

Inhibitory effect of serotonin derivatives on high glucose-induced adhesion and migration of monocytes on human aortic endothelial cells

Rosaria Piga^{1*}, Yuji Naito², Satoshi Kokura³, Osamu Handa³ and Toshikazu Yoshikawa¹

¹Department of Inflammation and Immunology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan

²Department of Medical Proteomics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan

³Department of Biomedical Safety Science, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan

(Received 10 July 2008 – Revised 27 October 2008 – Accepted 29 October 2008 – First published online 18 February 2009)

Previous reports have shown that safflower-seed extract and its major antioxidant constituents, serotonin hydroxycinnamic amides, attenuated atherosclerotic lesion formation in apoE-deficient mice, as well as inflammation and aortic stiffness in human subjects. In the present report, we examined a still unknown cell-based mechanism of serotonin derivatives against the development of atherosclerosis, focusing our attention on their action against the increase of adhesion molecules and the release of chemotactic factors on human aortic endothelial cells, phenomena that represent the key events in the early stages of atherosclerogenesis. Serotonin derivatives *N*-(*p*-coumaroyl)serotonin and *N*-feruloylserotonin exerted an inhibitory effect on short-term high glucose-induced up-regulation of mRNA and protein of adhesion and migration factors, and the consequent adhesion and migration of monocytes to endothelial cells; they inhibited the activation of transcription factors such as NF- κ B, and the overproduction of the mitochondrial superoxide by acting as scavengers of the superoxide radical. In addition, serotonin derivative concentration inside the cells and inside the mitochondria was increased in a time-dependent manner. These results identify a mechanism of action of serotonin derivatives against endothelial damage at a cellular level, and underline their benefits against the disorders and complications related to reactive oxygen species.

Serotonin derivatives: Superoxide: Monocyte adhesion: Monocyte migration: Antioxidants

Dietary factors play significant aetiological roles in the development of several diseases. Many epidemiological studies have shown an inverse relationship between the consumption of diets rich in polyphenols, especially flavonoids, and cardiovascular risk⁽¹⁾. CVD in the Far East is significantly lower than that documented in the West, and atherosclerosis is the leading cause of morbidity and mortality in Western society⁽¹⁾.

Recently, some independent laboratories have focused their attention on other dietary compounds, such as serotonin derivatives, which may exert significant antioxidant effects both *in vitro* and *in vivo*. *N*-(*p*-coumaroyl)serotonin (CS) and *N*-feruloylserotonin (FS), two serotonin conjugates, were identified as the major and unique phenolic constituents of defatted safflower seeds^(2,3). These are members of the indole hydroxycinnamic acid amides, known to perform defensive functions in plants when damaged or under pathogenic attack, with serotonin (5-HT), *p*-coumaric acid (*p*-ca) and ferulic acid (fa) representing the components of their structure (Fig. 1)⁽⁴⁾. The hydroxycinnamic acid amides of serotonin, including CS and FS, which

are synthesised by serotonin *N*-hydroxycinnamoyltransferase, are present in several wild-growing plants and their seeds, such as safflower (*Carthamus tinctorius* L.) used in herbal medicine in Eastern countries, and worldwide mainly for edible oil production^(3,4), konnyaku (*Amorphophallus konjac* K. Koch) widely used as a traditional food in all over Japan⁽⁵⁾, Japanese barnyard millet (*Echinochloa utilis* Ohwi & Yabuno), widely used in Japan especially in cold districts because of its resistance to low temperatures and for its highly nutritious grains that make it a good substitute for rice⁽⁶⁾, *Centaurea nigra* L. and many species of the genus *Centaurea*, traditionally used for their antibacterial activity in the treatment of various ailments^(7–9), and maize bran and other grains used in a wide variety of ways, including home cooking to increase the fibre content of various foods⁽¹⁰⁾. Furthermore, their presence was also detected in several vegetables⁽⁴⁾ widely used all over the world.

Some of their activities have been already reported; they possess antibacterial and free radical-scavenging activities^(5–7,10), show antioxidative activity on plasma and liver lipid status⁽¹¹⁾,

Abbreviations: CS, *N*-(*p*-coumaroyl)serotonin; fa, ferulic acid; FS, *N*-feruloylserotonin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAEC, human aortic endothelial cells; HG, 25 mM-D-(+)-glucose (high glucose); 5-HT, serotonin; MCP-1, monocyte chemoattractant protein-1; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; *p*-ca, *p*-coumaric acid; ROS, reactive oxygen species; α -Toc, α -tocopherol; VCAM-1, vascular cell adhesion molecule-1.

* **Corresponding author:** Rosaria Piga, fax +81 75 252 3721, email rosariapiga@tiscali.it

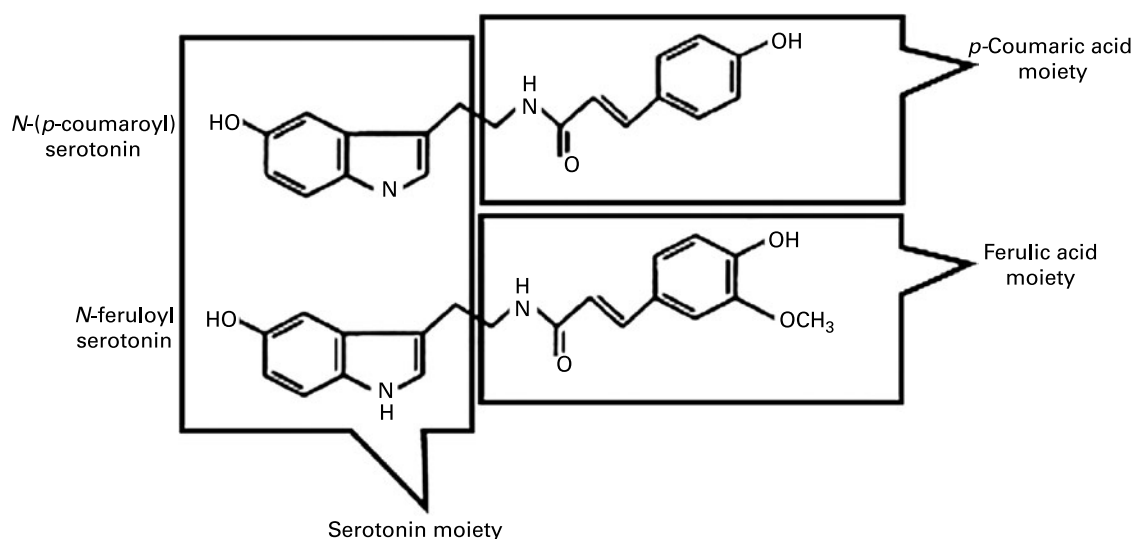


Fig. 1. Chemical structure of *N*-(*p*-coumaroyl)serotonin and *N*-feruloylserotonin.

