Biosci. Biotechnol. Biochem., **75** (12), 2394–2397, 2011

Note

The Level of Orally Ingested Vitamin C Affected the Expression of Vitamin C Transporters and Vitamin C Accumulation in the Livers of ODS Rats

Yasuko SONE,[1] Etsuko UETA,[2] Satoru KODAMA,[3] Yasuko SANNOUMARU,[4] Noriko MIYAKE,[5] Hirohito SONE,[3] Yoko FUJIWARA,[6] Yuzuru OTSUKA,[6] Kazuo KONDO,[6] Masahiro INAGAKI,[7] Eiji NAMBA,[2] Tadao KURATA,[8] and Emiko SUZUKI[6,†]

[1]*Institute of Environmental Science for Human Life, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[2]*Tottori University Faculty of Medicine, 86 Nishimachi, Yonago, Tottori 683-8503, Japan*
[3]*Institute of Clinical Medicine, Department of Internal Medicine, University of Tsukuba, 3-2-7 Miyamachi, Mito, Ibaraki 310-0015, Japan*
[4]*Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan*
[5]*Department of Modern Home Economics, Tokyo Kasei Gakuin University, 22 Sanban-cho, Chiyoda-ku, Tokyo 102-8341, Japan*
[6]*Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[7]*Faculty of Arts and Sciences at Fujiyoshida, Showa University, 4562 Kamiyoshida, Fujiyoshida, Yamanashi 403-0005, Japan*
[8]*Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi, Kanagawa 243-0292, Japan*

Received April 19, 2011; Accepted August 31, 2011; Online Publication, December 7, 2011
[doi:10.1271/bbb.110312]

We investigated the effects of vitamin C administration on vitamin C-specific transporters in ODS/ShiJcl-*od/od* rat livers. The vitamin C-specific transporter levels increased in the livers of the rats not administered vitamin C and decreased in the livers of those administered vitamin C at 100 mg/d, indicating that these transporter levels can be influenced by the amount of vitamin C administered.

Key words: 2-*O*-α-D-glucopyranosyl-L-ascorbic acid; L-ascorbic acid; ODS/ShiJcl-*od*/*od* (ODS) rat; sodium-dependent vitamin C transporter

L-Ascorbic acid (AsA) accumulates at high concentrations (mmol/kg) in human tissues,[1)] suggesting that it is important for the maintenance of tissue function. In several tissues, AsA transport is controlled by specific mechanisms to maintain AsA levels. These mechanisms include active transport and facilitated diffusion, which are mediated by distinct classes of membrane proteins, including sodium-dependent vitamin C transporters (SVCT) and facilitative glucose transporters (GLUT).[2,3)] SVCT has strong affinity for AsA and two isoforms encoded by solute carrier family 23 member 1 (SVCT1, NM_005847.4 and NM_152685.3 for the gene, NP_005838.3 and NP_689898.2 for the protein) and solute carrier family 23 member 2 (SVCT2, NM_005116.5 and NM_203327.1 for the gene, NP_005107.4 and NP_976072.1 for the protein).[2)] SVCT1 is predominantly expressed in epithelial cells, including those of the liver, intestine, and kidney, and can transport AsA in quantities exceeding the internal requirement of these cells.[2)] SVCT2 is localized to metabolically active specialized cells such as those of the brain, eye, and placenta,[2,4)] where it has been implicated in the maintenance of intracellular AsA levels that are vital to neuronal function and protection against oxidative stress.[5)] Effective serum AsA concentrations are controlled by low levels,[6)] and AsA supplementation or deprivation influences AsA transport in osteoblasts[7)] and astrocytes.[8)] Savini *et al.* found substrate-mediated translational control for SVCT2 in platelets,[9)] and MacDonald *et al.* found that AsA uptake and SVCT1 expression decreased significantly in Caco-2 TC7 cells exposed to AsA.[10)] Karaczyn *et al.* found that loss of intracellular AsA was compensated for by active AsA uptake resulting from a marked increase of SCVT2 expression in human lung epithelial cells.[11)] Another study reported that AsA depletion enhanced the expression of SVCT1 and SVCT2 and the uptake of AsA in the livers of SMP30/GNL knockout mice.[12)] SVCT2 mRNA levels were increased by the presence of fetal bovine serum and epidermal growth factor in human trophoblast cell line,[13)] and by glucocorticoids,[14)] zinc,[15)] and calcium or phosphate ions in osteoblasts.[16)] SVCT2 expression is downregulated during the differentiation of rat and mouse muscle cells.[17)] Previous studies have focused on the implications and applicability of high-dose intravenous AsA administration in cancer therapy. Totarget tumor cells selectively and kill them, high concentrations of AsA (0.3–20 mmol/L) are required.[18)] Therefore, the mechanisms of AsA transport at high AsA levels ought to be studied. The effect of AsA levels on the expression levels of vitamin C

