Proanthocyanidin promotes free radical-scavenging activity in muscle tissues and plasma

Kazumi Masuda, Tatsuya Hori, Kai Tanabe, Yutaka Kano, Aki Hirayama, and Sohji Nagase

Abstract: The present study was carried out to clarify the effect of oral administration of proanthocyanidin (PA) on radical-scavenging activity in muscle and plasma using electron spin resonance (ESR). Eight-week-old male Wistar rats were orally administered with 3 doses per day of 1 mL of 0.05% (PA0.05), 0.5% (PA0.5) or 5% (PA5) PA for 1 week. Control animals received the same volume of distilled water. We also examined the effect of a single dose of 0.5% PA. Blood and muscle were collected from rats 1 h after the final administration. Scavenging activity against superoxide anions in the plasma and m. soleus (Sol), m. plantaris (Pla), deep and surface areas of the m. gastrocnemius (GasD and GasS, respectively) and myocardium (Hrt) was determined using ESR with the spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide The scavenging activity in plasma for all groups given PA was 34%–44% higher than the control ($p < 0.05$). The scavenging activity in Hrt, Sol and GasD increased by up to 50% compared with the control and tended to increase depending on the dose of PA ($p < 0.05$). The impact of a single dose of PA was undetectable in all tissues. These results suggested that 1 week of oral PA improves the radical-scavenging activity in both plasma and muscle, especially in highly oxidative muscle. A single dose of PA was insufficient to improve the antioxidative capacity of muscle tissues.

Key words: antioxidative activity, electron spin resonance, myocardium, phytochemical, superoxide dismutase.

Résumé : Cette étude se propose d'élucider l'effet de l'administration per os de la proanthocyanidine (PA) sur l'activité antiradicalaire dans le muscle et le plasma au moyen de la résonance paramagnétique électronique (ESR). On administre durant 1 semaine 3 doses par jour de 1 mL de PA par voie buccale à des rats Wistar âgés de 8 semaines selon les concentrations suivantes : 0,05 % (PA0,05), 0,5 % (PA0,5) or 5 % (PA5). Les animaux témoins reçoivent un même volume d'eau distillée. Nous analysons aussi l'effet d'une seule dose de 0,5 % PA. On prélève des échantillons de sang et de tissu musculaire une heure après la dernière administration du PA. On analyse par ESR en présence d'un piège de spin, le 5,5-diméthyl-1-pyrroline-*N*-oxyde, l'activité antiradicalaire exercée contre les anions de superoxyde dans le plasma et dans les muscles soléaire (Sol) et plantaire (Pla), dans les parties profonde et superficielle du jumeau (GasD et GasS, respectivement) et dans le cœur. Dans tous les groupes de rats, l'activité antiradicalaire dans le plasma est de 34 %–44 % plus élevée que chez les rats témoins ($p < 0,05$). Comparativement au groupe témoin, l'activité antiradicalaire dans le cœur, le Sol et le GasD augmente jusqu'à 50 % et semble augmenter en fonction de la dose de PA ($p < 0,05$). L'effet d'une seule dose de PA est indétectable dans tous les tissus. D'après ces observations, l'administration de PA durant 1 semaine améliore l'activité antiradicalaire tant dans le plasma que dans le muscle, notamment dans le muscle à forte capacité oxydative. Une seule dose de PA ne semble pas suffisante pour améliorer la capacité antioxydative des tissus musculaires.

Mots-clés : capacité antioxydative, résonance paramagnétique électronique, myocarde, phytochimique, superoxyde dismutase.

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Introduction

Proanthocyanidin (PA) was discovered in 1947 as a polyphenol extracted from grape seeds (cf. Masquelier et al. 1979). Red wine is rich in PA and might explain the "French Paradox", which is the fact that morbidity rates from atherosclerosis and coronary heart disease are low in France despite a high intake of saturated fats (Renaud and Lorgeril 1992). Flavonoids derived from grape seeds have potent antioxidation properties (Ariga and Hamano 1990; Murray and Pizzorno 1999). These properties confer antiox-

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idant protection against low-density lipoprotein (LDL) in vitro (Bagchi et al. 1997; Bombardelli et al. 1997; Saint-Cricq De Gaulejac et al. 1999; Teissedre et al. 1996; Yamaguchi et al. 1999), which in turn reduces the incidence of coronary artery disease (Chatterjee et al. 1997; Pataki et al. 2002). Indeed, PA has been prescribed in Europe to treat vascular disorders such as arteriosclerosis, venous insufficiency, and varicose veins (Murray and Pizzorno 1999).

Aerobic energy production generates reactive oxygen species (ROS) in myocytes, and the amount of ROS increases 10- to 20-fold during physical exercise (Sjödin et al. 1990). However, free radical concentrations, lipid peroxidation, and mitochondrial damage are increased in tocopherol-deficient animals after exhaustive exercise (Davies et al. 1982). Thompson et al. (2001) also implied that prolonged ascorbic acid supplementation attenuates DOMS (delayed-onset muscle soreness) induced by intermittent exercise. Since PA has greater antioxidative capacity than either tocopherol or ascorbic acid (Yamaguchi et al. 1999), its beneficial effect might extend from the cardiovascular system to skeletal muscle functions if PA is absorbed into myocytes. However, little information is available about the mechanisms of absorption, distribution, and metabolism of digested PA, since PA is a high-molecular-weight polymer comprising monomeric units of flavan-3-ol ((+)-catechin and (–)-epicatechin) (Bravo 1998). Furthermore, whether myocytes absorb PA or whether PA affects antioxidant capacity in striated muscle remains unknown. We thus investigated whether oral PA increases antioxidative capacity in muscle tissues.

