) transport out of the cell and maintains physiologically relevant GSH/GSSH redox balance.

AIMS

In our experiments we searched for oxidative stressors effects (cadmium, H_2O_2, chromium) on the *X. dendrorhous* carotenogenesis and *Schizosaccharomyces pombe* glutathione redox system. We also studied the impact of carotenoids produced by *X. dendrorhous* yeast on the plasma membrane dynamics and how they can contribute to the defense system against oxidants.

METHODS

The **carotenoid** producing *X. dendrorhous CBS 6938* and its 4 carotenoid mutants (*C27*, *C29*, *C30*, *C31)* was studied.

- Carotenoids were extracted from the cell by DMSO (dimethyl-sulphoxide)
- HPLC was used to determine the carotenoid quantity and their composition ratio produced by strains, and examined how these values are changed by cadmium and the effect of H_2O_2. Data represent the mean of five independent experiments. *p* values were calculated using the Student's t-test.
- EPR spectra were recorded with an ESP 300E spectrometer (Bruker, Germany) equipped with an ER 412VT temperature regulator. The EPR spectra from the fatty acid spin label 5-SASL incorporated into the membranes were taken in the temperature range 0-30 °C.

In *S. pombe chr1-66T* **Cr(VI)** tolerant mutant glutathion reductase *pgr1*$^+$ gene mutation occurred:

- The *in vivo* generation and reduction of Cr(V) in *S. pombe* was followed by electron paramagnetic resonance (EPR) spectroscopy. To calculate the concentration of Cr(V), the double integral of the Cr(V) signal was

compared with a nitroxide free radical solution of known concentration (10 μM) The *in vivo* and *in vitro* formation of ˙OH was measured by using the spin trap 0.1 M N-*tert*-butyl-*α*-phenyl nitrone (PBN).

RESULTS AND PRESENTATION OF NOVEL FINDINGS

The **carotenoid** producer *CBS 6938 X. dendrorhous* parental strain and their 4 mutagenesis stable carotenoid synthesis altered mutants (*C27, C29, C30* and *C31*) were studied.

- We found that the parental strain *CBS 6938* and the *C31*-mutant contained a higher proportion of polar (astaxanthin, cis-astaxanthin and canthaxanthin) carotenoids. The *C29* and *C30* mutant strains produced significantly more non-polar (β-cryptoxanthint-, β-carotene) carotenoids. The white mutant *C27* strain did not produce detectable amounts of carotenoids, including phytoene synthesis is the likely path of mutation, which is the result of a lack of carotenoid intermediates.

- Subsequently, the affect of relative sensitivity of carotenogenesis by ROS-generated using cadmium and H_2O_2 was examined by HPLC. The resulting chain reaction induced by stressors leads to peroxyl radicals, •OH and appearance of free oxygen, are involved in the antioxidant function of carotenoids. However the amount of carotenoids produced slightly increased (with the exception of the *C27*and *C30* mutant) in both stressor effect, but this was more pronounced in the case of cadmium treatment. The increased amount of carotenoids means that cadmium and H_2O_2 actively participate in the elimination of free radicals, reducing the adverse consequences.

  Cadmium increases the polar carotenoids levels, while the H_2O_2, elevates the non-polar carotenoids level. Thus, it is assumed that, since the cadmium and H_2O_2 treatment result in different free radicals which are degraded by different antioxidant processes, in which the different levels of carotenogensis will induce. The results conclude that the astaxanthin

are typically involved in reducing the harmful effect of cadmium-induced free radicals (especially •OH) [except for the other redox (glutathion redox) systems]. The β-carotene and β-cryptoxanthin more efficiently reduce the effect of the consequences of H_2O_2 treatment.

C30 mutant was an exception because in both cases it only minimally increased or retained the total carotene level and stress reduced astaxanthin and β-carotene level. In contrast β-cryptoxanthin ratio greatly increased under the effect of cadmium and H_2O_2 treatment.

C27 albino mutant did not produce carotenoids, it was protected against the free radicals produced by activation of other antioxidant system (glutathione redox system).

The carotenogenesis and its involvement in dynamically adapted antioxidant system provide the right mix of stable level of carotenoid oxidation for protection of *Xantophyllomyces* cell against antioxidation. As required it increases or decreases the amount of particular carotenoids or affects their rates. From these results, we concluded that there is a link between the structure and reactivity of the carotenoids, as well as the type of free radical induction trigger.

