Cytotoxicity of water-soluble fullerene in vascular endothelial cells

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Yamawaki, Hideyuki, and Naoharu Iwai. Cytotoxicity of water-soluble fullerene in vascular endothelial cells. Am J Physiol Cell Physiol 290:C1495–C1502, 2006. First published January 11, 2006; doi:10.1152/ajpcell.00481.2005.—Nanoscale materials are presently under development for diagnostic (nanomedicine) and electronic purposes. In contrast to the potential benefits of nanotechnology, the effects of nanomaterials on human health are poorly understood. Nanomaterials are known to translocate into the circulation and could thus directly affect vascular endothelial cells (ECs), causing vascular injury that might be responsible for the development of atherosclerosis. To explore the direct effects of nanomaterials on endothelial toxicity, human umbilical vein ECs were treated with 1–100 μg/ml hydroxyl fullerene [C60(OH)24; mean diameter, 7.1 ± 2.4 nm] for 24 h. C60(OH)24 induced cytotoxic morphological changes such as cytosolic vacuole formation and decreased cell density in a dose-dependent manner. Lactate dehydrogenase assay revealed that a maximal dose of C60(OH)24 (100 μg/ml) induced cytotoxic injury. Proliferation assay also showed that a maximal dose of C60(OH)24 inhibited EC growth. C60(OH)24 did not seem to induce apoptosis but caused the accumulation of polyubiquitinated proteins and facilitated autophagic cell death. Formation of autophagosomes was confirmed on the basis of Western blot analysis using a specific marker, light chain 3 antibody, and electron microscopy. Chronic treatment with low-dose C60(OH)24 (10 μg/ml for 8 days) inhibited cell attachment and delayed EC growth. In the present study, we have examined, for the first time, the toxicity of water-soluble fullerenes to ECs. Although fullerenes changed morphology in a dose-dependent manner, only maximal doses of fullerenes caused cytotoxic injury and/or death and inhibited cell growth. EC death seemed to be caused by activation of ubiquitin-autophagy cell death pathways. Although exposure to nanomaterials appears to represent a risk for cardiovascular disorders, further in vivo validations are necessary.

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OsO₄ solution at 4°C for 2 h. After dehydration in an ethanol gradient (50–100% for 10 min each), samples were embedded in EPON812 at 60°C for 2 days. Ultrathin sections (80 nm) were stained using uranyl acetate and lead citrate. Sections were examined using an electron microscope (model JEM2000EX; JEOL, Tokyo, Japan) at 100 kV.

LDH cytotoxicity assay. Cytotoxicity assay was performed using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) in accordance with the instructions of the manufacturer. Briefly, after treating HUVECs at ~90% confluence in six-well plates with C₆₀(OH)₂₄ (1–100 μg/ml) for 24 h, culture medium was collected. Lactate dehydrogenase (LDH), a stable cytosolic enzyme released during cell lysis, was measured at 490-nm absorbance using a standard 96-well plate reader. Cytotoxicity was expressed relative to basal LDH release in untreated control cells.

Proliferation assay. Proliferation assay was performed using a Cell Counting-8 kit (Dojindo Laboratories, Kumamoto, Japan) according to the instructions of the manufacturer. Briefly, after treating HUVECs at ~30% confluence in 12-well plates with C₆₀(OH)₂₄ (1–100 μg/ml) for 24 h, water-soluble tetrazolium salt (WST-8) was added for 3 h and culture medium was collected. Conversion of WST-8 into formazan by living cells (active mitochondria) was measured using a standard 96-well plate reader at 450-nm absorbance. Total numbers of living cells were compared with untreated control samples.

Western blot analysis. Western blot analysis was performed as described previously (29). Proteins were obtained by homogenizing HUVECs with Triton X-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, and 0.1% protease inhibitor mixture; Nacalai Tesque, Kyoto, Japan). Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL). Equal amounts of proteins (15 μg) were separated by SDS-PAGE (7.5% and 14%) and transferred onto nitrocellulose membrane (Pall, Ann Arbor, MI). After being blocked with 3% BSA, membranes were incubated with primary antibody (1:1,000 dilution) at 4°C overnight and membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the ECL system (Amersham Biosciences, Little Chalfont, UK). Experiments were performed three or more times, and equal loading of protein was ensured by measuring total actin expression.