Electron spin resonance (ESR) and spin trapping can identify and quantify the ROS involved in a mixture (Green et al. 1979; Rosen and Klebanoff 1979), and its biological applications have been extended to investigations of muscle tissues (Masuda et al. 2003; Tanabe et al. 2006). The present study examined the effect of oral PA on radical-scavenging activity in various muscles and in plasma and estimated PA contents in muscle tissues.

Materials and methods

Animals

All procedures performed in the present study conformed to the *Fundamental guidelines for proper conduct of animal experiment and related activities in academic research institutions* (published by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Male Wistar rats (n = 21; Sankyo Lab Service Corporation, Japan), aged 8 weeks (body mass, 269 ± 13 g), were housed in a temperature-controlled room (22 ± 2 °C) with a 12 h light : 12 h dark photoperiod. A standard diet (Rat Chow, Oriental Yeast Co., Japan) and water were provided ad libitum. The rats were randomly assigned to 5 groups depending on the concentration of PA administered over 1 week: 0% (control, n = 4), 0.05% (PA0.05, n = 5), 0.5% (PA0.5, n = 4), 5% (PA5, n = 4), and a single dose of 0.5% (PA0.5acute, n = 4).

Oral administration of PA

Kikkoman Corporation (Tokyo, Japan) provided PA (Gravinol-SL), which was prepared as follows. Grape seeds were washed with water for 2 h and then extracted with water and ethanol under reflux for 2 h. The extract was condensed to remove solvents, then the concentrate was passed through cellulose powder and Celite® (Yamakoshi et al. 2002). Gravinol-SL contained 81% PA, which consisted of 6.6% dimers, 5.0% trimers, 2.9% tetramers, 74.8% oligomers, 6.6% monomeric flavanols (2.53% (+)-catechin, 2.17% (–)-epicatechin, 1.37% (–)-epigallocatechin, 0.5% (–)-epigallocatechin gallate), 2.24% moisture, 1.06% protein, and 0.8% ash (Iwasaki et al. 2004).

We dissolved Gravinol-SL in water to prepare 0.05%, 0.5%, and 5% PA before each feeding time. During a 1-week period, 1 mL of either PA or distilled water (control) was intragastrically administered to the rats by direct stomach intubation via the esophagus using a feeding tube (Terumo, Japan). Either PA at various concentrations or distilled water was administered once each in the morning, noon, and night and thus the animals received a total volume of 3 mL·d^{-1}. Rats in the PA0.5acute group were administered once with 0.5% PA 1 h before dissection. All rats fasted for 12–14 h before dissection.

Plasma and tissue preparation

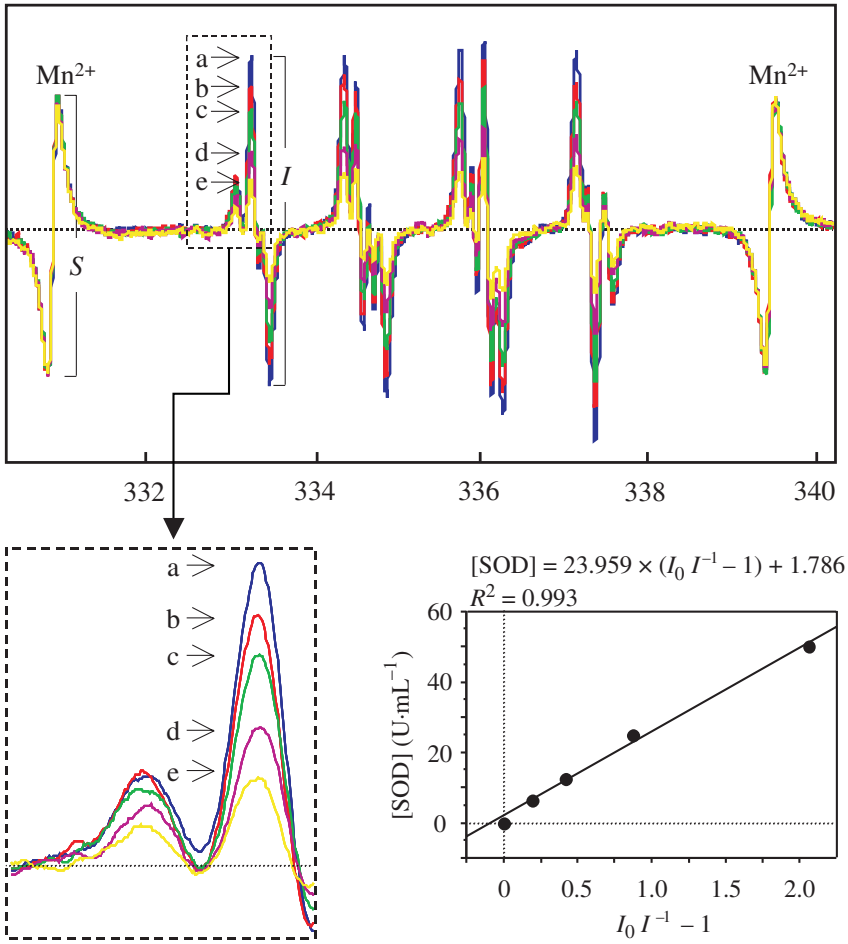
After the 1-week experimental period, blood and muscle samples were collected under pentobarbital sodium (70 mg·kg^{-1} i.p.) anesthesia. Blood was collected from the abdominal aorta using ethylene diamine tetraacetic acid (EDTA) syringes. Plasma separated from the blood by centrifugation at 1000g for 20 min at 4 °C was stored at –80 °C. The m. soleus (Sol), m. plantaris (Pla), deep and surface areas of the m. gastrocnemius (GasD and GasS, respectively), and heart (Hrt) were quickly isolated, freed from connective tissue, immediately weighed, frozen in liquid nitrogen, and then stored at –80 °C until the ESR analysis.