- Subsequently, we examined the impact and dynamics of carotenoid membrane located in the *X. dendrorhous* plasma membrane using spin-labeled EPR technique. The spin-labeled plasma membrane mobility is strongly influenced by temperature and properties of carotenoids located in the membrane. In astaxanthin rich plasma membrane strains the labeled rotational freedom began to decrease in lower temperature began as more β-carotene containing. Astaxanthin increases the order of lipid hydrocarbon of the plasma membrane while the β-carotene decreases the membrane order by shifting the lipid chains. Thus, we can say that the astaxanthin containing membranes are more rigid than β-carotene containing membrane. We found that the astaxanthin has rigidifying, structure stabilizing effect on the membrane lipid chains, while the β-carotene has fluidized and relaxing effects on the plasma membrane. The polar heads of carotenoids anchor the inner and

outer surface of the double membrane stretched the carotenoids in the membrane perpendicular to its long axis. This leads to a decrease in the fluidity of biological membranes and reduces the intermolecular space leading to a condense structure and consequently reduces the trespassing of particular molecules across the plasma membrane exercising its antioxidant effect. In contrast, β-carotene and β-cryptoxanthin do not bond between the lipids heads, which would results in the space and motility reduction because the fatty acids are slightly repelled from each other in a well-ordered, stable, network membrane. Thus, the carotenoids with their different binding to the membrane lipid region constitute more fluid or more rigid plasma membrane structure. Thus, various types of carotenoids, their properties, their location and their orientation defines and regulates the membrane dynamics, providing an additional security system for the *X. dendrorhous* against oxidation harmful effects. Therefore we conclude that astaxanthin protects more effectively against the lipid peroxidation than β-carotene, which as evidenced by the HPLC analysis of the results, explains the excess amount of astaxanthin produced under the effect of cadmium. This means that cadmium can induce lipid peroxidation, and their degrading effect is more effectively reduced by of astaxantin.

- A remarkable correlation was noted between the carotenoids polarity of strains and temperature phase transition. The non-polar carotenoids increasing the phase transition temperature, while the polar reduce it. We concluded that the astaxanthin reduces the phase transition temperature by distraction of organization of the lipid bilayer polar head and the β-carotene does not disturb the membrane surface. The results of this study showed that the strain, which contained largest amount of "most polar" carotenoids (astaxanthint) have lowest phase transition temperature. On the other hand where highest temperature measured there was maximum "most non-polar" carotenoids (β-carotene). Consequently, the HPLC retention time of polarity of carotenoids correlated with the phase transition temperature.

The **Cr (VI) compounds** cytotoxic and genotoxic effects were examined on *S. pombe* parental strain *6chr*$^{+}$ and Cr(VI)-tolerant *chr1-66T* mutants. The following established:

- The time course of Cr(V) formation and reduction monitored by EPR spectroscopy indicated that both strains reduced Cr(VI) to Cr(III) through Cr(V), but the elimination of Cr(V) in the Cr(VI)-tolerant *chr1-66T* mutant was much slower than in *6chr*$^{+}$ cells. The decreased reduction capacity of the Cr(VI)-tolerant mutant was also demonstrated *in vitro*. In this case, disrupted cells of both strains were exposed to 2.0 mM $K_2Cr_2O_7$ for 5 min; again, a significantly lower Cr(V) concentration was detected in the *chr1-66T* mutant than in the *6chr*$^{+}$ strain. These experimental data supported the idea that there was a causal connection between the decreased reduction capacity of *chr1-66T* cells and their lower bioaccumulation of Cr(VI).

- Taking into account the low intracellular H_2O_2 concentration of chrome-tolerant mutant, the elevated level of $O_2^{\bullet-}$ concentration implies that in Fenton-type reaction $O_2^{\bullet-}$ has no significant role, especially for the H_2O_2, which through the Fenton reaction contributes significantly to the chrome-tolerance.
- NADPH given to Cr(VI) treated cells, the tolerant mutant Cr(V) concentrations showed a 2.8-fold increase, while the *6chr*$^{+}$ strains the NADPH did not increase substantially the Cr(V) amount (59.0 → 59.3 μM). If the exposed strain was treated by H_2O_2 as well as with Cr(VI) and NADPH, then the chromium(V) concentration reduced in both strains due to the Fenton reaction. The amount of $^{\bullet}OH$ radicals, similar to Cr(V) amount, was lower in the tolerant mutant *chr1-66T* in each compiled test conditions. It is therefore assumed that the G6PD enzyme produced by a greater amount of NADPH is not sufficient to counteract the GR enzymatic activity reduction due to reduced capacity. Thus, it appears likely that in addition

to the GSH the GR / NADPH reduction system plays a decisive role in the Cr (VI) → Cr (III) reduction process.

