Adverse effects of fullerenes on endothelial cells:
Fullerol C₆₀(OH)₂₄ induced tissue factor and ICAM-1 membrane expression and apoptosis in vitro

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Abstract: We studied the effects of a C₆₀ water suspension at 4 µg/mL (nC₆₀) and the water soluble fullerol C₆₀(OH)₂₄ at final concentrations of 0–100 µg/mL on human umbilical vein endothelial cells (HUVECs) in culture. We found that a 24 hr treatment of HUVECs with C₆₀(OH)₂₄ at 100 µg/mL significantly increased cell surface expression of ICAM-1 (CD54) (67 ± 4% CD54+ cells vs. 19 ± 2% CD54+ cells in control; p < 0.001). In addition, this treatment induced the expression of tissue factor (CD142) on HUVECs (54 ± 20% CD142+ cells vs. 4 ± 2% CD142+ cells in control; p = 0.008) and increased exposure of phosphatidylserine (PS) (29 ± 2% PS+ cells vs. 12 ± 5% PS+ cells in control; p < 0.001). Analysis of cell cycle and DNA fragmentation (TUNEL) showed that both nC₆₀ and C₆₀(OH)₂₄ caused G1 arrest of HUVECs and C₆₀(OH)₂₄ induced significant apoptosis (21 ± 2% TUNEL+ cells at 100 µg/mL of C₆₀(OH)₂₄ vs. 4 ± 2% TUNEL+ cells in control; p < 0.001). We also demonstrated that both nC₆₀ and C₆₀(OH)₂₄ induced a rapid concentration dependent elevation of intracellular calcium [Ca²⁺]. This could be inhibited by EGTA, suggesting that the source of [Ca²⁺] in fullerene stimulated calcium flux is predominantly from the extracellular environment. In conclusion, fullerol C₆₀(OH)₂₄ had both pro-inflammatory and pro-apoptotic effects on HUVECs, indicating possible adverse effects of fullerenes on the endothelium.

Keywords: endothelial cells, fullerenes, tissue factor, apoptosis, ICAM-1, flow cytometry

Introduction
Carbon fullerenes have enormous practical potential in the field of biomedical nanotechnology. Fullerenes and carbon nanotubes have already had a profound impact on the development of diagnostic biosensors, drug delivery nanosystems, and imaging nanoprobes for intravascular use. In addition, fullerenes can be used as components in a variety of plastics, including filtration membranes (Bosi et al 2003; Endo et al 2004; Bianco et al 2005; Sinha and Yeow 2005; Djordjevic et al 2006; Lacerda et al 2006; Polizu et al 2006; Harrison and Atala 2007). It has been reported that hydroxylated fullerene derivatives have potent antioxidant properties (Dugan et al 1996; Tsai et al 1997; Huang et al 1998; Straface et al 1999; Wang et al 1999; Jin et al 2000). Furthermore, the cytoprotective effects of fullerene derivatives in oxidative stress, mostly by free radical scavenging, have been demonstrated in different cell lines in vitro, and also on animal models, in vivo (Lin et al 1999; Tsao et al 1999; Lai et al 2000; Gharbi et al 2005; Daroczi et al 2006). It has been shown that fullerene derivatives have anti-viral and anti-bacterial properties. Also, their photodynamic cytotoxicity has been employed in experimental anti-tumor therapy and pathogen inactivation...
was prepared by continuous stirring 400 mg of C60 powder in a concentration of 4 (nC60) (C60 of 99.5% purity from SES Res., Houston, TX, USA) (Millli-Q H2O) was prepared in parallel by continuous stirring way as nC60 suspension. For 2 weeks at room temperature and manipulated the same way. The supernatant was diluted with the culture medium to a final concentration of 1–100 µg/mL. As a control, the fullerene vehicle (Millili-Q H2O) was prepared in parallel by continuous stirring for 2 weeks at room temperature. After stirring, the suspension was centrifuged twice at 4000 g (4°C) with fullerenol C60(OH)24 or a C60 fullerene water suspension (nC60) on cultured human umbilical vein endothelial cells.

