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Highly hydroxylated fullerene localizes at the cytoskeleton and inhibits oxidative stress in adipocytes and a subcutaneous adipose-tissue equivalent

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ABSTRACT

Adipose tissue is a crucial site for pathologic changes in obesity/metabolic syndrome-related diseases. Interaction between adipogenesis and reactive oxygen species (ROS) in adipose tissue involving chronic low-grade inflammation is postulated to be causal in the development of insulin resistance and other metabolic consequences. We used different culture systems to investigate the relationship between ROS and adipogenesis at three levels: within adipocytes, during adipocyte–monocyte interactions, and in a subcutaneous adipose tissue model. The effects of highly hydroxylated fullerene (HHF; $C_{60}(OH)_{36}$) on adipogenesis-accompanying oxidative stress and inflammatory changes were examined using these three systems. We demonstrated that H_2O_2 stimulates lipid accumulation in 3T3-L1 preadipocytes, and lipid uptake causes ROS generation in OP9 preadipocytes, both of which were then markedly suppressed with HHF treatment. HHF significantly inhibited the adipogenic stimulant insulin-rich serum replacement (SR)-induced triacylglycerol accumulation, ROS production, and macrophage activation in cultured OP9 cells and an OP9–U937 monocyte-like cell coculture system. H_2O_2-induced intracellular ROS production in OP9 adipocytes was also notably inhibited by HHF. We developed a three-dimensional subcutaneous adipose-tissue equivalent (SATE) consisting of air-exposed cultures of HaCaT keratinocytes on an OP9 adipocyte-populated collagen gel in a culture insert. With SR stimulation and under suitable conditions, fat accumulation, ROS generation, and macrophage infiltration were observed in the SATE and significantly inhibited by HHF. By western blotting, we demonstrated that HHF localized at the cytoskeleton, which controls the transport of lipids. In conclusion, HHF is able to inhibit oxidative stress in adipocytes and adipogenesis-related macrophage activation in adipose tissues through its antioxidation.

Adipose tissue is a specialized connective tissue composed of adipocytes that function as the major storage site for fat in the form of triacylglycerol (TAG). Adipocytes originate from fibroblast-like precursor cells, named preadipocytes, which differentiate into adipocytes under the appropriate stimulatory conditions; mainly long-duration overfeeding. Overfeeding causes hyperplastic growth of preadipocytes/adipocytes and excessive adipogenesis, leading to obesity and other metabolic diseases. In obesity, reactive oxygen species (ROS) are generated from the overexpanded adipose tissue [1,2]. As a cellular second messenger, ROS products play a crucial role in adipogenesis, macrophage activation, and the secretion of various inflammatory cytokines, all of which trigger chronic systemic inflammation and are linked to insulin resistance, type 2 diabetes, cardiovascular diseases, and cancer [3–5]. Therefore, antioxidants are considered a potential tool for controlling the above-mentioned metabolic consequences. Studies on obesity and metabolic syndrome have generally been performed using animal experiments. Owing to ethical objections, however, alternative systems, such as bioengineered subcutaneous adipose tissue models, would be preferable for the screening of potential medicines.

Fullerene C_{60} and its derivatives have been characterized as powerful antioxidants. Chemical studies demonstrated that fullerenes have extremely high reactivity with radical species [6]. The addition of the hydroxyl radical to the double bonds of C_{60} is known to increase the water solubility and bioactivity of C_{60}. The hydroxylated derivatives of fullerene, $C_{60}(OH)_n$ have attracted much attention in biomedical research. Two polyhydroxylated C_{60} derivatives, $C_{60}(OH)_{12}$ and $C_{60}(OH)_nO_m$, $n = 18–20$, $m = 3–7$ hemiketal groups, decreased excitotoxic neuronal death after brief exposure to glutamate receptor agonists and reduced neuronal apoptosis induced by serum deprivation owing to their ROS-scavenging activity [7]. Fullerenol $C_{60}(OH)_{22}$ protected rat brain cerebral microvessel endothelial cells against nitric oxide (NO)-induced apoptosis,

Abbreviations: HHF, highly hydroxylated fullerene; ADC, adipogenic cocktail; SR, serum replacement; ROS, reactive oxygen species; SATE, subcutaneous adipose-tissue equivalent; TAG, triacylglycerol; NBT, nitroblue tetrazolium; DHE, dihydroethidium.

