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# Antioxidant Protection of Resveratrol and Catechin in *Saccharomyces cerevisiae*

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Moderate consumption of red wine reduces the risk of heart disease and extends lifespan, but the relative contribution of wine polyphenols to these effects is unclear. In this work, the capacity of resveratrol and catechin to protect the eukaryotic microorganism *Saccharomyces cerevisiae* against oxidative stress caused by different agents, hydrogen peroxide, carbon tetrachloride, and cadmium, was evaluated. Under all stress conditions, both polyphenols increased tolerance, although their protection was more evident under peroxide exposure. By using mutant strains deficient in specific antioxidant defense systems (superoxide dismutases, catalase, or glutathione), it was observed that increased $H_2O_2$ tolerance produced by both polyphenols was associated with catalase, as well as the rise in survival rates caused by resveratrol under $CCl_4$. The acquisition of tolerance was correlated with a reduction in lipid peroxidation, indicating that the antioxidant property of resveratrol and catechin involves protection against membrane oxidation.

**KEYWORDS: Catechin; resveratrol; oxidative stress; lipid peroxidation; *Saccharomyces cerevisiae***

## INTRODUCTION

Oxidative stress has been correlated with aging and diseases. On the other hand, phytochemicals present in fruits and vegetables may have antioxidant effects that protect from the oxidative damage arising from metabolic and exogenous sources (*1*). Although the protective effects have been primarily attributed to the well-known antioxidants, such as vitamins C and E and $\beta$-carotene, plant phenolics also seem to play a significant role. Grapes are rich in phenolic compounds, such as flavonoids (catechin, epicatechin, quercetin, anthocyanins, and procyanidins), and resveratrol (*2, 3*). Increasing evidence indicates the importance of wine consumption in the daily diet since it is supposed to be one of the explanations for the "French paradox"—the low incidence of heart disease and cancer in France in spite of high fat consumption (*4*).

Resveratrol (3,5,4′-trihydroxystilbene) is one of most important polyphenols found in red wine. It is associated with a surprising number of health benefits, most notably the mitigation of age-related diseases, including neurodegeneration, carcinogenesis, and atherosclerosis (*5*). Catechin is a flavan-3-ol, namely, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran) present mainly in white wine (*3*). It has been shown that catechin is very effective in blocking the growth of human cell lines originating from prostate (*6*) and breast (*7*) cancers and is also a potential antioxidant and antimutagenic agent.

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Despite the studies that showed the antioxidant properties of resveratrol and catechin, the molecular mechanisms of how they function in vivo remain unclear. These polyphenols show different bioavailabilities (*8*), which make it difficult to determine the antioxidant potential of each one. In addition, their protective effects have been reported to be more pronounced in vitro, using high, nonphysiological concentrations (*9*).

The bioavailability appears to differ greatly between the various polyphenols, and the most abundant polyphenols in our diet are not necessarily those that have the best bioavailability profile (*8*). Recently, we found that Wistar rats that consumed purple grape juice daily showed protection against oxidative stress, but the results obtained did not allow us to state which polyphenol was absorbed most or gave the best protection (*2*).

The aim of this work was to evaluate the mechanism by which resveratrol and catechin protect *Saccharomyces cerevisiae*, a useful model to screen in vivo for natural antioxidants: Its entire genome sequence has been elucidated, and it is a genetically tractable organism, amenable to modifications such as gene disruption or mutation, which facilitates the identification of gene targets of chemicals or drugs or stress, such as oxidative stress, response pathways (*10*). *S. cerevisiae* has similar antioxidant responses to mammals, and 30% of known genes involved in human disease have yeast orthologues, that is, functional homologues (*11*). Furthermore, by using this microorganism, the differences in bioavailability of polyphenols would be discarded. We tested different oxidative stresses, generated by carbon tetrachloride, hydrogen peroxide, or cadmium. The toxicity of $CCl_4$ results from its reductive dehalogenation by cytochrome P450 into trichloromethyl free radical, which readily

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interacts with molecular oxygen to form trichloromethyl peroxyl radicals (*12*). Both radicals are able to attack proteins and lipids or to remove hydrogen atoms from unsaturated lipids leading to membrane lipid peroxidation, cellular dysfunction, and finally cell necrosis (*13*). Cadmium is an environmental carcinogenic pollutant that inactivates several proteins involved in DNA repair systems and creates an oxidative stress that can result in additional DNA lesions (*14*). $H_2O_2$ can generate hydroxyl radical, the most reactive and toxic reactive oxygen species (ROS) (*15*).