ESR protocol

Scavenging activity against superoxide ($O_2^{\cdot -}$) was measured as described (Masuda et al. 2003; Tanabe et al. 2006). Briefly, muscle specimens were homogenized with 1.15% KCl buffer. The homogenate was diluted in 0.2 mmol·L^{-1} phosphate buffer (pH 7.4). The inhibition rate of ESR signals in a mixture of muscle homogenate and a free radical generating system was derived from the xanthine oxidase – hypoxanthine reaction as a direct measurement of muscle scavenging activity against $O_2^{\cdot -}$. The ESR (JES-TE25X, JEOL, Japan) settings were as follows: frequency, 9.419 GHz; power, 4.00 mW; field, 334.0 ± 5 mT; sweep time, 1.0 min; modulation, 0.079 mT; time constant, 0.1 s.

To determine scavenging activity against $O_2^{\cdot -}$, the reaction mixture included 50 μL of plasma and homogenate containing 2.5%–5% muscle, 5.5 mmol·L^{-1} diethylenetriamine pentaacetic acid (DETAPAC; an iron chelator), 2 mmol·L^{-1} hypoxanthine (6-hydroxypurine), 0.4 U·mL^{-1} xanthine oxidase, and 15 μL of 9.2 mol·L^{-1} 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trap agent. The ESR spectra were recorded 45 s after adding hypoxanthine at room temperature (23 °C). The blank spectrum was the control, and a standard curve of superoxide dismutase (SOD) activity was constructed based on the spectra generated using 6.25, 12.5, 25, and 50 U·mL^{-1} SOD (Fig. 1). Signal intensity (I/S) is expressed as the ratio of the peak located at the lowest magnetic field of the 4-line DMPO-OOH signal (I) to the signal

Fig. 1. Electron spin resonance (ESR) spectra of reaction mixture containing superoxide dismutase (SOD) at various concentrations. Spectrum intensity for the superoxide–DMPO adduct decreased as SOD concentrations increased. SOD concentrations (U·mL^{-1}) were as follows: *a*, 0; *b*, 6.25; *c*, 12.5; *d*, 25; *e*, 50. *S*, signal intensity of the Mn^{2+} (internal reference); *I*, signal intensity of DMPO-OOH. The scavenging activity against $O_2^{\cdot -}$ is calculated based on the *I*/*S* ratio.



intensity of internal standard Mn^{2+} (*S*) (Fig. 1). The I_0 corresponds to the DMPO-OOH signal intensity when the [SOD] = 0. Scavenging activity against $O_2^{\cdot -}$ was calculated as SOD activity based on the standard curve (Noda et al. 1997; Fig. 1):

$$[1] \qquad [\mathrm{SOD}] = k(I_0 \times I^{-1} - 1) + a$$

To reference the scavenging activity of the samples to that of the standard PA solution, the *I*/*S* ratios in the ESR were also determined using 0.001%, 0.005%, 0.025%, 0.05%, 0.125%, and 0.25% PA to generate a standard curve of [PA] versus the *I*/*S* ratios (Fig. 2):

$$[2] \qquad [\mathrm{PA}] = k\prime(I_0 \times I^{-1} - 1) + b$$

A simple equation showing the relationship between [PA] and [SOD] was calculated by solving eq. 1 and eq. 2 simultaneously. Based on this simple equation, the PA contents in the muscle and plasma samples were estimated as an expression of SOD-scavenging activity against $O_2^{\cdot -}$.

Statistical analysis

All data are expressed as means ± SD. Variables among groups were compared using the one-way analysis of variance (ANOVA). Tukey–Kramer's post-hoc test was conducted if the ANOVA indicated a significant difference. The level of significance was set at $p < 0.05$.

Results

Morphological analysis

Table 1 summarizes the body mass and mean wet tissue masses. Body mass did not significantly differ among groups. When body mass among the groups was analyzed excluding PA0.5acute, the PA5 rats gained 3% body mass, which was significantly lower than that of the other experimental groups ($p < 0.01$ vs. control and PA0.05). Muscle (especially Sol) tended to weigh less in the PA5 than in the control and PA0.5acute groups ($p < 0.01$ vs. control). No significant differences were found in other muscles among the groups.

$O_2^{\cdot -}$-scavenging activity in plasma and muscle tissue

The $O_2^{\cdot -}$-scavenging activity in the Hrt was significantly higher among the PA0.05, PA0.5, and PA5 groups than either control or PA0.5acute ($p < 0.01$ ~0.05, Fig. 3*e*). The

Fig. 2. Electron spin resonance (ESR) spectra of reaction mixture containing proanthocyanidin (PA) at various concentrations. Spectrum intensity for the PA–DMPO adduct decreased as PA concentrations increased. PA concentrations were as follows: *a*, 0.001%; *b*, 0.005%; *c*, 0.025%; *d*, 0.05%; *e*, 0.125%; *f*, 0.25%. *S*, signal intensity of the Mn^{2+} (internal reference); *I*, signal intensity of DMPO-OOH. The scavenging activity against $O_2^{\cdot -}$ was calculated based on the *I*/*S* ratio.

