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Inhibitory Actions of Several Natural Products on Proliferation of Rat Vascular Smooth Muscle Cells Induced by Hsp60 from *Chlamydia pneumoniae* J138

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Atherosclerosis is a vascular disorder involving inflammation, a narrowed vascular lumen in the entire tunica intima, and reduced elasticity of the arterial wall. It has been found that Hsp60 from *Chlamydia pneumoniae*, an obligate bacterial pathogen associated with atheroma lesions, mimics human Hsp60, thereby causing attacks by immune cells on stressed endothelial cells expressing endogenous Hsp60 on their surface. Furthermore, Hsp60 from *C. pneumoniae* has been shown to promote the growth of vascular smooth muscle cells (VSMCs). To explore probes that can be used for studying signal transduction elicited by the chlamydial Hsp60, we have tested several natural products for their inhibitory actions on the Hsp60-induced proliferation of rat arterial smooth muscle cells. Sesamol, vanillyl alcohol, and *trans*-ferulic acid exhibited moderate inhibitory actions on the Hsp60-induced cell proliferation; zerumbone, humulene, and caryophylene effectively inhibited it at low concentrations with IC_{50} values of 529, 122, and 110 nM, respectively. The results indicated that the 11-membered alicyclic ring is favorable for interactions with receptors involved in the Hsp60-induced VSMC proliferation.

KEYWORDS: ***Chlamydia pneumoniae*; heat shock protein 60; chaperonin; atherosclerosis; vascular smooth muscle cell; humulene; caryophylene; zerumbone; sesamol**

INTRODUCTION

According to the vital statistics of the Ministry of Health and Welfare of Japan, the leading cause of death from disease is malignant tumors (cancer, sarcoma), followed by cardiac diseases and cerebrovascular diseases. The total number of deaths from cardiac and cerebrovascular diseases is 280 000 per year, which is almost the same as the number of deaths from malignant tumors. It is estimated that about 12% of males and about 8% of females aged 45−74 years currently have disorders in coronary arteries such as atherosclerosis, and these percentages are rapidly increasing. Therefore, the prevention of such vascular diseases as well as diabetes and hypertension is very important.

Atherosclerosis is a disorder involving immune inflammation of arterial walls mediated by foam cells that stem from blood-derived monocytes and macrophages (*1*). The inflammation results in a narrowed vascular lumen, thereby causing heart disease and stroke. Although elevated levels of oxidized low-density lipoprotein, modified lipoprotein, and homocyteine have been proposed as risk factors for atherosclerosis, it has been recently demonstrated that the atherogenesis is likely to be associated with microbial infection (*2*). Of the candidate causal pathogens, an obligate bacterium *Chlamydia pneumoniae* is most correlated with atherosclerosis (*3*−*6*). In support of a tight link with bacterial infection, the application of an antibiotic azithromycin was shown to prevent atherosclerosis (*7*).

Hsp60, referred to as chaperonin 60 or GroEL, is able to refold and prevent the aggregation of denatured polypeptides in the presence of co-chaperonin or Hsp10 (*8*, *9*). Besides such intracellular chaperone functions, chlamydial Hsp60s are able to activate human immune cells to release cytokines (*10*). Interestingly, Hsp60 from *C. pneumoniae* was shown to co-localize with infiltrating macrophages in the atheroma (*11*). Once immune cells interact with the bacterial Hsp60, they attack not only the bacterial pathogen but also stressed endothelial cells expressing endogenous Hsp60s on their cell surface due to the cross-reactivity of the antigens (*12*).

In addition to activating the immune system, Hsp60 from *C. pneumoniae* has been shown to directly promote the growth of vascular smooth muscle cells (VSMCs) (*13*). Although Hsp60-induced cell proliferation was shown to be mediated by Toll-like receptor (TLR) 4 (*13*), there is evidence that the binding

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affinity of chlamydial Hsp60 for macrophages dose not differ from that for macrophages lacking TLR4 (*14*), suggesting that TLR4 plays an important role in the Hsp60-induced signal transduction but does not interact directly with this protein elicitor.