## MATERIALS AND METHODS

**Chemical Reagents.** $H_2O_2$ was purchased from Merck; dimethyl sulfoxide (DMSO), resveratrol, and catechin were acquired from Sigma-Aldrich. Media components were obtained from Difco.

***S. cerevisiae* Strains and Growth Conditions.** Wild-type (WT) strain BY4741 (*MATa*, *his3*, *leu2*, *met15*, and *ura3*) and its isogenic mutants *sod1*Δ, *sod2*Δ, *ctt1*Δ, and *gsh1*Δ, harboring the genes *SOD1*, *SOD2*, *CTT1*, or *GSH1*, respectively, interrupted by gene *KanMX4*, were acquired from Euroscarf (Frankfurt, Germany). Stocks of yeast strains were maintained on solid 2% YPD (1% yeast extract, 2% glucose, 2% peptone, and 2% agar); in the case of the mutant strains, the medium also contained 0.02% geneticine. For all experiments, cells were grown in liquid YPD medium using an orbital shaker at 28 °C and 160 rpm with the ratio of flask volume/medium of 5/1.

**In Vivo Antioxidant Analysis.** Yeast cells at the midlong phase ($10^6$ cells/mL) were reinoculated in fresh medium (the initial cell concentration was $10^5$ cells/mL), containing or not the antioxidant agent (10 μg/mL catechin or resveratrol), and incubated, for 1 h, at 28 °C/160 rpm. To choose the doses of the polyphenols used in the adaptive treatments, cells were exposed to increased concentrations of resveratrol or catechin and then spotted adjacently on YPD agar plates incorporating $CCl_4$, peroxide, or cadmium. The concentration chosen was the lowest that could improve cell growth as compared to cohorts exposed to stress without being treated with polyphenol. Both cultures, treated or not with polyphenol, were subjected to oxidative stress (2.5 mM $H_2O_2$, 10 mM $CCl_4$, or 2.5 mM $CdSO_4$) at 28 °C/160 rpm for 1 h. Cell viability was analyzed by plating, in triplicate, on solid YPD medium, after proper dilution. Plates were incubated at 28 °C for 72 h, and the colonies were counted. The number of colonies in each plate was between 150 and 200. Tolerance was expressed as a percentage of survival (*16*).

**Detection of Lipid Peroxidation.** Cells (50 mg) were centrifuged at 2000*g* for 2 min and washed twice with distilled Millipore purified water. The pellets were resuspended in 0.5 mL of 10% trichloroacetic (w/v), and 1.5 g of glass beads was added. The samples were lysed by six cycles of 20 s agitation on a vortex followed by 20 s on ice. Extracts were centrifuged at 2000*g* for 3 min, and the supernatant was mixed with 0.1 mL of 0.1 M EDTA and 0.6 mL of 1% (w/v) thiobarbituric acid in 0.05 M NaOH. The reaction mixture was incubated in a boiling water bath for 15 min, and after the mixture was cooled, the absorbance was measured at 532 nm (*17*).

**Intracellular Oxidation.** The oxidant-sensitive probe 2′7′-dichlorofluorescein diacetate was used to measure intracellular oxidation (*18*). Fluorescence was measured using a Photo Technology International (PTI) spectrofluorimeter set at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. A fresh 5 mM stock solution of 2′,7′-dichlorofluorescein diacetate dissolved in ethanol was added to the culture (the final concentration was 10 μM) and incubation at 28 °C continued for 15 min to allow uptake of the probe. The culture was divided according to treatment (with or without polyphenol). After 1 h, the oxidative agent ($CCl_4$, $Cd^{2+}$, or $H_2O_2$) was added. Thereafter, 50 mg of cells was harvested by centrifugation and washed twice with water. The pellets were resuspended in 500 μL of water, and 1.5 g of glass beads was added. The samples were lysed by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. The supernatant solutions were obtained after centrifugation at 25000*g* for 5 min and diluted 6-fold with water, and then, the fluorescence was measured. As a control, the fluorescence was analyzed in cells that had not been exposed to oxidative stress.