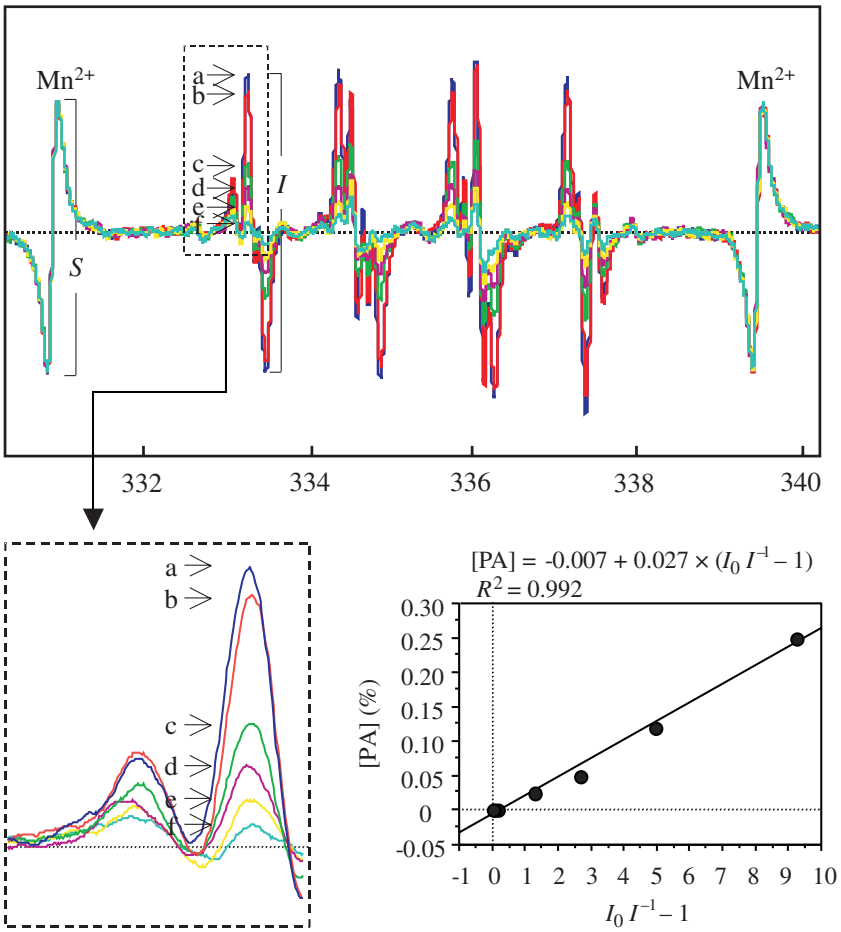


Table 1. Summary of body mass and wet tissue mass of experimental rats.

		Body mass (g)			Wet tissue mass (mg)				
	n	Pre	Post	Diff (%)	Hrt	Sol	Gas	Pla	Kidney
Control	4	243.3±10.3	267.3±12.3	+9.9±0.5	621.2±36.4	95.8±3.0	1260.0±20.1	262.1±10.1	966.7±69.7
PA0.05	5	246.8±6.1	273.0±4.5	+10.6±1.3	611.0±16.6	92.3±4.2	1320.3±62.7	279.7±19.5	884.4±39.5
PA0.5	4	265.0±12.3	282.3±2.1	+6.5±4.2	605.4±19.2	89.9±3.0	1355.1±43.4	290.0±15.1	909.2±67.4
PA5	4	258.3±10.3	268.3±11.9	+3.9±0.9[ab]	613.6±31.7	86.7±1.0[a]	1286.0±42.9	269.8±10.6	918.3±30.8
PA0.5acute	4	246.3±6.9	251.7±6.2	+2.2±0.6	594.5±30.3	97.1±4.8	1155.3±43.5	240.9±6.6	970.9±92.4

Note: Values are means ± SD. Pre, before experiment; post, after experiment; diff, % difference; PA, proanthocyanidin; Hrt, heart; Sol, soleus; Gas, gastrocnemius; Pla, plantaris.

[a] $p < 0.01$ vs. control.
[b] $p < 0.01$ vs. PA0.05 (statistical comparison excluded PA0.5acute).

