British Journal of Pharmacology (1998) 123, 1097–1102

The possible mechanisms of the antiproliferative effect of fullerenol, polyhydroxylated C_{60}, on vascular smooth muscle cells

Liang-Huei Lu, [1]Yuan-Teh Lee, Huei-Wen Chen, [2]Long Y. Chiang & [3]Huei-Chen Huang

Department of Pharmacology, and [1]Department of Internal Medicine, College of Medicine, and [2]Center for Condensed Matter Sciences, National Taiwan University, Taipei, Taiwan

1 The possible mechanisms of the antiproliferative effect of polyhydroxylated fullerene (fullerenol), a novel free radical trapper, were studied in rat vascular smooth muscle cells (A7r5 cells) and compared with the effect of ascorbic acid.

2 Fullerenol-1 and ascorbic acid inhibited the proliferative responses in a number of cells, including rat aortic smooth muscle cells (A7r5 cells), human coronary artery smooth muscle cells, and human CEM lymphocytes (CEM cells) in a concentration dependent manner.

3 At the concentration range of 10^{-6} to 10^{-2} M, fullerenol-1 and ascorbic acid concentration-dependently inhibited the proliferative responses stimulated by serum in A7r5 cells. Fullerenol-1 was more potent than ascorbic acid.

4 The production of O_2^- induced by alloxan, a diabetogenic compound, was reduced by fullerenol-1 (10^{-4} M) in the presence of A7r5 cells.

5 The cytosolic protein kinase C activity of A7r5 cells stimulated by phorbol ester was reduced by 10^{-3} M fullerenol-1, but not ascorbic acid (10^{-4}–10^{-2} M) and fullerenol-1 at lower concentrations (10^{-6}–10^{-4} M).

6 In contrast, the membraneous protein tyrosine kinase activity of A7r5 cells stimulated by foetal calf serum was significantly reduced by fullerenol-1 (10^{-6}–10^{-3} M) and ascorbic acid (10^{-4}–10^{-2} M). Again, the inhibitory activity of fullerenol-1 was greater than that of ascorbic acid.

7 Our results demonstrate that fullerenol-1 and ascorbic acid exhibit inhibitory effects on transduction signals in addition to their antioxidative property. It is suggested that the antiproliferative effect of fullerenol-1 on vascular smooth muscle cells may partly be mediated through the inhibition of protein tyrosine kinase.

Keywords: Antiproliferative effect; fullerenol; ascorbic acid; antioxidant; protein tyrosine kinase; protein kinase C; smooth muscle cells (vascular)

Introduction

Atherosclerosis and post-angioplasty restenosis are characterized by the abnormal accumulation of vascular smooth muscle cells, inflammatory cells, and extracellular matrix proteins (Lundergan *et al*., 1991; Ross, 1993). The proliferative responses of both vascular smooth muscle cells (SMC) and mononuclear cells are important in the pathogenesis of atherosclerosis and restenosis. We have previously shown that some hydroxylated plant phenols, curcumin (diferuloylmethane), esculetin (6,7-dihydroxycoumarin), scoparone (6,7-dimethoxycoumarin) and baicalein (5,6,7-trihydroxyflavone), exhibited antiproliferative effects in cultured vascular SMC and mononuclear cells (Huang *et al*., 1992a,c; 1993; 1994a,b). Recently, we found that fullerenols also reduced the proliferative responses of vascular SMC and mononuclear cells. Fullerenol-1 used in this study is one of the water-soluble polyhydroxylated fullerene C_{60} derivatives with a simple chemical composition of 60 carbons and multiple hydroxy groups (Figure 1) (Chiang *et al*., 1992). Fullerenic truncated-cage molecules represent an intriguing new class of carbons, which possess high conjugated olefins with π-electron interactions in a spherical molecular orbital. In the case of reactive unpaired electron-containing molecules, such as hydroxyl and superoxide radicals, the attack of these reactants on fullerenic compounds results in multiple additions on the olefinic moieties of C_{60} to the formation of corresponding functionalized fullerene derivatives. The combination of the moderate electron affinity of fullerenol and its allylic hydroxy functional groups makes fullerenol a suitable candidate for application as a free radical remover of water-soluble antioxidant in biological systems (Chiang *et al*., 1995). In this study, the effect of fullerenol-1 on the proliferative response of vascular smooth muscle cells was investigated and compared to that of ascorbic acid. Ascorbic acid (vitamin C) is a water-soluble, chain breaking antioxidant that can regenerate α-tocopherol from its chromanoxyl radical form (Levine, 1986). Low plasma and tissue concentrations of ascorbate have been identified as a risk factor for atherosclerosis (Carew *et al*., 1987). We have previously shown that scoparone and esculetin, the plant antioxidants, inhibited protein tyrosine kinase activity in connection with the reduction of proliferative response of vascular smooth muscle cells (Huang *et al*., 1992a; 1993). It was of interest to investigate the possibility that fullerenol-1 and ascorbic acid exert their effects by molecular mechanisms in addition to their intrinsic antioxidative radical absorption properties.