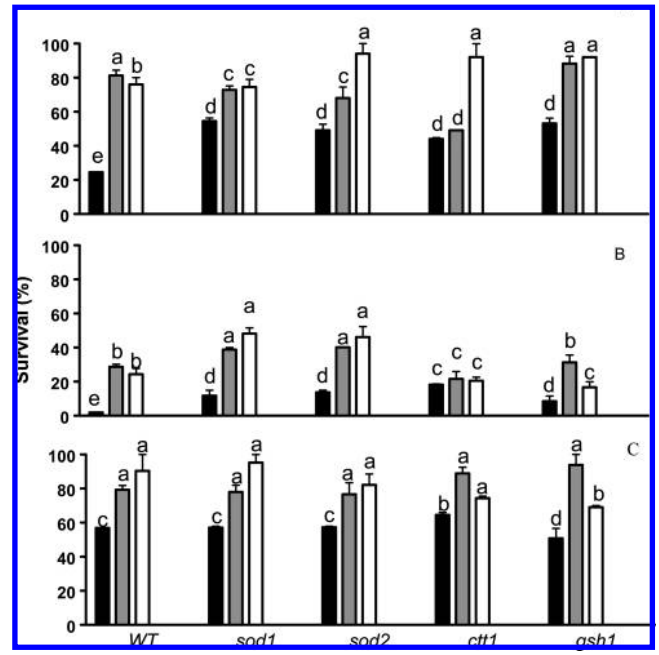


**Figure 1.** Effect of resveratrol and catechin on survival rates of cells stressed with 10 mM $CCl_4$ (**A**), 2.5 mM $H_2O_2$ (**B**), or 2.5 mM $CdSO_4$ (**C**). Black bars mean that cells were directly stressed, gray bars mean that cells were adapted with resveratrol and stressed, and white bars mean that cells were adapted with catechin and stressed. Data represent the means ± SDs of at least three independent experiments. Each stress was analyzed separately to determine statistical differences (different letters mean statistically different results in each oxidative stress group; $p < 0.05$).

**Statistical Analyses.** The statistics were done by means of analysis of variance and Tukey's test using the SPSS 12.0 package. The latter denotes homogeneity between experimental groups at $p < 0.05$. In all figures and tables, different letters mean statistically different results.

## RESULTS AND DISCUSSION

**Resveratrol and Catechin Increase Oxidative Stress Tolerance in *S. cerevisiae*.** According to **Figure 1**, 2.5 mM $H_2O_2$ was the most toxic stress for all strains tested, followed by $CCl_4$ and $Cd^{2+}$. This result was expected since $H_2O_2$ generates the most toxic and highly reactive hydroxyl radical, against which the organisms have no defense (*15*). Cells of the WT strain acquired tolerance to all stresses when preadapted with resveratrol or catechin. The increased tolerance caused by each polyphenol was similar, and the best protection was observed under peroxide.

Molecular studies have revealed that phenolics can modulate the cell response by interacting with a wide spectrum of molecular targets, such as protein kinases, transcription factors NF-κβ and c-JUN, and antioxidant detoxifying enzymes (*5*). To investigate the involvement of some antioxidant defense system in the mechanism of acquisition of tolerance, mutant strains deficient in superoxide dismutase, catalase, or glutathione synthesis were used. Free radical scavenging enzymes such as catalase and superoxide dismutase are the first line of cell defense against oxidative injury. The equilibrium between these enzymes is a major process for the effective removal of ROS (*19*). *S. cerevisiae* possesses two isoforms of superoxide dismutase (the cytosolic Sod1 and the mitochondrial Sod2) and two isoforms of catalase, although only the cytosolic Ctt1 seems