Materials and methods

Fullerenes

The water soluble poly-hydroxylated fullerene derivative fullerenol C60(OH)24 (at final concentrations of 1–100 µg/mL) from MER Corp., Tuscon, AZ was used for all experiments. In addition, the water nanocrystalline suspensions of insoluble C60 (nC60) (C60 of 99.5% purity from SES Res., Houston, TX, USA) was prepared by continuous stirring 400 mg of C60 powder in 400 mL of Milli-Q water for 2 weeks at room temperature. After stirring, the suspension was centrifuged twice at 4000 g for 20 minutes to remove larger aggregates of undissolved fullerene. The final supernatant which contained 21 µg/mL of nC60 was collected, and stored at 4 °C. The concentration of nC60 was determined by extracting the C60 from the suspension by toluene and analyzing the absorbance at 336 nm, as described by Lyon et al (2006). In tissue culture experiments, the supernatant was diluted with the culture medium to a final concentration of 4 µg/mL. As a control, the fullerene vehicle (Millili-Q H2O) was prepared in parallel by continuous stirring for 2 weeks at room temperature and manipulated the same way as nC60 suspension.

Analysis of hydrodynamic size distribution of fullerene particles using Dynamic Light Scattering (DLS)

The Malvern Zetasizer Nano ZS instrument (Southborough, MA) with a back scattering detector was used for measuring the hydrodynamic size (diameter) in batch mode (no fractionation) at 25 °C in a low volume quartz cuvette. The relative particle volume is used as the size class for histograms of the size distribution (the diameter is plotted on the abscissa and the particle volume on the ordinate). The average diameter (weighted by particle volume) of the largest peak in the volume distribution is reported as the hydrodynamic size, along with its standard deviation and the percentage of the total volume contained in the largest peak. The nC60 sample concentration was 21 µg/mL as described in the stock preparation (see above). The hydroxylated C60 derivative (C60(OH)24) was weighed and dissolovded in 10 mM NaCl to give a final concentration of 2 mg/mL and filtered through a 0.02µm filter (Anotop 10 Plus, Whatman International Ltd., Maidstone, England). A minimum of twelve measurements were made per sample.

Human umbilical vein endothelial cells (HUVECs)

HUVECs (2nd passage) from Cambrex (Walkersville, MD), were cultured in either 6 or 24 well plates (Becton Dickinson Labware, Franklin Lakes, NJ) with EGM-2 media containing 2% fetal bovine serum and supplements (Cambrex). Cells were used for experiments when they reached 80%–90% confluence (~48 hrs in culture). HUVECs were washed and incubated for 24 hrs at 37 °C with fullerenol C60(OH)24 or fullerene nC60 in water diluted one to five with the EGM-2 medium. After incubation, cells were harvested with 20 mM HEPES buffer with 100 mM NaCl, 0.5% BSA and 10 mM EDTA, pH 7.4. After harvesting, the cells were centrifuged (300 g for 5 min), washed with Hanks’ balanced salt solution with 0.35% bovine serum albumin (HBSS/BSA) and used for analysis.

Antibodies

Cy-Chrome conjugated anti-human monoclonal antibody (mAb) to CD54 (ICAM-1, clone HA58), phycoerythrin-conjugated (PE) anti-human mAb to CD142 (tissue factor, clone HTF-1), isotype matched controls, annexin V (FITC-conjugated), and an APO-BRDU kit were purchased from BD Pharmingen (San Diego, CA). All antibodies were titrated to ensure saturating concentrations.

Labeling of endothelial cells

Approximately 10^5 cells were resuspended in 50 µL of HBSS/BSA and incubated with saturating concentration of the mAbs or annexin V. Non-labeled cells and cells incubated with either relevant isotype controls or with annexin V in the presence of 20 mM EDTA were prepared as controls.
After 20 min incubation at room temperature, the suspension of labeled cells was diluted with 2 mL of HBSS/BSA and centrifuged (300 × g for 5 min). Sedimented cells were resuspended in 0.5 mL of HBSS/BSA and analyzed using flow cytometry.

**Flow cytometry of endothelial cells**

Cell samples were analyzed as described previously (Simak et al. 2002; Simak, Holada, and Vostal et al. 2002). Briefly, a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with CELLQuest software, with forward scatter (FSC) and side scatter (SSC) in linear mode was used. Populations of intact cells were gated according to their light-scattering characteristics in order to exclude debris, and 10,000 gated cells were analyzed for each sample. The total percentage (%) of CD54+, CD142+, and PS+ (annexin V-binding) cells was evaluated. Apoptosis (TUNEL) was analyzed using the APO-BRDU kit following the manufacturer’s instructions (BD Pharmin-gen, San Diego, CA). In addition, cell cycle analysis was performed using ModFit software (Verity Software House, Topsham, ME).