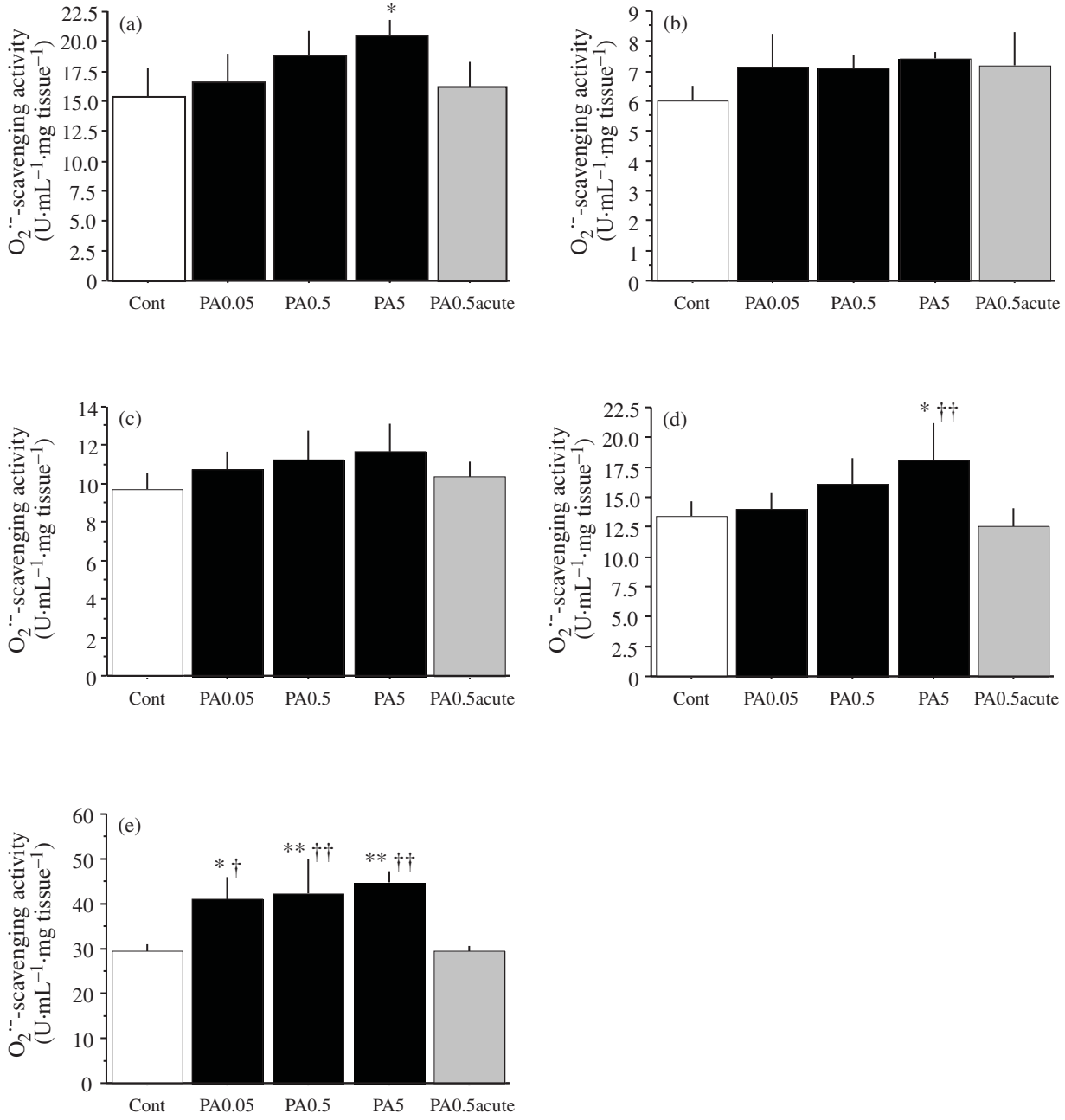
increase ranged from 39.1%–52.7% depending on the amount of PA administered. The $O_2^{\cdot -}$-scavenging activity in Sol also tended to increase according to the amount of PA (range, 4.5%–35.4%, and was significantly higher in the PA5 group than in the control and PA0.5acute groups ($p < 0.01$ to 0.05, Fig. 3*d*). The scavenging activity in GasD from the PA5 group was also about 33% higher than that from the control ($p < 0.05$, Fig. 3*a*). The $O_2^{\cdot -}$-scavenging activities of GasS and Pla were not significantly affected by PA (Figs. 3*b*, 3*c*). However, the scavenging activity of the plasma was significantly higher in all experimental groups compared with the control ($p < 0.05$, Fig. 4). The relative difference ranged from 34.2%–44.0%.

In addition, a single dose of 0.5% PA (PA0.5acute) affected the scavenging activity of the plasma ($p < 0.01$ vs. control; Fig. 4), but not in any muscle tissue (Fig. 3).

Estimated PA content in plasma and muscle tissues

The present study demonstrated to estimate the PA content in samples as relative scavenging activity of SOD

Fig. 3. Proanthocyanidin administration and $O_2^{\cdot -}$-scavenging activity of muscle tissues. Values are means ± SD. PA, proanthocyanidin; acute, single dose. (*a*) Gastrocnemius deep portion (GasD), (*b*) gastrocnemius surface portion (GasS), (*c*) plantaris (Pla), (*d*) soleus (Sol), (*e*) heart (Hrt). Asterisks indicate significant differences vs. control (*, $p < 0.05$; **, $p < 0.01$) and daggers indicate vs. PA0.5acute (†, $p < 0.05$; ††, $p < 0.01$).



against $O_2^{\cdot -}$ using a standard curve of PA concentration against the *I/S* ratios of the ESR (Table 2). The estimated PA contents in the Hrt samples from the PA0.5 and PA5 groups were 0.014%–0.019%, which was significantly higher than those of the control and the PA0.5acute ($p < 0.05$; Table 2) groups. The Sol and GasD in the PA5 contained 0.01% and 0.006% PA, respectively, which were also significantly higher than those of control and (or) PA0.5acute ($p < 0.05$). The estimated plasma PA contents in the PA0.05, PA0.5, PA5, and PA0.5acute groups were 0.025%–0.033% and all were significantly higher than that of the control ($p < 0.05$).

Discussion

Physiological significance of the oral PA administration for muscle tissue

The present study examined the effect of acute and chronic (1 week) PA administration on the radical-scavenging activity of various muscle tissues and plasma. The main findings of the present study were that oral PA promoted radical-scavenging activity in cardiac and skeletal muscle tissues (especially red portions), as well as in plasma, and that the acute administration of PA affected radical-scavenging activity in plasma, but not in muscle tissues.

Although the mechanism through which myocytes absorb oral PA is unknown, the evidence presented here showed that PA promotes the antioxidative capacity of the myocardium and in specific skeletal muscles, suggesting that PA is distributed in the myocytes. These unique findings should be of interest to biochemists and nutritional researchers.

Effect of the chronic administration of PA

The present study found that the 1 week of oral PA administration increased the radical-scavenging activity in myocardium, skeletal muscles, and plasma (Figs. 3 and 4). These effects of PA were evident in muscles with highly oxidative metabolic properties, such as the Hrt, Sol, and GasD, suggesting that PA plays a key role in reducing oxidative stress derived from muscle oxidative metabolism during muscle activity (Sjödin et al. 1990). Also, the $O_2^{\cdot -}$-scavenging activity of Hrt was promoted even at a concentration of PA below that at which the activities of Sol and GasD were not affected (Fig. 3*e*), suggesting that PA has more beneficial impact on myocardium to reduce the incidence of coronary artery disease (Chatterjee et al. 1997; Pataki et al. 2002).

