α-Mangostin protects endothelial cell against H₂O₂-induced senescence via p38/Sirt1/MnSOD pathway

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ABSTRACT
Cardiovascular diseases (CVDs) are a major cause of mortality. Increased CVD in aging is associated with vascular endothelial dysfunction due to cell senescence, which is activated by oxidative stress. α-Mangostin is a pure substance from mangosteen (Garcinia mangostana L.) and has been shown to have anti-oxidant properties. However, the effect of α-mangostin on senescence in endothelial cells has not been widely studied. This study evaluated the effect of α-mangostin on H₂O₂-induced endothelial senescence. The concentrations of H₂O₂-induced senescent signaling and α-mangostin were determined by Western blot and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Intracellular reactive oxygen species (ROS) production was determined by 2', 7' dichlorodihydrofluorescein diacetate assay, and senescent cells were detected by β-galactosidase staining. Cell proliferation and nitric oxide (NO) production were determined by MTT and Griess assays. Senescent signaling was analyzed by Western blot. An H₂O₂ concentration of 50 µM was used to stimulate cell senescence. α-Mangostin doses of 0.1 and 1 µM were non-toxic and significantly reduced ROS formation and cell senescence, significantly increasing cell proliferation, mitochondrial membrane potential, and NO production. It significantly attenuated the phosphorylation of p38 MAPK and increased Sirt1 and MnSOD. This study found that α-mangostin suppresses oxidative stress and prevents H₂O₂-induced endothelial senescence by inhibiting p38 mitogen -activated protein kinase (MAPK) and stimulating Sirt1 and MnSOD.

INTRODUCTION
Vascular aging is an important risk factor for cardiovascular diseases (CVDs) which is associated with endothelial dysfunction. One process that has been connected to aging and vascular pathologies is cellular senescence. Senescence is an arrest of cellular growth induced by stress stimuli such as inflammation, hyperglycemia, and oxidative stress. Excessive production of reactive oxygen species (ROS) stimulates vascular senescence, leading to decreased nitric oxide (NO) production, resulting in attenuated NO bioavailability and enhanced ROS. In addition, senescent cells can secrete chemokines and pro-inflammatory cytokines [1]. Several studies also demonstrated that ROS-induced senescence is involved in mitochondrial dysfunction, leading to ROS production [2,3]. Mitochondrial ROS production also activates inflammation and suppresses NO bioavailability. ROS stimulates senescence through the activation of p53/p21, p16, and suppression of Sirt1 [4–6]. Moreover, several studies have shown that p38 mitogen -activated protein kinase (MAPK) is involved in endothelial senescence [7–9].

α-Mangostin is the most xanthone substance that is extracted from mangosteen (Garcinia mangostana L.). It has been shown to have anti-oxidant properties [10,11], anti-tumour effects [12–14], anti-inflammatory effects [15,16], anti-apoptosis effects [13,17], and anti-angiogenesis effects [18–21]. The study demonstrated that α-mangostin suppressed ultraviolet B-induced apoptosis and senescence in keratinocytes [22]. Recently, a study showed mangosteen peel extracts have...
anti-oxidant, anti-elastase, and anti-collagenase activities [23]. The molecular effect of α-mangostin on H$_2$O$_2$-induced endothelial senescence has not been widely studied. The aim of this study is to determine the effect of α-mangostin on H$_2$O$_2$-induced endothelial senescence.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), penicillin and streptomycin, Dulbecco’s Modified Eagle’s Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin-ethylenediaminetetraacetic acid, were purchased from Gibco-Invitrogen (Grand Island, NY, USA). 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carboxylic acid iodide (JC-1), Griess and nitrite were obtained from Merck Millipore. 2′, 7′ dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and the senescence assay kit was purchased from Biovision (Milpitas, California, USA). Sirt1 (catalog No. 2310), p21 (catalog No. 2947), acetylated p53 (catalog No. 2570), p53 (catalog No. 2524), p16 (catalog No. 80772), MnSOD (catalog No. 13194), and β-actin (catalog No. 3700) were obtained from Cell Signaling Technology (Danvers, MA, USA). Phospho-p38 (catalog No. Sc17852) and p38 MAPK (catalog No. Sc535) were purchased from Santa Cruz Biotechnology (CA, USA). Secondary antibodies against mouse (catalog No. AP124P) or rabbit (catalog No. AP132P) and enhanced chemiluminescence were purchased from Merck Millipore.