increase the proliferation of fibroblasts⁽¹²⁾, inhibit the production of pro-inflammatory cytokines⁽¹²⁾, inhibit LDL oxidation in apoE-deficient mice⁽²⁾ and show beneficial effects against cardiovascular risk in healthy human volunteers⁽¹³⁾. Despite this, there are no reports describing a cell-based mechanism of these serotonin derivatives against endothelial damage; their protective effect in this system at a cellular level is still unknown.

Short-term high glucose exposure, even in terms of hours, could represent a dangerous stressor in the early stages of atherosclerogenesis, suggesting that its dangerous effect could also affect healthy individuals⁽¹⁴⁾. Monocyte–endothelial cell interactions play a pivotal role in the initiation and progression of an inflammatory process induced by several stressors, including hyperglycaemia, making human aortic endothelial cells (HAEC) and the human monocyte U-937 cell line suitable for this kind of approach.

Therefore, in order to look into the still unknown cellular mechanism and effect of serotonin derivatives, the present study followed a cell-based approach to underline the potential properties of CS and FS, focusing on their action against the increase of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1).

Materials and methods

Cells

HAEC (Clonetics, San Diego, CA, USA) were routinely maintained at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂ in endothelial cell growth medium (EBM-2; Clonetics) containing 5.5 mM-D-(+)-glucose, and supplemented with endothelial growth medium 2 (EGM-2) kit (Clonetics). Therefore, HAEC growing in their EBM-2 medium containing 5.5 mM-D-(+)-glucose required for their survival were used as control cells and considered as untreated. For all experiments, cells were used up to the sixth passage.

Human monocyte line U-937 (American Type Culture Collection, Rockville, MD, USA) was grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen

Life Technologies, Carlsbad, CA, USA) containing 10 % fetal calf serum, streptomycin (100 µg/ml), penicillin (100 IU/ml) and fungizone (250 ng/ml) at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂.

Cell viability was assessed by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Cell Counting Kit; Dojindo, Kumamoto, Japan) as previously described⁽¹⁵⁾.

Enzyme-linked immunosorbent assay

HAEC were grown on a ninety-six-well plate and pretreated for 24 h with CS or FS (kindly provided by Ajinomoto Co., Kawasaki, Japan) at different concentrations (from 0.1 to 10 µM), or 40 µM-α-tocopherol (α-Toc) (positive control), or 10 µM-5-HT, -*p*-ca or -*fa* that represent the components of the structure of CS and FS (all from Sigma, St Louis, MO, USA), then stimulated with 25 mM-D-(+)-glucose (HG) (Sigma) for 4 h. Enzyme-linked immunoassay was used to assess VCAM-1 expression on HAEC as previously described⁽¹⁶⁾. MCP-1 protein level in the cell culture supernatant fraction was examined using a human MCP-1 Immunoassay kit (Biosource International, Camarillo, CA, USA) according to the manufacturer's protocol.

Glucose uptake by human aortic endothelial cells

Glucose uptake was performed as previously described⁽¹⁷⁾ using a fluorescent D-glucose analogue 2-[*N*-(7-nitrobenx-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG; Invitrogen Life Technologies), used to monitor glucose uptake in living cells. HAEC were treated with 2-NBDG for 1 h in the simultaneous presence or absence of 10 µM-CS or -FS. Untreated HAEC were used as the control group. Fluorescence was observed using the Cytomics FC500 Flow Cytometry System (Beckman Coulter, Fullerton, CA, USA).

Monocyte adhesion and transmigration assay

The adhesion assay was performed as previously described⁽¹⁸⁾. HAEC were pretreated with 10 µM-CS, -FS, -5-HT, -*p*-ca or

-fa or 40 μM - α -Toc for 24 h, then stimulated with HG for 4 h. Subsequently, 2',7'-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR, USA)-labelled U-937 (1×10^6 cells/ml, 100 μl) were added to each well, in the same media with HG in the presence or absence of the antioxidant used, and incubated at 37°C for 30 min. The same treatment was performed for the transmigration assay, using transwell membranes and adding labelled monocytes into the upper compartment as previously described⁽¹⁹⁾. 2',7'-Bis (2-carboxyethyl)-5(6)-carboxyfluorescein fluorescent intensity of adherent and transmigrated U-937 was quantified using the previously described method⁽²⁰⁾.

Isolation of mRNA and real-time PCR

HAEC were pretreated with 10 μM -CS or -FS for 24 h, then stimulated with HG for 3 h. Total RNA was isolated with the acid guanidinium phenol chloroform method using an Isogen kit (Nippon Gene, Tokyo, Japan). Primers were as follows: for VCAM-1, sense 5'-CCCTTGACCCGGCTG-GAGATT-3', antisense 5'-CTGGGGCAACATTGACATA-AAGTG-3'; for MCP-1, sense 5'-CGCTCCAGCATGAAA-GTCT-3', antisense 5'-GGAATGAAGGTGGCTGCTATG-3'; for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCCTGTTGCTGTA-3'. In order to quantify VCAM-1 and MCP-1 gene expression, the VCAM-1 and MCP-1 mRNA level was normalised by the total RNA content ($\mu\text{g}/\mu\text{l}$) and GAPDH mRNA level.

NF- κ B activation assay

HAEC were treated with HG for 2 h in the simultaneous presence or absence of 10 μM -CS, 10 μM -FS, 10 μM -thenoyltrifluoroacetone (Aldrich, St Louis, MO, USA), a complex II inhibitor, 0.5 μM -*m*-chlorophenylhydrazine (Sigma), a mitochondrial membrane proton gradient inhibitor, or 100 μM -Mn(III)5,10,15,20-tetrakis(*N*-methyl-pyridinium-2-yl)porphyrin (Sigma), a synthetic cell-permeable Mn porphyrin-based superoxide dismutase mimetic. Subsequently, HAEC nuclear extraction was performed using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. NF- κ B on nuclear extracts was detected using a TransAM NF- κ B p65 Kit (Active Motif) according to the manufacturer's protocol.