**Intracellular free Ca²⁺ assay**

The acute effect of fullerenes on intracellular free Ca²⁺ concentration was studied in HUVEC loaded with a Ca²⁺-sensitive probe (FURA-2-AM). Changes in fluorescence in individual cells (n = 100) were monitored at 340 nm and 380 nm excitation (the rate of data capture was 170/min) using a Nikon inverted epi-fluorescence/phase microscope equipped with a low-light level integrating CCD camera with a microphotometer assembly (InCyt I/P-2 TM Imaging and Photometry System, Intracellular Imaging Inc., Cincinnati, OH). Intracellular [Ca²⁺], in real time was calculated from the ratio of emission detected at 510 nm at two excitation wavelengths (340 nm and 380 nm) and by comparison to a standard curve established for these settings using buffers of known free [Ca²⁺] with the InCyt Im2 software.

**Statistical analyses**

Results are presented as means ± standard deviations (SD). Kruskal-Wallis One Way Analysis of Variance on Ranks and multiple comparisons using Student-Newman-Keuls method or Dunett’s method was used where appropriate (SPSS 12; SPSS, Chicago, IL). A value of p < 0.05 was considered statistically significant.

**Results**

**Hydrodynamic size distribution of fullerene particles in nC₆₀ and C₆₀(OH)₂₄ preparations**

The mean size distribution by volume and the mean hydrodynamic diameter of nC₆₀ and C₆₀(OH)₂₄ are shown in Figure 1. Based on the size distributions of particles present in the nC₆₀ water suspension (Figure 1A), the major water soluble component is about 224 nm in size. The small peak at about 5000 nm corresponds to approximately 2% of the total particle volume, and may be due to aggregates. Since unmodified nC₆₀ is not immediately water soluble, we stirred C₆₀ in water for two weeks, which resulted in a nanocrystalline suspension of stable aggregates (nC₆₀). nC₆₀ refers to an unknown number ‘n’ of C₆₀ molecules agglomerating to form the suspension. The formed clusters contain unmodified C₆₀ in their centers, surrounded by partially hydroxylated C₆₀ on the outside (Fortner et al. 2005). In contrast, the fullerenol C₆₀(OH)₂₄ is very soluble in water. Based on its size distribution by volume (Figure 1B), only one peak at 4.3 nm was observed, as expected for the hydroxylated C₆₀ derivative.

**High dose fullerenol C₆₀(OH)₂₄ induced elevated HUVEC surface expression of ICAM-1 (CD54), tissue factor (CD142), and phosphatidylserine (PS)**

HUVECs were cultured for 24 hrs with different concentrations of fullerenol C₆₀(OH)₂₄, (1, 10, 50, and 100 µg/mL), fullerene nC₆₀ at 4 µg/mL, or with the vehicle. Flow cytometry was used to analyze the difference in expression of several surface markers. Figure 2 shows that the 24 hr treatment of HUVECs with 100 µg/mL C₆₀(OH)₂₄ significantly increased cell surface expression of ICAM-1 (CD54) (67 ± 4% CD54+ cells vs 19 ± 2% CD54+ cells in control; p < 0.001). This treatment also induced expression of tissue factor (CD142) on HUVECs (54 ± 20% CD142+ cells vs 4 ± 2% CD142+ cells in control; p = 0.008). In addition, an increase in exposed phosphatidylserine (PS, detected by Annexin V) (29 ± 2% PS+ cells vs 12 ± 5% PS+ cells in control; p < 0.001) was identified. However, treating the HUVECs with lower concentrations of fullerenol C₆₀(OH)₂₄ or nC₆₀ at 4 µg/mL did not induce a significant increase of surface expression of CD54, CD142 or PS.
Both fullerene C$_{60}$ and fullerenol C$_{60}$(OH)$_{24}$ induced G1 arrest and a high dose of fullerenol induced significant apoptosis (TUNEL) in HUVECs