Plant material and extraction

α-Mangostin was kindly provided by Prof. Primchanien Moongkarni, Faculty of Pharmacy, Mahidol University, Thailand. The extraction of α-mangostin was performed as in previous studies [24,25]. α-Mangostin was dissolved with dimethylsulfoxide (DMSO).

Cell culture

EA.hy926 (ATCC number CRL-2922), human endothelial cell lines, were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in 5% CO$_2$. At 70% confluence, the cells were treated with a low serum medium (LSM) (DMEM supplemented with 1% FBS, 10 U/ml penicillin, and 10 U/ml streptomycin) for at least 6 hours before subsequent experiments. The dose and time course of H$_2$O$_2$-induced cell senescence signaling were determined using Western blot. Cells were treated with 200 µM H$_2$O$_2$ for 60 minutes, and then the medium was replaced with LSM for 5, 15, 30, and 60 minutes 3, 6, 12, 24, and 48 hours. Also, H$_2$O$_2$ was used at doses of 25, 50, 100, and 200 µM for 60 minutes, and the medium was then replaced with LSM for the times stated above to detect p21 and Sirt1 signaling.

Cytotoxicity assay

The concentration of α-mangostin was determined using an MTT assay. Cells were treated with 0.1, 1, 2, and 4 µM α-mangostin for 24 hours. After treatment, the cells were incubated with MTT for 2 hours. Finally, the formazan crystal was dissolved by adding DMSO. The optical density was determined using a microplate reader (SpectraMax iD3, Molecular Devices) at 540 nm.

Senescence associated β-galactosidase assay

Cells were treated with α-mangostin or Vitamin C (Vit C) for 30 minutes and then treated with 50 µM H$_2$O$_2$ for 60 minutes. The medium was then replaced with LSM for 24 hours. Senescent cells were detected using the β-galactosidase assay according to the manufacturer’s protocol. Four images were randomized using a microscope with 200× magnification.

Intracellular ROS assay

Cells were treated with 0.1 and 1 µM of α-mangostin or Vit C for 30 minutes and then incubated with 20 µM DCF-DA for 20 minutes. Consequently, cells were incubated with 50 µM H$_2$O$_2$ for 30 minutes. Quantification of the fluorescence intensity was immediately detected by a microplate reader at 504 nm excitation and 529 nm emission.

Proliferation assay

Cells were cultured and then pretreated with 0.1 and 1 µM α-mangostin at or Vit C for 30 minutes. After that, the cells were treated with 50 µM H$_2$O$_2$ for 60 minutes. Consequently, cells were incubated in LSM for 24 hours. MTT assay was performed as the protocol stated above.

NO production assay

After treatment, the medium was replaced with LSM without phenol red for 24 hours. The media were determined nitrite concentration by Griess reagent. Optical density (OD) was evaluated using a microplate reader at a wavelength of 540 nm. The nitrite concentration was determined according to the nitrite standard.

Mitochondrial membrane potential assay

The fluorescent probe JC-1 was used for monitoring the mitochondrial membrane potential to determine mitochondrial function. The lipophilic, cationic JC-1 dye can enter into the mitochondria and then accumulate, forming reversible complexes called J-aggregates. Thus, healthy cells stain with JC-1 and then spontaneously form red fluorescent J-aggregates. By contrast, in senescent cells, JC-1 can enter the mitochondria but cannot form J-aggregates. After treatment, the medium was replaced with LSM for 24 hours. Cells were incubated with 20 µM JC-1 for 30 minutes. The four images were randomly taken using a fluorescent microscope with 200× magnification (Zeiss Axio observer). The fluorescent intensity was analyzed with ImageJ software. The red/green fluorescent intensity ratio was then calculated.

