1 *Article*

2 The Protective Effect of Chlorogenic Acid on
3 Vascular Senescence via Nrf2/HO-1 Pathway

4 **Yoshiko Hada [1], Haruhito A. Uchida [2]*, Nozomu Otaka [1,3], Yasuhiro Onishi [1], Shugo Okamoto [1],**
5 **Mariko Nishiwaki [1], Rika Takemoto [1], Hidemi Takeuchi [1] and Jun Wada [1]**

6 [1] Department of Nephrology, Rheumatology, Endocrinology and Metabolism, Okayama University
7 Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.;
8 yuzuro7@gmail.com (Y.H.); nomu2129@gmail.com (N.O.); oocyst0024@gmail.com (Y.O.);
9 shubo422@gmail.com (S.O.); m.tsuchida.okayama.u@gmail.com (M.N.) ; rika-t@md.okayama-u.ac.jp (R.T.);
10 takeuchih@okayama-u.ac.jp (H.T.); junwada@okayama-u.ac.jp (J.W.)
11 [2] Department of Chronic Kidney Disease and Cardiovascular Disease, Okayama University Graduate School
12 of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan; hauchida@okayama-u.ac.jp (H.A.U.)
13 [3] Department of Human Resource Development of Dialysis Therapy, Okayama University Graduate School
14 of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan; nomu2129@gmail.com (N.O.)
15 * Correspondence: hauchida@okayama-u.ac.jp (H.A.U.)

17 **Abstract:** The world faces the serious problem of aging. In this study, we aimed to investigate the
18 effect of chlorogenic acid (CGA) on vascular senescence. C57/BL6 female mice of 14 ± 3 months old
19 were infused with either AngII or saline subcutaneously for 2 weeks. These mice were administered
20 CGA of 20 or 40 mg/kg/day, or saline via oral gavage. AngII infusion developed vascular senescence,
21 which was confirmed by SA-β-gal staining. CGA administration attenuated vascular senescence in
22 dose-dependent manner, in association with the increase of Sirt1 and eNOS, and with the decrease
23 of p-Akt, PAI-1, p53 and p21. In vitro study, with or without pre-treatment of CGA, HUVECs were
24 stimulated with H_2O_2 for an hour, then cultured in the absence or presence of 0.5-5.0 μM CGA for
25 indicated time. Endothelial cell senescence was induced by H_2O_2, which was attenuated by CGA
26 treatment. Pre-treatment of CGA increased Nrf2 in HUVECs. After H_2O_2 treatment, translocation of
27 Nrf2 into nucleus and subsequent increment of HO-1 were observed earlier in CGA-treated cells.
28 Furthermore, HO-1 inhibitor canceled the beneficial effect of CGA on vascular senescence in mice.
29 In conclusion, CGA exerts a beneficial effect on vascular senescence, at least partly, dependent on
30 Nrf2/HO-1 pathway.

31 **Keywords:** chlorogenic acid; vascular senescence; nuclear factor erythroid 2-related factor 2; heme
32 oxygenase-1

33

34 1. Introduction

35   Lifespan of the people in the world are getting longer. Since the pace of population ageing
36 around the world is getting faster, the prevalence of age-related diseases is in an upward trend. Thus,
37 aging raises socioeconomic problem for countries around the world. In this sense, our society will
38 face an enormous economic challenge in the decades to come. Consequently, it is an urgent need to
39 find apt interventions that slow down aging and reduce or delay the incidence of debilitating age-
40 related diseases.
41   Among senescence-related conditions, vascular senescence has been identified as an important
42 factor underlying diseases such as hypertension, stroke, and arteriosclerosis. [1-3] Vascular
43 senescence not only reduces function of the affected organ, but also exacerbates insufficiency due to
44 chronic inflammation. [4] Therefore, aging of the vasculature plays a central role in senescence-
45 related diseases. [5,6] Among the vascular component cells, vascular endothelial cells (ECs) play a

pivotal role in maintaining vascular homeostasis and healthy respond to physical and chemical stimuli. Thus, therapies targeting endothelial senescence would have important clinical implications for the treatment of senescence-related diseases including cardiovascular diseases. [7,8] Furthermore, three main routes lead to senescence: telomere-dependent replicative senescence, oncogene-induced senescence and stress-induced (premature) senescence. [9] The mechanisms of senescence are very complex, and thus remain poorly understood. Since many cell types never exhaust their maximum replicative potential during lifespan and fail to present senescence, premature senescence is likely the most important inducer of cellular senescence in vivo. [10] Although cellular models of senescence provide valuable mechanistic information, they can lead to limited data because they may not replicate in vivo biology. In this point, animal models are useful tools for an investigation for aging, allowing us to find conserved pathways that may be involved in human aging. Activation of renin-angiotensin-system induced by AngII can act as a key player in cell and organ senescence. [11,12] Interestingly, AngII-affected genes were abundant in vascular senescence pathway. [13] In deed, many papers have reported vascular senescence induced by AngII. [14-16]

Polyphenols are compounds which are contained in many fruits, vegetables, and beverages including tea or wine. [17] In addition, a few of polyphenols are widely accepted to suppress vascular changes associated with aging. [18-20] Coffee is the very popular beverage in the world and has been consumed for their attractive flavors and physiological effects. Several studies have reported that a relationship between the consumption of coffee and the potential health benefits which might be correlated with the polyphenols contained. [21-23] Recent investigations have demonstrated that coffee showed 24% of risk reduction in total mortality in subjects who consumed 3–4 cups a day compared with in non-drinkers, additionally, the consumption of coffee was inversely associated with mortality from heart disease and cerebrovascular disease. [24] One of the most abundant polyphenols in coffee is chlorogenic acid. [25] Moreover, it has been found that chlorogenic acid has anti-oxidant, [26] anti-inflammatory, [26] anticancer, [27] antidiabetic, [28] antihypertensive, [29] and antineurodegenerative activities. [30][31] Despite these promising and diverse antisenescence actions, investigations addressing the effect of CGA on senescence are scarce.

Nrf2 was cloned in 1994, by Kan et al. as a factor that binds to the NE-F2 coupling array in the globulin gene expression control region. [32] Recently, Nrf2 has been successively reported to play important roles in detoxification, oxidative stress, and inflammation. Attention is focused on the role of Nrf2 in aging research, since several evidences revealed that Nrf2 attenuated oxidative stress and inflammation, both of which are known to lead aging. Moreover, Nrf2 has been demonstrated to play a role in slowing aging processes by mediating the beneficial effects of many manipulations that extend longevity and health span. [33,34] Several polyphenols, such as resveratrol and curcumin, have been reported to attenuate aging via the Nrf2/HO-1 pathway. [35-37] In addition, CGA has been reported to enhance Nrf2/HO-1 pathway in several cells. [38]

Therefore, this study is aimed to test the hypothesis that CGA protects against vascular senescence and to explore the role of Nrf2/HO-1 pathway in vivo and in vitro.

2. Results

2.1. CGA Inhibits AngII-induced Vascular Senescence

To investigate the effect of CGA on vascular senescence in vivo, CGA was administered to mice. No significant difference was found in body weight and heart rate among the saline/saline, the saline/CGA, the AngII/saline, and the AngII/CGA groups. (Table 1) Systolic blood pressure (SBP) was notably higher in the AngII/saline group compared with the saline/saline and the saline/CGA groups. (Table 1)

Table 1. Characteristics of the study mice

	saline / saline	saline / CGA high	Ang II / saline	AngII / CGA low	AngII / CGA high
N	4	4	4	4	4
BW (g)	33.5 ± 5.8	34.2 ± 5.1	29.9 ± 1.4	28.0 ± 1.4	31.6 ± 3.7

| HR (bpm) | 648 ± 28 | 662 ± 7 | 682 ± 43 | 660 ± 86 | 702 ± 23 |
| SBP (mmHg) | 104 ± 7 | 107 ± 9 | 129 ± 5 * # | 121 ± 13 | 119 ± 7 |

N: number, BW: body weight, HR: heart rate, SBP: systolic blood pressure, Data present mean ± SD, *$p < 0.05$ vs saline / saline, # $p < 0.05$ vs saline / CGA high (One-way ANOVA on Rank).

Aorta was dissected from these mice and the phenotype of senescence with and without CGA administration was evaluated by senescence associated-β-galactosidase (SA-β-gal) assay. SA-β-gal staining increased in AngII-infused mice compared to saline-infused mice. The mice treated with CGA suppressed AngII-induced senescence in ECs in dose-dependent manner. (Figure 1)


saline / saline   saline / CGA high   AngII / saline   AngII / CGA low   AngII / CGA high
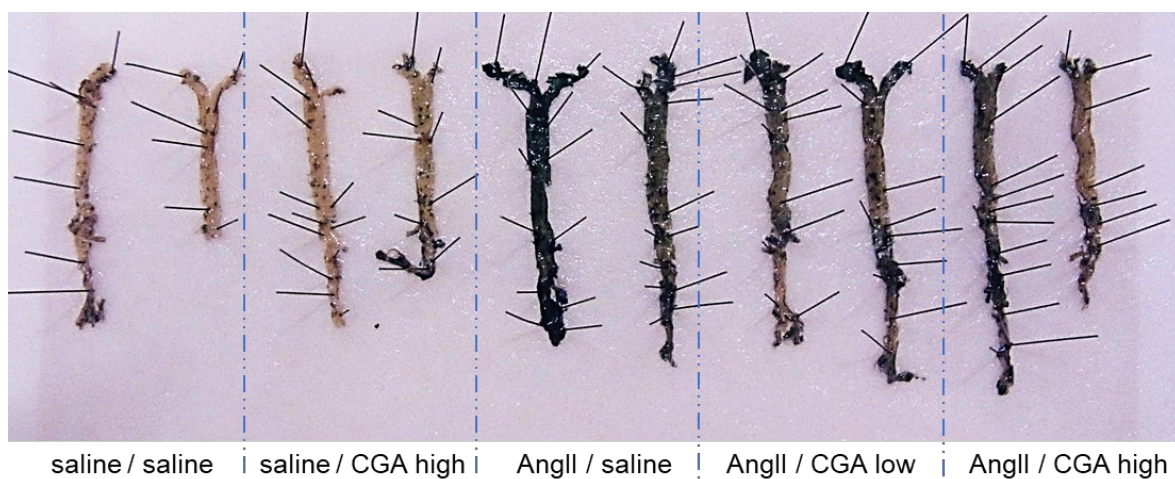
Figure 1. Effects of CGA on vascular senescence. SA-β-gal staining of aorta with or without CGA low (20mg/kg/day) or high (40 mg/kg/day) at 14 day after AngII infusion are shown.