Determination of intracellular reactive oxygen species

HAEC were stimulated with HG for 1 h in the simultaneous presence or absence of the compounds described for the adhesion and NF- κ B assay. Intracellular reactive oxygen species (ROS) were detected using the fluorescent probe 2',7'-dichlorofluorescein diacetate as described earlier⁽²¹⁾ and RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR, USA). Mitochondria were labelled using the MitoTracker Green FM (Molecular Probes). The MitoTracker Green FM stain in conjunction with a RedoxSensor Red CC-1 stain was used to verify the distribution of the oxidised product, according to the manufacturer's protocol. Mitochondrial superoxide was detected using a MitoSOX mitochondrial

superoxide indicator (Molecular Probes). Cells were observed using the FV1000 fluoview BX61 (Olympus, Center Valley, PA, USA).

HPLC analysis

HAEC were treated with 20 μM -CS or -FS for the specified periods (0, 24, 48, 72, 96 h). Untreated HAEC were used as the control group. Cells were then collected, and a sample was used for mitochondria extraction performed using a Mitochondria Isolation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Subsequently, CS and FS were extracted with methanol-PBS (4:1, v/v) and were measured by HPLC as described earlier⁽²²⁾. In order to quantify the concentration, CS and FS levels were normalised by the total protein content ($\mu\text{mol}/\text{mg}$ protein).

Data analysis

All data of at least three separate experiments were expressed as mean values with their standard errors. Two-way ANOVA was used to assess the differences between multiple groups using the GraphPad Prism 4 program (GraphPad Software, Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Effects of N-(p-coumaroyl)serotonin and N-feruloylserotonin on short-term high glucose-induced vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 and mRNA expression in human aortic endothelial cells

HG treatment for 4 h caused a significant four-fold increase in the surface expression of VCAM-1 protein (Fig. 2(A)) and a significant three-fold increase of MCP-1 protein release (Fig. 2(B)) in the cell culture supernatant fraction. Pretreatment of HAEC with CS and FS for 24 h decreased VCAM-1 expression and MCP-1 release in a concentration-dependent manner, with a significant inhibition at 5 and 10 μM . 40 μM - α -Toc had a similar effect to 10 μM -CS and -FS, while treatment with 10 μM -5-HT, -*p*-ca or -fa resulted in a non-significant reduction of VCAM-1 expression and MCP-1 release, which appeared to be significant only for 5-HT. These findings were further confirmed by mRNA expression analysis for VCAM-1, MCP-1 and GAPDH genes in HAEC by real-time PCR (Fig. 3(A)). Pretreatment of HAEC with 10 μM -CS or -FS for 24 h resulted in a significant decrease of HG-induced mRNA expression for VCAM-1 and MCP-1. The protection exerted by CS and FS was not the result of a prevention of cell glucose uptake, as shown by the flow cytometry analysis results. Cells treated with CS and FS with the addition of the fluorescent D-glucose analogue 2-NBDG exhibited the same fluorescence as that shown on cells treated with 2-NBDG alone (Fig. 3(B)).

Effects of N-(p-coumaroyl)serotonin and N-feruloylserotonin on short-term high glucose-induced monocyte adhesion and migration to human aortic endothelial cells

Incubation of HAEC with HG for 4 h caused an approximate three-fold increase in adhesion of U-937 compared with the

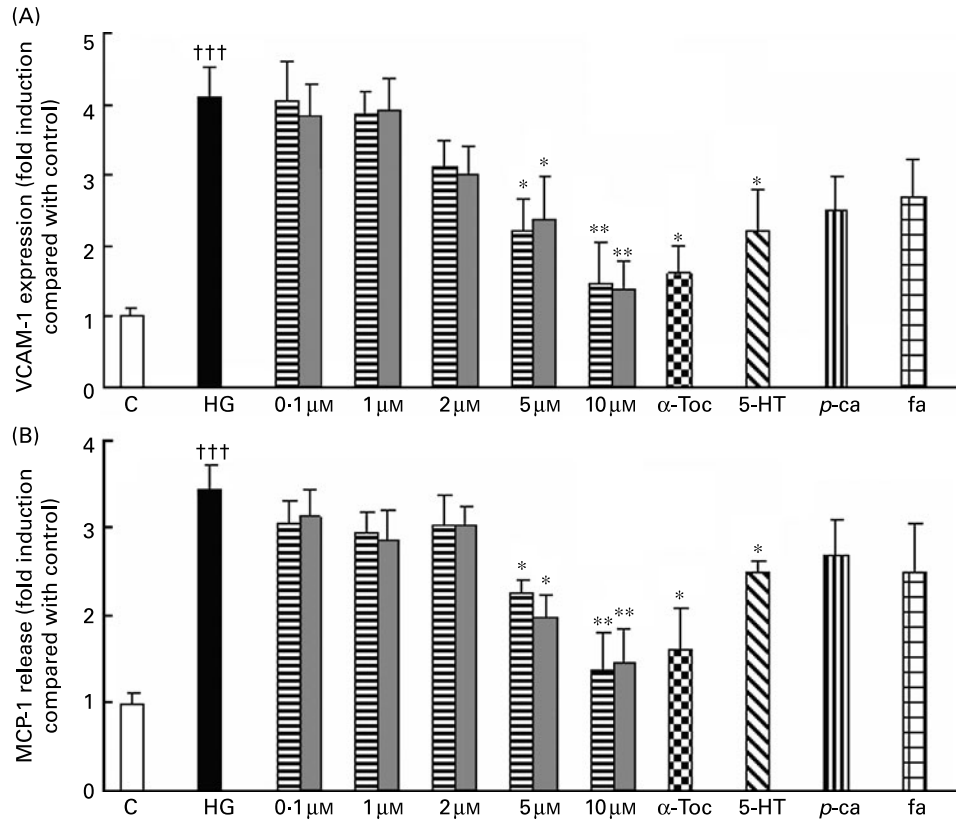


Fig. 2. Effects of *N*-(*p*-coumaroyl)serotonin (▨) and *N*-feruloylserotonin (▩) on short-term high-glucose (HG; 25 mM-D-(+)-glucose)-induced (A) vascular cell adhesion molecule-1 (VCAM-1) protein expression and (B) monocyte chemoattractant protein-1 (MCP-1) protein release, normalised to total cell protein (optical density arbitrary units/μg protein for VCAM-1 and ng/μg protein for MCP-1). C, control; α-Toc, α-tocopherol; 5-HT, serotonin; *p*-ca, *p*-coumaric acid; fa, ferulic acid. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that for HG alone: * $P < 0.05$, ** $P < 0.01$. ††† Mean value was significantly different from that for the control condition ($P < 0.001$).