Methods

Measurement of cell proliferation

A7r5 cells (rat aortic smooth muscle cells) were grown in Dulbecco's modified Eagle's medium (supplemented with 10%

[3]Author for correspondence at: Department of Pharmacology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan.

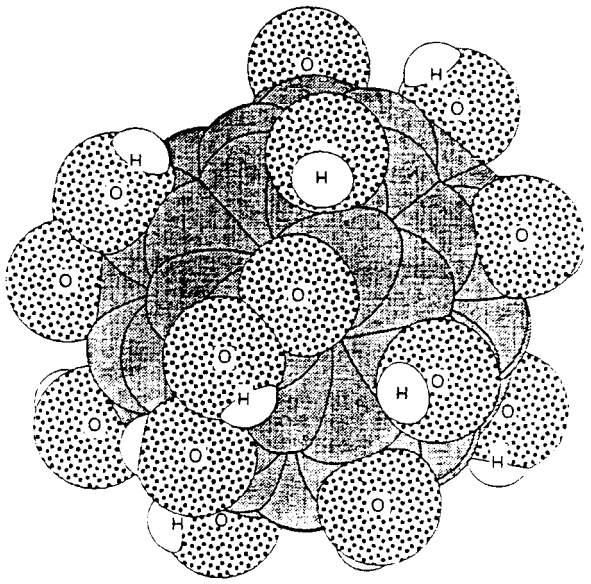

Figure 1 The structure of fullerenols, water-soluble polyhydroxylated fullerene derivatives.

v/v foetal calf serum, 100 u ml^{-1} penicillin, and 10^{-6} g ml^{-1} streptomycin) and human coronary artery smooth muscle cells (human CASMC) in medium containing SmBM (Clonetics Corporation's BulletKit containing growth factors and antimicrobials). The proliferative responses of A7r5 cells and human CASMC were determined by the uptake of tritiated thymidine (Huang *et al*., 1992b). Before all the experiments, confluent smooth muscle cells were rendered quiescent by culturing for 48 h (with one medium change after 24 h) in 0.5% v/v foetal calf serum (FCS) instead of 10%. In human CASMC (2.5×10^4 cells/well), 5% v/v FCS together with the test compound were then added to the medium for 24 h before [^{3}H]-thymidine (0.2 μCi/well) was included in the medium. Twenty-four hours after the addition of [^{3}H]-thymidine, the cells were harvested and the amount of [^{3}H]-thymidine incorporated into DNA was counted in a liquid scintillation counter. In A7r5 cells (10^4 cells/well), 5% v/v FCS and the test compound were added to the medium for 16 h before [^{3}H]-thymidine (1 μCi/well) was included in the medium. Four hours after addition of [^{3}H]-thymidine, the cells were harvested and the [^{3}H]-thymidine incorporated was counted. CEM cells (human CEM lymphocytes) were cultured in RPMI-1640 medium containing 10% v/v FCS. Assessment of tumour cell proliferation was performed by the [^{3}H]-thymidine incorporation assay as described previously (Huang *et al*., 1994a; Watabe *et al*., 1984). The test compound was added to the tumour cell suspension (5×10^3 cells/well). Reactions were incubated in a CO_2 incubator at 37°C for 0.5 h before 1 μCi [^{3}H]-thymidine was added to each well. After an additional 4 h of incubation, cells were harvested and [^{3}H]-thymidine incorporated to cells was counted.

Each experiment was performed in triplicate and repeated 5 or 6 times. The inhibitory activities of the test compounds are expressed as percentage of the untreated control values. The concentration evoking 50% maximal inhibition (IC_{50}) was calculated for each experiment.

Cell viability was determined with the trypan blue dye exclusion method. After addition of the test compound for 20 h, cells were harvested from the dishes with 0.1% w/v trypsin solution and the viability was examined by the trypan blue dye exclusion test. The cell number was estimated by microscopic cell counting with a haemocytometer.

Measurement of free radical scavenging activity

An established method of cytochrome C reduction by superoxide in vascular rings as described previously (Heim *et al*., 1991; Huang 1992c) was modified to measure superoxide production in cultured vascular smooth muscle cells. A7r5 cells (2×10^6 cells ml^{-1}) were incubated for 10 min at 37°C before the experiments. Cytochrome C (37.5 μM) was used to assay for O_2^- and NEM (3 mM) was added after the test period to prevent further time-dependent reduction of cytochrome C. The O_2^- production was stimulated by adding alloxan immediately after the addition of cytochrome C to the incubation medium for 10 min. In order to establish that O_2^- was responsible for the reduction of cytochrome C, some samples were incubated in the presence of superoxide dismutase (SOD) (150 u ml^{-1}) from bovine erythrocytes (Sigma). Reduction of the cytochrome C was completely inhibited in the presence of SOD. The amount of O_2^- produced is expressed as nmol min^{-1}. The effect of the test compound was observed by adding the compound 10 min before the addition of alloxan.