2.2. Treatment with CGA Attenuates H_2O_2-induced Cellular Senescence in HUVECs

Next, to examine the preferable effect of CGA in vitro, HUVECs were treated with H_2O_2 to induce senescence. H_2O_2 increased the number of 8-hydroxy-2'-deoxyguanosine (8-OHdG) positive cells, suggesting that DNA damage level increased in HUVECs. Treatment with CGA reduced the number of 8-OHdG positive cells in dose-dependent manner. (Figure 2(a)) In addition, H_2O_2 induced flattened morphology and increased SA-β-gal activity. Treatment with CGA attenuated SA-β-gal activity and restored the morphological appearance of senescence in dose-dependent manner. (Figure 2(b)) Furthermore, H_2O_2 reduced cell proliferation. (Figure 2(c)) Treatment with CGA of 1.0 μM abrogated the suppression of cell proliferation by H_2O_2. However, CGA of 5.0 μM led severe DNA damage, flattened morphology, enhanced SA-β-gal activity, and reduced cell proliferation, indicating toxicity. Thus, 0.5 and 1.0 μM concentrations of CGA were used for further experiments. To investigate the effect of CGA without H_2O_2, HUVECs were exposed to different concentrations of CGA for 3 days, then the protein expression of Sirtuin 1 (Sirt1) and endothelial nitric oxide synthase (eNOS) were assessed. The expression of Sirt1 and eNOS increased in dose dependent manner of CGA. (Figure 2(d))

(a)
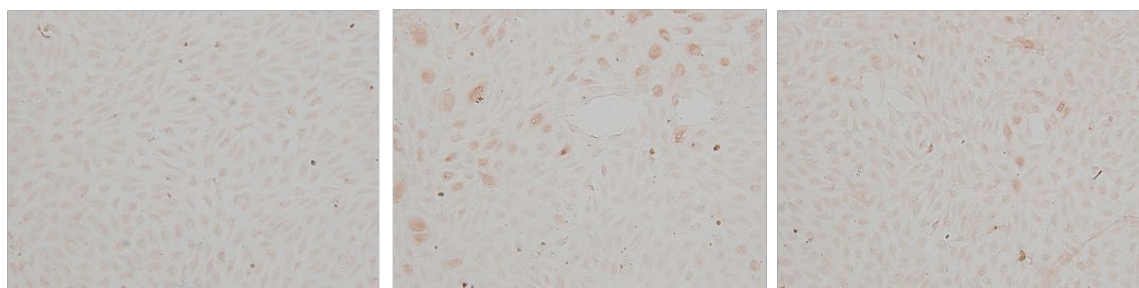
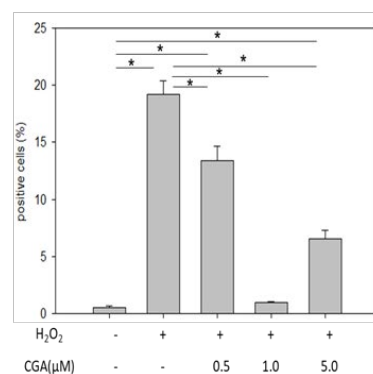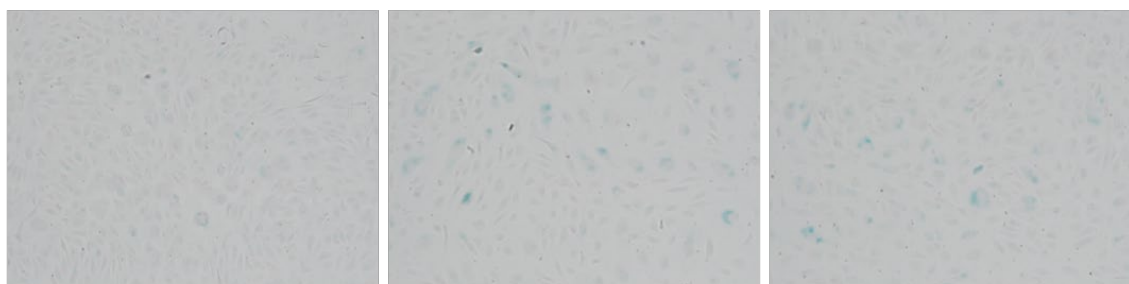

H₂O₂	-	+	+
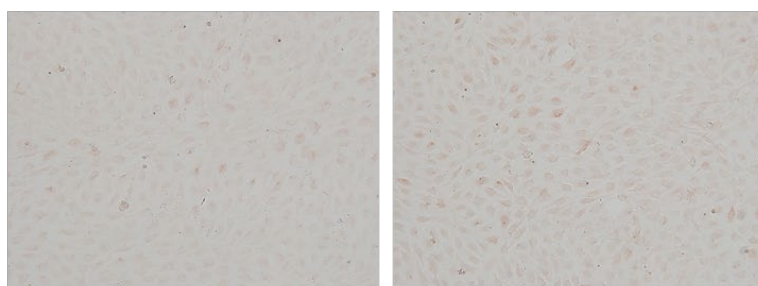
H_2O_2 -   +   +

CGA(μM)   -   -   0.5

H_2O_2 +   +

CGA (μM)   1.0   5.0
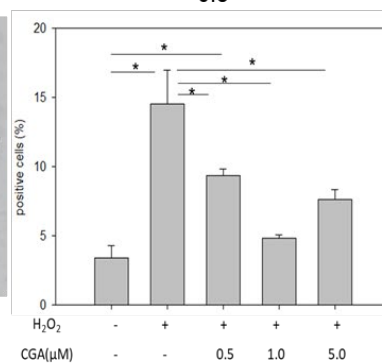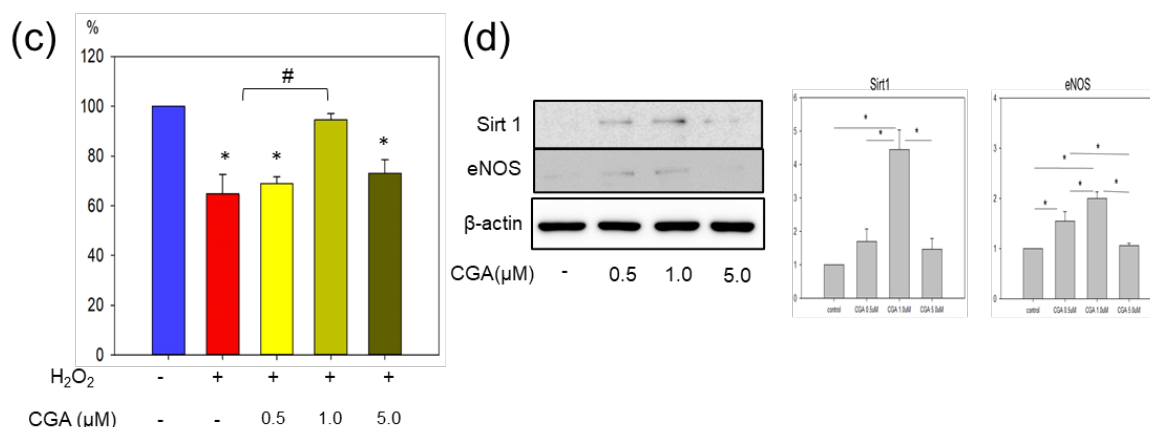
1

(b)

H_2O_2 -   +   +

CGA(μM)   -   -   0.5
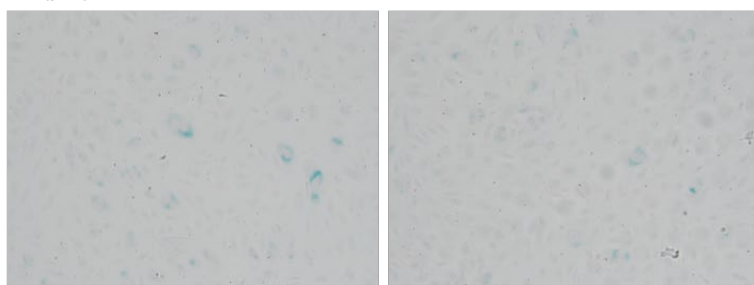
H_2O_2 +   +

CGA (μM)   1.0   5.0

2

Figure 2. Effects of CGA on HUVECs. (a,b) Immunostainings of (a) 8-OHdG, (b) SA-β-gal staining, and (a,b) morphological changes *$p < 0.05$ (n=6) Each bar presents the mean ± SE of six experiments; (c) Cell proliferation was determined using CCK-8 kit. *$p < 0.05$ vs CGA-/H_2O_2-, # $p < 0.05$ (n=3) Each bar presents the mean ± SE of three experiments; (d) Protein expression of Sirt1 and eNOS in CGA-treated HUVECs. *$p < 0.05$ (n=3) Values represent the means ± SE of three experiments.