adhesion of U-937 to control HAEC (Fig. 4(A)), and an approximately three-fold increase in transmigration of U-937 to the lower compartment compared with the transmigration of U-937 to control HAEC (Fig. 4(B)). Pretreatment of HAEC with 10 μM-CS or -FS for 24 h resulted in a significant decrease of HG-induced adhesion and migration of U-937 to HAEC. 40 μM-α-Toc had a comparable effect to 10 μM-CS or -FS, while treatment with 10 μM-5-HT, *p*-ca or -fa resulted in a non-significant reduction of monocyte adhesion and migration.

Effect of N-(*p*-coumaroyl)serotonin and *N*-feruloylserotonin on short-term high glucose-induced NF-κB activation

HG treatment for 2 h induced a significant increase of the NF-κB binding activity of approximately 2.6-fold when compared with control HAEC (Fig. 5). This HG-induced effect was prevented by all the antioxidants used.

Effect of N-(*p*-coumaroyl)serotonin and *N*-feruloylserotonin on short-term high glucose-induced intracellular reactive oxygen species

The HG-induced NF-κB activation on HAEC was preceded by a significant increase in the intracellular ROS levels (Fig. 6(A)) after treatment of HAEC with HG for 1 h, which

was significantly reduced by CS and FS. 40 μM-α-Toc had a similar effect to 10 μM-CS and -FS, while treatment with 10 μM-*p*-ca or -fa resulted in a non-significant reduction of ROS fluorescence, which appeared to be significant only for 5-HT. The distribution of the oxidised product (Fig. 6(B)) showed red fluorescence of ROS in green mitochondria (lower images); HG-treated HAEC showed yellow areas because of co-localisation of green and red fluorescence as a result of ROS in mitochondria (HG, lower images). Co-localisation was not present in the control, or in any other treated group (lower images). These results were confirmed by the MitoSOX fluorescence, specific for mitochondrial superoxide, that was highly present on HG-treated HAEC (HG, upper image), but not relevant in any other treated group (upper images).

N-(*p*-coumaroyl)serotonin and *N*-feruloylserotonin concentration inside human aortic endothelial cells and mitochondria

Treatment with 20 μM-CS from 0 to 96 h resulted in a time-dependent increase of CS concentration inside the cells and the mitochondria; the increase was significant after 48 h inside the cells (6.9 μmol CS/mg protein) and after 72 h inside the mitochondria (4.6 μmol CS/mg protein) (Fig. 7). A similar pattern was obtained for FS detection (data not shown).

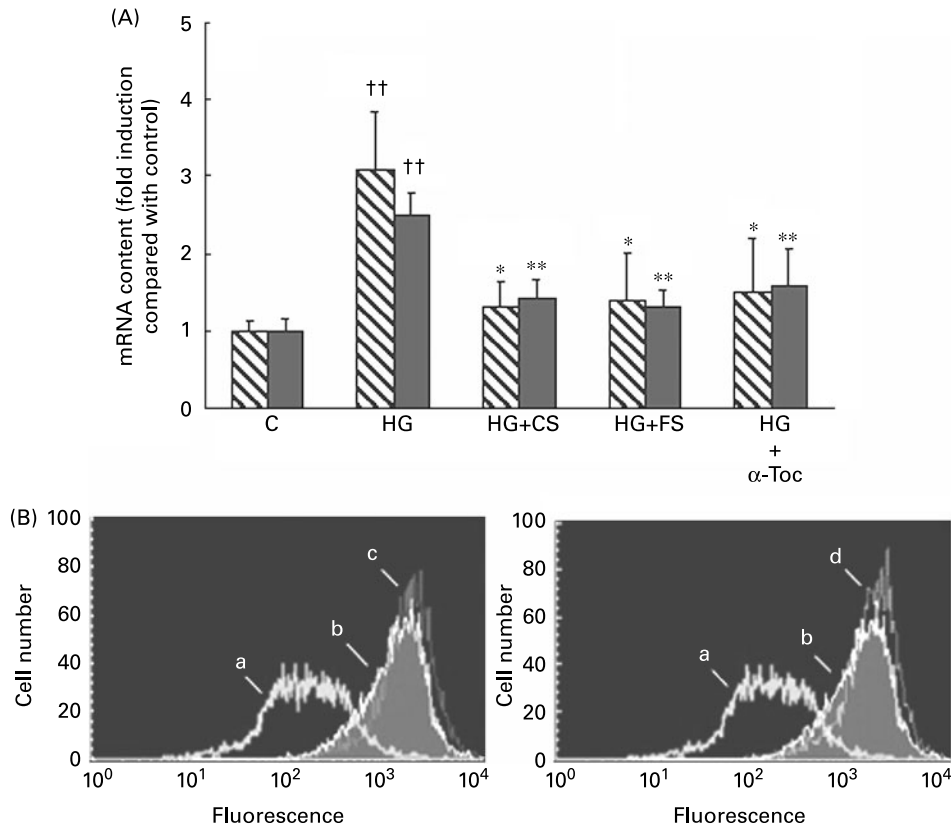


Fig. 3. Effects of *N*-(*p*-coumaroyl)serotonin (CS) and *N*-feruloylserotonin (FS) on short-term high-glucose (HG; 25 mM-D-(+)-glucose)-induced mRNA expression in human aortic endothelial cells (HAEC), and HAEC glucose uptake. (A) Vascular cell adhesion molecule-1 (VCAM-1; ▨) and monocyte chemoattractant protein-1 (MCP-1; ▩) mRNA level, normalised by the total RNA content ($\mu\text{g}/\mu\text{l}$) and glyceraldehyde 3-phosphate dehydrogenase mRNA level. C, control; α -Toc, α -tocopherol. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that for HG alone: * $P < 0.05$, ** $P < 0.01$. †† Mean value was significantly different from that for the control condition ($P < 0.01$). (B) Cell glucose uptake, as shown by flow cytometry analysis. Trace a, control condition; trace b, 2-[*N*-(7-nitrobenx-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG); trace c, CS + 2-NBDG; trace d, FS + 2-NBDG.