Analysis of the cell cycle by flow cytometry showed that both nC$_{60}$ at 4 µg/mL and C$_{60}$(OH)$_{24}$ at concentrations 10-100µg/mL caused significant G1 arrest in HUVECs after 24 hours of treatment (Figure 3). In addition, fullerenol C$_{60}$(OH)$_{24}$ at 100 µg/mL induced significant apoptosis after 24 hours treatment of HUVECs, as compared to control cells treated with the vehicle (21 ± 2% TUNEL$^+$ cells at 100 µg/mL of C$_{60}$(OH)$_{24}$ vs. 4 ± 2% TUNEL$^+$ cells in control; p < 0.001) (Figure 4). Treating the HUVECs with lower concentrations of fullerenol C$_{60}$(OH)$_{24}$ or nC$_{60}$ at 4 µg/mL did not induce significant increase of TUNEL$^+$ cells.

Both fullerene nC$_{60}$ and fullerenol C$_{60}$(OH)$_{24}$ induced acute Ca$^{2+}$ influx in HUVECs

The acute effect of fullerenes on intracellular free Ca$^{2+}$ concentration [Ca$^{2+}$], was studied, using a ratio fluorometry in HUVECs. Cells were loaded with a Ca$^{2+}$-sensitive probe FURA-2AM. We demonstrated that both nC$_{60}$ and C$_{60}$(OH)$_{24}$ induced a rapid concentration dependent elevation of [Ca$^{2+}$], nC$_{60}$ caused a continuous increase in [Ca$^{2+}$], and was about ten times more potent compared to C$_{60}$(OH)$_{24}$ (Figure 5). This activity of both C$_{60}$(OH)$_{24}$ and nC$_{60}$ could be inhibited by EGTA, suggesting that the source of [Ca$^{2+}$] in fullerene stimulated calcium flux is predominantly from the extracellular environment.
Figure 2 Flow cytometric analysis of HUVECs treated for 24 hrs with fullerenol C_{60}(OH)_{24} (100 µg/mL) or the vehicle (CTRL). Data are presented as means ± SD (n = 3).

Figure 3 Cell cycle analysis of HUVECs treated 24 hrs with fullerenol C_{60}(OH)_{24} (100 µg/mL) or the vehicle (CTRL). Representative histograms of 3 experiments are shown.
Figure 4 Flow cytometric analysis of TUNEL assay of HUVECs treated for 24 hrs with fullerol C_{60}(OH)_{24} (100 µg/mL) or the vehicle (CTRL). Representative double fluorescence plots of 3 experiments are shown.

A

\[ \text{[Ca}^{2+}]_i \text{ (nM)} \]

\[ \text{EGTA pre-incubation + C}_{60}(\text{OH})_{24} \]

\[ \text{TIME (s)} \]
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B

% of responding cells

Δ[Ca^{2+}]_i (nM)

0 10 20 30 40 50 60 70 80 90

C_60(OH)_{24} (μg/mL)

C

nC_{60}

EGTA pre-incubation + nC_{60}

TIME (s)
Figure 5 Analysis of C₆₀(OH)₂₄ and nC₆₀ induced intracellular Ca²⁺ increase (∆[Ca²⁺]ᵢ) in HUVECs. (A) The upper tracing shows the time course of C₆₀(OH)₂₄-induced ∆[Ca²⁺]ᵢ in a representative cell. The lower tracing shows a time course in a representative cell in a Ca²⁺ free solution (containing 1 mM EGTA). Arrows indicate when C₆₀(OH)₂₄ (final conc. 80 µg/mL) was added. There was no response observed when the fullerenol vehicle was added (not shown). (B) A dose-response curves for the effect of C₆₀(OH)₂₄ on ∆[Ca²⁺]ᵢ and the percentage of cells responded. Mean values of 30 cells ± SD are shown. (C) The upper tracing shows the time course of nC₆₀-induced ∆[Ca²⁺]ᵢ in a representative cell. The lower tracing shows a time course in a representative cell in a Ca²⁺ free solution (containing 1 mM EGTA). Arrows indicate when nC₆₀ (final conc. 10 µg/mL) was added. There was no response observed when the nC₆₀ vehicle was added (not shown). (D) A dose-response curves for the effect of nC₆₀ on ∆[Ca²⁺]ᵢ and the percentage of cells responded. Mean values of 30 cells ± SD are shown.