2.3. CGA Exerts Favorable Effect on Senescence-related Markers

Exposure to H_2O_2 decreased the expressions of Sirt1 and eNOS, although not significantly. (Figure 3)　Contrary to expectations, treatment with CGA 0.5 μM significantly increased the expressions of Sirt1 more than the CGA untreated groups, however treatment with CGA 1.0 μM restored the expressions of Sirt1 no significantly. ($p = 0.06183$)　On the other hand, exposure to H_2O_2 significantly increased the expressions of plasminogen activator inhibitor-1 (PAI-1), p53, and p21, however, treatment with CGA significantly suppressed their expressions almost in dose-dependent manner. (Figure 3)
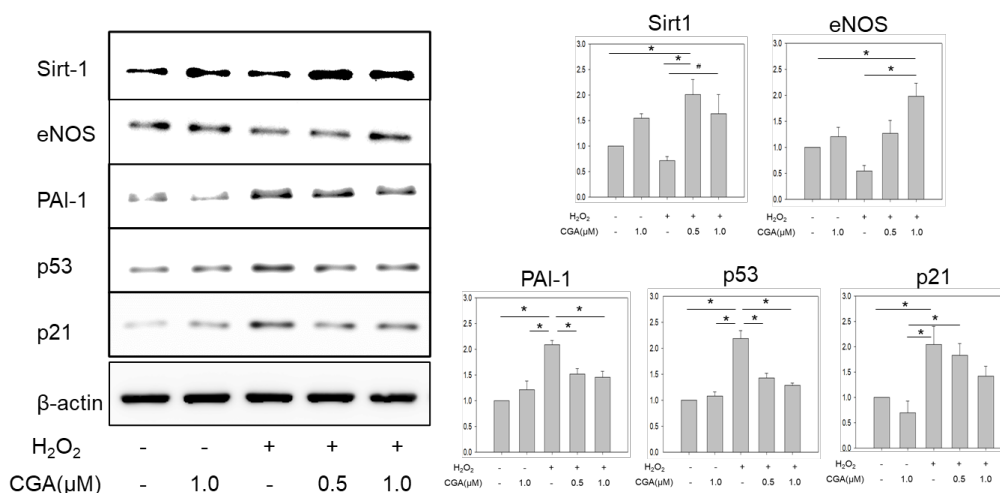

Figure 3. Effects of CGA on the senescence related molecules. Protein expression of Sirt1, eNOS, PAI-1, p53, and p21, Quantitative analyses of the results. *$p < 0.05$, # $p = 0.06183$, (n=6).　Values represent the means + SE of three experiments.

2.4. CGA induces Nrf2 and HO-1 expression

To further investigate the anti-senescence mechanism of CGA, HUVECs were exposed to different concentrations of CGA for 3 days. the expression of Nrf2 and HO-1 were examined. Not CGA of 0.5 μM but of 1.0 μM significantly increased protein level of Nrf2. (Figure 4(a)) However,

1   mRNA levels of Nrf2 and keap1 showed no significant changes at each indicated time after the
2   stimulation of H_2O_2 treatment (Supplementary Figure A1), suggesting that CGA may induce the
3   expression of Nrf2 at the post-transcriptional level but not at the transcriptional level. No significant
4   change in HO-1 protein was detected. (Figure 4(a)) Therefore, 1.0 μM concentrations of CGA was
5   used for the following experiments. Next, after 3 days exposure to CGA or vehicle, HUVECs were
6   stimulated with/without H_2O_2 for 1 hour. Regarding Nrf2, the translocation into the nucleus was
7   observed only in CGA+/H_2O_2+ group at 1 hour after stimulation with H_2O_2. (Figure 4(b)) However,
8   this translocation significantly increased in CGA-/H_2O_2+ group compared with CGA+/H_2O_2+ group.
9   Six hours after stimulation with H_2O_2, Nrf2 was detected only in the cytoplasm in the CGA+ groups
10   irrespective of co-incubation with H_2O_2. (Figure 4(b)) Regarding HO-1, mRNA level was determined
11   by qPCR at the indicated time. (Figure 4(c)) HO-1 expression increased significantly in CGA+/H_2O_2-
12   and CGA-/H_2O_2+ group, compared with CGA-/H_2O_2- group at 0 hour. HO-1 expression tended to
13   increase more in CGA+/H_2O_2+ group than CGA-/H_2O_2+ group. ($p = 0.08380$) Given these observation,
14   CGA induced HO-1 expression irrespective of co-incubation with H_2O_2. A half hour and an hour after
15   H_2O_2 stimulation, HO-1 expression increased more in CGA-/H_2O_2+ group than CGA+/H_2O_2+ group.
16   CGA also increased protein expression of HO-1, (Figure 4(d)) as well as mRNA expression. However,
17   3 hours after H_2O_2 stimulation, HO-1 protein level increased only in CGA-/H_2O_2+ group, compared
18   with all other groups. Further, protein expression of p-Akt increased in CGA-/H_2O_2+ group compared
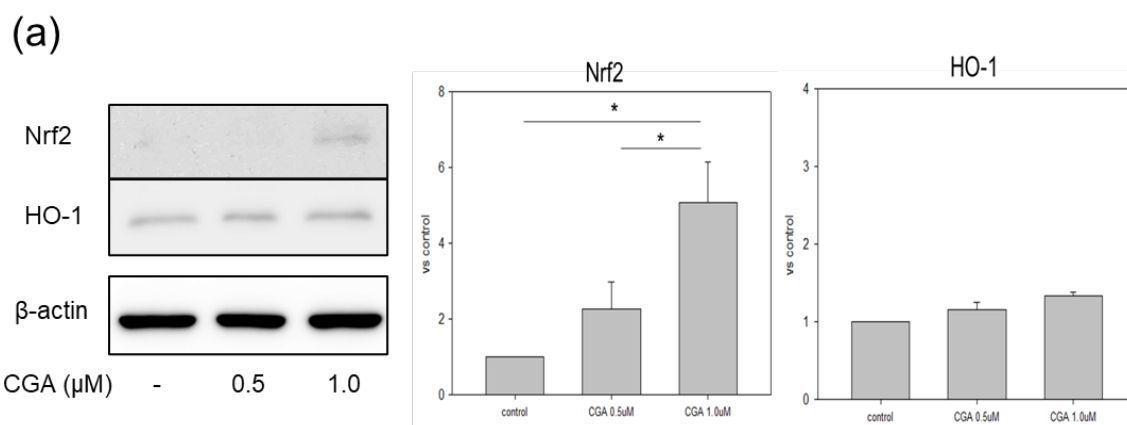
19   with all other groups. (Figure 4(e))
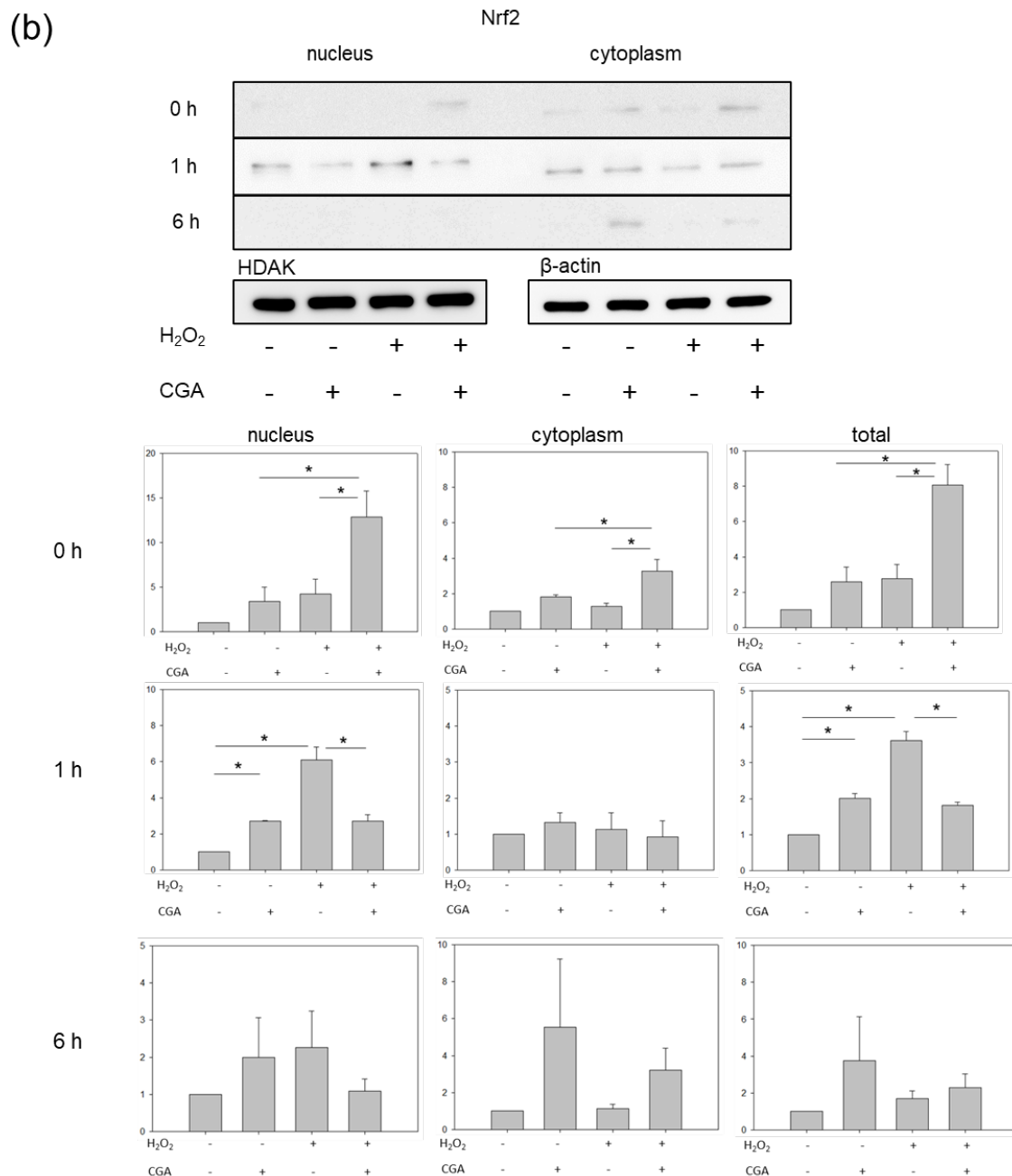
1
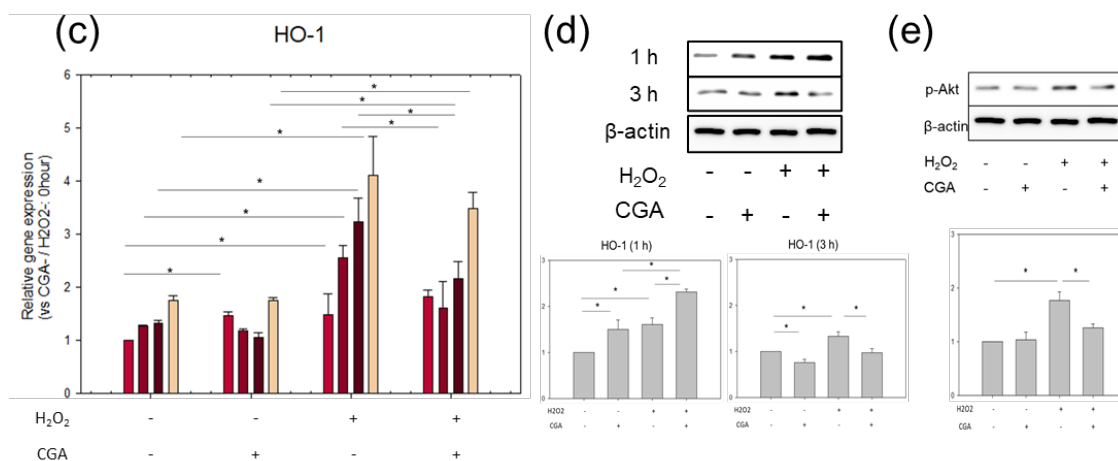
2