Discussion

CS and FS, two serotonin conjugates, have been identified from safflower seeds⁽³⁾, konnyaku⁽⁵⁾ and Japanese barnyard millet⁽⁶⁾. They exhibit antioxidant and anti-inflammatory activities^(3–7,10), together with many species of the genus *Centaurea*, traditionally used for their antibacterial activity in the treatment of various ailments⁽⁷⁾, such as cornflower (*Centaurea cyanus* L.)⁽⁸⁾ and sweet sultan (*Centaurea moschata* L.)⁽⁹⁾. In addition to these already mentioned plants and their effects, CS and FS have been detected in some vegetables, such as paprika and green onions, which contain CS, and tomatoes, cherry tomatoes, hot peppers, paprika, lettuce, Chinese cabbage, chicory and spinach, which contain FS⁽⁴⁾. Finally, maize bran, which is an important byproduct of the maize dry milling industry, is rich in several functional lipid constituents, including serotonin hydroxycinnamic acids, possessing antioxidant and anti-melanogenic activities⁽¹⁰⁾. Although it has been reported that CS and FS were protective against LDL oxidation and atherogenesis in experimental animals and in human subjects^(2,13), to the best of our knowledge, cell-based studies on their action against endothelial damage have not been reported.

The migration of circulating monocytes into the subendothelial space is one of the crucial events in early atherogenesis.

VCAM-1 and MCP-1 are well-known factors involved in this process; VCAM-1 mediates the adhesion of leucocytes to activated endothelium⁽²³⁾, MCP-1 is a powerful monocyte chemoattractant, both *in vivo* and *in vitro*⁽²⁴⁾, and both are expressed by endothelial cells in early atherosclerotic lesions and involved in monocyte adhesion and recruitment to early lesions⁽¹⁴⁾. In accordance with the protective effect of CS and FS against atherogenesis as previously reported *in vivo*^(2,13), we found that the pretreatment of HAEC with CS or FS reduced the HG-stimulated HAEC expression of VCAM-1 and MCP-1 in a concentration-dependent manner, as well as the adhesion and migration of U-937. We also compared these inhibitory effects with the effect of 40 μM - α -Toc, which is the level achieved in plasma by consuming 133.4 mg (200 IU) α -Toc supplements/d^(7,25,26), and with the effect of 5-HT, *p*-ca and fa, which represent the components of the structure of CS and FS; CS and FS protection was higher than that of 5-HT, *p*-ca, and fa alone in the experimental condition used, suggesting that their inhibitory effect may be due to both 5-HT and *p*-ca or fa moieties acting together, and was also comparable with the protective effect of 40 μM - α -Toc, used as a positive control. These treatments did not affect cell viability (data not shown). In addition, similar results were obtained with the simultaneous treatment of HAEC with CS and FS plus HG without 24 h CS or FS pretreatment (data not shown), suggesting that

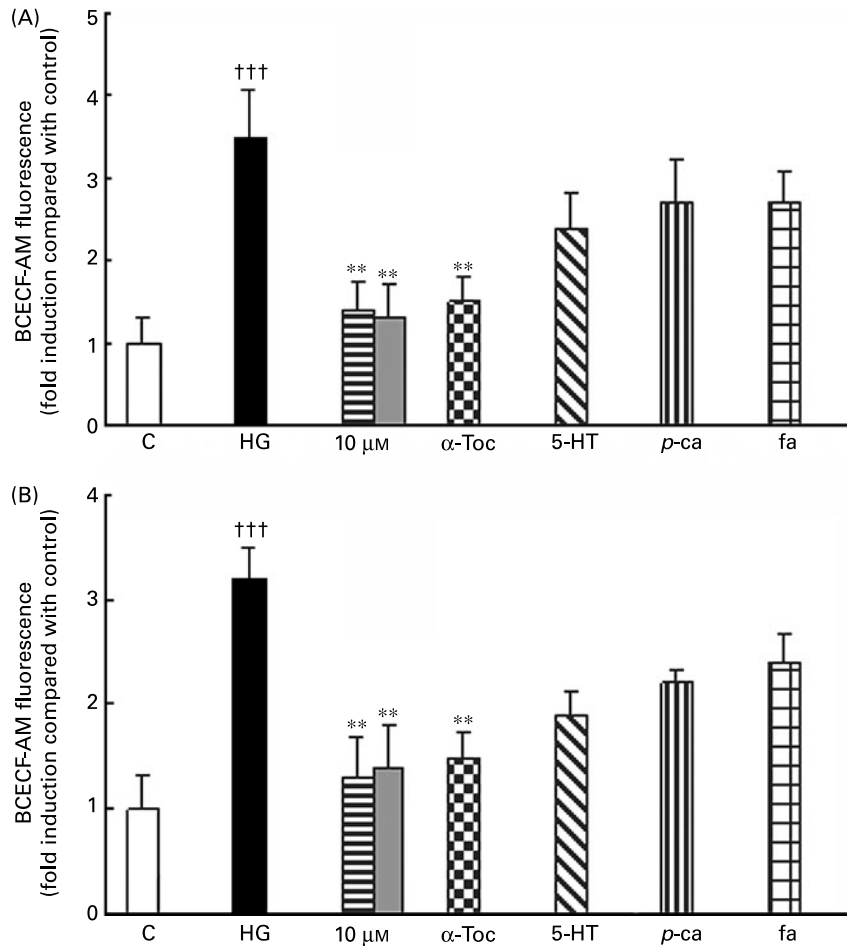


Fig. 4. Effects of *N*-(*p*-coumaroyl)serotonin (▨) and *N*-feruloylserotonin (▩) on short-term high-glucose (HG; 25 mM-D-(+)-glucose)-induced U-937 adhesion (A) and migration (B) to human aortic endothelial cells (HAEC). (A) 2',7'-Bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) fluorescent intensity of adherent cells. (B) BCECF-AM fluorescent intensity of migrated cells to the lower compartment. C, control; α-Toc, α-tocopherol; 5-HT, serotonin; *p*-ca, *p*-coumaric acid; fa, ferulic acid. Values are means, with their standard errors represented by vertical bars. ** Mean value was significantly different from that for HG alone ($P < 0.01$). ††† Mean value was significantly different from that for the control condition ($P < 0.001$).