In this study, to develop useful probes for understanding the mechanism of the signal transduction elicited by extracellular Hsp60s, we have evaluated actions of various natural compounds on the proliferation of rat vascular smooth muscle cells induced by Hsp60 from *C. pneumoniae*. The results indicate zerumbone and its related compounds may become lead compounds for developing novel probes.

MATERIALS AND METHODS

Natural Products. Zerumbone (Z) was steam distilled and purified from essential oil of the rhizomes of *Zingiber zerumbet* (*15*). Humulene (HL) and caryophylene (CA) were distilled and purified from essential oil of *Eugenia caryophylla*. Eugenol (Eug) from citrus fruit peels (*16*, *17*), sesamol (Ses) from sesames, vanillyl alcohol (VanAlc) from vanilla bean, and *trans*-ferulic acid (FerA) from *Celosia argentea* L. (*18*) were isolated and purified from 70% ethanol−water extract. Anacardic acid monoene (AM) and triene (AT) were isolated and purified from methanol extract of cashew nuts (*19*).

Gene Cloning. The gene encoding Hsp60-1 (GroEL1) of *C. pneumoniae* (CP-Hsp60-1) was amplified by PCR using primers C.P.Gro1-N (5′-GGGAATTCCATATGGCAGCGAAAAATATTAAA-TATAATG-3′) and C.P.Gro1-C (5′-CCGCTCGAGGTAGTCCATTC-CTGCGCTTGGC-3′). PCR was carried out using 1 unit of KOD -Plus- (TOYOBO, Osaka, Japan), 2 ng of the *C. pneumoniae* (strain J138, see ref *20*) genomic DNA as template, 0.2 μM primers (C.P.Gro1-N and C.P.Gro1-C), and 0.2 mM dNTP mixture in a 50 μL solution for 30 cycles of 15 s at 94 °C for denaturing, 30 s at 53 °C for annealing, and 90 s at 68 °C for DNA extension. After gel purification using a low melting point agarose gel, the isolate fragment was digested by *Nde*I and *Xho*I, and cloned into the *Nde*I and *Xho*I sites of a vector pET22b (+) (Novagen, WI).

Expression and Purification of CP-Hsp60. Recombinant CP-Hsp60-1, fused with additional amino acids (leucine, glutamic acid, and six histidine residues) from the vector pET-22b (+) at the carboxyl terminus, was expressed at 28 °C in *Escherichia coli* BL21 (DE3) (Novagen) according to the manufacturer's directions. The crude recombinant protein was purified by a Ni^{2+}-nitrilotriacetic acid (NTA) column (Novagen) followed by gel filtration chromatography using a Superdex 200 HR 10/30 column (Amarsham Biosciences, NJ) with phosphate-buffered saline (PBS). The protein concentration was determined by the Bradford method (*21*) using bovine serum albumin as the standard. The purity of the protein was checked by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (*22*). Proteins in the gel were stained with Coomassie Brilliant Blue R-250.

Cell Cultures. Rat VSMCs were obtained by explant from the SD rat artery. Some of the cells were kindly provided by Dr. Ken-ichiro Hayashi (Department of Neurochemistry and Neuropharmacology, Medical School of Osaka University, Japan). VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, penicillin, streptomycin, and fungizone at 37 °C, and were used after 2−10 passages (*23*).

Cell Proliferation Assay. Rat VSMCs (1 mL, 1×10^5 cells) plated in 24-well plates were incubated for 1 day in the presence of 10% FCS and starved in DMEM without FCS for 48 h. The compounds were then applied for 1 h prior to co-application with 10 μg/mL CP-Hsp60-1. After incubation for 48 h, the cells were enumerated using a hemocytemeter. Unless otherwise noted, tests were made in quadruplicate and the IC_{50}, concentration (M) required to reduce the proliferation of VSMCs induced by CP-Hsp60-1 by 50%, was determined for active compounds such as Z, HL, and CA.