Measurement of protein kinase C activity

The protein kinase C activity of A7r5 cells were measured as previously described (Huang *et al*., 1993). A7r5 cells were lyzed in ice-cold buffer A containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 300 mM sucrose, 2 mM phenylmethylsulphonylfluoride and 23 μM leupeptin by a sonicator. After centrifugation at 2,500 *g* for 5 min, the supernatant was centrifuged at 100,000 *g* for 1 h, and the resulting supernatant was retained as the cytosol fraction. After ultracentrifugation, the pellets were washed with buffer B (buffer A without sucrose) by centrifugation (12,000 *g*, 15 min), resuspended in buffer B with 1% v/v Triton X-100, and resonicated. After a 1 h incubation at 4°C, the homogenate was centrifuged at 100,000 *g* for 1 h, and the supernatant thus obtained was the membrane fraction. Protein kinase C activity was measured with a protein kinase C assay kit (Amersham) by a modification of a mixed micelle assay (Hannun *et al*., 1985), activating the enzyme with 39 μM phorbol-12-myristate-13-acetate. After removal of protein by acid precipitation, labelled peptide was bound to a phosphocellulose disc and phosphorylation of the peptide was measured by a scintillation counter. The protein content was determined according to the method of Lowry *et al*. (1951). The results are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}.

Measurement of protein tyrosine kinase activity

The membranous protein tyrosine kinase activity of A7r5 cells was measured as previously described (Huang *et al*., 1993). A7r5 cells were lyzed in ice-cold extraction buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 50 kiu aprotinin and 1 mM phenylmethylsulphonylfluoride by a sonicator. The suspension was centrifuged first at 800 *g* for 10 min and then at 100,000 *g* for 1 h. The final pellet was resuspended in solubilizing buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $Mg(C_2H_3O_2)_2$, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, 1 mM dithiothreitol and 30 μM Na_3VO_4 and resonicated. After a 1 h incubation at 4°C, the homogenate was

centrifuged at 100,000 *g* for 1 h. The supernatant thus obtained was the membrane fraction. The protein tyrosine kinase activity in the resultant supernatant was assayed with a protein tyrosine kinase assay kit (Gibco) by incubating with the synthetic peptide substrate, RR-SRC (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly), in the presence of [γ-^{32}P]-ATP, and activating enzyme with 5% v/v FCS. After removal of protein by acid precipitation, labelled peptide was bound to a phosphocellulose disc and phosphorylation of the peptide was measured by a scintillation counter. The protein content was determined according to the method of Lowry *et al*. (1951). The results are expressed as pmol^{32}P incorporated mg^{-1} protein min^{-1}.

Materials

Water-soluble fullerenol-1 (Figure 1), a polyhydroxylated fullerene derivative was synthesized by a sequence of reactions involving electrophilic addition of nitronium tetrafluoroborate ($NO^+BF_4^-$) onto fullerene molecules in the presence of organocarboxylic acids as the key-step as described previously (Chiang *et al*., 1992). Rat aortic smooth muscle cells (A7r5 cells) and human CEM lymphocytes (CEM cells; human lymphoblastoid leukaemia cells) were purchased from the American Type Culture Collection (Rockville, MD). Human coronary artery smooth muscle cells (human CASMC) and smooth muscle growth medium, SmBM (BulletKit containing growth factors and antimicrobials), were purchased from Clonetics Corporation (San Diego, CA). L-Ascorbic acid (vitamin C), alloxan, cytochrome C (horse heart, type III), superoxide dismutase (SOD, bovine erythrocytes), N-ethylmaleimide (NEM), aprotinin, leupeptin, phenylmethylsulfonyl fluoride, dithiothreitol, genistein (4′,5,7-trihydroxyisoflavone) and H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine 2HCl) were purchased from Sigma Chemicals Co. (St. Louis, MO). Foetal calf serum, Dulbecco's modified Eagle's medium (DMEM) and RPMI medium were purchased from Gibco Lab. (Grand Island, NY). [Methyl-^{3}H]-thymidine (5 Ci mmol^{-1}) and [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) were purchased from Amersham Co. (Buckinghamshire, U.K.).

Statistical analysis

The data are expressed as mean $\pm$ s.e.mean. *P* values less than 0.05 were considered significant (ANOVA analysis and Student's *t* test).

