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Antioxidative Activities of Wine and Grape Polyphenols

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Lipid peroxidation of biological membranes damages the membrane structures and functions, resulting various cellular dysfunctions. Mitochondria are the most susceptible targets of the lipid peroxidation. The effects of wine and grape polyphenols on mitochondrial peroxidation were investigated. Anthocyanidins (cyanidin, melvidin and pelargonidin) were effective to prevent respiratory chain linked and non-enzymic lipid peroxidation in mitochondria. Flavan-3-ol (epicatechin) protected respiratory enzyme activities against NADPH-dependent peroxidation. Resveratorol was also effective in preventing mitochondrial peroxidation induced by dihydroxyfumarate. These protective activities of polyphenolic compounds in wines and grapes against mitochondrial injury would provide beneficial influence to human health.

Key words: wine, grape, polyphenol, antioxidative activity, mitochondria

Introduction

There has been an increasing interest in the contribution of free radical reactions participated in reactive oxygen species to the overall metabolic perturbations that result in tissue injury and disease. Reactive oxygen species are generated in specific organelles of cells under normal physiological conditions. The reduction of molecular oxygen to water proceeds by a series of single electron transfers, therefore, highly reactive intermediates such as superoxide anion, hydrogen peroxides and hydroxyl radical are generated in mitochondria[1)]. The defence mechanisms against these reactive oxygen species include radical scavenging enzymes and cellular antioxidants. A critical balance exists between the generation and detoxication of reactive oxygen species in cells. However, diseases, aging and chemical environments such as drugs, pesticides, herbicides and various of pollutants can disrupt this balance by inhibition of the cellular antioxidant defences and/or by stimulation of the formation of reactive oxygen species.

These reactive oxygen species can abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids. It is suggested lipid peroxidation may be a common pathogenic mechanism because it is considered a basic mechanism involved in reversible and irreversible cell and tissue damage[2)]. Lipid peroxidation of biolobical membranes damages the membrane structures and functions not only by degrading the highly unsaturated fatty acids but also forming breakdown products that can result in other types of

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membrane damages and disturbances elsewhere. Cellular damage due to lipid peroxidation causes serious derangements such as ischemia-reperfusion injury, coronary arteriosclerosis, diabetes mellitus and neurodegenerative diseases[3], and is associated with aging[4].

In living systems, dietary antioxidants such as a-tocopherol, ascorbic acid, carotenoids, flavonoids, and other phenolics may be effective in protection from oxidative damages There is substantial evidence that a diet rich in fruit and vegetables may reduce the risk of aging and oxidative stress associated with diseases[5]. Grapes and wines are known as polyphenol-rich foods[6].) Various types of phenolic compounds were found in commercial wines and grapes; benzoic acids such as gallic acid, syringic acid, gensitic acid, and *p*-hydroxybenzoic acid, phenylpropanoids such as cinnamic acid, caftaric acid, caffeic acid, *p*-coumaric acid and ferulic acid, flavonols such as rutin, quercetin and myricetin, anthocyanins such as cyanidin-3-glucoside, fravan-3-ols including catechin and epicatechin, procyanidin dimers B_1, B_3, B_4, B_6, B_8, and trimer C_3, stilbenes including reveratorol and viniferin[7-9].

This article deals with wine and grape polypnenols as antioxidants in mitochondrial peroxidation processes, because mitochondria are the most common sources of reactive oxygen species[10]. Polyphenols in grapes and wines mentioned here were presented in Figure 1.