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depolymerization of cytoskeleton, and nuclear damage and also accelerated cell repair [8]. Fullerenol $C_{60}(OH)_{24}$ has a direct NO-quenching activity to prevent NO-induced decrease in catalase, glutathione transferase, and glutathione peroxidase activities in the enucleated fraction of interstitial testicular cells of adult rats [9]. A study of the genotoxic and antigenotoxic potential of fullerenol $C_{60}(OH)_{24}$ showed that fullerenol in a wide range of concentrations (11–221 μM) has no genotoxicity and can protect mitomycin C-damaged CHO-K1 cells [10].

In vivo studies give more evidence about the antioxidative activity of fullerenol. Fullerenol $C_{60}(OH)_{18-20}$ can preventively and therapeutically scavenge the free radicals that are massively induced during ischemia–reperfusion injury of the small intestine in dogs [11]. Fullerenol $C_{60}(OH)_{24}$ prevents doxorubicin (Dox)-induced chronic cardio- and hepatotoxicity in rats with colorectal cancer through the reduction of oxidative stress [12]. Another similar study showed that fullerenol $C_{60}(OH)_{24}$ also has a preventive effect on Dox-caused acute cardiotoxicity in rats [13]. However, most of the biological research of hydroxylated fullerenes has focused on fullerenols with the hydroxyl group (OH) below a number of 30. Little is known about the bioactivity of highly hydroxylated fullerenes (over 30 OH groups). Data on chemical properties showed that highly hydroxylated fullerenes have higher water solubility and possibly higher bioavailability than normal fullerenols because of the additional hydroxyl groups [14].

We have demonstrated that highly hydroxylated fullerenes have excellent antioxidative abilities as demonstrated by electron spin resonance (ESR) and β-carotene bleaching assay [15].

In this study, we investigate the biological activity and cellular localization of the antioxidant highly hydroxylated fullerene (HHF) $C_{60}(OH)_{36}$, using monolayer preadipocytes/adipocytes, an adipocyte–macrophage coculture system, and as a reconstructed subcutaneous adipose tissue model, which represent three levels of the relationship between ROS and adipogenesis: the cellular level, the cell–cell interaction level, and the tissue level, respectively.

Materials and methods

Reagent

HHF ($C_{60}(OH)_{36} \cdot 8H_2O$) was kindly provided by Professor Takumi Oshima and Dr. Ken Kokubo of Osaka University [14].

Propagation of cells

3T3-L1 mouse fibroblasts (IFO50416; Japanese Collection of Research Bioresources, Osaka, Japan) were cultured in 3T3-L1 propagation medium: DMEM with 10% bovine serum (Trace Scientific Ltd., Melbourne) and 1% penicillin/streptomycin/amphotericin B (Gibco, Invitrogen).

OP9 mouse stromal cells (RCB1124; Riken Cell Bank, Saitama, Japan) were grown in the OP9 propagation medium: MEM-α with 20% fetal bovine serum (FBS) (Trace Scientific Ltd.), 2 mM L-glutamine (Sigma), and 1% penicillin/streptomycin/amphotericin B (Gibco, Invitrogen).

Human skin epidermal keratinocytes (HaCaT) were kindly provided by Professor Norbert E. Fusenig of Deutsches Krebsforschungszentrum (Heidelberg, Germany) [16] and cultivated as previously described [17].

U937 monocyte-like cells (IFO50038; Japanese Collection of Research Bioresources) were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin/amphotericin B.