RESULTS AND DISCUSSION

Assay Conditions. To establish an assay for the proliferation of rat VSMCs elicited by CP-Hsp60-1, we evaluated the effects of the time of starvation before treatment with the protein and the time of treatment with the chlamydial protein on the growth of VSMCs.

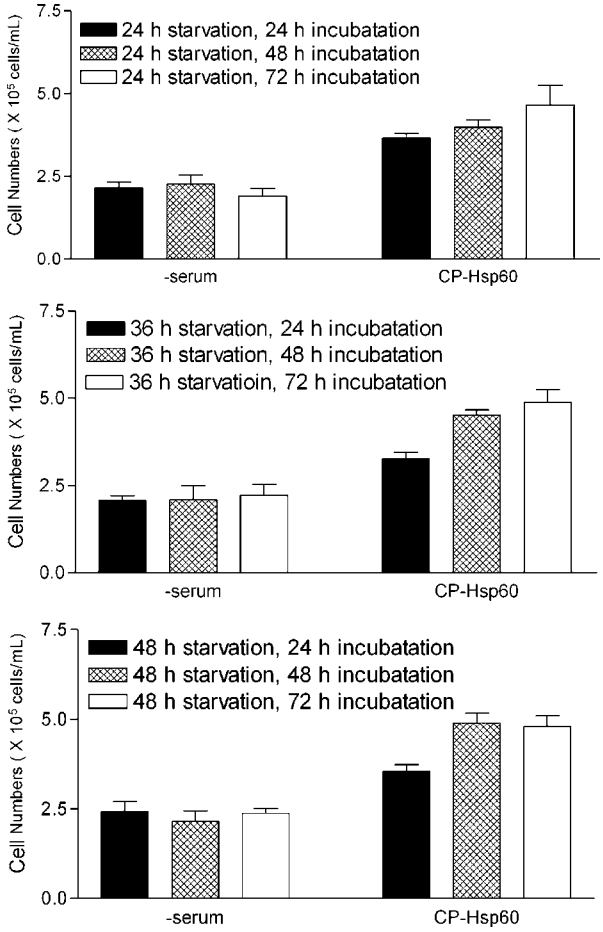

Figure 1. Effects of time of starvation and treatment with Hsp60-1 from *C. pneumoniae* on growth of rat vascular smooth muscle cells. The recombinant Hsp60-1 expressed by *E. coli* was applied at 10 μg/mL. Each data point represents mean ± SEM of four experiments.

Figure 1 shows the cell growth in 24, 36, and 48 h starvation groups, which were then cultured in a serum-deficient but Cp-Hsp60-1-supplemented medium for 24, 36, and 48 h after starvation. The longer the culture period in the presence of Cp-Hsp60-1, the more VSMCs proliferated. The growth of VSMCs measured after starvation for 48 h was higher than that after starvation for 24 h. Taking these results into consideration, the effects of the natural compounds on the Hsp60-induced cell growth were evaluated by co-applying them with the chlamydial protein for 48 h after 48 h-starvation.

Concentration-Dependent Proliferation of Rat VSMCs by CP-Hsp60-1. Rat VSMCs were exposed to CP-Hsp60-1, and the cell proliferation was measured after 48 h of incubation ($n = 4$). CP-Hsp60-1 was added to the medium at final concentrations of 0.1, 1.0, 5.0, 10, 20, and 50 μg/mL. As the control, PBS (−), which does not include CP-Hsp60-1, was added to the culture medium. There were no significant differences in the proliferation of VSMCs treated with 0.1, 1.0, and 5.0 μg/mL CP-Hsp60-1 and the control. When exposed to 10, 20, and 50 μg/mL CP-Hsp60-1, however, the number of cells was 2.3-, 3.2-, and 3.7-fold larger than the control, respectively (**Figure 2**). The cell proliferation reached almost the maximal level on treatment with CP-Hsp60-1 at 20 μg/mL. By contrast, when the recombinant Hsp60-1 was either heated at 100 °C for 30 min, or treated with proteinase K at 200 μg/mL for 2 h at 37