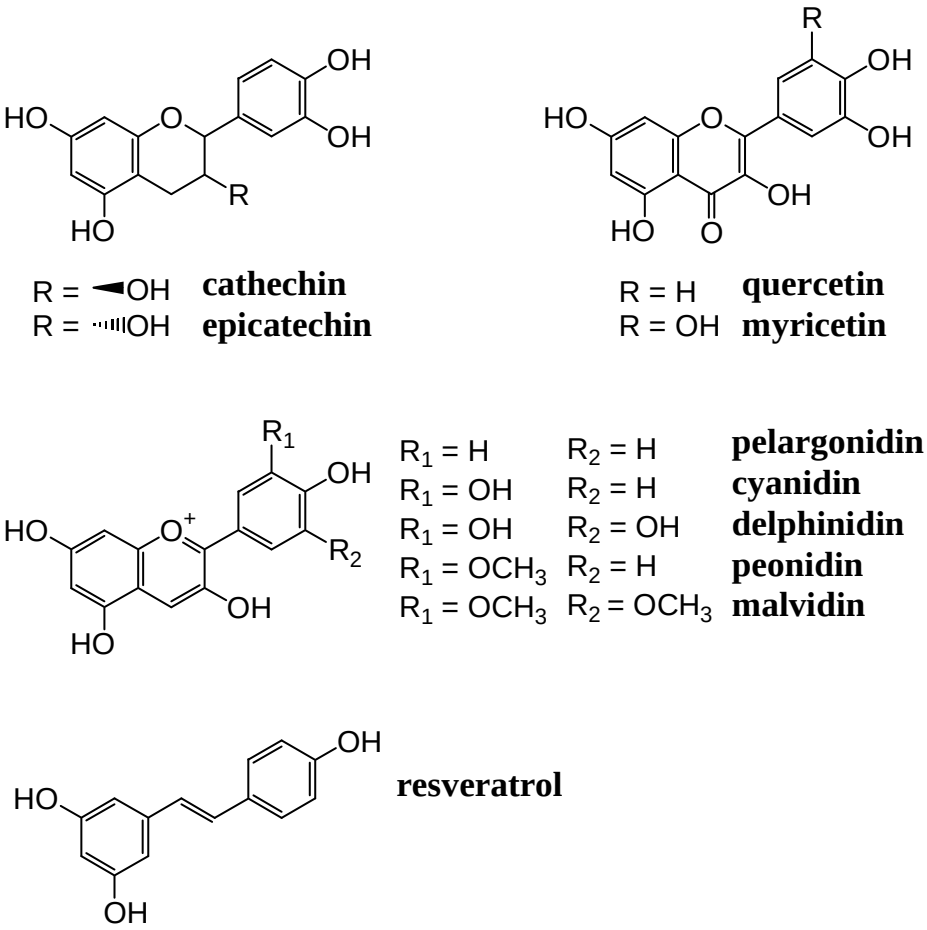

Figure 1. Phenolic Compounds in Wines and Grapes.

Materials and Method

Preparation of mitochondria. Livers of Wistar male rat weighing 100-150 g were removed quickly and dropped into ice-cold 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA. Mitochondria were obtained as a pellet after centrifugation at 15,000 x *g* and then resuspended in 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (pH 7.2). Submitochondrial particles were prepared by sonication of mitochondrial suspension for 1 min at 4°C[11].

Measurement of lipid peroxidation. Rat liver submitochondrial particles (equivalent 0.3 mg protein) were incubated at 37°C in 1 mL of reaction mixture containing 50 mM HEPES-NaOH (pH 7.0), 2 mM ADP, 0.1 mM $FeCl_3$, 10 μM rotenone and 0.1 mM NADH. The reaction was initiated by the addition of NADH. After 5 min, 2 mL of TCA-TBA-HCl reagent (15 % w/v trichloroacetic acid; 0.375 % thiobarbituric acid; 0.25 N HCl) and 90 μL of 2 % butylated hydroxytoluene (BHT) were added to the reaction mixture. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1,000 x *g* for 10 min. The absorbance of thiobarbituric acid (TBA) reactive substances in the supernatant was determined at 535 nm. Ascorbate-induced mitochondrial lipid peroxidation was measured in a solution consisted of 50 mM HEPES buffer (pH 7.4), 20 mM KCl, 10 μM $FeSO_4$, 0.2 mM ascorbate and mitochondrial suspension at 37°C for 20 min. The formation of TBA reactive substances was determined by the same method as described above [12].