1   **Figure 4.** Effects of CGA on the expression of Nrf2-related proteins and mRNA. (a) Protein
2   expression of Nrf2 and HO-1 in CGA-treated HUVECs (n=3) *$p < 0.05$ Values represent the
3   means + SE of three experiments.; (b) Protein expression of Nrf2 in the nucleus and cytoplasm
4   in CGA-treated HUVECs after stimulation with H_2O_2 (n=3) . *$p < 0.05$ Values represent the means
5   + SE of three experiments.; (c) mRNA (n=3) *$p < 0.05$ Each bar presents the mean + SE of three
6   experiments and (d) protein expression of HO-1 (n=3) Values represent the mean + SE of three
7   experiments.; (e) Proteins expression of p-Akt. (n=3).   Values represent the means + SE of three
8   experiments.

9   *2.5. CGA Attenuates Senescence of Vascular Endothelial Cells through Nrf2/HO-1 pathway.*

10   To investigate whether CGA attenuates vascular EC senescence through Nrf2/HO-1 pathway, a
11   specific HO-1 inhibitor (zinc protoporphyrin IX, ZnPP) were administered to AngII-induced mice.
12   Body weight and pulse rate were unaltered among all groups.   SBP was notably higher in the
13   AngII/saline group compared with the saline/saline group. (Table 2) The favorable effect of CGA on
14   vascular senescence was canceled by ZnPP in vivo. (Figure 5(a))

15
**Table 2.** Characteristics of the study mice

	saline / saline	AngII / saline	AngII / CGA	AngII / CGA / ZnPP
N	5	5	5	5
BW (g)	29.1 ± 5.5	28.7 ± 1.5	29.1 ± 3.8	27.2 ± 2.2
HR (bpm)	643 ± 62	663 ± 63	629 ± 99	564 ± 104
SBP (mmHg)	109 ± 9	127 ± 5*	113 ± 9	112 ± 9

16   N: number, BW: body weight, HR: heart rate, SBP: systolic blood pressure, Data present mean ±
17   SD. *$p < 0.05$ vs saline / saline (One-way ANOVA on Rank).

18   To further elucidate whether the upregulation of HO-1 induced by CGA confers attenuation of
19   senescence, the effect of ZnPP was also examined in vitro. ZnPP showed augmented cellular
20   senescence irrespective of CGA. (Figure 5(b)) Moreover, the protein expression of Sirt1, eNOS, PAI-
21   1 and p21 in aorta were examined among each group. Treatment of CGA increased Sirt1 and eNOS
22   but reduced PAI-1 and p21. ZnPP treatment cancelled all these effects of CGA. (Figure 5(c)) Taken
23   together, these results clearly show that CGA attenuates vascular EC senescence through Nrf2/HO-1
24   pathway.

(a)