Differentiation to adipocytes

3T3-L1 or OP9 preadipocytes were plated at 30,000 cells/well in a 24-well plate and then were differentiated into adipocytes using three methods as follows:

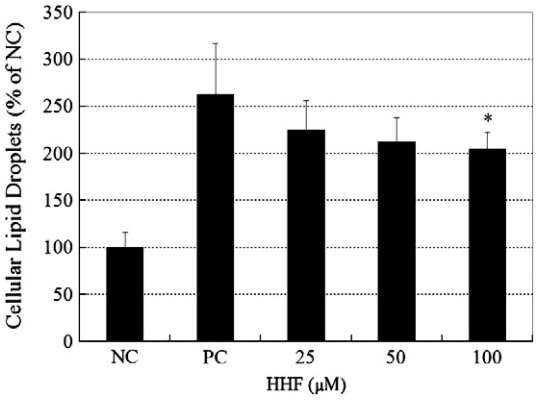

Fig. 1. Effect of HHF on cellular oxidative stress during adipogenesis in 3T3-L1 cells. 3T3-L1 cells were cultured with or without HHF during ADC-induced differentiation. At the end of cultivation, cellular lipid droplets were measured with oil red O staining. Each concentration point with bar represents the mean ± SD of three independent experiments. NC, negative control (nondifferentiated cells); PC, positive control (ADC-differentiated cells); $^*p<0.05$ (versus PC).

Adipogenic cocktail (ADC) method

This method was performed as previously described for 3T3-L1 cells [18]. In brief, 3T3-L1 cells were grown to confluence and then cultured for 2 additional days in 3T3-L1 adipocyte medium: DMEM with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin/amphotericin B. Cells were then cultured for 4 days in differentiation medium (DM) 1: DMEM with 10% FBS, 175 nM insulin, 0.25 μM dexamethasone (Dex), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 2 mM L-glutamine, and 1% penicillin/streptomycin/amphotericin B. The cells were cultured for 3 additional days in DM2: DM1 lacking Dex and IBMX. From then on, the cells were maintained in 3T3-L1 adipocyte medium for 2 additional days.

H_2O_2 stimulation method

3T3-L1 cells were grown to confluence and then cultured for 2 additional days in the propagation medium described above. The cells were then cultured for 6 additional days in H_2O_2 medium: DMEM with 10% FBS, 0.5–5 μM H_2O_2, 2 mM L-glutamine, and 1% penicillin/streptomycin/amphotericin B. At the end of the culture period, cellular lipid droplets were measured.

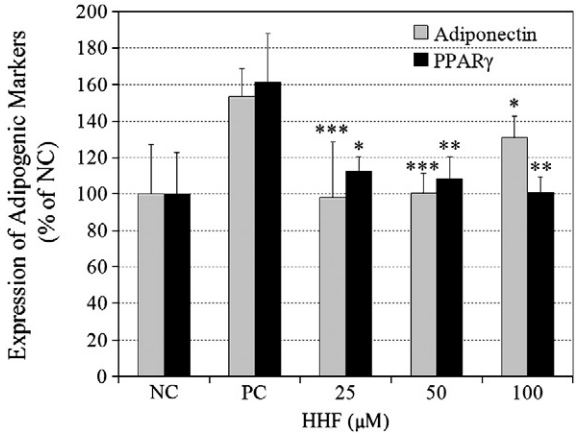

Fig. 2. Effect of HHF on the expression of adipogenic markers during adipogenesis in 3T3-L1 cells. 3T3-L1 cells were treated as described for Fig. 1. At the end of cultivation, the expression of adiponectin and PPARγ proteins was identified with immunofluorescence staining and quantitatively analyzed using ACT-II software as described under Materials and methods. Each concentration point with bar represents the mean ± SD of three independent experiments. NC, negative control (nondifferentiated cells); PC, positive control (ADC-differentiated cells); $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (versus PC).