† To whom correspondence should be addressed. Tel/Fax: +81-35-978-5770; E-mail: emiko.suzuki@ocha.ac.jp

Abbreviations: AA-2G, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid; AsA, L-ascorbic acid; H_2O_2, hydrogen peroxide; NAC, *N*-acetylcysteine; ODS rat, osteogenic disorder Shionogi rat; SVCT, sodium-dependent vitamin C transporter

transporters is not completely clear. The exact mechanisms involved in regulating the expression of vitamin C transporters, by redox state in particular, remain unknown. Understanding the effects of AsA levels, which are closely related to the redox state, on the regulation of vitamin C transporters is important, and this understanding can provide further information on the bioavailability, required levels, and functions of AsA. Hence the effect of the AsA ingestion level on the expression levels of the vitamin C transporters (SVCT1 and SVCT2) was examined.

ODS/ShiJcl-od (ODS) rats used in this study spontaneously lack L-gulono-γ-lactone oxidase, a key enzyme in AsA biosynthesis, like humans. The effects of the AsA concentration in the culture medium on the expression of SVCT1 and SVCT2 in primary hepatocytes were also observed. The effects of an oxidant (hydrogen peroxide, H_2O_2), a reductant (*N*-acetylcysteine, NAC), and a stable AsA derivative (2-*O*-α-D-glucopyranosyl-L-ascorbic acid, AA-2G) on SVCT levels were also measured. Because AsA is generally easy to degrade in a neutral medium, AA-2G was also used in our experiments as a stable derivative of AsA. This derivative has vitamin C activity after enzymatic hydrolysis to AsA by α-glucosidase.[19]

6-week-old male ODS rats (Clea Japan, Tokyo) were individually housed in a temperature- and humidity-controlled room ($22 \pm 1\,^{\circ}C$, 55% relative humidity) on a 12-h light/dark cycle. They were fed an AIN76 purified diet (Clea Japan, Tokyo) without AsA,[20] and were allowed free access to water and food. All the rats were given 5 mg/d of AsA by oral gavage for the pre-experimental period, and were then divided into three groups: the 0 mg group was not administered additional AsA ($n = 7$), the 5 mg group was orally administered 5 mg/d of AsA ($n = 7$), and the 100 mg group was administered 100 mg/d of AsA ($n = 7$). The 5 mg/rat/d level and a concentration of 0.05 mmol/L (used for the hepatocyte experiments, described below) were considered to be the normal AsA level. At the end of the experiment, the rats were sacrificed under diethyl ether anesthesia, and their tissues were removed immediately. All experiments reported here received prior approval from the Animal Care Advisory Committee of Ochanomizu University. The animals were maintained in accordance with the guidelines of Ochanomizu University for the Care and Use of Laboratory Animals. Primary hepatocytes were prepared from 10-week-old male ODS rats as described in a previous report.[20] The hepatocytes were incubated for 24 h for AsA measurement and for 48 h for SVCT1 level measurement after addition with the following: 0, 0.05, or 5 mmol/L AsA (Wako Pure Chemical Industries, Osaka); 0, 0.05, or 5 mmol/L AA-2G (Hayashibara Biochemical Laboratories, Okayama); 0.1, 1, or 10 mmol/L NAC (Sigma Aldrich, St. Louis, MO); and 0.1, 0.5, or 1 mmol/L H_2O_2 (Sigma Aldrich, St. Louis, MO). mRNA expression levels were quantified by quantitative reverse transcription polymerase chain reaction, as described in a previous report.[20] SYBR Premix Ex Taq (Takara Bio, Shiga) and TaqMan Gene Expression Assays (Rn00568971_m1, SVCT1; Rn99999916_s1, GAPDH) (Applied Biosystems, Carlsbad, CA) were used. The primer sequences for PCR were described in a previous report.[20] Each relative mRNA level was normalized to the expression of GAPDH. The AsA levels of rat liver and the hepatocytes were also measured by high-performance liquid chromatography with electrochemical detection, as previously described.[20] The plasma membranes from the livers ($n = 6$ or 7) were isolated by Emmelot's methods, with slight modifications.[21] The extracted membrane protein was immunoblotted as described in a previous report.[20] Anti-SVCT1 (sc-9921; Santa Cruz Biotechnology, Santa Cruz, CA), immunoglobulin G-type (STAR88P; Daiichi Pure Chemicals, Tokyo) and human SVCT1 (sc-9924 P; Santa Cruz Biotechnology, Santa Cruz, CA) were used. The density of SVCT1 was analyzed with a LAS-3000 (Fujifilm, Tokyo) and Multi Gauge Ver 3.0 (Fujifilm, Tokyo), and was expressed in comparative units with the background as the baseline amount. The results given in the text are expressed as means $\pm$ standard deviation (SD), and were analyzed using the StatView 5.0 software (SAS Institute, Cary, NC). Significant differences among the three groups were evaluated by ANOVA and Fisher's protected least significant differences test. Differences were considered significant at p-values of < 0.05.