saline / saline   AngII / saline   AngII / CGA   AngII / CGA / ZnPP
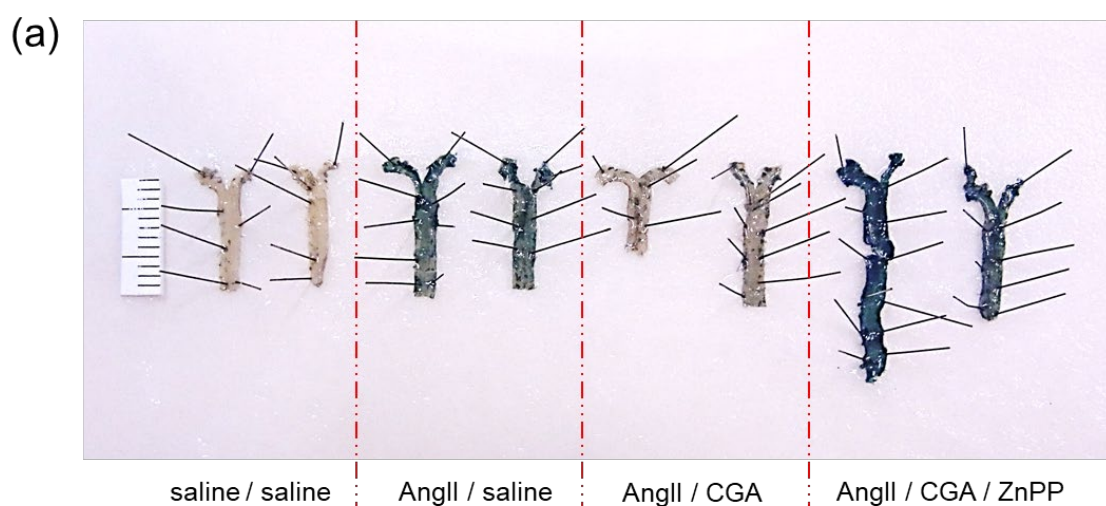
(b)
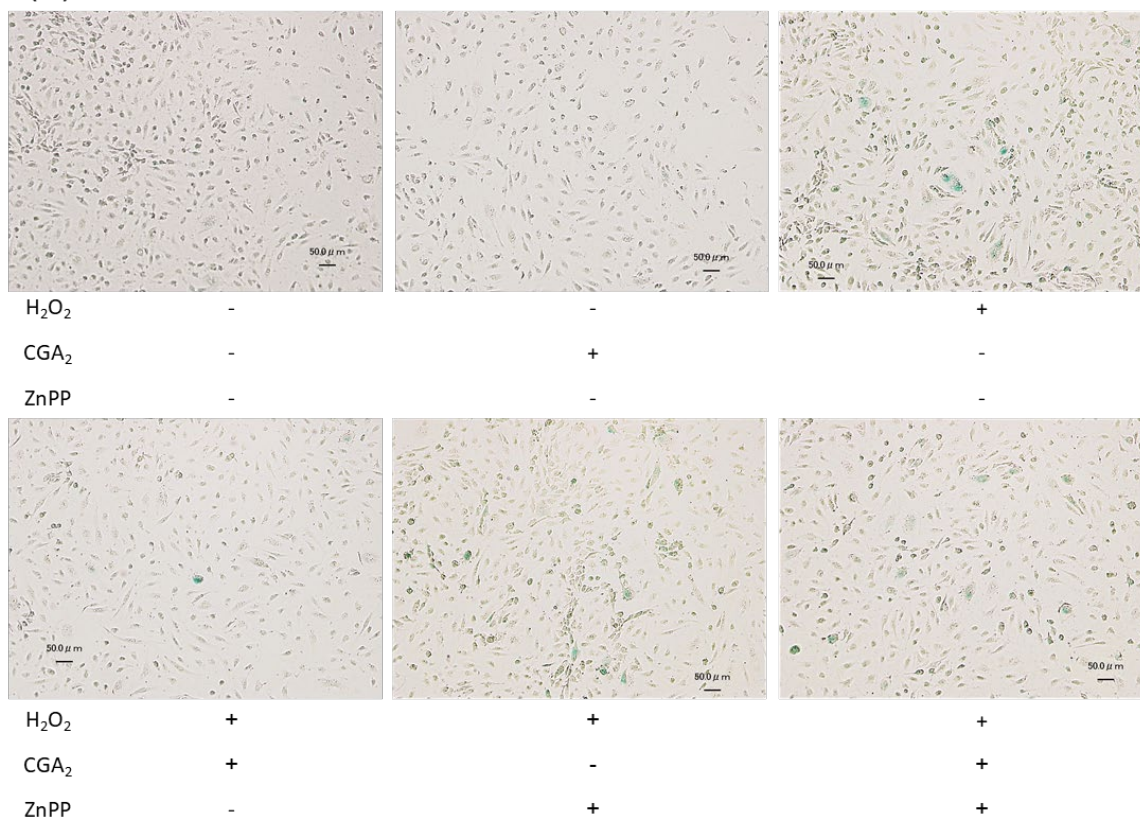
(c)
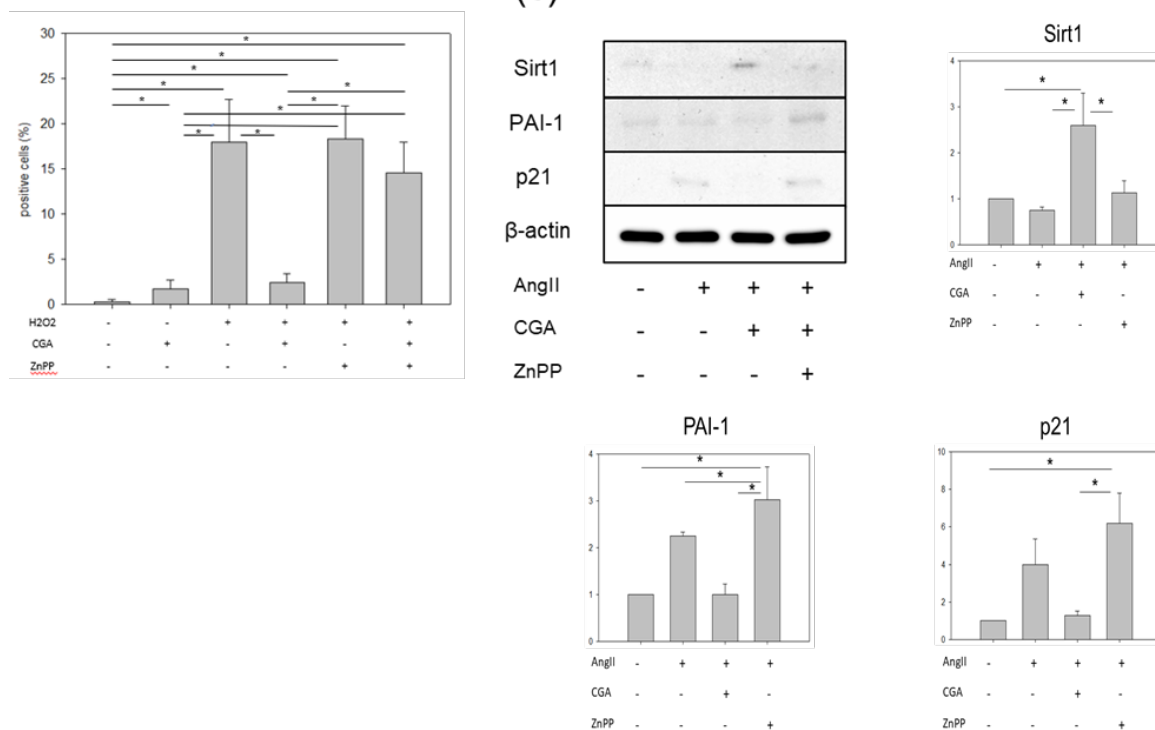
1
2
3 **Figure 5.** Effect of HO-1 inhibitor on vascular senescence in vivo and in vitro. (a) SA-β-gal
4 staining of aorta with or without CGA, and/or i.p. injection of ZnPP, and/or AngII for 14 days;
5 (b) SA-β-gal staining of HUVECs co-incubated with or without H_2O_2, and/or CGA, and/or ZnPP.

1 The scale bar indicates 50 μm. Each bar presents the mean + SE of three experiments (n=6) *p <
2 0.05; (c) Protein expression of Sirt1, eNOS, PAI-1 and p21 in aortas. (n=3). *p < 0.05   Values
3 represent the means + SE of three experiments.

4 **3. Discussion**

5   In this study, we found for the first time that CGA attenuated vascular senescence, in association
6 with the increase of Sirt1 and eNOS, and with the decrease of p-Akt, PAI-1, p53 and p21. Furthermore,
7 in vivo and vitro study, we revealed that ZnPP canceled the anti-senescence effect of CGA. Thus, our
8 study demonstrated that CGA inhibited endothelial senescence by regulating Nrf2/HO-1 pathway.
9   AngII is a commonly used as an oxidative stress model in animal study. Chronic infusion of
10 AngII induces oxidative-associated vascular senescence. [39] Since many reports on mice with
11 senescence induced by AngII has been published, we used the AngII-induced model to examine
12 vascular senescence in vivo. To enhance oxidative stress in vitro, we used H_2O_2 to treat HUVECs,
13 leading to cellular senescence. Recent investigations have demonstrated that CGA activates Sirt1 in
14 ECs. [40] In the present study, as expected, CGA increased Sirt1 and eNOS expression. These
15 molecules are closely associated with vascular senescence, therefore, CGA has, in part at least, an
16 anti-aging effect. Since eNOS upregulated by CGA is a target downstream of activated Akt/PKB, the
17 expression of p-Akt was examined. Although it was assumed that the phosphorylation status of Akt
18 showed a similar tendency of the expression of eNOS, the result was different than expected. Akt has
19 been activated with H_2O_2, and CGA canceled the activation of Akt. Activation of the Akt not only
20 leads to the excessive production of ROS, but also increases expression of HO-1. The breakdown of
21 the original redox homeostasis by high levels of intracellular oxidation or anti-oxidation, is probably
22 an important factor for senescence. Pretreatment of CGA may restore the original intracellular redox
23 homeostasis by modulating AKT1 phosphorylation. Previously, a different kind of polyphenols,
24 Mogroside V, was reported to attenuate the activation of Akt. [41]
25   Similarly, resveratrol is a plant polyphenol that activates Sirt1 and eNOS. [40,42,43] Activation
26 of the Nrf2 and Sirt1 signaling pathways by resveratrol ameliorated the cellular unfavorable
27 condition, such as renal injury due to oxidative stress and mitochondrial dysfunction caused by aging.
28 [44] Resveratrol detained age-related cognitive decline through Sirt1. [45] Thus, resveratrol has the
29 anti-aging effect. In this sense, polyphenols may be commonly equipped with anti-aging effect.
30 However, it is still unclear whether the upregulation of Sirt1 and eNOS by CGA is caused by the
31 same mechanism as resveratrol does. Further studies are needed to elucidate the precise mechanism
32 how CGA regulate Sirt1 and eNOS expression. Basically, polyphenols exist in nature, are non-
33 invasive properties, and seem to have minimal side effects for human being, although the toxicity of
34 a few polyphenols at high concentrations have been reported in vitro study, as seen in the present
35 study. [46]
36   Nrf2 is a transcription factor responsible for the regulation of cellular redox balance and
37 protective antioxidant and phase II detoxification responses in mammals. [47,48] We hypothesized
38 that CGA may enhance the levels of phase II enzymes. In this study, we examined the several mRNA
39 expression of typical phase II enzymes, Superoxide dismutase 1(SOD1), Catalase (CAT), NAD(P)H:
40 quinone oxidoreductase 1 (NQO-1), Glutamate-cysteine ligase catalytic subunit (GCLC), and
41 Glutamate-cysteine ligase modifier subunit (GCLM), by qPCR, however, failed to find any significant
42 changes in most of the mRNA levels other than HO-1. It is known that HO-1 has the highest capability
43 to diminish oxidative stress among the genes induced by Nrf2, and plays an important role to prevent
44 many diseases caused by oxidative stress, like cardiovascular diseases. [49,50] This was because we
45 focused Nrf2/HO-1 pathway regarding the anti-aging effect of CGA on vascular senescence in this
46 study.
47   We further investigated the link between CGA and Nrf2/HO-1 pathway. In vitro study clearly
48 demonstrated that treatment with CGA enhanced Nrf2 in the cytoplasm due to inhibition of its
49 degradation by CGA. This result suggests that CGA makes these cells ready to bear oxidative stress.
50 Therefore, after stimulation with H_2O_2, Nrf2 translocated into the nucleus faster in the CGA-treated
51 cells, compared to those without CGA. Consequently, translocated Nrf2 could increase HO-1 mRNA

and protein early. This increased HO-1 could promptly diminish oxidative stress, resulting in the attenuation of the vascular senescence. The fact that a specific HO-1 inhibitor canceled the beneficial effect of CGA, confirmed the impact of CGA on the regulation of HO-1 in anti-aging. Taken together, the beneficial effects of CGA on endothelial cell senescence were, at least partly, dependent on Nrf2/HO-1 pathway. Previously, a different kind of polyphenols, fisetin, was reported to inhibit the degradation of Nrf2. [46] It appears that this property might be commonly inhered in polyphenols.