Activity assay for 20S proteasome. Activity of the 20S proteasome was determined using the 20S proteasome assay kit (Calbiochem, San Diego, CA) according to the instructions of the manufacturer. Cells were lysed in Triton X-based lysis buffer as described above. The assay mixture contained 178 μl of reaction buffer (25 mM HEPES and 0.5 mM EDTA, pH 7.6), 10 μl of substrate [10 μM Suc-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC)], 2 μl of SDS (0.03%), and 10 μl of cell lysate (10 μg of protein). After incubation for 30 min at 37°C, the fluorescence of liberated AMC was measured using excitation and emission wavelengths at 340 and 450 nm, respectively.

Microarray analysis. Total RNA was isolated from HUVECs treated with or without C₆₀(OH)₂₄ (100 μg/ml, 24 h) using an RNeasy Kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. Only samples with A₂₆₀/A₂₈₀ between 1.7 and 2.2 (measured in 10 mM Tris•HCl, pH 7.6) were considered suitable for use. Hybridization samples were prepared according to the GeneChip Expression Analysis Technical Manual (701021, Rev. 5, section 2,

Fig. 1. Representative photomicrographs of human umbilical vein endothelial cells (HUVECs) treated with hydroxyl fullerene [C₆₀(OH)₂₄]. HUVECs at ~90% confluence were treated with C₆₀(OH)₂₄ (A and E: 0 μg/ml; B: 1 μg/ml; C: 10 μg/ml; D and F: 100 μg/ml) for 24 h. Scale bars, 50 μm (A–D) and 100 μm (E and F).
“Eukaryotic Sample and Array Processing,” chapt. 1, “Eukaryotic Target Preparation”; http://www.affymetrix.com/support/technical/manuals.affx). Total RNA (2 μg) was amplified for each sample. Next, cRNA (30 μg) was fragmented in 40 μl of 1× fragmentation buffer. Hybridization cocktails were made as described in the GeneChip Expression Analysis Technical Manual (701021, Rev. 5, section 2, chapt. 2, “Eukaryotic Target Hybridization”) and hybridized to human genome U133 plus2.0 chips at 60 rpm and 45°C for 16 h using the Hybridization Oven 640 110 V (no. 800138; Affymetrix, Santa Clara, CA). Human genome U133 plus2.0 chips (Affymetrix) comprise 54,000 probe sets and provide comprehensive coverage of the transcribed human genome on a single array to analyze expression levels of >47,000 transcripts and variants, including 38,500 well-characterized human genes plus ~6,500 new genes. GeneChips were stained with streptavidin-phycoerythrin using a Fluidics Station 450 (00-0079; Affymetrix). After being washed extensively, GeneChips were scanned using a GeneChip Scanner 3000 (00-0074; Affymetrix). Data were analyzed using GeneChip Operating Software version 1.1 (no. 690036; Affymetrix) according to GeneChip Expression Analysis Data Analysis Fundamentals (chapt. 4, “First-Order Data Analysis and Data Quality Assessment”; and chapt. 5, “Statistical Algorithms Reference”; http://www.affymetrix.com/support/technical/manuals.

Fig. 2. Fullerene-induced cytotoxic injury in HUVECs. HUVECs at ~90% confluence were treated with C_{60}(OH)_{24} (1–100 μg/ml for 24 h). Culture medium was then collected. A: lactate dehydrogenase (LDH) released into the supernatant was measured using a commercially available kit. Cytotoxicity was expressed relative to basal LDH release in controls (n = 4–11). B: living cell number was calculated using water-soluble tetrazolium salt (WST-8), n = 3; **P < 0.01 vs. controls.

Fig. 3. Fullerene-inhibited cell growth in HUVECs. HUVECs at ~30% confluence were treated with C_{60}(OH)_{24} (1–100 μg/ml) for 24 h. A: representative photomicrographs are shown (a: Start; b: 0 μg/ml; c: 1 μg/ml; d: 10 μg/ml; and e: 100 μg/ml; all for 24 h). Scale bar, 100 μm. B: total number of living cells was counted using WST-8. Results are shown relative to controls, n = 6; **P < 0.01 vs. controls.
To allow comparison, all chips were scaled to a target intensity of 500 on the basis of all probe sets on each chip. Comparison of GeneChip array data was obtained using custom analysis services (Kurabo Industries, Osaka, Japan). Kurabo Industries is the authorized service provider for Affymetrix Japan (Tokyo, Japan). Genes that were significantly upregulated (top 100 genes; see Supplemental Table 1; http://ajpcell.physiology.org/cgi/content/full/00481.2005/DC1) or downregulated (top 100 genes; Supplemental Table 2) in two independent experiments are summarized. Microarray data were deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GenBank accession no. GSE3364).