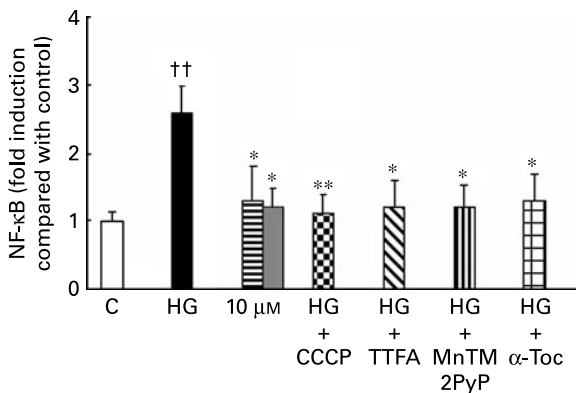


Fig. 5. Effect of *N*-(*p*-coumaroyl)serotonin (▨) and *N*-feruloylserotonin (▩) on short-term high-glucose (HG; 25 mM-D-(+)-glucose)-induced NF-κB binding activity. C, control; CCCP, *m*-chlorophenylhydrazine; TTFA, thenoyltrifluoroacetone; MnTM2PyP, Mn(III)5,10,15,20-tetrakis(*N*-methyl-pyridinium-2-yl) porphyrin; α-Toc, α-tocopherol. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that for HG alone: * $P < 0.05$, ** $P < 0.01$. †† Mean value was significantly different from that for the control condition ($P < 0.01$).

pretreatment with CS and FS is not an essential requirement on the inhibitory effect of CS and FS against VCAM-1 and MCP-1 increase. Our observations are novel and provide a potential mechanism by which CS and FS reduce the risk of atherosclerosis *in vivo*.

Because NF-κB is activated by reactive oxygen species, and VCAM-1 and MCP-1 expression is also regulated by NF-κB^(14,27), the radical scavenging activity of CS and FS may contribute to the inhibition of the expression of VCAM-1 and MCP-1. Furthermore, previous reports⁽²⁸⁾ showed a hyperglycaemia-increased activation of NF-κB that was prevented by thenoyltrifluoroacetone, *m*-chlorophenylhydrazine, and by an overexpression of the Mn superoxide dismutase, the mitochondrial form of this antioxidant enzyme. This suggests that the superoxide is the reactive oxygen radical produced by this mechanism, that the mitochondria is the site of hyperglycaemia-induced ROS, and that hyperglycaemia activates NF-κB through a ROS-dependent pathway. Therefore, we tested the effect of CS and FS in comparison with the effect of Mn(III)5,10,15,20-tetrakis(*N*-methyl-pyridinium-2-yl) porphyrin, thenoyltrifluoroacetone and *m*-chlorophenylhydrazine