Body weight and total food intake did not differ among the groups during the experimental period. There was no reduction in mean body weight following AsA deficiency. The mean liver weight of the 0 mg group was slightly low on day 14 as compared with the other groups. The AsA concentrations in the livers increased significantly depending on the amount of AsA administered (Table 1). AsA levels in the plasma were the highest in the 100 mg group. Intracellular AsA concentrations in primary hepatocytes (Table 2) also increased significantly depending on the AsA concentration added

Table 1. AsA Concentrations in the Tissues of ODS Rats

Administered amount (mg per d)	AsA concentration (mg per 100 g tissue)		
	0 mg AsA	5 mg AsA	100 mg AsA
Liver	2.13 ± 1.44^{a}	4.20 ± 1.62^{b}	6.83 ± 0.96^{c}
Plasma	0.009 ± 0.002^{a}	0.043 ± 0.008^{a}	0.200 ± 0.015^{b}

[1]Values are means $\pm$ SD ($n = 7$).
[2]Within a row, values are not significantly different unless the superscripts contain a common letter ($p < 0.05$).

Table 2. Intracellular AsA Concentrations in Primary Hepatocytes

	Intracellular AsA concentration (ng per 100,000 cells)		
AsA	0 mmol/L	0.05 mmol/L	5 mmol/L
	1.02 ± 0.82^{a}	16.24 ± 1.45^{b}	1806.1 ± 127.2^{c}
AA-2G	0 mmol/L	0.05 mmol/L	5 mmol/L
	0.46 ± 0.11^{a}	21.17 ± 8.36^{a}	422.6 ± 35.8^{b}
H_2O_2	0.1 mmol/L	0.5 mmol/L	1 mmol/L
	0.60 ± 0.08	0.48 ± 0.07	0.38 ± 0.19
NAC	0.1 mmol/L	1 mmol/L	10 mmol/L
	0.49 ± 0.14	0.42 ± 0.20	0.44 ± 0.05

[1]Values are means $\pm$ SD ($n = 4$).
[2]Within a row, values are not significantly different unless the superscripts contain a common letter ($p < 0.01$).