**Table 1.** Effect of Resveratrol and Catechin on Lipid Peroxidation

| stress | treatment | WT | *ctt1* |
|---|---|---|---|
| $CCl_4$ | without polyphenol | 1.5 ± 0.2[a] a[b] | 1.6 ± 0.1 a |
| | resveratrol | 0.9 ± 0.1 b | 0.5 ± 0.0 c |
| | catechin | 0.9 ± 0.1 b | 1.4 ± 0.0 d |
| $H_2O_2$ | without polyphenol | 2.6 ± 0.1 a | 2.7 ± 0.1 a |
| | resveratrol | 1.1 ± 0.1 b | 1.5 ± 0.0 c |
| | catechin | 1.2 ± 0.1 b | 1.0 ± 0.2 b |
| Cd | without polyphenol | 1.2 ± 0.1 a | 1.4 ± 0.1 c |
| | resveratrol | 0.8 ± 0.1 b | 0.4 ± 0.0 d |
| | catechin | 0.6 ± 0.0 b | 0.7 ± 0.2 b |

[a] The results were expressed as a ratio between lipid peroxidation levels of stressed, adapted or not with polyphenol, and nonstressed cells. Data represent the means ± SDs of at least three independent experiments. [b] Each stress was analyzed separately to determine statistical differences (different letters mean statistically different results at $p < 0.05$).

to be directly involved in oxidative stress (*20*). The second line of defense consists of nonenzymatic scavengers. The most important one is glutathione (GSH), present from bacteria to higher eukaryotes and whose synthesis in *S. cerevisiae* requires the enzymes Gsh1 and Gsh2 (*21*).

Under our experimental conditions, cells fermented glucose, and thus, some of the intracellular antioxidants were absent or present at very low concentrations. Catalase and superoxide dismutase activities and glutathione levels increase significantly only when cells are breathing (*22*). Therefore, although we did not test it in this study, we can assume that if a mutant strain deficient in a specific antioxidant system is not able to acquire tolerance after the adaptive treatment, this might mean that the protection mechanism caused by polyphenol involves the induction of this antioxidant.

Catechin was capable of increasing the tolerance of all mutant strains to $CCl_4$, indicating that both Sods, Ctt1, and GSH are not necessary for this adaptive treatment (**Figure 1A**). On the other hand, the protection caused by resveratrol against $CCl_4$ seems to need the cytosolic catalase, since the *ctt1* strain was not able to acquire tolerance when adapted with resveratrol. Ctt1 seems also to be involved in the protection mechanism achieved by both polyphenols against $H_2O_2$ (**Figure 1B**). Against cadmium stress, both polyphenols were capable of increasing the survival rates of all mutants, although in the *gsh1* strain the protection caused by catechin was slightly less effective than in WT (**Figure 1C**).

**Levels of Lipid Peroxidation and Intracellular Oxidation.** Next, intracellular oxidation and lipid peroxidation were analyzed to understand how catechin and resveratrol protect cells against the oxidative damage caused by $CCl_4$, $H_2O_2$, and $Cd^{2+}$. One of the targets of free radical attack is the membrane, leading to lipid peroxidation, cell leakage, and death. **Table 1** shows that all stresses increased the levels of lipid peroxidation. Both polyphenols showed similar capacities of reducing the oxidative damage to membrane. Peroxide stress was most aggressive to the membrane, which is in accordance with the low survival rates (**Figure 1**). However, the treatment with polyphenols practically inhibited the increase in lipid peroxidation caused by peroxide in WT (the increase fell from 160% to around 10%). $CCl_4$ and $Cd^{2+}$ produced a more modest increase in lipid peroxidation (50 and 20%, respectively), which was suppressed by resveratrol and catechin. The protection conferred by resveratrol and catechin against membrane oxidation appears to be directly correlated to the acquisition of tolerance, since the greatest increase in survival rates was reached during peroxide exposure. While the phenols increased almost 20-fold the tolerance to peroxide, reducing to around 10% the rise in the lipid peroxidation levels, under carbon tetrachloride and cadmium stresses, the increase in the survival rates did not exceed 3-fold, and the reduction in the levels of peroxidation was smaller.