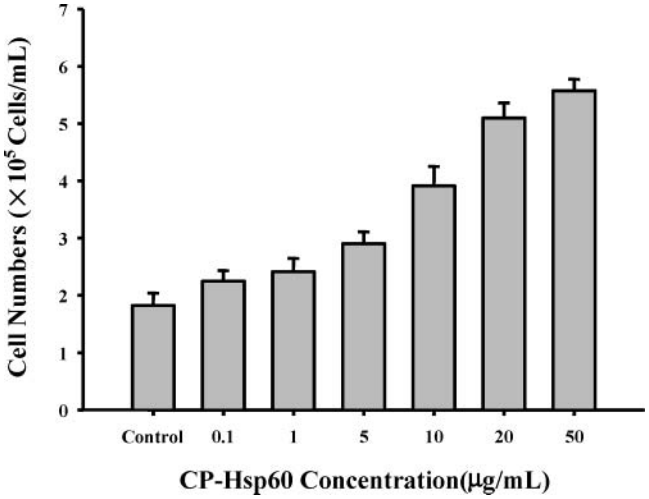

Figure 2. Dose-dependent actions of recombinant Hsp60-1 from *C. pneumoniae* on growth of rat vascular smooth cells. Each data point represents mean ± SEM of four experiments.

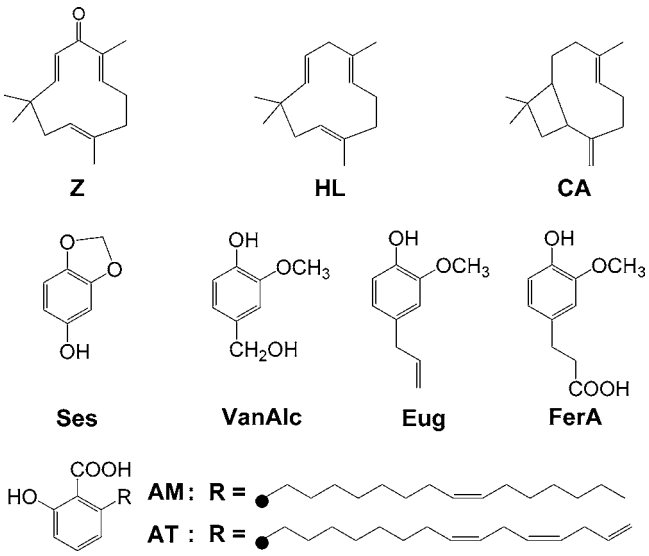

Figure 3. Chemical structures of natural compounds tested for their actions on proliferation of vascular smooth muscle cells induced by Hsp60-1 from *C. pneumoniae*.

°C, no cell proliferation was observed (data not shown), suggesting that the VSMC growth was due to an action of the recombinant CP-Hsp60-1 preparation.

Actions of Compounds on Cell Proliferation. Compounds that have been shown to have physiological activities against cancer cells were used for assaying inhibitory effects on the proliferation of VSMCs. The compounds isolated from natural materials (**Figure 3**) were zerumbone (Z) obtained from *Zingiber zerumbet*, eugenol (Eug) obtained from citrus fruit peels, sesamol (Ses) obtained from sesames, and anacardic acid monoene (AM) and triene (AT) obtained from cashew nuts. Dimethyl sulfoxide (DMSO) was used as the solvent to prepare stock solutions of these compounds. The DMSO concentration in the assay medium was 1% (v/v), irrespective of the presence or absence of the compounds. Prior to co-application of CP-Hsp60-1 with the compounds, the cells were treated for 1 h with the same concentration of the compounds. Among the tested compounds (**Figure 3**), Ses, VanAlc, FerA, and AM applied at 10 μg/mL showed weak or moderate inhibitory action on the cell proliferation induced by CP-Hsp60-1, whereas Z completely suppress it at this concentration (**Figure 4**). By