Western blot analysis

After treatment, the medium was replaced with LSM for an indicated time. Cytosolic protein was extracted using radioimmunoprecipitation assay buffer and separated using gel electrophoresis. Subsequently, separated proteins on the gel were transferred to a polyvinylidene difluoride membrane. The
membrane was incubated with primary antibodies (1:1,000) at 4°C overnight and incubated with secondary antibodies (1:2,000) at room temperature for 60 minutes. The bands of protein were detected by using enhanced chemiluminescence and then placed in the Gel Doc XR+ system. Quantification of the band density was analyzed with ImageJ software.

**Statistical analysis**

All data are expressed as mean ± SD. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey test for comparisons of multiple groups. The statistically significant was considered if the p-value was less than 0.05.

**RESULTS**

**H2O2-induced senescence**

Blotting was used to determine the time and concentration of H2O2-induced cellular senescent signaling, therefore 200 µM H2O2 increased acetylated p53/p53 and p21 at 24 hours and p16 expression at 48 hours and decreased Sirt1 expression at 3 hours. p21 expression was the highest, and Sirt1 expression was the lowest in H2O2 treatment at 50 and 100 µM (Fig. 1). Hence, 50 µM H2O2 was used in further experiments.

**Cytotoxicity of α-mangostin**

MTT assay was used to determine the non-toxic concentrations of α-mangostin. α-Mangostin concentrations greater than 2 µM significantly decreased the number of viable cells. The surviving cells treated with 0.1–1 µM of α-mangostin for 24 hours were not significantly changed (Fig. 2). Therefore, 0.1 and 1 µM α-mangostin were used in the following experiments.

**α-Mangostin reduces H2O2-induced cell senescence**

β-galactosidase is a marker of cell senescence. After treatment of 50 µM H2O2 for 1 hour and then replaced with LSM for 24 hours, cells were significantly increased in β-galactosidase stain positive about 50%. Both α-mangostin + H2O2 and Vit C + H2O2 groups were significantly attenuated in β-galactosidase stain positive (Fig. 3).

**α-Mangostin attenuates H2O2-induced intracellular ROS production**

Next, we determined the effect of α-mangostin on H2O2-induced intracellular ROS formation by DCF-DA staining assay. α-Mangostin and Vit C significantly reduced H2O2-induced ROS. α-Mangostin at 0.1 and 1 µM and Vit C 200 µM reduced H2O2-induced intracellular ROS production by 6, 8, and 8%, respectively (Fig. 4).

**α-Mangostin increases cell proliferation**

Cell growth arrest is a common marker of cellular senescence. We determined cell proliferation using an MTT assay. H2O2 decreased endothelial proliferation significantly. Only 1 µM α-mangostin significantly increased H2O2-suppressed proliferation (Fig. 5).

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**Figure 1.** Effects of H2O2 on cell senescent signaling. (A) Cells were treated with 200 µM H2O2 at various times to determine p21, p53, Sirt1, and p16. (B) Cells were treated with various concentrations of H2O2 for the indicated times of 24 hours to determine p21 and 3 hours for Sirt1.

**Figure 2.** The effect of α-mangostin on cell viability. EA.hy926 cells were treated with various α-mangostin concentrations and assessed cell viability using MTT assay (n = 3, **p < 0.01, ***p < 0.001 versus control).
α-Mangostin increases NO production

Basically, healthy endothelial cells release NO, primary endothelium-derived autacoids, to regulate vascular walls in a quiescent state. We determined the effect of α-mangostin on NO production by using Griess assay. Cells treated with \( H_2O_2 \) significantly decreased in NO formation by 10%. Both α-mangostin + \( H_2O_2 \) and Vit C + \( H_2O_2 \) groups enhanced NO production. Only 1 µM α-mangostin and 200 µM Vit C significantly increased \( H_2O_2 \)-suppressed NO production (Fig. 6).

α-Mangostin prevents mitochondrial dysfunction

Next, we determined the effect of α-mangostin on \( H_2O_2 \)-induced mitochondrial dysfunction by JC-1 staining. Cells treated with \( H_2O_2 \) significantly decreased mitochondrial membrane potential. α-Mangostin at 0.1 and 1 µM and Vit C 200 µM significantly increased \( H_2O_2 \)-suppressed cell proliferation (Fig. 5).
C 200 µM significantly increased mitochondrial membrane potential by 57%, 65%, and 78%, respectively (Fig. 7).