Catechins from tea, anthocyanins from blueberries, and curcumin from curry are the famous polyphenols. Coffee contains many kinds of polyphenols as much as red wine. [25] A recent cohort study revealed that the cardioprotective effect of low dosage of resveratrol (10 mg/day) in the patients with stable coronary artery disease. [51] Red wine contains 1-75 mg of trans-resveratrol / L. [52] Therefore, to obtain the significant effect of resveratrol like above, it is necessary to drink 1.5 L or more of red wine. As mentioned earlier, individuals who habitually drink at least 3–to-4 cups of coffee a day have a reduced risk of mortality, heart disease, cerebrovascular disease, and respiratory disease [19]. A diet rich in polyphenols, at least CGA, may slow down aging, decrease the risk of age-related diseases, and improve the quality of life of the people in the world.

In conclusion, our study demonstrated that CGA inhibited endothelial senescence both in vivo and in vitro by regulating Nrf2/HO-1 pathway. CGA may be a new therapeutic target to attenuate endothelial senescence. CGA may help in developing apt intervention therapies that decelerate the pace of aging as well as diminish or defer the prevalence of age-related diseases.

4. Materials and Methods

4.1. Mice and Study Protocol

C57/BL6 female mice of 14 ± 3 months old were purchased from The Jackson Laboratory (Bar Harbor, Cat. No. 000664). All mice were maintained in a barrier facility, and ambient temperature ranged from 20°C to 24°C. Mice were fed diet and water ad libitum. The mice were divided into 5 groups; saline/saline (n=4), saline/CGA (n=4), saline/AngII (n=4), CGA low/AngII (n=4), and CGA high/AngII (n=4). Saline or AngII (1,000 ng/kg/min, Bachem, Cat. No. H-1705-0100) was infused via Alzet mini-osmotic pumps (Alzet, Model 2002) for 14 days. Mini-osmic pumps were implanted subcutaneously on the right flank, as described previously. [53,54] In the CGA (SIGMA, Cat. No. C3878) low and high groups, the mice were administered 20 or 40 mg/kg/day CGA via oral gavage for 14 days from the initial day of AngII infusion. In the other groups, the mice were given saline via oral gavage for 14 days from the initial day of AngII infusion. The mice were sacrificed at 14 day after AngII infusion. The aorta was removed after systemic perfusion with phosphate-buffered saline for histological examination. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical sciences (OKU-2018875).

4.2. Blood Pressure Measurement

SBP and pulse rate were measured by sphygmomanometry using a tail cuff system (Visitech Systems, BP-2000) following a published protocol. [55] Conscious mice were introduced into a small holder mounted on a thermostatically controlled warming plate and maintained at 37°C during measurement.

4.3. SA-β-gal Staining

Cellular Senescence Assay Kit (CELL BIOLABS, INC. Cat. No.CBA-230) was used throughout according to the company's instructions.

4.4. Cell Culture and Treatment

HUVECs were purchased from Lonza (Lonza, Cat. No. C2519A 01127: multi donor) and were grown in endothelial growth medium (EGM™-2 Bullet Kit™ Medium: Lonza Cat. No.CC-3162).

Population doubling levels were calculated as described previously, [56] and all experiments were performed at population doubling levels of 7 to 9. HUVECs were grown in 75 cm^2 collagen coated flask to 80 % confluence for 3 days in the absence or presence of 0.5 - 5.0 μM CGA. The media changed at 0 and 2 day (pre-treatment). Then, HUVECs were washed 3 times and stimulated for 1 hour with 100 μM H$_2$O$_2$. After the stimulation, HUVECs were cultured with EGM-2 in the absence or presence of 0.5 - 5.0 μM CGA for appropriate time.

4.5. Cellular Senescence

To valuate cellular senescence, HUVECs were plated at 1.0×10^4 cells/well into an 8-well chamber slide (Iwaki, Cat. no. 5732-008) after pre-treatment with or without CGA and stimulated by H$_2$O$_2$, as described herein. Then, HUVECs were cultured with EGM-2 in the presence or absence of 0.5 - 5.0 μM CGA for appropriate time. The morphological change of cells was observed by a phase-difference microscope. SA-β-gal activity was analyzed by staining as described above. Immunoperoxidase staining was performed to evaluate oxidative stress in HUVECs using 8-OHdG antibody (Japan Institute for the Control of Aging NIKKEN SEIL, Cat. No. clone N45.1). Reactivity of the antibodies with tissue antigens was detected using AEC and ImmPACT AEC HRP Substrate (Vector Laboratories, SK-4200) as described previously. [53,54]

4.6. Cell Viability

HUVECs were plated at 5.0×10^3 cells/well into a 96 well plate (Corning, Cat. no. 354407) after pre-treatment with CGA and stimulated by H$_2$O$_2$, as described above. After the stimulation, HUVECs were cultured with EGM-2 containing these compounds for 3 days. The cell viability in the cell proliferation and cytotoxicity assays was determined by the Cell Counting Kit-8 (CCK-8) (Dojin, Cat. no. 347-07621) according to the company's instructions.

4.7. Western Blotting

Whole cell proteins were extracted from HUVECs or aortic tissue using lysis buffer (Cell Signaling, Cat. No. 9803). Nuclear and cytoplasmic proteins were extracted from HUVECs using a nuclear extract kit (ACTIVE MOTIF, Cat. No. 40010) according to the manufacturer's instructions. Each sample was loaded onto 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane, immunoblotted with primary antibodies (Sirt-1; Cell Signaling Cat. No. 9475S, eNOS; Cell Signaling Cat. No. 9572S, PAI-1; Cell Signaling Cat. No. 11907S, p53; Cell Signaling Cat. No. 9282S, p-Akt; Cell Signaling Cat. No. 4060S, Nrf2; abcam Cat. No. ab62352, HO-1; abcam Cat. No. ab13243, HDAC; Cell Signaling Cat. No. 34589, and β-actin; Sigma Cat. No. A5441). Membranes were then incubated with appropriate secondary antibodies, and immune complexes were visualized on chemiluminescence (Merck Millipore, Cat. no. WBLUF0100, Cat. no. WBLUC0100) and quantified using a General Electric Imager (GE Healthcare, LAS 4000 mini). [53,54]

4.8. Real-Time Polymerase Chain Reaction

mRNAs were extracted from HUVECs using RNeasy Mini kits (Qiagen, Cat. No. 74104). Reverse transcription was performed using iScript cDNA synthesis kit (Bio Rad, Cat. No. 1708891). PCR reactions were performed with an ABI Step One Real-Time PCR System (Applied Biosystems, Quant Studio3) using Fast SYBR Green Real-time PCR Mixture (Applied Biosystems, Cat. No. 4385612). [53] Primers for Nrf2, kelch-like ECH-associated protein 1 (keap1), HO-1, SOD1, CAT, NQO-1, GCLC, GCLM, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were available commercially (Takara Bio Inc.). Each sample was normalized to values for GAPDH mRNA expression (ΔΔCT method).

4.9. Reagents

Stock solutions of ZnPP (Wako Pure Chemical Industries, Cat. No. 167-13651), a HO-1 inhibitor was diluted with 0.25 M NaOH to make final concentration 10 μg/μL. For administration to mice, the

stock solutions were diluted with phosphate-buffered saline by 1% (v/v) for 20 μg/mouse. As the control of in vitro and in vivo treatments, vehicle was used; for in vitro experiment, 0.25 M NaOH was added to culture medium (final pH of culture medium was 7.6 ± 0.3); for in vivo treatment, 1% (v/v) 0.25 M NaOH were used (final pH of the injection solution was 8.3 ± 0.3). [57]

4.10. Treatment with ZnPP in vivo

The mice were divided into 4 groups; saline/saline (n=5), saline/AngII (n=5), CGA/AngII (n=5), and CGA/AngII/ZnPP (n=5). Saline or AngII (1,000 ng/kg/min) was infused via Alzet mini-osmotic pumps for 14 days. Mini-osmic pumps were implanted subcutaneously on the right flank, as described previously. [53,54] In the CGA group, the mice were administered 40 mg/kg/day CGA via oral gavage for 14 days from the initial day of AngII infusion. In other groups, mice were given saline via oral gavage for 14 days from the initial day of AngII infusion. ZnPP (20 μg/mouse) was injected into the peritoneal cavities every other day for 14 days from the initial day of AngII infusion. All mice were sacrificed at 14 day after AngII infusion. [57]

4.11. Treatment with ZnPP in vitro

To evaluate cellular senescence, HUVECs were plated at 1.0×10^4 cells/well into an 8-well chamber slide after pre-treatment with CGA and stimulated by H_2O_2 with or without ZnPP of 0.5 μM, as described herein. Then, HUVECs were cultured with EGM-2 in the absence and presence of 1.0 μM CGA with or without ZnPP of 0.5 μM for 24 hours. The morphological change of cells was evaluated by a phase-difference microscope. SA-β-gal activity was analyzed by staining as described previously.

4.12. Statistics

All statistical analyses were performed using Sigma Plot v14.0 (Systat Software Inc. California, USA). Data are presented as mean ± standard deviation or standard error of the mean where appropriate. Statistical significance between multiple groups was assessed by one-way or two-way analysis of variance followed by Holm-Sidak post hoc test or Student–Newman–Keuls post hoc test. A *p* value < 0.05 was considered statistically significant.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1. Supplemental Figure A1. mRNA expression of Nrf2, keap1, SOD1, CAT, NQO1, GCLC, and GCLM in CGA-treated HUVECs after stimulation with H_2O_2. (n=3) The X axis represents time (hour), and the Y axis represents relative gene expression (vs control: 0 hour). *: *p* < 0.05.