**Table 2.** Enhancement of Intracellular Oxidation Produced in Response to Peroxide or Carbon Tetrachloride Stress

| stress | rreatment | WT | *ctt1* |
|---|---|---|---|
| $CCl_4$ | without polyphenol | 4.9 ± 0.1[a] a[b] | 2.0 ± 0.1 b |
| | resveratrol | 3.3 ± 0.4 c | 2.6 ± 0.0 d |
| | catechin | 3.4 ± 0.3 c | 0.8 ± 0.1 c |
| $H_2O_2$ | without polyphenol | 12.2 ± 0.3 d | 1.6 ± 0.1 b |
| | resveratrol | 4.6 ± 0.3 c | 1.9 ± 0.4 b |
| | catechin | 10.2 ± 0.5 a | 2.1 ± 0.2 a |

[a] The results were expressed as a ratio between fluorescence of stressed, adapted or not with polyphenol, and nonstressed cells. Data represent the means ± SDs of at least three independent experiments. [b] Each stress was analyzed separately to determine statistical differences (different letters mean statistically different results at $p < 0.05$).

Because cytosolic catalase might be involved in the protection mechanism achieved by polyphenols (**Figures 1A**,**B**), peroxidation was also investigated in the *ctt1* mutant strain. However, the behavior of the mutant was similar to that of the WT strain. In the mutant, both polyphenols were able to reduce the levels of lipid peroxidation caused by all stresses, although the *ctt1* strain had not acquired tolerance after polyphenol treatment and peroxide stress or after catechin adaptation and carbon tetrachloride stress. These results indicate that Ctt1 is not involved in the protection conferred by catechin and resveratrol against lipid oxidation.

Polyphenols exhibit a wide range of biological effects (*23*); many of them have been attributed to their free radical scavenging activity. To determine whether resveratrol and catechin are responsible for the increase in tolerance by decreasing reactive oxidative species concentration, the level of intracellular oxidation was measured by using the fluorescent probe 2′,7′-dichlorofluorescein diacetate. This probe is widely used to evaluate the enhancement of reactive oxidative species after oxidative stress since, once inside the cell, it becomes susceptible to attack by reactive oxidative species, producing a more fluorescent compound (*24*). According to **Table 2**, after direct exposure of the WT strain to peroxide and $CCl_4$, there was an increase in intracellular oxidation. There was a greater increase after $H_2O_2$, which can be correlated with the higher sensitivity shown by cells under this stress condition (**Figure 1**). Cell exposure to 2.5 mM $Cd_2SO_4$ for 60 min did not increase the levels of intracellular oxidation (data not shown).

Similarly to what occurred with lipid peroxidation, both catechin and resveratrol decreased the levels of reactive oxidative species produced by peroxide or carbon tetrachloride (**Table 2**). After resveratrol treatment, the levels of reactive oxidative species produced in response to peroxide were almost 3-fold lower, suggesting that resveratrol has a high capacity to eliminate hydroxyl radicals formed by a Fenton reaction. In the absence of cytoplasmic catalase, the levels of reactive oxidative species produced by carbon tetrachloride were only reduced when the mutant was adapted with catechin, the same treatment that led to acquisition of tolerance of *ctt1* strain (**Figure 1A**). In the mutant, neither catechin nor resveratrol was able to reduce the increase in the levels of intracellular oxidation caused by peroxide; coincidentally, neither treatment increased the tolerance of this strain (**Figure 1B**). Considering these results, Ctt1 seems to contribute to the elimination of reactive oxidative species achieved by resveratrol under $CCl_4$ and $H_2O_2$ stresses as well as by catechin under $H_2O_2$ exposure.

The mutant deficient in Ctt1 showed an increase in intracellular oxidation caused by $CCl_4$ and $H_2O_2$ lower than the WT strain. This could be associated with super expression of other antioxidant systems as a form of compensation. Several other studies have shown that a deficiency in one antioxidant system is overcome by an increase in the remaining defense system (*25, 26*).