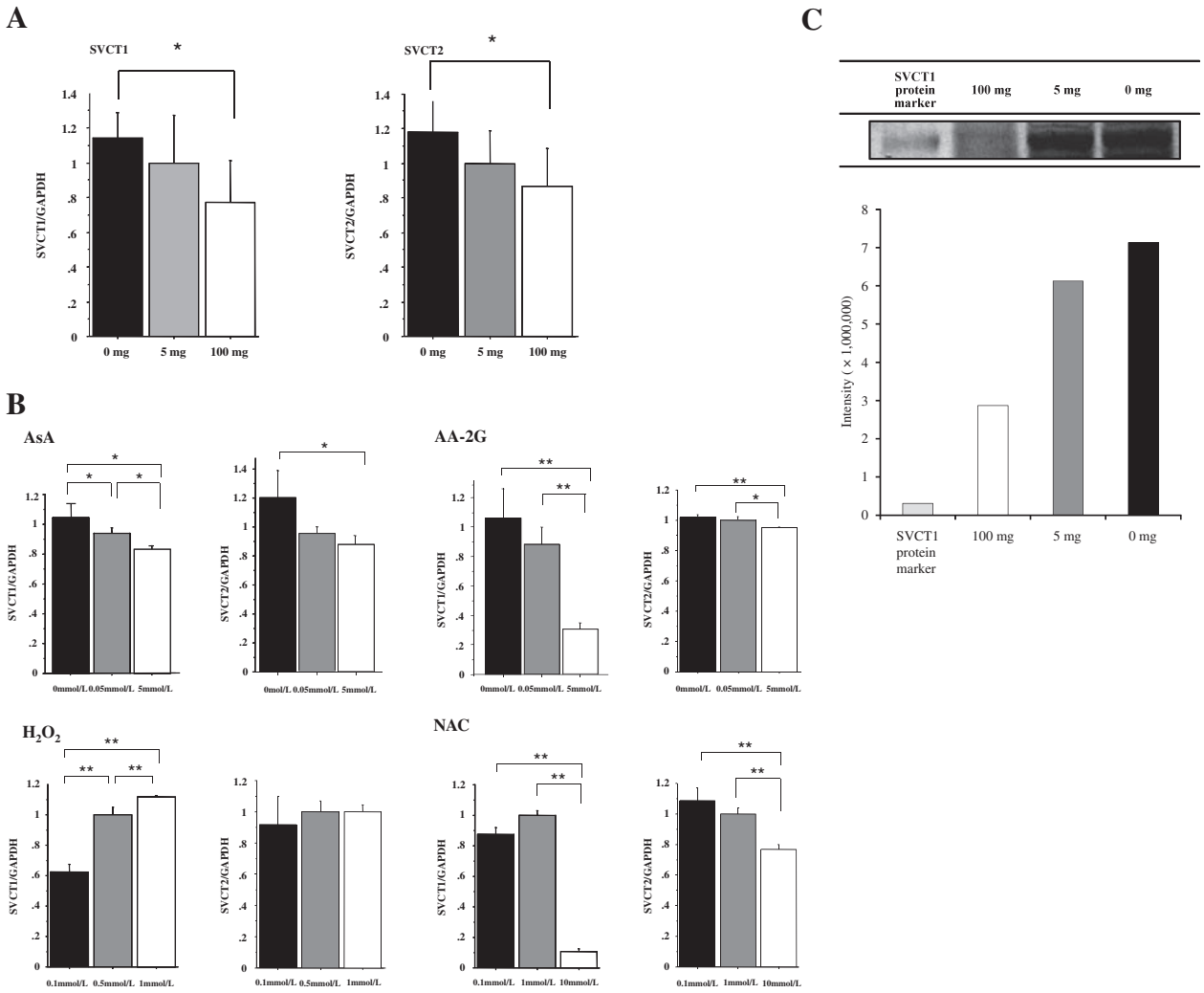

Fig. 1. Expression of SVCT1 and SVCT2 in Livers and Primary Hepatocytes of ODS Rats.

A, mRNA levels of SVCT1 and SVCT2 in the livers of the ODS rats administered 0, 5, or 100 mg/d of AsA (n = 6 or 7). Black bars represent the non(0 mg)-AsA-administered group, gray bars represent the 5 mg AsA-administered group, and white bars represent the 100 mg AsA-administered group. Values are means ± SD, n = 4. Asterisks indicate significant differences as compared with the control ($^{*}p < 0.05$). B, mRNA levels of SVCT1 and SVCT2 in ODS rat primary hepatocytes treated with AsA, AA-2G, H_2O_2, or NAC. Black bars represent the non(0 mmol/L)-AsA-added group, gray bars represent the 0.05 mmol/L AsA-added group, and white bars represent the 5.0 mM AsA-added group. Values are means ± SD, n = 4. Asterisks indicate significant differences as compared with control ($^{*}p < 0.05$; $^{**}p < 0.01$). C, Expression of SVCT1 in hepatic plasma membranes of ODS rats as analyzed by Western blotting. The sample amount for electrophoresis was normalized by the amount of protein. Results are expressed in comparative units using the background as reference.