Author Contributions: Conceptualization, Y.H. and H.A.U.; methodology, Y.H. and H.A.U.; software, Y.H.; validation, Y.H. and H.A.U.; formal analysis, Y.H., H.A.U., and H.T.; investigation, Y.H., H.A.U., N.O., Y.O.,S.O., M.N. and H.T.; resources, Y.H.; data curation, Y.H.; writing—original draft preparation, Y.H.; writing—review and editing, H.A.U., N.O., Y.O., S.O., M.N., R.T., H.T. and J.W.; visualization, Y.H.; supervision, J.W.; project administration, H.A.U.; funding acquisition, H.A.U. All authors have read and agreed to the published version of the manuscript.

Abbreviations

EC	Endothelial cell
AngII	Angiotensin II
CGA	Chlorogenic acids
Nrf2	Nuclear factor erythroid 2-factor 2
HO-1	Heme Oxygenase-1

SBP	Systolic blood pressure
SA-β-gal	Senescence associated-β-galactosidase
HUVEC	Human Umbilical Vein Endothelial Cell
8-OHdG	8-hydroxy-2'-deoxyguanosine
Sirt1	Sirtuin 1
eNOS	endothelial nitric oxide synthase
PAI-1	Plasminogen activator inhibitor-1
ZnPP	Zinc protoporphyrin IX
keap1	kelch-like ECH-associated protein 1
SOD1	Superoxide dismutase 1
CAT	Catalase
NQO1	NAD(P)H: quinone oxidoreductase 1
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit

References

1. Hill, J.M.; Zalos, G.; Halcox, J.P.; Schenke, W.H.; Waclawiw, M.A.; Quyyumi, A.A.; Finkel, T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* **2003**, *348*, 593-600, doi:10.1056/NEJMoa022287.

2. Vanhoutte, P.M.; Shimokawa, H.; Tang, E.H.; Feletou, M. Endothelial dysfunction and vascular disease. *Acta Physiol (Oxf)* **2009**, *196*, 193-222, doi:10.1111/j.1748-1716.2009.01964.x.

3. Urbich, C.; Dimmeler, S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* **2004**, *95*, 343-353, doi:10.1161/01.RES.0000137877.89448.78.

4. Wu, J.; Xia, S.; Kalionis, B.; Wan, W.; Sun, T. The role of oxidative stress and inflammation in cardiovascular aging. *Biomed Res Int* **2014**, *2014*, 615312, doi:10.1155/2014/615312.

5. Ghebre, Y.T.; Yakubov, E.; Wong, W.T.; Krishnamurthy, P.; Sayed, N.; Sikora, A.G.; Bonnen, M.D. Vascular Aging: Implications for Cardiovascular Disease and Therapy. *Transl Med (Sunnyvale)* **2016**, *6*, doi:10.4172/2161-1025.1000183.

6. Costantino, S.; Paneni, F.; Cosentino, F. Ageing, metabolism and cardiovascular disease. *J Physiol* **2016**, *594*, 2061-2073, doi:10.1113/JP270538.

7. Naylor, R.M.; Baker, D.J.; van Deursen, J.M. Senescent cells: a novel therapeutic target for aging and age-related diseases. *Clin Pharmacol Ther* **2013**, *93*, 105-116, doi:10.1038/clpt.2012.193.

8. Park, S.Y.; Kwon, O.S.; Andtbacka, R.H.I.; Hyngstrom, J.R.; Reese, V.; Murphy, M.P.; Richardson, R.S. Age-related endothelial dysfunction in human skeletal muscle feed arteries: the role of free radicals derived from mitochondria in the vasculature. *Acta Physiol (Oxf)* **2018**, *222*, doi:10.1111/apha.12893.

9. Kim, S.; Piao, S.; Lee, I.; Nagar, H.; Choi, S.J.; Shin, N.; Kim, D.W.; Shong, M.; Jeon, B.H.; Kim, C.S. CR6 interacting factor 1 deficiency induces premature senescence via SIRT3 inhibition in endothelial cells. *Free Radic Biol Med* **2020**, *150*, 161-171, doi:10.1016/j.freeradbiomed.2020.02.017.

10. Schosserer, M.; Grillari, J.; Breitenbach, M. The Dual Role of Cellular Senescence in Developing Tumors and Their Response to Cancer Therapy. *Front Oncol* **2017**, *7*, 278, doi:10.3389/fonc.2017.00278.

11. Romero, A.; San Hipolito-Luengo, A.; Villalobos, L.A.; Vallejo, S.; Valencia, I.; Michalska, P.; Pajuelo-Lozano, N.; Sanchez-Perez, I.; Leon, R.; Bartha, J.L., et al. The angiotensin-(1-7)/Mas receptor axis protects from endothelial cell senescence via klotho and Nrf2 activation. *Aging Cell* **2019**, *18*, e12913, doi:10.1111/acel.12913.

12. Mogi, M. Effect of renin-angiotensin system on senescence. *Geriatr Gerontol Int* **2020**, 10.1111/ggi.13927, doi:10.1111/ggi.13927.

13. Lv, S.J.; Ding, Y.N.; Pei, X.Y.; Zhao, X.; Hao, L.; Zhang, Z.Q.; Chen, H.Z.; Liu, P. Vascular Transcriptome Profiling Reveals Aging-Related Genes in Angiotensin Ⅱ-Induced Hypertensive Mouse Aortas. *Chin Med Sci J* **2020**, *35*, 43-53, doi:10.24920/003709.

14. Minamino, T.; Miyauchi, H.; Yoshida, T.; Ishida, Y.; Yoshida, H.; Komuro, I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* **2002**, *105*, 1541-1544, doi:10.1161/01.cir.0000013836.85741.17.

15. Shan, H.; Bai, X.; Chen, X. Angiotensin II induces endothelial cell senescence via the activation of mitogen-activated protein kinases. *Cell Biochem Funct* **2008**, *26*, 459-466, doi:10.1002/cbf.1467.

16. Li, R.; Mi, X.; Yang, S.; Yang, Y.; Zhang, S.; Hui, R.; Chen, Y.; Zhang, W. Long-term stimulation of angiotensin II induced endothelial senescence and dysfunction. *Exp Gerontol* **2019**, *119*, 212-220, doi:10.1016/j.exger.2019.02.012.

17. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **2004**, *79*, 727-747, doi:10.1093/ajcn/79.5.727.

18. Khurana, S.; Venkataraman, K.; Hollingsworth, A.; Piche, M.; Tai, T.C. Polyphenols: benefits to the cardiovascular system in health and in aging. *Nutrients* **2013**, *5*, 3779-3827, doi:10.3390/nu5103779.

19. Santilli, F.; D'Ardes, D.; Davì, G. Oxidative stress in chronic vascular disease: From prediction to prevention. *Vascul Pharmacol* **2015**, *74*, 23-37, doi:10.1016/j.vph.2015.09.003.

20. Suganya, N.; Bhakkiyalakshmi, E.; Sarada, D.V.; Ramkumar, K.M. Reversibility of endothelial dysfunction in diabetes: role of polyphenols. *Br J Nutr* **2016**, *116*, 223-246, doi:10.1017/S0007114516001884.

21. Huxley, R.; Lee, C.M.; Barzi, F.; Timmermeister, L.; Czernichow, S.; Perkovic, V.; Grobbee, D.E.; Batty, D.; Woodward, M. Coffee, decaffeinated coffee, and tea consumption in relation to incident type 2 diabetes mellitus: a systematic review with meta-analysis. *Arch Intern Med* **2009**, *169*, 2053-2063, doi:10.1001/archinternmed.2009.439.

22. Mostofsky, E.; Rice, M.S.; Levitan, E.B.; Mittleman, M.A. Habitual coffee consumption and risk of heart failure: a dose-response meta-analysis. *Circ Heart Fail* **2012**, *5*, 401-405, doi:10.1161/CIRCHEARTFAILURE.112.967299.

23. Bravi, F.; Bosetti, C.; Tavani, A.; Bagnardi, V.; Gallus, S.; Negri, E.; Franceschi, S.; La Vecchia, C. Coffee drinking and hepatocellular carcinoma risk: a meta-analysis. *Hepatology* **2007**, *46*, 430-435, doi:10.1002/hep.21708.

24. Saito, E.; Inoue, M.; Sawada, N.; Shimazu, T.; Yamaji, T.; Iwasaki, M.; Sasazuki, S.; Noda, M.; Iso, H.; Tsugane, S. Association of coffee intake with total and cause-specific mortality in a Japanese population: the Japan Public Health Center-based Prospective Study. *Am J Clin Nutr* **2015**, *101*, 1029-1037, doi:10.3945/ajcn.114.104273.

25. Fukushima, Y.; Ohie, T.; Yonekawa, Y.; Yonemoto, K.; Aizawa, H.; Mori, Y.; Watanabe, M.; Takeuchi, M.; Hasegawa, M.; Taguchi, C., et al. Coffee and green tea as a large source of antioxidant polyphenols in the Japanese population. *J Agric Food Chem* **2009**, *57*, 1253-1259, doi:10.1021/jf802418j.

26. Liang, N.; Kitts, D.D. Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions. *Nutrients* **2015**, *8*, doi:10.3390/nu8010016.

27. Belkaid, A.; Currie, J.C.; Desgagnés, J.; Annabi, B. The chemopreventive properties of chlorogenic acid reveal a potential new role for the microsomal glucose-6-phosphate translocase in brain tumor progression. *Cancer Cell Int* **2006**, *6*, 7, doi:10.1186/1475-2867-6-7.