Discussion
Our study showed that fullerenol induced pro-inflammatory activation of cultured HUVECs, which was indicated by the increase of CD54 (ICAM-1) and CD142 (TF) surface expression. The intercellular adhesive molecule 1 (ICAM-1), also referred to as CD54, is an adhesive receptor of the immunoglobulin gene superfamily. It is constitutively expressed at low levels on most types of vascular endothelial cells, and is highly upregulated on pro-inflammatory cytokine activated endothelium (Mutin et al 1997a). Tissue factor (TF), also referred to as CD142, is a cellular receptor and cofactor of activated factor VIIa. Tissue factor initiates activation of the plasma coagulation system, which leads to the generation of thrombin activity (Steffel et al 2006). Although functional TF is not exposed in significant levels on resting HUVECs, its expression is greatly increased after stimulation with pro-inflammatory cytokines and some other stimuli, similarly to ICAM-1 (Mutin et al 1997b). In addition, the observed increased exposure of phosphatidylserine (PS) on the plasma membrane of HUVECs indicates a disturbance of the membrane asymmetry, likely due to pro-apoptotic stimulation or other types of cell activation. Although the exposure of PS on cells incubated with the fullerenol vehicle (the Milli-Q water) was higher than expected, the difference in PS-surface exposure between vehicle- treated and fullerenol-treated cells was highly significant (P < 0.001). PS is an essential cofactor for activation complexes in the plasma coagulation system. Also, it is a signaling molecule for phagocytes (Zwaal et al 2005). Thus, our results show that the phenotype of endothelial cells treated with fullerenol become pro-inflammatory and pro-coagulant. It is important to further investigate whether fullerenes exhibit similar effects on the endothelium in vivo in animal models.
Our cell cycle analysis showed that both nC60 and C60(OH)24 induced G1 arrest in HUVECs. Polyhydroxylated fullerenol-1 was reported to inhibit the proliferative responses in a variety of cells, including smooth muscle cells and human lymphocytes, in a concentration dependent manner. The tested concentration range was of 10^4 to 10^2 M, which corresponds to approximately 1 µg/mL to 10 mg/mL. It was also shown that fullerene inhibited cytosolic protein kinase C activity. Therefore, it was suggested that the anti-proliferative effect of fullerenol-1 on vascular smooth muscle cells may partly be mediated through the inhibition of protein tyrosine kinase (Lu et al, 1998).

We found that C60(OH)24 induced apoptosis in HUVECs. This was clearly detected by TUNEL assay, which is a common method for detecting DNA fragmentation. In addition, the observed increase in PS exposure of fullerenol treated cells can be considered as supporting evidence of pro-apoptotic activation of HUVECs. Our finding contradicts the results of a recent study by Yamawaki and Iwai (Yamawaki and Iwai, 2006). These authors concluded that fullerene did not cause apoptosis based on the failure to demonstrate cleavage of caspase 3 and PARP. However, their study showed autophagic cell death in fullerene treated HUVECs. We may therefore speculate that both apoptotic and autophagic cell death may occur in fullerene treated endothelium.

To investigate a possible mechanism of fullerene action on endothelium, we demonstrated that both nC60 and C60(OH)24 induced influx of extracellular Ca2+ in HUVECs. This suggests that fullerenes may either activate some of the Ca2+ channels on the cell membrane, or that they may create new channels/pores to facilitate Ca2+ influx, or both. In either case, it results in an increase in intracellular Ca2+, which may induce activation and/or apoptotic changes of the cells.

In conclusion, the results of this pilot study show possible adverse effects of fullerenes on the endothelium. The hydroxyfullerene C60(OH)24 had both pro-inflammatory and pro-apoptotic effects on endothelial cells. In addition, cell cycle arrest and increase of intracellular Ca2+ was observed in cells treated with hydroxyfullerene C60(OH)24 and also with a low concentration of nC60. With the wide potential applications of fullerenes in mind, the safety hazards of these materials need to be thoroughly evaluated. We suggest that additional in depth studies should be performed to investigate possible adverse effects of fullerenes on the cardiovascular system in exposed populations.

Disclaimer

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

References