to the culture medium. The intracellular AsA levels in the groups treated with AA-2G were highest in the 5 mmol/L AA-2G group. These results indicate that the AsA concentrations in the liver and the plasma were reflective of the AsA ingestion dose. Pericellular AsA levels, at least in the hepatocytes of the ODS rats, were involved in AsA accumulation. Consequently, increased AsA levels in the rat livers might also be affected by an increase in AsA levels in the body derived from AsA ingestion. Treatment with NAC did not influence the AsA concentrations in hepatocytes. Although treatment with H_2O_2 slightly affected intracellular AsA concentrations in the hepatocytes, no significant differences among the groups were observed. The mRNA expression levels of SVCT1 and SVCT2 in the livers and hepatocytes increased in the 0 mg and 0 mmol/L groups (Fig. 1A and B) and were suppressed in the 100 mg and 5 mmol/L groups. Suppression in the hepatocytes was more intense in the AA-2G-supplemented groups than in the AsA-supplemented group. The difference in suppression of SVCT1 levels between the AsA and the AA-2G group may have been due to their stability in the medium. These results suggest that hepatic SVCTs levels in ODS rats can be influenced by AsA ingestion or the AsA level in the tissue. The SVCT1 protein levels in the rat livers in the 0 and 5 mg groups were higher than those in the rat livers in the 100 mg group (Fig. 1C). These results indicate that graded AsA supplementation decreased SVCT expression in the rat livers, suggesting that the AsA ingestion level is involved in regulation of AsA transportors. Previous reports have suggested that serum AsA concentrations are controlled by low levels.[16] Other studies have also indicated that AsA supplementation or deprivation influences AsA transport.[7,10,12] Our results also suggest that the peri-/intracellular AsA level is an important regulator of SVCT1 and SVCT2 expression in the hepatocytes of ODS rats. Because the SVCT1 transporter is predominantly expressed in epithelial cells of the liver, an increase in hepatic SVCT1 levels can contribute to

intracellular AsA accumulation in the liver. Hence we focused on the alteration of SVCT1 expression. We also measured the relative mRNA levels of SVCT2. The SVCT1 mRNA levels were influenced by treatment with NAC or H_2O_2 in the medium. Treatment with 10 mmol/L NAC decreased SVCT1 and SVCT2, and the treatment with H_2O_2 increased SVCT1 levels depending on the amount of H_2O_2 added, which ranged from 0.1 to 1.0 mmol/L. These results suggest that redox status can affect SVCT1 expression, resulting in the required balance of redox status by uptake of AsA. The presence of H_2O_2 at widely varying levels (in some cases, 100 µmol/L or more) has been reported in the aqueous and vitreous humors of humans and other animals.[22] Thus the H_2O_2-concentration-dependant rises in the SVCT1 levels in the tested range of H_2O_2 concentrations are biologically significant. On the other hand, NAC, a synthetic cysteine derivative, supplies cells with cysteine, thereby increasing cellular GSH levels. GSH is the most abundant natural cellular antioxidant. It is found at millimolar concentrations (1–10 mmol/L) in the body and plays an essential role in maintaining the cellular redox state.[23] GSH levels are regulated by the cellular availability of cysteine, the precursor for glutathione synthesis. For these reasons, treatment with 10 mmol/L NAC was used as a criterion of the redox state. Some research has suggested that achieving high levels of AsA by intravenous injection is feasible,[24] and physiological AsA concentrations (0.1 mmol/L) did not have any effect on tumor or normal cells.[25] In contrast, pharmacological AsA concentrations (0.3–20 mmol/L) comparable to those attained by intravenous administration target selectively and kill tumor cells *in vitro*.[25] It is important to understand AsA transport mechanisms at high AsA levels. An inability to maintain AsA levels in tissues and the consequent reduction in antioxidant capacity can result in an increased flux of harmful reactive oxygen species and reactive intermediates, which contribute to high incidence of degenerative diseases such as cancer and other lifestyle-related diseases. Further detailed investigation of the association between SVCT1 levels and redox regulation is required.