**Statistical analysis.** Data are means ± SE. Statistical evaluations were performed using an unpaired Student’s *t*-test. Values of *P* < 0.05 were considered statistically significant.

**RESULTS**

**Fullerene induces cytotoxic morphological changes in HUVECs.** To examine the direct effects on vascular ECs, cultured HUVECs were treated with C$_{60}$(OH)$_{24}$ for 24 h. Fullerenes (1–100 µg/ml) induced cytotoxic morphological changes in HUVECs such as vacuole formation in the cytosol and decreased cell density in a dose-dependent manner (Fig. 1, A–D). Figure 1, E (Control) and F (100 µg/ml C$_{60}$(OH)$_{24}$), represent low-magnification pictures, and cell density was clearly decreased after treatment with fullerene.

**Fullerene increases release of LDH from HUVECs.** To assess EC injury by fullerene quantitatively, we examined the effects of fullerenes on endothelial LDH release, a marker of cell death and injury of the plasma membrane. Although 10 µg/ml C$_{60}$(OH)$_{24}$ showed slight cytotoxic morphological changes (Fig. 1C), only the maximal concentration of C$_{60}$(OH)$_{24}$ (100 µg/ml, 24 h, *n* = 8) significantly increased LDH release into culture medium (Fig. 2A) (LDH increased 2.4 ± 0.2-fold vs. controls, *n* = 11; *P* < 0.01). To further explore the degree of cell injury after fullerene treatment, we calculated the living cell number using WST-8. A maximal dose of 100 µg/ml C$_{60}$(OH)$_{24}$ killed 58.0 ± 1.7% of cells (*n* = 3; *P* < 0.01 vs. controls) (Fig. 2B).

**Fullerene has antiproliferative effects on HUVECs.** To examine the effects of fullerene on cell growth, HUVECs at ~90% confluence were treated with C$_{60}$(OH)$_{24}$ (1–100 µg/ml) for 24 h and then the total number of living cells was measured using WST-8. HUVEC growth was inhibited by C$_{60}$(OH)$_{24}$ in a dose-dependent manner (Fig. 3A). Quantitative analysis (Fig. 3B) revealed that only the maximal concentration of 100 µg/ml C$_{60}$(OH)$_{24}$ significantly inhibited cell growth (64.2 ± 4.7%, *n* = 6; *P* < 0.01 vs. controls).

**Fullerene does not induce apoptosis in HUVECs.** We next examined whether fullerene induces apoptosis in vascular ECs. Serum starvation but not a maximal dose of C$_{60}$(OH)$_{24}$ (100 µg/ml, 24 h) induced cleavage of caspase-3 (17 kDa) and PARP (Fig. 4), which are markers for the activation of the apoptotic cascade (5, 20). This suggests that fullerene does not induce apoptosis in HUVECs. Notably, protein bands for both caspase-3 and PARP were not readily apparent after treatment with C$_{60}$(OH)$_{24}$ in the context of serum starvation.

**Fig. 4. Fullerene does not induce apoptosis in HUVECs.** After HUVECs at ~90% confluence were treated with 100 µg/ml C$_{60}$(OH)$_{24}$ for 24 h, total cell lysates were harvested. Cleaved caspase-3 (17 kDa; A) and poly(ADP-ribose) polymerase (PARP; B) expression were determined using Western blot analysis. Equal protein loading was confirmed using total actin antibody.

**Fig. 5. Fullerene treatment induces polyubiquitination in HUVECs.** After HUVECs at ~90% confluence were treated with C$_{60}$(OH)$_{24}$ (1–100 µg/ml) for 24 h, total cell lysates were harvested. A: accumulation of polyubiquitin was determined using Western blot analysis. Equal protein loading was confirmed using total actin antibody (*n* = 8). B: activity of the 20S proteasome was determined using a commercially available kit. Activity of 20S proteasomes was measured on the basis of fluorescence of liberated amino-4-methyl-coumarin (AMC) using excitation and emission wavelengths at 340 and 450 nm. Results shown are relative to controls (*n* = 3).
caspase-3 and PARP were weak in C60(OH)24-treated samples. This finding is consistent with fullerene-treated samples in other immunoblot analysis experiments. We speculate that this phenomenon is due to protein degeneration by fullerene.