Results

Antiproliferative effect

Cell proliferation was studied by measuring the incorporation of [^{3}H]-thymidine into DNA in different cells. The control values of [^{3}H]-thymidine incorporation in A7r5 cells (10^4 cells/well), human coronary artery smooth muscle cells (human CASMC, 2.5×10^4 cells/well), and CEM lymphocytes (5×10^3 cells/well) induced by 5% foetal calf serum was 7,266$\pm$755, 3,250$\pm$837, and 7,031$\pm$332 c.p.m./well, respectively. Exposure of different cells to the test antioxidants (10^{-6}–10^{-2} M), fullerenol-1 and ascorbic acid inhibited serum-induced proliferation in a concentration-dependent manner. As shown in Figure 2, both antioxidants concentration-dependently inhibited proliferative responses in A7r5 cells. At concentrations higher than 10^{-4} M, the inhibitory activity of fullerenol-1 was significantly greater than that of ascorbic acid. The IC_{50} and

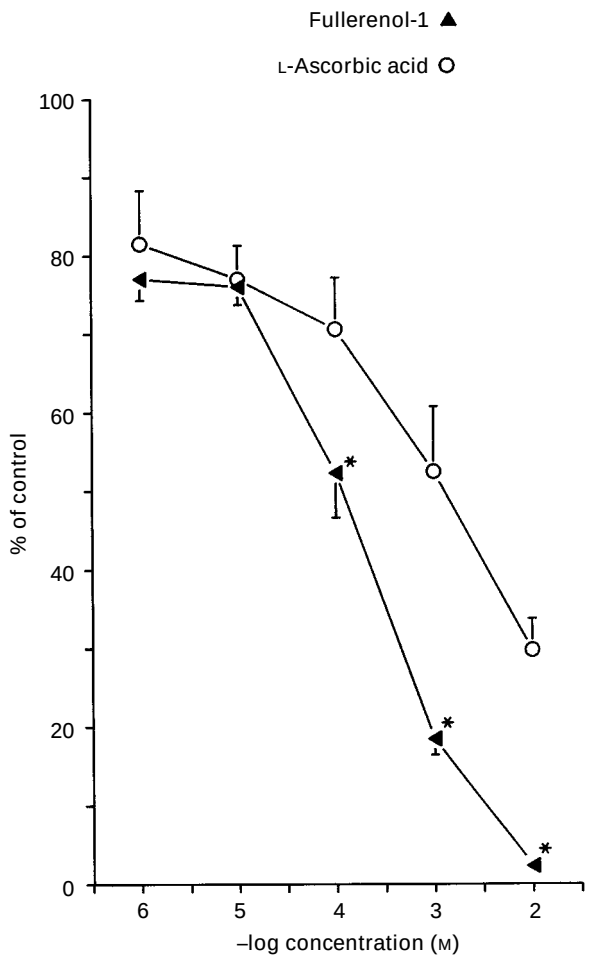

Figure 2 Effects of fullerenol-1 and ascorbic acid on the proliferative responses of A7r5 cells. Rat aortic smooth muscle cells (A7r5 cells, 10^4 cells/well) were stimulated with 5% v/v foetal calf serum. The inhibitory activities of test compounds are expressed as percentage of the controls stimulated with foetal calf serum in the absence of test compound (% of control). The control value was 7,266$\pm$755 c.p.m./well. Points with vertical lines represent the means and s.e.mean of triplicate experiments ($n=6$). *$P<0.05$, compared to the inhibitory activity of ascorbic acid.

Table 1 Antiproliferative activities of fullerenol-1 and ascorbic acid on various cells

	n	IC_{50} (M)	*Max. suppression* (at 10^{-2} M) (%)
Fullerenol-1			
A7r5 cells	6	$1.2\pm0.3\times10^{-4}$	97.7$\pm$0.8
Human CASMC	6	$3.2\pm1.2\times10^{-5}$	100.0$\pm$0.0
CEM cells	5	$6.4\pm2.0\times10^{-6}$	90.0$\pm$3.9
Ascorbic acid			
A7r5 cells	6	$1.5\pm0.5\times10^{-4}$	70.2$\pm$4.1
Human CASMC	5	$2.6\pm1.1\times10^{-4}$	95.0$\pm$2.0
CEM cells	5	$1.1\pm0.5\times10^{-3}$	94.1$\pm$0.8

The inhibitory effects of test compounds were determined by [^{3}H]-thymidine incorporation induced in various cells by 5% v/v foetal calf serum. The inhibitory activities are expressed as percentage of untreated control values stimulated by serum (% of control). The untreated control values of [^{3}H]-thymidine incorporation were 7,266$\pm$755 c.p.m./well for rat aortic smooth muscle cells (A7r5 cells), 3,250$\pm$837 c.p.m./well for human coronary artery smooth muscle (human CASMC), and 7,031$\pm$332 c.p.m./well for human CEM lymphocytes (CEM cells). Values are means$\pm$s.e.mean for triplicate experiments.

maximal suppression values in various cells are shown in Table 1.