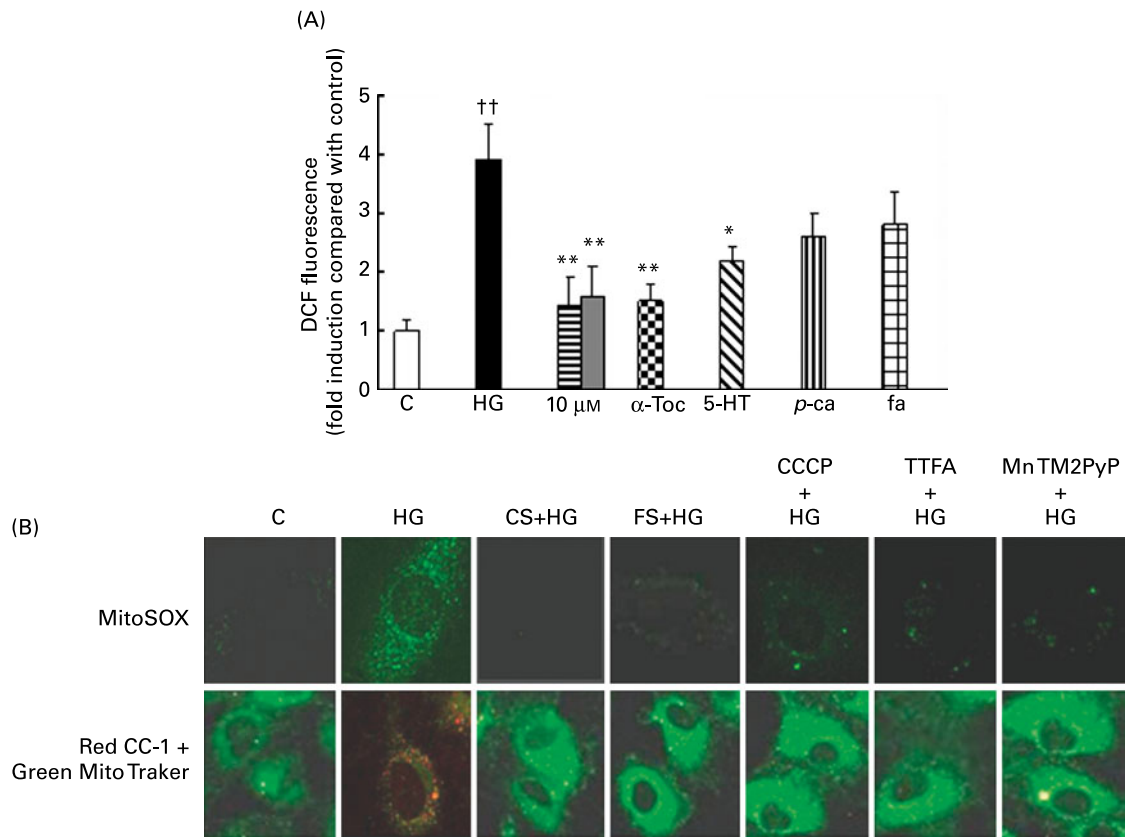


Fig. 6. Effect of *N*-(*p*-coumaroyl)serotonin (CS; ▨) and *N*-feruloylserotonin (FS; ▩) on short-term high-glucose (HG; 25 mm-D-(+)-glucose)-induced intracellular reactive oxygen species (ROS). (A) Intracellular ROS assessed by the fluorescence intensity of dichlorofluorescein (DCF) emission. C, control; α -Toc, α -tocopherol; 5-HT, serotonin; *p*-ca, *p*-coumaric acid; fa, ferulic acid. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that for HG alone: * $P < 0.05$, ** $P < 0.01$. †† Mean value was significantly different from that for the control condition ($P < 0.01$). (B) Intracellular ROS detected by RedoxSensor Red CC-1 (red fluorescence, lower images), mitochondria labelled by Green-Fluorescent MitoTracker Green FM (green fluorescence, lower images) and mitochondrial superoxide detected by MitoSOX (green fluorescence, upper images). CCCP, *m*-chlorophenylhydrazine; TTFA, thenoyltrifluoroacetone; MnTM2PyP, Mn(III)5,10,15,20-tetrakis(*N*-methyl-pyridinium-2-yl) porphyrin.

on short-term HG-induced NF- κ B and ROS in HAEC to clarify and compare their ability to penetrate inside the mitochondria and their role against the superoxide; furthermore, the distribution of the oxidised products was also determined.

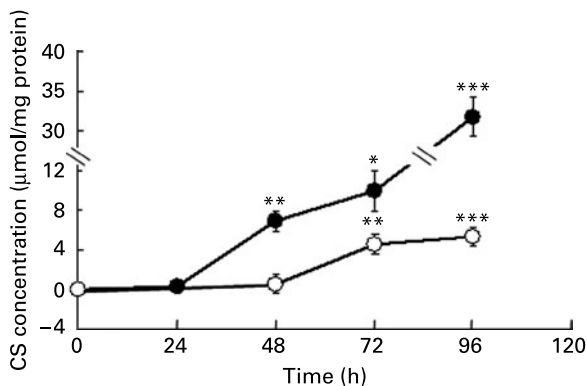


Fig. 7. *N*-(*p*-coumaroyl)serotonin (CS) concentration inside whole human aortic endothelial cells (●) and mitochondria (○). CS level was normalised by the total protein content (μ mol/mg protein). Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that at time 0: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Each compound completely inhibited NF- κ B activation and ROS production; the distribution of the mitochondrial superoxide revealed fluorescence of the mitochondrial superoxide only in HG-treated HAEC. These results indicated that CS and FS are acting on the superoxide inside the mitochondria, and that their scavenger activity against mitochondrial superoxide overproduction was effective in preventing the consequent activation of NF- κ B. In addition, the ability of CS and FS to reduce ROS was higher than that of 5-HT, *p*-ca, and fa alone^(25,29–31) in the experimental conditions used, suggesting that CS and FS antioxidant activity may be due to both 5-HT and *p*-ca or fa moieties acting together. In addition, ROS-scavenging activity by CS and FS was comparable with that exerted by 40 μ M- α -Toc.

As a consequence of the biological activities of these two serotonin derivatives, it is possible that they are acting as direct scavengers of the superoxide radical or by scavenging the peroxynitrite generated by the reaction of the superoxide with NO. Indeed, the intracellular ROS fluorescence detected using Red CC-1 and 2',7'-dichlorofluorescein diacetate, which are not specific for the superoxide, could also be due to the presence of peroxynitrite. The consequent dangerous peroxynitrite generation is then neutralised by the scavenging activity of CS and FS.

Since MitoSOX results confirmed an early uptake of CS and FS into the mitochondria, we quantified the concentration of

both compounds inside the HAEC and their mitochondria by HPLC. Even though the presence of CS and FS inside the cells was not detectable by HPLC after 10 μM treatment and 1–4 h incubation, the treatment of HAEC with 20 μM -CS or -FS from 24 to 96 h revealed a time-dependent increase of CS or FS content inside the cells and mitochondria, the increases significant after 48 h although detectable from 24 h. These results may suggest a potential role of CS and FS as protective agents against prolonged insults and may explain their protective effect *in vivo* due to the stability of their molecules, which do not show a time-dependent modification, transformation or degradation.

In conclusion, using a cell-based approach, the present study suggested a serotonin derivative mechanism against the increase of ROS-dependent VCAM-1 and MCP-1, due to the derivatives' strong antioxidant properties and scavenger abilities. The presence of CS and FS in several wild-growing plants and their seeds, as well as in grains and vegetables, suggests that they could be useful sources of natural antioxidants, and that they could be helpful in reducing the incidence of atherosclerosis and CVD especially in Western society where it is particularly high⁽¹⁾. Furthermore, they could also be taken into consideration as an alternative and/or preventive remedy against ROS-related dysfunctions.

Acknowledgements

The authors are employees of the Kyoto Prefectural University of Medicine and the work described in the present article has been funded by a Grant-in-Aid for Scientific Research (15390178 TY) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, from the Bio-oriented Technology Research Advancement Institution and from the Ministry of Agriculture, Forestry and Fisheries of Japan. All the authors designed the study. R. P. conducted the experiments and drafted the paper. Y. N., S. K., O. H. and T. Y. provided helpful technical advice and support in all the experiments carried out, and also supervised the entire work.

The authors are grateful to Professor Etsuo Niki, Professor Yoshiro Saito and Professor Yasukazu Yoshida, Human Stress Signal Research Center, AIST Kansai, Japan, for their assistance on HPLC analysis and critical discussion of data.

The authors declare that they have no other conflict of interest.