That PA affected specific muscle tissues was of particular interest (Fig. 3), and could be related to blood flow within the muscle tissue. The myocardium and slow-twitch oxidative muscles are rich in capillaries (Armstrong and Phelps 1984), and such slow-twitch oxidative muscles have greater intramuscular blood flow even under resting conditions, as well as during work (Laughlin and Armstrong 1982). Proanthocyanidin is a high-molecular-weight polymer consisting of monomeric flavan-3-ol ((+)-catechin and (–)-epicatechin) units and is both water and oil soluble (Ariga et al. 1988). These properties imply that through digestion and absorption into the blood circulation, PA could be transported to and gradually accumulate in muscle and endothelium membranes or diffuse into myocytes located in muscles such as Hrt, Sol, and GasD, which are sites of increased intramuscular blood flow.

Another possible mechanism involved in the promotion of radical-scavenging activity in specific muscle tissues could be a synergistic action of PA with other antioxidants such as ascorbic acid, *dl*-α-tocopherol, β-carotene, and SOD (Yamaguchi et al. 1999). Mitochondria-rich myocytes usually contain more SOD and glutathione peroxidase (GPX), and consequently have increased potential for $O_2^{\cdot -}$-scavenging ability (Ji 1993, 1994; Ji et al. 1992; Masuda et al. 2003). Our results showed that the $O_2^{\cdot -}$-scavenging activity of Hrt was almost double that of Sol or GasD under control conditions (Fig. 3). Again, the $O_2^{\cdot -}$-scavenging activity of Hrt was promoted even at a concentration of PA below that at which the activities of Sol and GasD were not affected (Fig. 3*e*). Thus, the impact of PA could be greater in tissues where the SOD content is intrinsically higher, as PA would act as an antioxidant per se, and also have a synergistic effect with other intrinsic antioxidants in myocytes.

The fractional amount of PA absorbed into muscle tissue could be quite low. The estimated level of urinary PA excretion in humans is only 0.1% of the ingested dose (Matsumoto et al. 2001). Indeed, the estimated [PA] in the present study corresponded to 0.006%–0.03% in muscle tissues and plasma where the radical-scavenging activity was

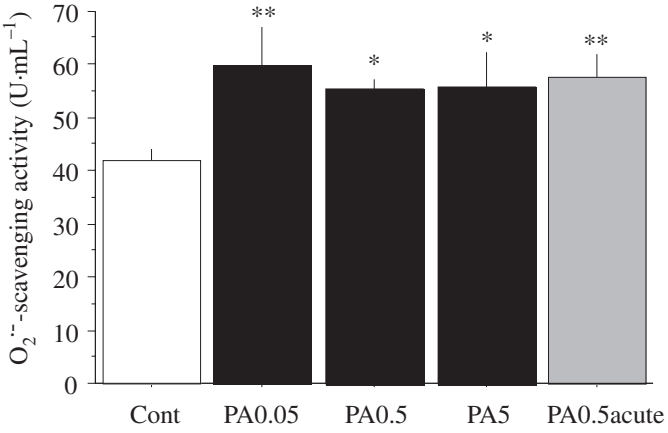


Fig. 4. Proanthocyanidin administration and $O_2^{\cdot -}$-scavenging activity of plasma. Values are means ± SD. $O_2^{\cdot -}$-scavenging activity significantly elevated in PA0.05, PA0.5, PA5, and PA0.5acute compared with control (*, $p < 0.05$; **, $p < 0.01$). PA, proanthocyanidin; acute, single dose.

increased (Table 2). Therefore, we speculated that a high daily intake of PA might be unnecessary. Red wine reportedly contains 1644 ± 260 mg·L^{-1} of PA (Sánchez-Moreno et al. 2003). If the present PA content was considered in terms of red wine consumption, then a 60 kg adult would have to consume approximately 0.2, 2.0, and 20 L·d^{-1} to approximate the PA0.05 (5.8 ± 0.1 mg·kg^{-1}·d^{-1}), PA0.5 (55.1 ± 1.7 mg·kg^{-1}·d^{-1}), and PA5 (569.9 ± 27.4 mg·kg^{-1}·d^{-1}) doses, respectively, required to promote tissue antioxidant activity.

Effect of acute treatment of PA

The acute administration of PA affected radical-scavenging activity in plasma, but not in muscle tissues. This finding indicated and emphasized that chronic PA administration would be necessary for absorption into Hrt, Sol, and GasD muscles and for improving radical-scavenging activities in these tissues. The effects of acute and chronic PA administration on $O_2^{\cdot -}$-scavenging activity clearly differed between plasma and muscle tissues (Figs. 3 and 4). The single dose of PA immediately and effectively altered radical-scavenging activity in the blood circulation (Fig. 4), supporting previous suggestions that oral PA positively impacts cardiovascular diseases (Koga et al. 1999; da Silva et al. 1998).

On the other hand, the notion that the present results were due to technical inconsistencies or artifacts was refuted. Isolated muscles such as Hrt, Sol, and GasD might have included blood in capillaries where PA could have lingered during sample preparation. If blood containing PA remained in muscle tissues, then radical-scavenging activity should have increased in Hrt, Sol, and GasD obtained from the PA0.5acute group. The present results, however, rebuffed artifactual and technical concerns.

Finally, an adult human weighing 60 kg would need to consume about 750 mL of red wine to ingest an amount of PA equivalent to that of the acute group in the present study (single dose: 20 mg·kg^{-1}) (Sánchez-Moreno et al. 2003) and promote radical-scavenging activity in the plasma.