Mitochondrial peroxidation and assay for enzyme activity. NADPH-dependent peroxidation of rat liver submitochondrial particles were acheived in a medium containing 0.1 M mannitol, 5 mM potassium phosphate (pH 7.4), 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM ADP and 0.3 mM $FeCl_3$ at 25°C. The reaction was started by the addition of 0.5 mM NADPH. At intervals during incubation, mitochondrial suspensions were taken out from the mixture and NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities were measured. The reductase activity was assayed by measuring the increase in the absorbance at 550 nm resulting from the reduction of cytochrome *c*. The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 5 mM NaN_3, 2.1 mg of oxidized cytochrome *c*, and 200 μM NADH or 20 mM sodium succinate in a total volume of 3 mL. [13]

Dihydroxyfumarate-induced mitochondrial peroxidation was carried out in a solution consisting of 50 mM phosphate buffer (pH 7.4), 0.1 mM $FeCl_3$, 1 mM ADP and 0.3 mM dihydroxyfumarate (DHF) at 30°C. At intervals during incubation, mitochondrial suspensions were taken out from the mixture and respiratory enzyme activities were measured as described above[14].

Results

Effect of anthocyanidins on mitochondrial lipid peroxidation. Redox reactions frequently occur in mitochondria, which are constantly susceptible to oxidative stress. Especially, inner membranes of mitochondria are at risk for lipid peroxidation, because mitochondria utilize oxygen at a high rate and inner membranes have a large content of polyunsaturated fatty acids, together with peroxidation catalysts such as iron and copper. It has been reported that NAD(P)H support enzymatically induced lipid peroxidation in submitochondrial particles in

the presence of an iron chelate[15].

The flesh peels of grapes and commercial red wines contain anthocyanins, which are hydrolyzed to generate anthocyanidin[16]. As shown in Table 1, anthocyanidins were effective to prevent NADH-dependent lipid peroxidation in mitochondria. Especially, cyanidin and melvidin completely inhibited mitochondrial lipid peroxidation at 30 μM. Lipid peroxidation linked with complex Ⅱ were also prevented by anthocyanidins. Succinate-dependent mitochondrial lipid peroxidation were completely inhibited by both pelargonidin and melvidin at 30 μM. Pelargonidin was a potent antioxidant against NADHP-dependent peroxidation, showed complete inhibition at 3 μM.

Ascorbate-induced nonenzymatic lipid peroxidation in mitochondria was also inhibited by all of compounds. Flavonols such as quercetin and myricetin showed antioxidative activities against microsomal lipid peroxidation as well as anthocyanidins (data not shown), however, had no effect against mitochondrial electron transport dependent peroxidation.

Table 1. Antioxidative Activities of Anthocyanidins in Mitochondria.

anthocyanidin	IC_{50} (μM)			
	NADH-dependent	succinate-dependent	NADPH-dependent	ascorbate-induced
pelargonidin	**16.8**	**14.5**	**4.3**	**65.9**
cyanidin	**14.7**	**67.2**	**14.8**	**47.0**
delphinidin	**86.2**	**71.7**	**71.8**	**15.7**
peonidin	**16.2**	**66.3**	**60.2**	**65.5**
malvidin	**15.8**	**16.8**	**65.8**	**62.7**

Effect of flavan-3-ols on mitochondrial functions. Various oxidative stresses affect the mitochondrial enzyme activities[17]. NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase are the most sensitive sites to mitochondrial peroxidative injury. NADPH-dependent lipid peroxidation in submitochondrial particles results in a remarkable loss of these enzyme activities. When rat liver mitochondria were incubated with Fe^{3+}-ADP/NADPH, membrane lipids were peroxidized and NADH- and succinate-cytochrome *c* reductase activities decreased; almost 80% loss of activities were observed for 90 and 30 min incubation, respectively.