**Fullerene induces accumulation of polyubiquitinated proteins in HUVECs.** Because activation of the ubiquitin-proteasome system represents another death pathway, protein polyubiquitination by fullerene was examined. C60(OH)24 (1–100 μg/ml, 24 h) induced protein polyubiquitination in a dose-dependent manner (Fig. 5A). Proteasome activity assay showed that 100 μg/ml C60(OH)24 (24 h) did not directly modify it (Fig. 5B) (1.09 ± 0.27-fold increase vs. controls; n = 3). Proteasome activity in cell lysates was suppressed almost completely by a proteasome inhibitor, MG132 (1 μM).

**Ultrastructural features of HUVECs: fullerene facilitates autophagic cell death.** We next performed ultrastructural analysis using transmission electron microscopy. The cytoplasm of untreated HUVECs (control) (Fig. 6A) contained small vesicles. In vascular smooth muscle cells, the formation of small vesicles reportedly occurs under normal physiological conditions to remove abnormal proteins and cytoplasmic macromolecules (15). Treatment of HUVECs with the maximal dose of C60(OH)24 (100 μg/ml, 24 h) caused extensive vacuolization and internalization of fullerene (Fig. 6B). Fullerene aggregates were observed primarily within autophagosome-like vesicles (Fig. 6, C and D). To show that vesicles represented autophagosomes, Western blot analysis was performed to detect LC3 II because conversion of LC3 I (cytosolic isoform) to LC3 II (membrane-bound form) are frequently used markers for autophagosomes (9, 32). Figure 7 clearly shows that 100 μg/ml C60(OH)24 (24 h) increased levels of the LC3 II isoform (n = 4).

**Fig. 6.** Ultrastructural features of HUVECs treated with C60(OH)24. HUVECs were treated without (A; control) or with 100 μg/ml C60(OH)24 (B–D) for 24 h. N, nucleus. Arrows indicate vacuoles with phagocytic function. Scale bars, 2 μm (A and B) and 500 nm (C and D).

**Fig. 7.** Fullerene induced the formation of autophagosomes in HUVECs. After HUVECs at ~90% confluence were treated with 100 μg/ml C60(OH)24 for 24 h, total cell lysates were harvested. Autophagosomes were detected using Western blot analysis with light chain (LC)3 antibody (n = 4). Top band represents cytosolic LC3 I, and bottom band represents membrane-bound LC3 II, a typical marker for autophagosomes. Equal protein loading was confirmed using total actin antibody.

**Chronic effects of low-dose fullerene on EC toxicity.** We examined the chronic effects of low concentrations of C60(OH)24 (1–10 μg/ml, up to 10 days) on EC toxicity. During this time, cells were subcultured three times (passages 3–6). Media containing fullerene were changed every 2 days. Chronic treatment with 1 μg/ml C60(OH)24 for 10 days had no significant effects on EC toxicity (data not shown). Figure 8 shows the morphological features of HUVECs treated without (control) or with 10 μg/ml C60(OH)24. Fullerene was treated soon after splitting cells from passages 3 to 4. On day 2, both control and fullerene-treated cells reached subconfluence, but fullerene-treated cells showed clear morphological changes such as cytosolic vacuole formation and spindlelike cell shape.
After the second passage, control cells reached confluence within 4 days. In contrast, the attachment of cells (day 3) was bad in fullerene-treated groups and cell growth speed was slow. On day 8, fullerene-treated cells reached confluence, but the shapes of the cells were bad (spindlelike) and vacuole formation was commonly observed, suggesting the possibility that fullerene-resistant types of cells survived and increased.

Microarray analysis. Finally, microarray analysis was performed using total RNA from HUVECs treated with the maximal dose of C_60(OH)_{24} (100 μg/ml, 24 h). Results from 2 independent samples are summarized in Supplemental Tables 1 and 2. Of note, although these were not top 100 genes, several genes related to the ubiquitin-proteasome system were significantly upregulated by fullerene [HECT (a COOH-terminal catalytic homologous to E6-AP-COOH terminus domain), C2, and WW domain containing E3 ubiquitin protein ligase 2 (ratio, 2.3 to 1), ubiquitin-specific protease 31 (ratio, 1.7 to 1), ubiquitin-specific protease 32 (ratio, 1.7 to 1), and ubiquitin-conjugating enzyme E2 (ratio, 1.5 to 1)].