In *S. pombe* chr1-66T Cr(VI) tolerant mutant glutathion reductase *pgr1*$^{+}$ gene mutation occurred, resulting in decrease:

- specific enzyme activity of GR and GSH content, which reduced the Cr(VI) reducing capacity and the Cr(VI) bioaccumulation,
- reduced GSH content and decreased GR, GPx and catalase-specific enzymatic activity increased sensitivity to oxidative stressors,
- decreased MnSOD activity leads to a low intracellular concentration of H_2O_2 which is an important element of Fenton type $^{\bullet}OH$ radical production.
- Finally, the Cr(VI) tolerance is the ultimate reason for reduced Cr(VI) reduction capacity resulted in low Cr(VI) uptake.

This finding is supported by the fact that if in the tolerant mutant transformation the GR-specific activity increased, then the Cr(VI) tolerance ends and the parental mutant strains show the same chromium sensitivity.

SUMMARY

In our study the plasma membrane **carotenoid** content of *X. dendrorhous* was examined by High Performance Liquid Chromatography and Electron Paramagnetic Resonance. The quantity and the quality of the membrane carotenoids and its structural and dynamic alteration were determined utilizing these two techniques.

I.1. We found that the parental strain *CBS 6938* and the *C31* mutant contained polar carotenoids (astaxanthin, cis-astaxanthin and canthaxanthin). The *C29* and *C30* mutant strains produced non polar carotenoids (β-cryptoxanthint, β-carotene).

I.2. As a result of Cd^{2+} and H_2O_2 treatment the amount of carotenoids produced slightly increased. This indicates that both stressors actively participate in the elimination of free radicals, reducing the adverse

consequences. The results conclude that the polar carotenoids are typically involved in reducing the harmful effect of cadmium-induced free radicals (especially $^{\bullet}OH$). The non polar carotenoids more efficiently reduce the effect of the consequences of H_2O_2 treatment. From these results, we concluded that there is a link between the structure and reactivity of the carotenoids, as well as the type of free radical induction trigger.

II.1. We concluded that the polar carotenoids reduce the phase transition temperature by distraction of organization of the lipid bilayer polar head and increase the order of lipid hydrocarbon of the plasma membrane. The non polar carotenoids do not disturb the membrane surface, however decrease the membrane order by shifting the lipid chains.

A correlation was noted between the carotenoids' polarity and phase transition temperature, which suggest that the more polar carotenoids reduce more effectively the higher phase transition temperature, than less polar or non polar carotenoids.

Base on our result, we demonstrated that the carotenoids have various effects on the plasma membrane which influenced by different factors, such as carotenoids polarity, their orientation within the plasma membrane, membrane character (content, thickness). By study of different factors further information can be achieved about the role of carotenoids in the plasma membrane.

The **Cr (VI) compounds** cytotoxic and genotoxic effects were examined on *S. pombe* parental strain *6chr*$^{+}$ and Cr(VI)-tolerant *chr1-66T* mutants by Electron Paramagnetic Resonance. We established that both strains reduced Cr(VI) to Cr(III) through Cr(V), but the elimination of Cr(V) in the Cr(VI)-tolerant *chr1-66T* mutant was much slower than in *6chr*$^{+}$ cells which were due to *chr1-66T* Cr(VI) tolerant mutant glutathion reductase *pgr1*$^{+}$ gene mutation resulting in:

- decrease specific enzyme activity of GR and GSH content, that reduced the Cr(VI) reducing capacity and the Cr(VI) bioaccumulation,

- decreased MnSOD activity leads to a low intracellular concentration of H_2O_2 which is an important element of Fenton type $^{\bullet}OH$ radical production.

This finding is supported by the fact that if in the tolerant mutant transformation the GR-specific activity increased, then the Cr(VI) tolerance ends and the parental mutant strains show the same chromium sensitivity.

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