The medicinal actions of resveratrol and catechin are mostly attributed to their antioxidant capacity and free radical scavenging potential, since oxidative stress is involved in aging as well as in the onset and evolution of more than 100 diseases (*27*). However, the true antioxidant effect of these polyphenols and the mechanisms by which they protect the organisms against oxidative stress have not yet been elucidated. The antioxidant potential of catechin and resveratrol has been investigated mainly through in vitro analyses, although several studies have shown that phenolics are extensively metabolized in vivo, resulting in significant alteration in their redox potentials (*28*). Therefore, it is essential to screen the efficacy of these compounds in vivo. The physiological significance of dietary antioxidants depends on their absorption and biotransformation mechanism. In animal models, it is more difficult to interpret the results, which warrants further investigation on the bioavailability of the polyphenols.

As previously observed (*25, 29, 30*), peroxide, carbon tetrachloride, and cadmium stresses caused damage in *S. cerevisiae* cells. Peroxide and carbon tetrachloride produced free radicals, verified by the increase in the levels of intracellular oxidation, and all stresses tested were able to attack the membrane, leading to lipid peroxidation. Both resveratrol and catechin increased the tolerance to all oxidative conditions, and their protective effects were similar (**Figure 1**).

Resveratrol and catechin reduced intracellular oxidation and lipid peroxidation, which could explain why cells acquired tolerance when adapted with these polyphenols. By reducing the ROS level, biomolecules become less prone to oxidation. The prevention of low-density protein (LDL) oxidation seems to protect against heart diseases, while the prevention of DNA oxidation diminishes genomic instability and the chances of developing cancer (*31*). Heart disease and cancer are the two leading causes of death worldwide.

The antioxidant properties of polyphenols seem to be associated with their capacity to donate hydrogen to free radicals, leading to the formation of stable molecules. Resveratrol and catechin reduced the levels of ROS produced in response to $H_2O_2$ or $CCl_4$ (**Table 2**). These stresses generate different free radicals, which contribute to the increase in the levels of intracellular oxidation. Cadmium stress, achieved by submitting cells to 2.5 mM $CdSO_4$ for 1 h, did not increase the level of intracellular oxidation but did induce lipid peroxidation (**Table 1**). The toxicity of this metal is associated with its attack against a membrane. Polyphenols are preferentially incorporated into membrane lipid bilayers and act as hydrogen donors, trapping free radicals and inhibiting the formation of lipid radicals (*5*). According to our results, both resveratrol and catechin reduced the level of lipid peroxidation caused by peroxide, carbon tetrachloride, and cadmium (**Table 1**).

While there has been a major focus on the antioxidant properties, there is an emerging view that polyphenols, and their in vivo metabolites, may affect signaling pathways that modulate cell response (*5*). According to the literature, the addition of polyphenols to commonly used cell culture media leads to the generation of substantial amounts of hydrogen peroxide (*32*). Such $H_2O_2$ generation could explain the increased tolerance to oxidative stress after adaptive treatments with polyphenols. Several studies show that treatment of yeast (and even human cells) with low concentrations of $H_2O_2$ induces adaptive responses, which protect cells from the lethal effects of a subsequent challenge with higher concentrations of oxidants (*25*). In silico data mining with Yeast Microarray Global Viewer (*33*) revealed that peroxide treatment preferentially activates genes involved with $H_2O_2$ degradation, such as *CTT1*.

According to our results, in the *ctt1* mutant strain, pretreatment with resveratrol or catechin did not increase tolerance to peroxide, nor did resveratrol induce tolerance to $CCl_4$. Catalase activity is very low in cells that are fermenting but increases linearly over a wide range of $H_2O_2$ concentrations, thereby maintaining a controlled intracellular peroxide concentration and avoiding oxidative damage to membranes, one of the main causes of several diseases and aging (*15*). Taken together, these results suggest that high levels of ROS could be reduced after resveratrol and catechin treatment, presumably by the activation of cellular defenses, like Ctt1. Therefore, we can conclude that if the same concentration of polyphenol is used and ignoring the differences in metabolism and permeability, both resveratrol and catechin achieved excellent protection against oxidative stress, which has been implicated in the etiology and progression of several acute and chronic disorders.

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