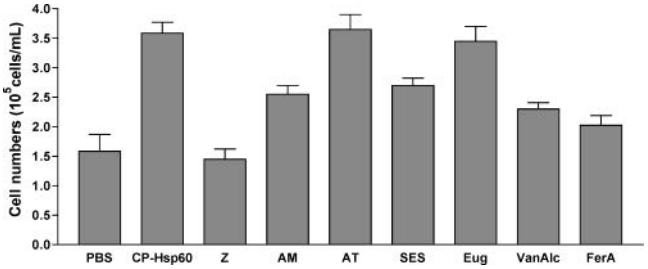

Figure 4. Actions of zerumbone (Z), sesamol (Ses), eugenol (Eug), vanillyl alcohol (VanAlc), *trans*-ferulic acid (FerA), anacardic acid monoene (AM), and triene (AT) on the proliferation of vascular smooth muscle cells induced by Hsp60-1 from *C. pneumoniae*. Each data point represents mean ± SEM of four experiments.

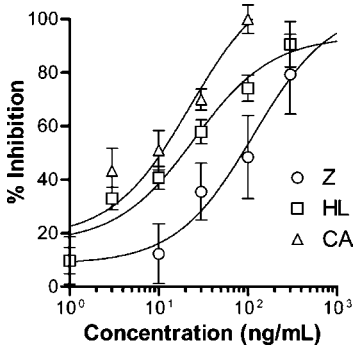

Figure 5. Dose-dependent inhibitory actions of zerumbone (Z, ○), humulene (HL, □), and caryophylene (CA, △) on the proliferation of rat smooth muscle cells induced by Hsp60-1 from *C. pneumoniae*. Each data point represents mean ± SEM of eight experiments. Experiments for Z were conducted separately from the test illustrated in **Figure 4**.

Table 1. Inhibitory Actions of Compounds on VSMC Proliferation Induced by CP-Hsp60-1

compd	IC_{50} (nM)	compd	IC_{50} (nM)	compd	IC_{50} (nM)
Z	530	HL	122	CA	110

contrast, Eug and AT was inactive on the cell proliferation. Based on the result, the inhibitory activity of Z-related compounds against the CP-Hsp60-1-induced cell proliferation was evaluated. The analogues of Z tested were humulene (HL), which lacks the carbonyl group, and caryophylene (CA), in which the double bonds in HL are cyclized (**Figure 3**). Z and its related compounds H and CA exhibited inhibitory actions even at 1 μg/mL (data not shown). Thus, as illustrated in **Figure 5**, the dose-inhibitory action relationships of Z, HL, and CA were investigated in detail. The inhibitory activity of HL and CA was more potent than Z. The IC_{50}, the concentration (M) required to reduce the growth of VSMCs induced by 10 μg/mL CP-Hsp60-1 by 50%, of the three compounds was calculated by the Hill equation. The IC_{50} values of Z, CA, and HL were 529, 122, and 110 nM, respectively (**Table 1**). At these IC_{50}s, all of these compounds had no significant effect on VSMC cultured in the absence of CP-Hsp60-1 (data not shown), suggesting that the inhibitory action at such low concentrations is selective for the CP-Hsp-1-induced events. The rank order of the inhibitory activity indicated that the carbonyl group at position 1 and olefinic bonds at positions 2 and 6 of Z are not essential and rather unfavorable for the inhibitory activity.

In summary, we have for the first time found by testing nine natural products that compounds having an 11-membered alicyclic ring and a trisubstituted benzene skeleton are effective

in reducing the proliferation of rat VSMCs induced by Hsp60-1 (GroEL1) of *Chamydia pneumoniae*. Although the modes of action of these inhibitory compounds are unknown, the discovery of compounds capable of influencing Hsp60-induced cell growth at low concentrations could lead to the development of novel and useful probes for understanding the cell signaling elicited by Hsp60s.

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