Cell viability was determined by the trypan blue dye exclusion method. After the addition of foetal calf serum and the test compound for 20 h, cells were harvested from the dishes and the viability was examined. The cell number was then counted. As compared to the control level stimulated by foetal calf serum, the effective antiproliferative concentrations for fullerenol-1 and ascorbic acid were 10^{-6} to 10^{-2} M. The cell number in untreated basal condition was 10^5 cells. As shown in Figure 3, higher concentrations ($\geqslant 10^{-3}$ M) of the test compounds significantly decreased the cell number as compared to the basal level. At concentrations lower than 10^{-3} M, the cell viability was not affected.

Effect on O_2^- production

Alloxan stimulated O_2^- production from intact vascular rings in a concentration-dependent manner (Heim *et al*., 1991; Huang *et al*., 1992c). In A7r5 cells, 10 mM alloxan significantly increased O_2^- production to $1{,}319.1 \pm 89.1$ nmol min^{-1} ($n=12$). In order to establish that O_2^- was responsible for the reduction of cytochrome C, some samples were incubated in the presence of superoxide dismutase (SOD). Reduction of the cytochrome C was completely inhibited in the presence of SOD (150 u ml^{-1}) ($n=6$). Fullerenol-1 (10^{-6}–10^{-4} M) tended to reduced O_2^- production stimulated by alloxan, and the reduction achieved significance at 10^{-4} M ($36.7 \pm 7.1\%$ inhibition, $n=5$) (Figure 4). However, ascorbic acid (10^{-4}–10^{-2} M) did not significantly affect O_2^- production by A7r5 cells challenged with alloxan.

Effect on protein kinase C activity

The cytosolic protein kinase C activity of A7r5 cells was 22.0 ± 1.4 pmol mg^{-1} protein min^{-1} (control, $n=16$) in the presence of 39 μM phorbol-12-myristate-13-acetate. The protein kinase C activity was inhibited by direct incubation of the cells with 10^{-3} M fullerenol-1 ($98.3 \pm 1.4\%$ inhibition, $n=5$), but not at lower concentrations (10^{-6}–10^{-4} M) (Figure 5). Protein kinase C activity was unaffected by ascorbic acid (10^{-4}–10^{-2} M) (Figure 5). In our assay system, 10^{-5} M H-7, a protein kinase C inhibitor, inhibited protein kinase C activity by $27.0 \pm 2.7\%$ ($n=5$).

Effect on protein tyrosine kinase activity

The increased protein tyrosine kinase activity in the membrane fraction stimulated by 5% v/v foetal calf serum was 3.2 ± 0.2 pmol mg^{-1} protein min^{-1} ($n=20$) in A7r5 cells. The increased protein tyrosine kinase activity stimulated by serum

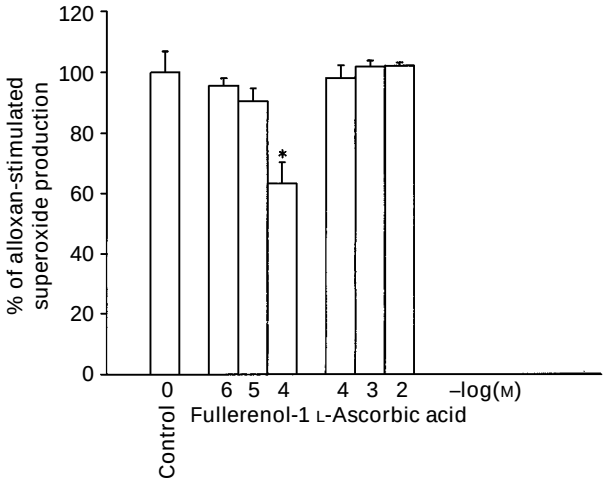

Figure 4 Effects of fullerenol-1 and ascorbic acid on alloxan-stimulated O_2^- production in A7r5 cells. A7r5 cells (2×10^6 cells ml^{-1}) were incubated with cytochrome C and 10 mM alloxan for 10 min. The effect of the test compound was observed by adding the compound 10 min before the addition of alloxan. The data are expressed as percentage of the O_2^- produced by the corresponding control group (experimental/control $\times$ 100%). Each column represents the mean $\pm$ s.e.mean ($n=5$). The mean alloxan-stimulated O_2^- concentration was $1{,}319.1 \pm 89.1$ nmol min^{-1} ($n=12$). *$P<0.05$, compared to alloxan-stimulated sample without test compound.

