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Gallic acid isolated from *Spirogyra* sp. improves cardiovascular disease through a vasorelaxant and antihypertensive effect

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ABSTRACT

In this study, we investigated the vasorelaxant and antihypertensive effects of gallic acid (GA), a polyphenol isolated from the green alga *Spirogyra* sp., to assess its suitability as a therapeutic for cardiovascular diseases (CVDs). We examined the effect of GA on endothelium-dependent vasorelaxation in human umbilical vein endothelial cells (HUVECs). GA increased nitric oxide (NO) levels by increasing phosphorylation of endothelial nitric oxide synthase (eNOS), and its effect on NO production was attenuated by pretreatment with the eNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). We also investigated its antihypertensive effect by examining GA-mediated inhibition of angiotensin-I converting enzyme (ACE). GA inhibited ACE with a half-maximal inhibitory concentration (IC_{50}) value of 37.38 ± 0.39 μg/ml. *In silico* simulations revealed that GA binds to the active site of ACE (PDB: 1O86) with a binding energy of −270.487 kcal/mol. Furthermore, GA clearly reduced blood pressure in spontaneously hypertensive rats (SHR) to an extent comparable to captopril. These results suggest that GA isolated from *Spirogyra* sp. exerts multiple therapeutic effects and has potential as a CVD treatment.

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1. Introduction

Cardiovascular diseases (CVDs) have a complex pathology and are defined by failure of vascular functions. Dysfunctional endothelium-dependent relaxation leads to chronic, abnormal elevation of vascular resistance and results in serious hypertension. As a result, CVDs were occurred in many different forms such as hyperlipidemia, atherosclerosis, coronary artery disease, arrhythmia, and heart failure

Abbreviations: GA, gallic acid; CVD, cardiovascular disease; NO, nitric oxide; HUVECs, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-arginine methyl ester; ACE, angiotensin-I converting enzyme; SHR, spontaneously hypertensive rats.

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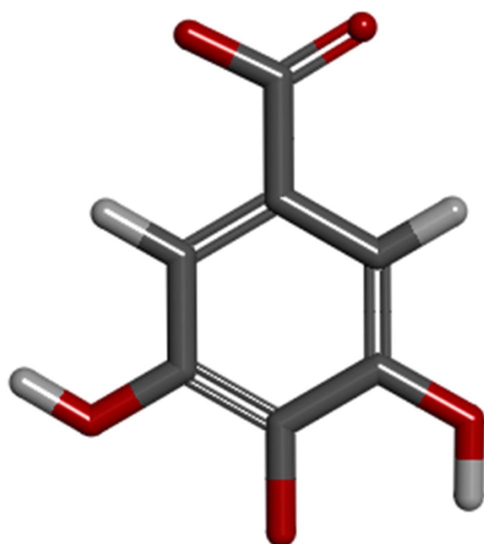

Fig. 1 – Structure of GA.

(Chen et al., 2010a; Giles et al., 2012; Hien et al., 2012). Therefore, proper endothelial function and vasorelaxation are important physiological phenomena related to many CVDs (Shou et al., 2012). An important means of treating CVD relates to the induction of vasorelaxation to reduce hypertension.

Some strong antioxidants including the red wine polyphenol resveratrol (Leikert et al., 2002), flavones (Chen et al., 2010b), and green tea (−)epicatechin (Huang et al., 1999) have been found to induce vasorelaxation *via* a mechanism related to nitric oxide (NO) production. According to the previous study, *Spirogyra* sp., a green alga commonly found in freshwater, contains various antioxidant galloyl derivatives such as gallic acid and methyl gallate (Han et al., 2012; Lee et al., submitted for publication). Especially, gallic acid (GA) is a common polyphenol, and because of its simple phenolic structure and antioxidant activity, it is used as a standard in assays of polyphenol content (Fig. 1).

In this work, we investigate the vasorelaxant effect of GA that was isolated from *Spirogyra* sp., and which possesses outstanding antioxidant activity. To investigate the vasorelaxant effect of GA, NO production and phosphorylation of endothelial nitric oxide synthase (eNOS) are measured in human umbilical vein endothelial cells (HUVECs), and its inhibition of phosphodiesterase 3 (PDE3) activity is using *in silico* analysis.

Additionally, we investigate the antihypertensive effect of GA for evaluating multiple effects on fundamental cause of CVDs. The inhibition of angiotensin I converting enzyme (ACE) is measured using a colorimetric method and simulated using *in silico* methods. Also, the effect of GA on the blood pressure is measured in spontaneously hypertensive rats (SHRs).

2. Materials and methods

2.1. Materials

Endothelial cell basal medium-2 (EBM-2) and endothelial cell growth medium-2 (EGM-2) bullet kit were purchased from Clonetics Inc. (San Diego, USA). Antibodies including phospho-eNOS (Ser^{1177}, cat no. #9572) and phospho-Akt (Ser^{473}, cat no. #9271) were purchased from Cell signaling Technology (Beverly, MA). Diamino fluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals and reagents used were of analytical grade.

2.2. GA isolated from Spirogyra sp.

Spirogyra sp. was collected in summer season, July in 2012, from several shallow pond in Kongju, Korea (36°20′34″, 127°12′28″). Dried *Spirogyra* sp. (20 g) was extracted three times for 3 h using 80% MeOH under sonication at room temperature. The extract was concentrated in a rotary vacuum evaporator and partitioned with ethyl acetate, and then the dried ethyl acetate fraction was stored in a refrigerator for high performance centrifugal partition chromatography (HPCPC) separation. The procedures of HPCPC experiments were described in Lee et al. (submitted for publication).

2.3. Measurement of NO production

HUVECs (Lonza, USA) were grown in EBM-2 supplemented with EGM-2 BulletKit and maintained in a humidified incubator at 37 °C with 5% CO_2. HUVECs from passages 3–6 were used in this study. HUVECs were starved in EBM-2 for the treatment period.

Time-dependent NO production was measured using a previously reported NO assay (Samarakoon et al., 2013). HUVECs (5×10^4 cells/well) were seeded in 24-well plates and were incubated with the test compound at a concentration of 25 μg/ml for 10–300 min. The accumulation of nitrite in the supernatant was assessed using Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid].

For more sensitive detection of NO at different concentrations of GA, NO levels were assessed using the NO-specific fluorescent probe, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA), which is converted to an intensely fluorescent triazole derivative *via* an NO-dependent mechanism (Itoh et al., 2000). HUVECs (2×10^4 cells/well) were seeded in 96-well plates and were incubated with or without the test compound at various concentrations for 1.5 h. Then, DAF-FM DA solution (5 μM) was added, and the cells were incubated at 37 °C for 1 h in the dark. After incubation, fluorescence was measured using a fluorescence plate reader (Genios Pro, Tecan, Austria) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

2.4. Western blot analysis

Phospho-eNOS (Ser^{1177}) and phospho-Akt (Ser^{473}) expressions were determined by Western blot analysis (Yamabe et al., 2007). 10 μg of protein samples were electrophoresed through 12% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The separated proteins were blocked with 5% skim milk solution, and then incubated with primary antibodies and goat anti-rabbit IgG HRP

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