Flavan-3-ols, catechin and epicatechin, are also physiological active compounds found in wines and grapes as well as anthocyanins[18]. As shown in Figure 2, epicatechin protected both enzyme activities against NADPH-dependent peroxidation. Catechin also exhibited almost same protective effect against mitochondrial peroxidation.

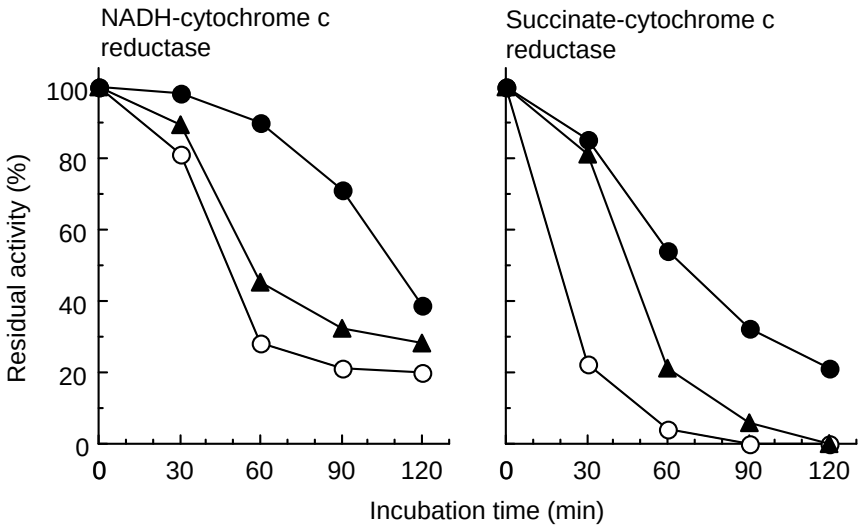

Fig. 2. Effects of Epicatechin on NADPH-dependent Oxidative Injury in Mitochondrial Respiratory Chain.

○, control; ●, 10,3 μM; ▲, 3,5 μM.

Effect of stilbene on mitochondrial functions. Mitochondrial respiratory chain generates superoxide anion and subsequently, hydrogen peroxide at the level of complex I and at the ubiquinone-cytochrome *b* segment. Lipid peroxides produced by hydroxy radical derived from hydrogen peroxide and superoxide anion affect mitochondrial function[19]. The autoxidation of dihydroxyfumarate (DHF) generates superoxide anion and hydrogen peroxide. Once formed, superoxide anion leads to the generation of hydroxy radical through non-enzymatic dismutation, which is catalyzed by Fe^{3+}-ADP. When mitochondrial suspensions were incubated with DHF, respiratory enzyme activities decreased. The addition of Fe^{3+}-ADP to the incubation mixture accelerated the loss of enzyme activities.

A stilbene, resveratorol, has various of physiological activities, and is rich in grape seeds and commercial wine[20]. As shown in Figure 3, resveratorol protected the enzyme activities of NADH- and succinate-cytochrome *c* reductase against dihydroxyfumarate-induced peroxidation. Resveratorol at 13.1 μM recovered the activity of NADH- cytochrome *c* reductase to non-peroxidized level.

Discussion

In living systems, various reactive oxygen species are generated and can cause cell damage. A major form of cellular oxidative damages is lipid peroxidation, which is initiated by reactive oxygen species through the extraction of a hydrogen atom from unsaturated fatty acids of membrane phospholipids. Membrane lipids are particularly susceptible to oxidation not only because of their high polyunsaturated fatty acid content but also because of their association in the cell membrane with enzymic and non-enzymic systems capable of generating free radical species. Mitochondria are the most susceptible targets of the lipid peroxidation, because of their high