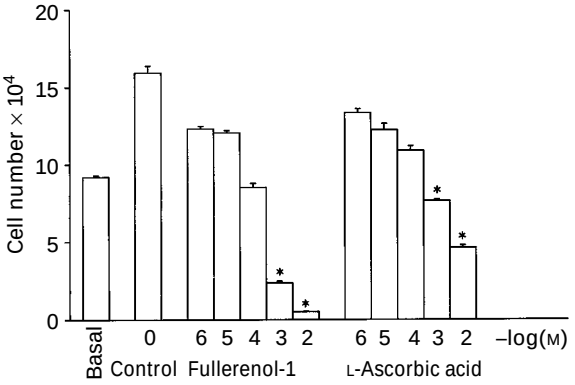

Figure 3 Effects of fullerenol-1 and ascorbic acid on A7r5 cells growth. Quiescent A7r5 cells (basal level, 10^5 cells) were stimulated with 5% v/v foetal calf serum. After addition of foetal calf serum for 20 h in the absence (control level) or presence of test compound, cells were harvested from the dishes with a 0.1% w/v trypsin solution and the viability examined by trypan blue dye exclusion test. The number of viable cells was estimated by microscopic cell counting with a haemocytometer. Each column represents the mean $\pm$ s.e.mean ($n=5$). *$P<0.05$, compared to the cell number in the basal condition (basal level, 10^5 cells).

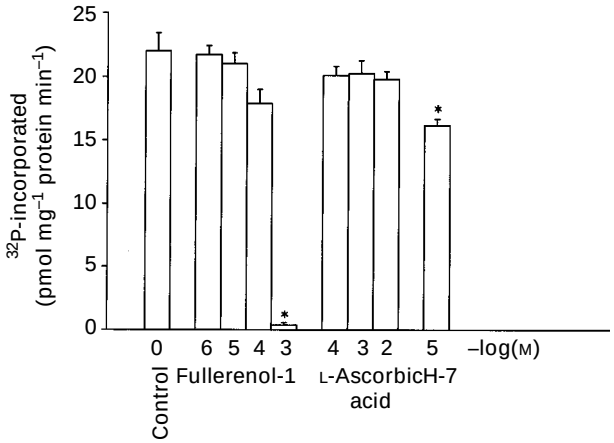

Figure 5 Effects of fullerenol-1 and ascorbic acid on protein kinase C activity in A7r5 cells. Subcellular fractions were prepared as described in Methods. The cytosolic protein kinase C was stimulated with 39 μM phorbol-12-myristate-13-acetate in the absence or presence of the test compound. The effects of the test compounds were compared to that of 10^{-5} M H-7 (a protein kinase C inhibitor). Protein kinase C activities are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}. Each column represents the mean $\pm$ s.e.mean ($n=5$). *$P<0.05$, when compared to the control value without test compound.

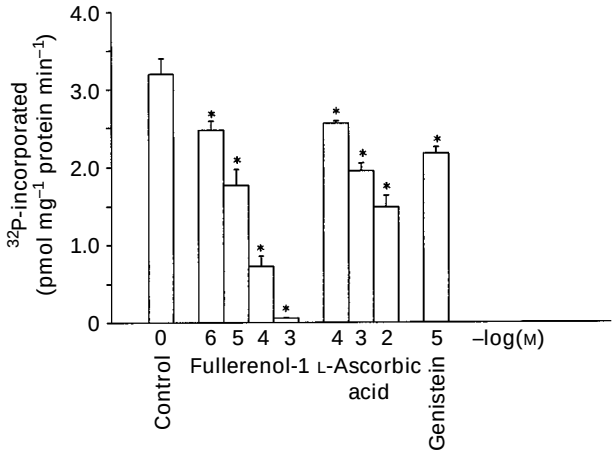

Figure 6 Effects of fullerenol-1 and ascorbic acid on protein tyrosine kinase activity in A7r5 cells. Subcellular fractions were prepared as described in Methods. The membranous protein tyrosine kinase was stimulated by 5% v/v foetal calf serum in the absence or presence of the test compound. The effects of the test compounds were compared to that of 10^{-5} M genistein (a protein tyrosine kinase inhibitor). Protein tyrosine kinase activities are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}. Each column represents the mean$\pm$s.e.mean ($n=5$). *$P<0.05$, when compared to the control value without test compound.

was reduced by fullerenol-1 ($10^{-6}-10^{-3}$ M) and ascorbic acid ($10^{-4}-10^{-2}$ M) ($n=5$) (Figure 6). The inhibition was $22.8\pm3.7\%$ and 98.2 ± 0.1 for 10^{-6} and 10^{-3} M fullerenol-1, $20.0\pm0.8\%$ and $53.4\pm4.8\%$ for 10^{-4} and 10^{-2} M ascorbic acid, respectively. In our assay system, 10^{-5} M genistein, a protein tyrosine kinase inhibitor, inhibited protein tyrosine kinase activity by $32.0\pm2.5\%$ ($n=5$).