Safety profile of the PA administration

The risk of chronic PA administration could be negligible

Table 2. Estimated amount of proanthocyanidin in muscle tissues and plasma.

	n	Hrt (%·mg^{-1})	Sol (%·mg^{-1})	GasD (%·mg^{-1})	GasS (%·mg^{-1})	Pla (%·mg^{-1})	Plasma (%)
Control	4	0.0000±0.0021	0.0000±0.0028	0.0000±0.0030	0.0000±0.0006	0.0000±0.0015	0.0000±0.0042
PA0.05	5	0.0144±0.0063[ab]	0.0012±0.0029	0.0013±0.0030	0.0013±0.0013	0.0017±0.0016	0.0333±0.0135[c]
PA0.5	4	0.0162±0.099[cd]	0.0056±0.0043	0.0039±0.0026	0.0012±0.0005	0.0026±0.0025	0.0249±0.0033[a]
PA5	4	0.0194±0.0030[cd]	0.0096±0.0064[ad]	0.0058±0.0018[a]	0.0016±0.0003	0.0034±0.0024	0.0255±0.0125[a]
PA0.5acute	4	0.0002±0.0013	–0.0017±0.0032	0.0009±0.0026	0.0013±0.0013	0.0011±0.0013	0.0291±0.0083[c]

Note: Values are means ± SD. PA, proanthocyanidin; Hrt, heart; Sol, soleus; GasD, gastrocnemius deep portion; GasS, gastrocnemius surface portion; Pla, plantaris; acute, single PA dose. The PA values were divided by the wet weight of the tissue.

[a]Significant differences vs. control $p < 0.05$.

[b]Significant differences vs. PA0.5acute $p < 0.05$.

[c]Significant differences vs. control $p < 0.01$.

[d]Significant differences vs. PA0.5acute $p < 0.01$.

for mammals at the levels demonstrated in the present study. A previous study found no evidence of either acute oral toxicity or mutagenicity in rats administered with 2 and 4 g·kg^{-1} doses of PA (Yamakoshi et al. 1999). These doses were considerably higher than those delivered in the present study (PA0.5acute group consumed an average of about 19.8 mg·kg^{-1}). Therefore, the risk of acute PA toxicity was considered negligible in the present study.

The rats were administered with 3 doses of PA at various concentrations for 7 d, so the cumulative doses were 10.5, 105, and 1050 mg (PA0.05, PA0.5, and PA5, respectively) and the total daily amounts of consumed PA were 5.8 ± 0.1, 55.1 ± 1.7, and 569.9 ± 27.4 mg·kg^{-1}·d^{-1}, respectively. Based on a study that administered dietary PA to rats at ratios of 0.02%, 0.2%, and 2% *w*/*w* for 90 days (food and water were ad lib, Yamakoshi et al. 1999), the average daily PA consumption was 13.3 ± 0.4, 129.1 ± 3.5, and 1409.8 ± 49.8 mg·kg^{-1}·d^{-1} in the 0.02%, 0.2%, and 2% group, respectively. That study found no toxicity or abnormal hematological values, leukocyte differentials, clinical chemistry values, or urinalysis values, even though body mass slightly decreased in the group given the highest dose (Yamakoshi et al. 1999). We administered the rats with about one-third of the amount of PA given by Yamakoshi et al. (1999) and found no differences in kidney weight. These results indicated that the chronic administration of 0.05%–5% PA conferred little risk to the rats.

However, the body mass of the PA5 group was lower than that of other groups (Table 1). This might be due to PA interfering with appetite, but that conclusion remains speculative; together with risk assessment of PA from physiological and pathological aspects, this aspect requires further study.

Conclusion

The present study shows that oral PA administration for 1 week increased free radical-scavenging activity in plasma, as well as in cardiac and skeletal muscle tissues. Furthermore, the effect of oral PA was greater on muscle tissues with more oxidative than non-oxidative metabolic properties, suggesting that PA plays a key role in reducing oxidative stress derived from muscle metabolism during activity. Further study is required to define the mechanisms of PA absorption into muscle cells and the specific localization of PA within muscle tissues.

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References

Ariga, T., and Hamano, M. 1990. Radical scavenging action and its mode in procyanidins B-1 and B-3 from azuki beans to peroxyl radicals. Agric. Biol. Chem. **54**: 2499–2504.

Ariga, T., Koshiyama, I., and Fukushima, D. 1988. Antioxidative properties of procyanidins B-1 and B-3 from azuki beans in aqueous systems. Agric. Biol. Chem. **52**: 2717–2722.

Armstrong, R.B., and Phelps, R.O. 1984. Muscle fiber type composition of the rat hindlimb. Am. J. Anat. **171**: 259–272. doi:10.1002/aja.1001710303. PMID:6517030.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Tran, M.X., and Stohs, S.J. 1997. Oxygen free radical scavenging abilities of vitamins C and E, and a grape seed proanthocyanidin extract in vitro. Res. Commun. Mol. Pathol. Pharmacol. **95**: 179–189. PMID:9090754.

Bombardelli, E., Morazzoni, P., and Carini, M. 1997. Biological activity of procyanidins from Vitis vinifera L. Biofactors, **6**: 429–431.

Bravo, L. 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. **56**: 317–333. PMID:9838798.

Chatterjee, S.S., Koch, E., Jaggy, H., and Krzeminski, T. 1997. In vitro and in vivo studies on the cardioprotective action of oligomeric procyanidins in a crataegus extract of leaves and blooms. Arzneimittelforschung, **47**: 821–825. PMID:9324931.

da Silva, D.E.L., Piskula, M.K., Yamamoto, N., Moon, J.H., and Terao, J. 1998. Quercetin metabolites inhibit copper ion induced lipid peroxidation in rat plasma. FEBS Lett. **430**: 405–408. doi:10.1016/S0014-5793(98)00709-1. PMID:9688581.

Davies, K.J., Quintanilha, A.T., Brooks, G.A., and Packer, L. 1982. Free radicals and tissue damage produced by exercise. Biochem. Biophys. Res. Commun. **107**: 1198–1205. doi:10.1016/S0006-291X(82)80124-1. PMID:6291524.

Green, M.R., Hill, H.A., Okolow-Zubkowska, M.J., and Segal, A.W. 1979. The production of hydroxyl and superoxide radicals by stimulated human neutrophils-measurements by EPR spectroscopy. FEBS Lett. **100**: 23–26. doi:10.1016/0014-5793(79)81123-0. PMID:220087.