References

1. Tunstall-Pedoe H, Kuulasmaa K, Amouyel P, *et al.* (1994) Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation* **90**, 583–612.
2. Koyama N, Kuribayashi K, Seki T, *et al.* (2006) Serotonin derivatives, major safflower (*Carthamus tinctorius* L.) seed antioxidants, inhibit low-density lipoprotein (LDL) oxidation and atherosclerosis in apolipoprotein E-deficient mice. *J Agric Food Chem* **54**, 4970–4976.
3. Zhang HL, Nagatsu A, Watanabe T, *et al.* (1997) Antioxidative compounds isolated from safflower (*Carthamus tinctorius* L.) oil cake. *Chem Pharm Bull (Tokyo)* **45**, 1910–1914.
4. Ly D, Kang K, Choi JY, *et al.* (2008) HPLC analysis of serotonin, tryptamine, tyramine, and the hydroxycinnamic acid amides of serotonin and tyramine in food vegetables. *J Med Food* **11**, 385–389.
5. Niwa T, Murakami K, Ohtake T, *et al.* (2002) Peroxynitrite scavenging activities of aromatic compounds isolated from Konnyaku, *Amorphophallus konjac* K.Koch. *Biosci Biotechnol Biochem* **66**, 1386–1388.
6. Watanabe M (1999) Antioxidative phenolic compounds from Japanese barnyard millet (*Echinochloa utilis*) grains. *J Agric Food Chem* **47**, 4500–4505.
7. Kumarasamy Y, Middleton M, Reid RG, *et al.* (2003) Biological activity of serotonin conjugates from the seeds of *Centaurea nigra*. *Fitoterapia* **74**, 609–612.
8. Sarker SD, Laird A, Nahar L, *et al.* (2001) Indole alkaloids from the seeds of *Centaurea cyanus* (Asteraceae). *Phytochemistry* **57**, 1273–1276.
9. Sarker SD, Savchenko T, Whiting P, *et al.* (1997) Moschamine, *cis*-moschamine, moschamindole and moschamindolol: four novel indole alkaloids from *Centaurea moschata*. *Nat Prod Lett* **9**, 189–191.
10. Choi SW, Lee SK, Kim EO, *et al.* (2007) Antioxidant and anti-melanogenic activities of polyamine conjugates from corn bran and related hydroxycinnamic acids. *J Agric Food Chem* **55**, 3920–3925.
11. Cho SH, Lee HR, Kim TH, *et al.* (2004) Effects of defatted safflower seed extract and phenolic compounds in diet on plasma and liver lipid in ovariectomized rats fed high-cholesterol diets. *J Nutr Sci Vitaminol* **50**, 32–37.
12. Kawashima S, Hayashi M, Takii T, *et al.* (1998) Serotonin derivative, *N*-(*p*-coumaroyl) serotonin, inhibits the production of TNF- α , IL-1 α , IL-1 β , and IL-6 by endotoxin-stimulated human blood monocytes. *J Interferon Cytokine Res* **18**, 423–428.
13. Koyama N, Suzuki K, Furukawa Y, *et al.* (2008) Effects of safflower seed extract supplementation on oxidation and cardiovascular risk markers in healthy human volunteers. *Br J Nutr* (epublication ahead of print version 1 July 2008).
14. Piga R, Naito Y, Kokura S, *et al.* (2007) Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells. *Atherosclerosis* **193**, 328–334.
15. Ishiyama M, Tominaga H, Shiga M, *et al.* (1996) A combined assay of cell viability and *in vitro* cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol Pharm Bull* **19**, 1518–1520.
16. Yoshida N, Manabe H, Terasawa Y, *et al.* (2000) Inhibitory effects of vitamin E on endothelial-dependent adhesive interactions with leukocytes induced by oxidized low density lipoprotein. *Biofactors* **13**, 279–288.
17. Zou C, Wang Y & Shen Z (2005) 2-NBDG as a fluorescent indicator for direct glucose uptake measurement. *J Biochem Biophys Methods* **64**, 207–215.
18. Reddy S, Hama S, Grijalva V, *et al.* (2001) Mitogen-activated protein kinase phosphatase 1 activity is necessary for oxidized phospholipids to induce monocyte chemotactic activity in human aortic endothelial cells. *J Biol Chem* **276**, 17030–17035.
19. Ryu JW, Hong KH, Maeng JH, *et al.* (2004) Overexpression of uncoupling protein 2 in THP1 monocytes inhibits β 2 integrin-mediated firm adhesion and transendothelial migration. *Arterioscler Thromb Vasc Biol* **24**, 864–870.
20. Chen JW, Chen YH, Lin FY, *et al.* (2003) Ginkgo biloba extract inhibits tumor necrosis factor- α -induced reactive oxygen species generation, transcription factor activation, and cell adhesion molecule expression in human aortic endothelial cells. *Arterioscler Thromb Vasc Biol* **23**, 1559–1566.

21. Takahashi M, Shibata M & Niki E (2001) Estimation of lipid peroxidation of live cells using a fluorescent probe, diphenyl-1-pyrenylphosphine. *Free Radic Biol Med* **31**, 164–174.
22. Yoshida Y, Hayakawa M, Habuchi Y, *et al.* (2006) Evaluation of the dietary effects of coenzyme Q *in vivo* by the oxidative stress marker, hydroxyoctadecadienoic acid and its stereoisomer ratio. *Biochim Biophys Acta* **1760**, 1558–1568.
23. Albelda SM, Smith CW & Ward PA (1994) Adhesion molecules and inflammatory injury. *FASEB J* **8**, 504–512.
24. Gerszten RE, Garcia-Zepeda EA, Lim YC, *et al.* (1999) MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* **398**, 718–723.
25. Kaliora AC, Dedoussis GV & Schmidt H (2006) Dietary antioxidants in preventing atherogenesis. Review. *Atherosclerosis* **187**, 1–17.
26. Liu L, Zubik L, Collins FW, *et al.* (2004) The antiatherogenic potential of oat phenolic compounds. *Atherosclerosis* **175**, 39–49.
27. Collins T, Read MA, Neish AS, *et al.* (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF- κ B and cytokine-inducible enhancers. Review. *FASEB J* **9**, 899–909.
28. Nishikawa T, Edelstein D, Du XL, *et al.* (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* **404**, 787–790.
29. Kajimoto G, Ikebe Y, Yoshida H, *et al.* (1979) Studies on the antioxidative activities of metabolites of several amino acids. *J Jpn Soc Food Nutr* **32**, 41–46.
30. Luceri C, Giannini L, Lodovici M, *et al.* (2007) *p*-Coumaric acid, a common dietary phenol, inhibits platelet activity *in vitro* and *in vivo*. *Br J Nutr* **97**, 458–463.
31. Rozek A, Achaerandio I, Almajano MP, *et al.* (2007) Solid foodstuff supplemented with phenolics from grape: antioxidant properties and correlation with phenolic profiles. *J Agric Food Chem* **55**, 5147–5155.