Discussion

The present results demonstrated for the first time, that fullerenol-1, a water-soluble fullerene C_{60} derivative, concentration-dependently inhibited the proliferative responses of vascular smooth muscle cells and mononuclear cells, including rat aortic smooth muscle cells (A7r5 cells), human coronary artery smooth muscle cells, and human CEM lymphocytes. Fullerenol-1 was more potent than ascorbic acid. Atherosclerosis and post-angioplasty restenosis are characterized by the abnormal accumulation of vascular smooth muscle cells, inflammatory cells, and extracellular matrix proteins (Lundergan *et al*., 1991; Ross, 1993). Thus, fullerene C_{60} derivatives may be useful in the study and treatment of atherosclerosis and restenosis. In the present study, the superoxide radical production induced by alloxan in vascular smooth muscle cells was suppressed by fullerenol-1. The diabetogenic drug, alloxan, has been shown to stimulate O_2^- production which is inhibited by superoxide dismutase (SOD) (Cohen & Heikkila, 1974; Grankvist, 1981; Heim *et al*., 1991; Huang *et al*., 1992c). It appears possible that fullerenol-1 may act as a free radical scavenger. As O_2^- inactivates the endothelium-derived relaxing factor (EDRF) and SOD protects against such inactivation (Gryglewski *et al*., 1986), fullerenol-1 may partly act through suppression of O_2^-, thus protecting EDRF and leading to vascular dilatation and antiatherogenesis (Glasser *et al*., 1996). In atherosclerosis, the oxidation of low-density lipoprotein (LDL) is important in the pathogenesis of cardiovascular disease (Berliner *et al*., 1995). Thus, another mechanism by which antioxidants may contribute to the reduction in risk of cardiovascular disease is through antioxidant protection of LDL.

Epidemiological studies indicate that antioxidant vitamins may protect against atherosclerosis (Gey *et al*., 1987). Low plasma and tissue concentrations of ascorbate have been identified as a risk factor for atherosclerosis. Plasma ascorbate has been shown to be inversely correlated with coronary disease mortality (Carew *et al*., 1987). The present results demonstrate that ascorbic acid inhibited the proliferative responses of vascular smooth muscle cells and CEM lymphocytes. However, the *in vitro* antiproliferative effects of vitamin C in cell lines were found to be less potent than fullerenol-1. Fullerenol-1 and ascorbic acid should exhibit certain molecular mechanisms in addition to their intrinsic antioxidative radical absorption properties, leading to the inhibition of cell proliferation. Our results further show that fullerenol-1 may inhibit proliferative responses via inhibition of transduction signals leading to DNA synthesis. There are several signalling pathways for growth factors and mitogens (Powis & Kozikowski, 1991). In the present study, the effects of fullerenol-1 and ascorbic acid on protein tyrosine kinase and protein kinase C activities were investigated. Receptors for growth factors such as platelet-derived growth factor and epidermal growth factor are protein tyrosine kinase that, when activated by ligand binding, phosphorylate themselves as well as other proteins to affect the processes in the nucleus directly linked to cell proliferation. Tyrosine kinase activity has also been found to be associated with other growth factors and oncoproteins, such as insulin, *src*, *erbB*, *fms*, *yes*, *neu*, *sis*, *abl*, *fes*, and *ras* (Bishop, 1983). On the other hand, peptides such as vasopressin and bombesin act partly through cell surface receptors, coupled to a guanine nucleotide binding protein to activate a specific phospholipase C that hydrolyzes phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate and diacyglycerol. Diacylglycerol then activates protein kinase C (Powis & Kozikowski, 1991). Our results show that fullerenol-1 and ascorbic acid concentration-dependently reduced protein tyrosine kinase activity, but affected protein kinase C activity much less, indicating that fullerenol-1 and ascorbic acid may act partly through inhibition of protein tyrosine kinase to reduce the smooth muscle cell proliferation.

In summary, we found that fullerenol-1 exhibited a more potent antiproliferative effect in vascular smooth muscle and mononuclear cells than ascorbic acid. Our results further demonstrated that fullerenol-1 and ascorbic acid inhibit transduction signals in addition to their antioxidative activity. It is suggested that the antiproliferative effect of fullerenol-1 may partly be mediated through inhibition of the growth-related signal, protein tyrosine kinase. Ascorbic acid may also partly act through the inhibition of protein tyrosine kinase. Thus, fullerene C_{60} derivatives may be useful in the study and treatment of atherosclerosis and post-angioplasty restenosis.

This investigation was supported by grants from the National Science Council and Department of Health, Taiwan.

References

BERLINER, J.A., NAVAB, M., FOGELMAN, A.M., FRANK, J.S., DEMER, L.L., EDWARDS, P.A., WATSON, A.D. & LUSIS, A.J. (1995). Atherosclerosis: basic mechanisms. oxidation, inflammation, and genetics. *Circulation,* **91,** 2488 – 2496.