Acknowledgments

We thank Mr. Masanobu Watanabe of Act-Science, Inc., for helpful advice and assistance with our high-performance liquid chromatography methods. This project was funded in part by a Grant-in-Aid for Young Scientists from the Japan Society for the Promotion of Science.

References

1) Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, and Graumlich JF, *Proc. Natl. Acad. Sci. USA*, **93**, 3704–3709 (1996).
2) Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang YX, Brubaker RF, and Hediger MA, *Nature*, **399**, 70–75 (1999).
3) Vera JC, Rivas CI, and Fischbarg J, *Nature*, **364**, 79–82 (1993).
4) Takanaga H, Mackenzie B, and Hediger MA, *Pflugers Arch.*, **447**, 677–682 (2004).
5) Qiu S, Li L, Weeber EJ, and May JM, *J. Neurosci. Res.*, **85**, 1046–1056 (2007).
6) Padayatty SJ, Sun H, Wang YH, Riordan HD, Hewitt SM, Katz A, Wesley RA, and Levine M, *Ann. Intern. Med.*, **140**, 533–537 (2004).
7) Dixon SJ and Wilson JX, *J. Bone Miner. Res.*, **7**, 675–681 (1992).
8) Wilson JX, Jaworski EM, Kulaga A, and Dixon SJ, *Neurochem. Res.*, **15**, 1037–1043 (1990).
9) Savini I, Catani MV, Arnone R, Rossi A, Frega G, del Principe D, and Avigliano L, *Free Radic. Biol. Med.*, **42**, 608–616 (2007).
10) MacDonald L, Thumser AE, and Sharp P, *Br. J. Nutr.*, **87**, 97–100 (2002).
11) Karaczyn A, Ivanov S, Reynolds M, Zhitkovich A, Kasprzak KS, and Salnikow K, *J. Cell. Biochem.*, **97**, 1025–1035 (2006).
12) Amano A, Aigaki T, Maruyama N, and Ishigami A, *Arch. Biochem. Biophys.*, **496**, 38–44 (2010).
13) Biondi C, Pavan B, Dalpiaz A, Medici S, Lunghi L, and Vesce F, *Mol. Hum. Reprod.*, **13**, 77–83 (2007).
14) Fujita I, Hirano J, Itoh N, Nakanishi T, and Tanaka K, *Br. J. Nutr.*, **86**, 145–149 (2001).
15) Wu X, Itoh N, Taniguchi T, Nakanishi T, Tatsu Y, Yumoto N, and Tanaka K, *Arch. Biochem. Biophys.*, **420**, 114–120 (2003).
16) Wu X, Itoh N, Taniguchi T, Hirano J, Nakanishi T, and Tanaka K, *Biochem. Biophys. Res. Commun.*, **317**, 1159–1164 (2004).
17) Savini I, Catani MV, Duranti G, Ceci R, Sabatini S, and Avigliano L, *Free Radic. Biol. Med.*, **38**, 898–907 (2005).
18) Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, and Levine M, *Proc. Natl. Acad. Sci. USA*, **102**, 13604–13609 (2005).
19) Yamamoto I, Suga S, Mitoh Y, Tanaka M, and Muto N, *J. Pharmacobiodyn.*, **13**, 688–695 (1990).
20) Sone Y, Ueta E, Sannomaru Y, Miyake N, Sone H, Otsuka Y, Kondo K, Kurata T, and Suzuki E, *J. Biochem. Mol. Toxicol.*, in press.
21) Emmelot CJ, Bos EL, and Benedetti P, *Biochim. Biophys. Acta*, **90**, 126 (1964).
22) Spector A, Ma W, and Wang RR, *Invest. Ophthalmol. Vis. Sci.*, **39**, 1188–1197 (1998).
23) Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, and Telser J, *Int. J. Biochem. Cell Biol.*, **39**, 44–84 (2007).
24) Padayatty SJ, Riordan HD, Hewitt SM, Katz A, Hoffer LJ, and Levine M, *CMAJ*, **174**, 937–942 (2006).
25) Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, and Levine M, *Proc. Natl. Acad. Sci. USA*, **102**, 13604–13609 (2005).