Iwasaki, Y., Matsui, T., and Arakawa, Y. 2004. The protective and hormonal effects of proanthocyanidin against gastric mucosal in-

jury in Wistar rats. J. Gastroenterol. **39**: 831–837. doi:10.1007/s00535-004-1399-5. PMID:15565401.

Ji, L.L. 1993. Antioxidant enzyme response to exercise and aging. Med. Sci. Sports Exerc. **25**: 225–231. PMID:8450725.

Ji, L.L. 1994. Exercise-induced oxidative stress in heart. *In* Exercise and oxygen toxicity. *Edited by* C.K. Sen and L. Packer. Elsevier, Amsterdam, the Netherlands. pp. 249–267.

Ji, L.L., Fu, R., and Mitchell, E.W. 1992. Glutathione and antioxidant enzymes in skeletal muscle: effect of fiber type and exercise intensity. J. Appl. Physiol. **73**: 1854–1859. PMID:1474061.

Koga, T., Moro, K., Nakamori, K., Yamakoshi, J., Hosoyama, H., Kataoka, S., and Ariga, T. 1999. Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. J. Agric. Food Chem. **47**: 1892–1897. doi:10.1021/jf9810517. PMID:10552467.

Laughlin, M.H., and Armstrong, R.B. 1982. Muscular blood flow distribution patterns as a function of running speed in rats. Am. J. Physiol. Heart Circ. Physiol. **243**: 296–306. PMID:7114239.

Masquelier, J., Michaud, J., Laparra, J., and Dumon, M.C. 1979. Flavonoids and pycnogenols. Int. J. Vitam. Nutr. Res. **49**: 307–311. PMID:528157.

Masuda, K., Tanabe, K., Kuno, S., Hirayama, A., and Nagase, S. 2003. Antioxidant capacity in rat skeletal muscle tissues determined by electron spin resonance. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **134**: 215–220. PMID:12568799.

Matsumoto, H., Inaba, H., Kishi, M., Tominaga, S., Hirayama, M., and Tsuda, T. 2001. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J. Agric. Food Chem. **49**: 1546–1551. doi:10.1021/jf001246q. PMID:11312894.

Murray, M., and Pizzorno, J. 1999. Procyanidolic oligomers. *In* The textbook of natural medicine. *Edited by* M. Murray and J. Pizzorno. Churchill Livingstone, London, UK. pp. 899–902.

Noda, Y., Anzai, K., Mori, A., Kohno, M., Shinmei, M., and Packer, L. 1997. Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. Biochem. Mol. Biol. Int. **42**: 35–44. PMID:9192083.

Pataki, T., Bak, I., Kovacs, P., Bagchi, D., Das, D.K., and Tosaki, A. 2002. Grape seed proanthocyanidins improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. Am. J. Clin. Nutr. **75**: 894–899. PMID:11976164.

Renaud, S., and Lorgeril, D.M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet, **339**: 1523–1526. doi:10.1016/0140-6736(92)91277-F. PMID:1351198.

Rosen, H., and Klebanoff, S.J. 1979. Hydroxyl radical generation by polymorphonuclear leukocytes measured by electron spin resonance spectroscopy. J. Clin. Invest. **64**: 1725–1729. PMID:227939.

Saint-Cricq De Gaulejac, N., Provost, C., and Vivas, N. 1999. Comparative study of polyphenol scavenging activities assessed by different methods. J. Agric. Food Chem. **47**: 425–431. doi:10.1021/jf980700b. PMID:10563911.

Sánchez-Moreno, C., Cao, G., Ou, B., and Prior, R.L. 2003. Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with nontraditional wines obtained from highbush blueberry. J. Agric. Food Chem. **51**: 4889–4896. doi:10.1021/jf030081t. PMID:12903941.

Sjödin, B., Westing, Y.H., and Apple, F.S. 1990. Biochemical mechanisms for oxygen free radical formation during exercise. Sports Med. **10**: 236–254. PMID:2247725.

Tanabe, K., Masuda, K., Hirayama, A., Nagase, S., Kono, I., and Kuno, S. 2006. Effect of spontaneous exercise on antioxidant capacity in rat muscles determined by electron spin resonance. Acta Physiol. **186**: 119–125. doi:10.1111/j.1748-1716.2005.01515.x. PMID:16497189.

Teissedre, P.L., Frankel, E.N., Waterhouse, A.L., Peleg, H., and German, J.B. 1996. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. J. Sci. Food Agric. **70**: 55–61. doi:10.1002/(SICI)1097-0010(199601)70:1<55::AID-JSFA471>3.0.CO;2-X.

Thompson, D., Williams, C., McGregor, S.J., Nicholas, C.W., McArdle, F., Jackson, M.J., and Powell, J.R. 2001. Prolonged vitamin C supplementation and recovery from demanding exercise. Int. J. Sport Nutr. Exerc. Metab. **11**: 466–481. PMID:11915781.

Yamaguchi, F., Yoshimura, Y., Ariga, T., and Nakazawa, H. 1999. Free radical scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H_2O_2/NaOH/DMSO system. J. Agric. Food Chem. **47**: 2544–2548. doi:10.1021/jf9806762. PMID:10552524.

Yamakoshi, J., Kataoka, S., Koga, T., and Ariga, T. 1999. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. Atherosclerosis, **142**: 139–149. doi:10.1016/S0021-9150(98)00230-5. PMID:9920515.

Yamakoshi, J., Saito, M., Kataoka, S., and Kikuchi, M. 2002. Safety evaluation of proanthocyanidin-rich extract from grape seeds. Food Chem. Toxicol. **40**: 599–607. PMID:11955665.