BISHOP, J.M. (1983). Cellular oncogenes and retroviruses. *Ann. Rev. Biochem.,* **52,** 301 – 354.

CAREW, T., SCHWENKE, D.C. & STEINBERG, D. (1987). Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 7725 – 7729.

CHIANG, L.Y., LU, F.J. & LIN, J.T. (1995). Free radical scavenging activity of water-soluble fullerenol. *J. Chem. Soc. Chem. Commun.,* 1283 – 1284.

CHIANG, L.Y., UPASANI, R.B. & SWIRCZEWSKI, J.W. (1992). Versatile nitronium chemistry for C_{60} fullerene functionalization. *J. Am. Chem. Soc.,* **114,** 10154 – 10157.

COHEN, G. & HEIKKILA, R.E. (1974). The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J. Biol. Chem.,* **249,** 2447 – 2452.

GEY, K.F., BRUBACHER, G.B. & STAHELIN, H.B. (1987). Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. *Am. J. Clin. Nutr.,* **45,** 1368 – 1377.

GLASSER, S.P., SELWYN, A.P. & GANZ, P. (1996). Atherosclerosis: risk factors and the vascular endothelium. *Am. Heart, J.* **131,** 379 – 384.

GRANKVIST, K. (1981). Alloxan-induced luminol luminescence as a tool for investigating mechanisms of radical-mediated diabetogenicity. *Biochem. J.* **200,** 685 – 690.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factors. *Nature,* **320,** 454 – 456.

HANNUN, Y.A., LOOMIS, C.R. & ROBERT, M.B. (1985). Activation of protein kinase C by Triton X-100 mixed micelles containing diacyglycerol and phosphatidylserine. *J. Biol. Chem.,* **260,** 10039 – 10043.

HEIM, K.F., THOMAS, G. & RAMWELL, P.W. (1991). Superoxide production in the isolated rabbit aorta and effect of alloxan, indomethacin and nitrovasodilators. *J. Pharmacol. Exp. Ther.,* **256,** 537 – 541.

HUANG, H.C., HSIEH, L.M., CHEN, H.W., LIN, Y.S. & CHEN, J.S. (1994a). Effects of baicalein and esculetin on transduction signals and growth factors expression in T-lymphoid leukemia cells. *Eur. J. Pharmacol. Mol. Pharmacol.,* **268,** 73 – 78.

HUANG, H.C., HUANG, Y.L., CHANG, J.H., CHEN, C.C. & LEE, Y.T. (1992a). Possible mechanism of immunosuppressive effect of scoparone (6,7-dimethoxycoumarin). *Eur. J. Pharmacol.,* **217,** 143 – 148.

HUANG, H.C., JAN, T.R. & YEH, S.F. (1992b). Inhibitory effect of curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. *Eur. J. Pharmacol.,* **221,** 381 – 384.

HUANG, H.C., LAI, M.W., WANG, H.R., CHUNG, Y.L., HSIEH, L.M. & CHEN, C.C. (1993). Antiproliferative effect of esculetin on vascular smooth muscle cell: Possible roles of signal transduction pathways. *Eur. J. Pharmacol.,* **237,** 39 – 44.

HUANG, H.C., LEE, C.R., WENG, Y.I., LEE, M.C. & LEE, Y.T. (1992c). Vasodilator effect of scoparone (6,7-dimethoxycoumarin) from a Chinese herb. *Eur. J. Pharmacol.,* **218,** 123 – 128.

HUANG, H.C., WANG, H.R. & HSIEH, L.M. (1994b). Antiproliferative effect of baicalein, a flavonoid from Chinese herb, on vascular smooth muscle cells. *Eur. J. Pharmacol.,* **251,** 91 – 93.

LEVINE, M. (1986). New concepts in the biology and biochemistry of ascorbic acid. *N. Engl. J. Med.,* **314,** 892 – 902.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265 – 275.

LUNDERGAN, C.F., FOEGH, M.L. & RAMWELL, P.W. (1991). Peptide inhibition of myointimal proliferation by angiopeptin, a somatostatin analogue. *J. Am. Coll. Cardiol.,* **17,** 132B – 136B.

POWIS, G. & KOZIKOWSKI, A. (1991). Growth factor and oncogene signalling pathways as targets for rational anticancer drug development. *Clin. Biochem.,* **24,** 385 – 397.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* **362,** 801 – 809.

WATABE, S., SENDO, F., KIMURA, S. & ARAI, S. (1984). Activation of cytotoxic polymorphonucleuar leukocytes by in vivo administration of a streptoccal preparation, OK432. *J. Natl. Cancer Inst.,* **72,** 1365 – 1370.

(Received May 2, 1997
Revised December 1, 1997
Accepted December 3, 1997)