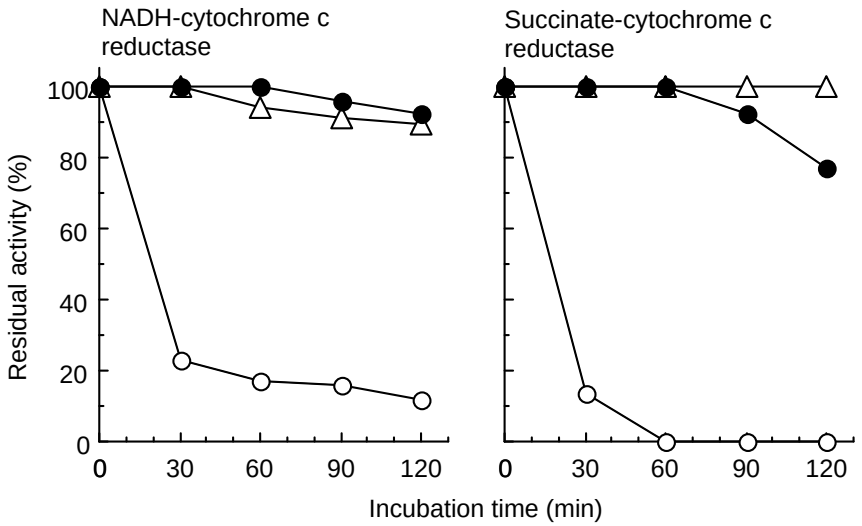

Fig. 3. Effects of Resveratorol on DHF-induced Oxidative Injury in Mitochondrial Respiratory Chain.

○, control (with DHF); ●, resveratorol at 13.1 μM; △, without DHF.

contents of polyunsaturated fatty acids and of the source of oxygen radicals by electron transport chain[10].

In this article, characteristic antioxidative polyphenols in wines and grapes were mentioned placing the focus on mitochondrial peroxidation. Grapes, wines and their polyphenols attract public attention by their potentially positive effect on human health[21]. Anthocyanidins, flavan-3-ols and stilbenes found in wines and grapes were effective not only in preventing membrane lipid peroxidation linked mitochondrial electron transport system, but also in protecting mitochondrial functions against oxidative injury. Mitochondrial damage due to lipid peroxidation causes various diseases[3] and associated with aging[4]. Epicatechin was reported to act as a positive regulator of mitochondrial structure/function endpoints and redox balance control systems in skeletal and cardiac muscles of dystrophic[22]. Certain anthocyanins can act as electron acceptors at complex I in mitochondria, and bypass ischemia-induced inhibition, resulting in increased ATP production after ischemia[23]. Resveratrol has protective effects against calcium-induced reduction of the respiratory rate in mitochondria[24]. Thus, wine and grape polyphenols affect various mitochondrial functions.

Beyond antioxidative activities in mitochondria and protective properties on mitochondrial function, anthocyanins[25], flavan-3-ols[26] and resveratrol[27] possess a variety of physiological effects. The polyphenolic compounds in wines and grapes would provide potentially beneficial influence to human health.

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ワイン・ブドウに含まれるポリフェノールの抗酸化活性

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生体膜脂質の過酸化は膜構造及び機能に障害を与え、細胞の機能低下を導く。ミトコンドリアは、特に活性酸素の生成が盛んであり、それに伴う内膜の過酸化のターゲットとなる。本稿では、ワイン及びブドウに含まれるポリフェノール化合物のラット肝ミトコンドリアにおける抗酸化作用を検討した。アントシアニジンはミトコンドリアの電子伝達系に依存した脂質過酸化及び非酵素的な脂質過酸化を抑制した。エピカテキンは NADPH 依存性の呼吸酵素の活性低下を抑制した。レスベラトロールも DHF により誘導される酸化障害からミトコンドリアの機能を保護した。これらの効果から、ワイン及びブドウのポリフェノールが我々の健康に寄与することが期待される。

キーワード：ワイン、ブドウ、ポリフェノール、抗酸化作用、ミトコンドリア