DISCUSSION

The major findings of the present study are that water-soluble fullerene directly affects vascular ECs to cause cytotoxic injury or cell death and inhibition of cell growth. To the best of our knowledge, this study provides the first demonstration of the direct effects of water-soluble fullerene on vascular endothelium. In other human cells, including dermal fibroblasts, liver carcinoma cells (HepG2), neuronal astrocytes, and T-lymphocytes (Jurkat cells), recent reports have noted that water-soluble fullerene shows cytotoxic effects, presumably via production of reactive oxygen species (22, 24). Notably, several types of water-soluble fullerene derivatives are available [e.g., hydroxyl fullerene used herein, dendritic C_60 monadduct (22), malonic acid C_60 (22) and nano-C_60 (24) (basically pristine C_60)], and cytotoxicity to cells varies depending on the fullerene subtype used, presumably because of surfactant chemistry, including a balance between hydrophobicity and hydrophilicity (3).

Some novel mechanistic insights of this study are that fullerene causes EC injury or cell death by increasing the accumulation of polyubiquitinated proteins in the cytosol and facilitating excessive autophagic cell death. EC injury and death are closely related to the initiation of atherosclerosis (14, 23). Furthermore, through nitric oxide production, ECs offer important protective functions against ischemic heart disease, including myocardial infarction, by inhibiting platelet aggregation (16) and lowering blood pressure (27). We thus propose that exposure to nanomaterials is a potential risk for cardiovascular disease, including atherosclerosis and ischemic heart disease. However, because quantitatively significant EC toxicity from water-soluble fullerene was observed only at high dosage, further validations (particularly in vivo) are needed.

We recently found that carbon black (CB), a chemically inert carbon nanoparticle present in diesel exhaust particles...
including metals (TiO$_2$, SiO$_2$, Co, Ni, polyvinyl chloride), on report (21) examined the effects of several nanomaterials, mechanisms remain unclear, our findings indicate that the related ECs. Furthermore, CB did not cause accumulation of oxygenase-1, and prostaglandin endoperoxide synthase 2. inflammatory mediators, including E-selectin, ICAM-I, IL-8, heme revealed that CB stimulated the induction of several proinflam-

tosis. Because angiogenesis is crucial to the maintenance of vascular integrity by forming collateral vessels in response to tissue ischemia (11), fullerene inhibition of EC growth may be related to the progression of ischemic heart disease. In addition to cell death and injury, fullerene also inhibited EC growth. Impair-

ment of EC growth may be related to impairment of angiogenesis. Because angiogenesis is crucial to the maintenance of vascular integrity by forming collateral vessels in response to tissue ischemia (11), fullerene inhibition of EC growth may be related to the progression of ischemic heart disease. Collectively, the present findings support the concept that exposure to fullerene could be a risk for atherosclerosis and ischemic heart disease.

The present study used 1–100 µg/ml fullerene concentrations for in vitro experiments. The pathophysiological concentra-
tions of fullerene are barely known. In addition to engi-

eered nanomaterials, traffic-derived nanoparticles are known to represent a risk for cardiovascular disorders, including atherosclerosis and ischemic heart disease (2, 4). The maximal concentration of particulate matter <2.5 µm in Chongqing, one of the biggest cities in China, was ~700 µg/m$^3$ (daily average) (26), indicating that an individual could inhale ~10,000 µg of particulate matter during the course of 24 h there. This value is equivalent to ~1 µg/ml when the extra-
cellular fluid volume is 12 L in a 60-kg person. The fullerene dosage used in this study was up to 100-fold higher than this level.

The kinetics of water-soluble fullerene in vivo have not yet been completely determined. Normally, inhaled microparticles are cleaned off by alveolar macrophages via phagocytosis. However, this is not applicable to nanoparticles (2, 19), which appear to translocate to extrapulmonary sites via blood and lymph and thus reach other tissues (19). Using radiolabeled water-soluble fullerene administered intravenously to rats, Yamago et al. (28) demonstrated that most fullerenes moved rapidly to the liver (within 1 h) and then were distributed to various other tissues, including spleen, lung, kidney, heart, and brain. Extraction seems slow, and >90% was retained in the body 1 wk later, raising concern about chronic toxic effects. Although we could not clearly observe EC cytoxicity after acute treatment with low-dose fullerene (10 µg/ml, 24 h), treatment for 8 days seems to enhance toxicity (Fig. 8). The effects of chronic exposure to low-dose fullerene in vivo need to be examined, particularly with regard to the cardiovascular system.

In summary, in the present study, we examined the direct effects of water-soluble fullerene on vascular endothelial cells to explore the potential toxicity of fullerene in humans, especially regarding the cardiovascular systems. We found that fullerene causes cytoxic injury or cell death in vascular ECs, indicating that exposure to fullerene could represent a risk for atherosclerosis and ischemic heart disease. Because cytotoxicity by water-soluble fullerene occurs only at high doses, further validation experiments using blood vessels and animal models are warranted.

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