28. Meng, S.; Cao, J.; Feng, Q.; Peng, J.; Hu, Y. Roles of chlorogenic Acid on regulating glucose and lipids metabolism: a review. *Evid Based Complement Alternat Med* **2013**, *2013*, 801457, doi:10.1155/2013/801457.

29. Zhao, Y.; Wang, J.; Ballevre, O.; Luo, H.; Zhang, W. Antihypertensive effects and mechanisms of chlorogenic acids. *Hypertens Res* **2012**, *35*, 370-374, doi:10.1038/hr.2011.195.

30. Lee, K.; Lee, J.S.; Jang, H.J.; Kim, S.M.; Chang, M.S.; Park, S.H.; Kim, K.S.; Bae, J.; Park, J.W.; Lee, B., et al. Chlorogenic acid ameliorates brain damage and edema by inhibiting matrix metalloproteinase-2 and 9 in a rat model of focal cerebral ischemia. *Eur J Pharmacol* **2012**, *689*, 89-95, doi:10.1016/j.ejphar.2012.05.028.

31. Dhingra, D.; Gahalain, N. Reversal of Reserpine-induced Orofacial Dyskinesia by Chlorogenic Acid in Rats. *Pharmacologia* **2016**, *7*, 272-277, doi:10.5567/pharmacologia.2016.272.277.

32. Moi, P.; Chan, K.; Asunis, I.; Cao, A.; Kan, Y.W. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci U S A* **1994**, *91*, 9926-9930, doi:10.1073/pnas.91.21.9926.

33. Ishii, T.; Itoh, K.; Ruiz, E.; Leake, D.S.; Unoki, H.; Yamamoto, M.; Mann, G.E. Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ Res* **2004**, *94*, 609-616, doi:10.1161/01.Res.0000119171.44657.45.

34. Sykiotis, G.P.; Bohmann, D. Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Dev Cell* **2008**, *14*, 76-85, doi:10.1016/j.devcel.2007.12.002.

35. Kim, E.N.; Lim, J.H.; Kim, M.Y.; Ban, T.H.; Jang, I.A.; Yoon, H.E.; Park, C.W.; Chang, Y.S.; Choi, B.S. Resveratrol, an Nrf2 activator, ameliorates aging-related progressive renal injury. *Aging (Albany NY)* **2018**, *10*, 83-99, doi:10.18632/aging.101361.

36. Bellaver, B.; Souza, D.G.; Bobermin, L.D.; Souza, D.O.; Gonçalves, C.A.; Quincozes-Santos, A. Resveratrol Protects Hippocampal Astrocytes Against LPS-Induced Neurotoxicity Through HO-1, p38 and ERK Pathways. *Neurochem Res* **2015**, *40*, 1600-1608, doi:10.1007/s11064-015-1636-8.

37. Takano, K.; Tatebe, J.; Washizawa, N.; Morita, T. Curcumin Inhibits Age-Related Vascular Changes in Aged Mice Fed a High-Fat Diet. *Nutrients* **2018**, *10*, doi:10.3390/nu10101476.

38. Han, D.; Chen, W.; Gu, X.; Shan, R.; Zou, J.; Liu, G.; Shahid, M.; Gao, J.; Han, B. Cytoprotective effect of chlorogenic acid against hydrogen peroxide-induced oxidative stress in MC3T3-E1 cells through PI3K/Akt-mediated Nrf2/HO-1 signaling pathway. *Oncotarget* **2017**, *8*, 14680-14692, doi:10.18632/oncotarget.14747.

39. Yang, D.; Xiao, C.; Long, F.; Wu, W.; Huang, M.; Qu, L.; Liu, X.; Zhu, Y. Fra-1 plays a critical role in angiotensin II-induced vascular senescence. *FASEB J* **2019**, *33*, 7603-7614, doi:10.1096/fj.201801671RRRR.

40. Tsai, K.L.; Hung, C.H.; Chan, S.H.; Hsieh, P.L.; Ou, H.C.; Cheng, Y.H.; Chu, P.M. Chlorogenic Acid Protects Against oxLDL-Induced Oxidative Damage and Mitochondrial Dysfunction by Modulating SIRT1 in Endothelial Cells. *Mol Nutr Food Res* **2018**, *62*, e1700928, doi:10.1002/mnfr.201700928.

41. Li, Y.; Zou, L.; Li, T.; Lai, D.; Wu, Y.; Qin, S. Mogroside V inhibits LPS-induced COX-2 expression/ROS production and overexpression of HO-1 by blocking phosphorylation of AKT1 in RAW264.7 cells. *Acta Biochim Biophys Sin (Shanghai)* **2019**, *51*, 365-374, doi:10.1093/abbs/gmz014.

42. Xia, N.; Daiber, A.; Forstermann, U.; Li, H. Antioxidant effects of resveratrol in the cardiovascular system. *Br J Pharmacol* **2017**, *174*, 1633-1646, doi:10.1111/bph.13492.

43. Wallerath, T.; Deckert, G.; Ternes, T.; Anderson, H.; Li, H.; Witte, K.; Forstermann, U. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* **2002**, *106*, 1652-1658, doi:10.1161/01.cir.0000029925.18593.5c.

44. Eun Nim Kim, Ji Hee L., Min Young Kim, Tae Hyun Ban, In-Ae Jang, Hye Eun Yoon, Cheol Whee Park, Yoon Sik Chang, Bum Soon Choi. Resveratrol, an Nrf2 activator, ameliorates aging-related progressive renal injury. *AGING* **2018**.

45. Cao, W.; Dou, Y.; Li, A. Resveratrol Boosts Cognitive Function by Targeting SIRT1. *Neurochem Res* **2018**, *43*, 1705-1713, doi:10.1007/s11064-018-2586-8.

46. Zhang, H.; Zheng, W.; Feng, X.; Yang, F.; Qin, H.; Wu, S.; Hou, D.X.; Chen, J. Nrf2-ARE Signaling Acts as Master Pathway for the Cellular Antioxidant Activity of Fisetin. *Molecules* **2019**, *24*, doi:10.3390/molecules24040708.

47. Kensler, T.W.; Wakabayashi, N.; Biswal, S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* **2007**, *47*, 89-116, doi:10.1146/annurev.pharmtox.46.120604.141046.

48. Mitsuishi, Y.; Motohashi, H.; Yamamoto, M. The Keap1-Nrf2 system in cancers: stress response and anabolic metabolism. *Front Oncol* **2012**, *2*, 200, doi:10.3389/fonc.2012.00200.

49. Ndisang, J.F. Synergistic Interaction Between Heme Oxygenase (HO) and Nuclear-Factor E2- Related Factor-2 (Nrf2) against Oxidative Stress in Cardiovascular Related Diseases. *Curr Pharm Des* **2017**, *23*, 1465-1470, doi:10.2174/1381612823666170113153818.

50. Loboda, A.; Damulewicz, M.; Pyza, E.; Jozkowicz, A.; Dulak, J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cell Mol Life Sci* **2016**, *73*, 3221-3247, doi:10.1007/s00018-016-2223-0.

51. Magyar, K.; Halmosi, R.; Palfi, A.; Feher, G.; Czopf, L.; Fulop, A.; Battyany, I.; Sumegi, B.; Toth, K.; Szabados, E. Cardioprotection by resveratrol: A human clinical trial in patients with stable coronary artery disease. *Clin Hemorheol Microcirc* **2012**, *50*, 179-187, doi:10.3233/ch-2011-1424.

52. Klinge, C.M.; Blankenship, K.A.; Risinger, K.E.; Bhatnagar, S.; Noisin, E.L.; Sumanasekera, W.K.; Zhao, L.; Brey, D.M.; Keynton, R.S. Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells. *J Biol Chem* **2005**, *280*, 7460-7468, doi:10.1074/jbc.M411565200.

53. Umebayashi, R.; Uchida, H.A.; Kakio, Y.; Subramanian, V.; Daugherty, A.; Wada, J. Cilostazol Attenuates Angiotensin II-Induced Abdominal Aortic Aneurysms but Not Atherosclerosis in Apolipoprotein E-Deficient Mice. *Arterioscler Thromb Vasc Biol* **2018**, *38*, 903-912, doi:10.1161/ATVBAHA.117.309707.

54. Okuyama, M.; Uchida, H.A.; Hada, Y.; Kakio, Y.; Otaka, N.; Umebayashi, R.; Tanabe, K.; Fujii, Y.; Kasahara, S.; Subramanian, V., et al. Exogenous Vasohibin-2 Exacerbates Angiotensin II-Induced Ascending Aortic Dilation in Mice. *Circulation Reports* **2019**, *1*, 155-161, doi:10.1253/circrep.CR-19-0008.

55. Uchida, H.A.; Kristo, F.; Rateri, D.L.; Lu, H.; Charnigo, R.; Cassis, L.A.; Daugherty, A. Total lymphocyte deficiency attenuates AngII-induced atherosclerosis in males but not abdominal aortic aneurysms in apoE deficient mice. *Atherosclerosis* **2010**, *211*, 399-403, doi:10.1016/j.atherosclerosis.2010.02.034.

56. Maciag, T.; Hoover, G.A.; Stemerman, M.B.; Weinstein, R. Serial propagation of human endothelial cells in vitro. *J Cell Biol* **1981**, *91*, 420-426, doi:10.1083/jcb.91.2.420.

57. Hirai, K.; Sasahira, T.; Ohmori, H.; Fujii, K.; Kuniyasu, H. Inhibition of heme oxygenase-1 by zinc protoporphyrin IX reduces tumor growth of LL/2 lung cancer in C57BL mice. *Int J Cancer* **2007**, *120*, 500-505, doi:10.1002/ijc.22287.