**α-Mangostin suppresses H$_2$O$_2$-induced senescence through p38 MAPK, Sirt1, and MnSOD**

The effect of α-mangostin on the downstream signaling of cell senescence was determined. α-Mangostin significantly decreased phosphorylation of p38 MAPK. α-Mangostin also significantly increased Sirt1 and MnSOD expression (Fig. 8).
The main finding of this study is the protective effect of α-mangostin on endothelial senescence induced by oxidative stress. We have shown that (1) exogenous ROS-induced intracellular ROS production is inhibited by α-mangostin; (2) H$_2$O$_2$-induced senescence resulting in decreased cell proliferation and NO bioavailability is inhibited by α-mangostin; (3) H$_2$O$_2$ induced a reduction in the mitochondrial membrane potential is restored by α-mangostin; and (4) H$_2$O$_2$-induced phosphorylation of p38 MAPK and suppressed expression of Sirt1 and MnSOD is reversed by α-mangostin.

Age-related degeneration in function occurs in normal physiology and pathological diseases, including hypertension and CVDs. Vascular disease is associated with endothelial dysfunction, impaired angiogenesis, and arterial stiffness and remodeling. Recently, several studies have been shown that endothelial cell senescence associated with age-related vascular pathologies. Senescence is a process whereby the irreversible arrest of cell growth in response to various stresses, including oxidative stress, occurs. Oxidative stress-induced cell senescence damages the DNA and mitochondria and stimulates cytosolic stress response kinases such as p38 and JNK MAPK. ROS induces endothelial senescence through stimulation of p53/p21 and p16 expression and suppression of Sirt1 [26]. It has been shown that Sirt1 suppresses oxidative stress-induced cell senescence by the activation of MnSOD [27].

α-Mangostin, a polyphenolic xanthone derivative, is a potent anti-oxidant property [28]. Our previous studies showed that α-mangostin attenuated ROS formation by hypoxia induced in Bovine retinal endothelial cells [18] and high glucose induced in human umbilical vein endothelial cell (HUVEC) [17]. This study showed α-mangostin inhibited H$_2$O$_2$-induced endothelial senescence and reduced intracellular ROS production. We also demonstrated that α-mangostin significantly reversed the reduction of cell proliferation and NO production induced by H$_2$O$_2$. These results indicated that α-mangostin ameliorates cell senescence and consequently increases cell proliferation and NO generation. In addition, our study found that α-mangostin inhibited p38 MAPK and increased Sirt1 and MnSOD; however, α-mangostin did not change the expression of p53/p21 and p16 (data not shown). Recently, a study by Tousian et al. [29], demonstrated that α-mangostin decreased high glucose-induced endothelial senescence via inhibition of p21 and p53 and increase of Sirt1 expression. It is probable that different stimulations may activate distinct downstream signaling.

Mitochondria is the major source of adenosine triphosphate (ATP) production in cardiac myocytes and other cells. By contrast, endothelial mitochondria play a pivotal role in cellular signaling [30]. Excessive ROS activates mitochondrial dysfunction, resulting in increased mitochondrial ROS production. Several studies have shown that H$_2$O$_2$ stimulates cell senescence by inducing mitochondrial dysfunction, including loss of mitochondrial membrane potential [31,32]. This study found that α-mangostin significantly restored the reduction in mitochondrial membrane potential. Previously, it has been shown that α-mangostin reduced retorone-induced mitochondrial dysfunction in neuroblastoma cells [32]. Other study also demonstrated that α-mangostin attenuated isoproterenol-induced cardiac mitochondrial dysfunction in rats [33]. These results indicated that α-mangostin can protect against mitochondrial dysfunction.

This study has demonstrated that α-mangostin has anti-oxidant and anti-senescent actions on endothelial cells through p38 MAPK, Sirt1, and MnSOD pathways. α-Mangostin may be used as a natural substance to prevent CVDs.

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