

Involvement of Heme Oxygenase-1 in Kaempferol-Induced Anti-Allergic Actions in RBL-2H3 Cells

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Abstract—Kaempferol is one of the most commonly found dietary flavonoids. The exposure to kaempferol is known to inhibit degranulation from mast cells, but the inhibitory mechanism of degranulation has not been clarified yet. In this study, we investigated the involvement of heme oxygenase (HO)-1 in the anti-allergic action of kaempferol against degranulation in rat basophilic leukemia (RBL-2H3) cells. Our results demonstrate upregulation of HO enzymatic activity after short (15 min) exposure to kaempferol, followed by the induction of HO-1 expression in protein. The involvement of HO-1 in the kaempferol-induced inhibition of degranulation was confirmed using tin protoporphyrin IX (SnPP), a HO-1 inhibitor. These findings strongly suggest that kaempferol exerts anti-allergic actions via activation of the HO-1.

KEY WORDS: flavonoids; heme oxygenase-1; kaempferol; mast cells; degranulation.

INTRODUCTION

Flavonoids are low molecular weight compounds found in many plants [1–3] and exhibit anti-oxidant,

anti-allergic, and anti-inflammatory activities [1, 3]. As intrinsic components of fruits, vegetables and beverages such as wine and tea, more than 4,000 different flavonoids are known to date [3]. Flavonoids have benzoic rings (A and B rings) and those rings are adapted by three carbons (C ring) [3]. Various activities of flavonoids depend on their structure, including the degree of hydroxyl substitution (–OH) [2, 3].

Mast cells play an important role in early and late phase allergic reactions [4]. Activation of mast cells carried out in response to specific allergens, a variety of biological substances (neuropeptides and certain cytokines), and pharmacological compounds (calcium ionophore, compound 48/80), resulted in the prompt release of preformed mediators, such as histamine and serotonin, together with *de novo* synthesized lipid mediators, such as leukotrienes and prostaglandins, and some cytokines [1, 3–5]. These substances contribute to the development of allergic inflammation. [1, 3–5]

HO-1 is a rate-limiting enzyme in the degradation of heme to produce biliverdin, which is further converted to the antioxidant, bilirubin, by biliverdin reduc-

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Abbreviations: ARE, antioxidant response element; Nrf2, NF-E2-related factor-2; SnPP, tin protoporphyrin IX; HO, heme oxygenase; CO, carbon monoxide.

tase, free iron, and carbon monoxide (CO) [6, 7]. Current understanding of HO-1 has demonstrated that HO-1 is important in a wide variety of physiological and pathological processes, even in maintaining cellular homeostasis, in addition to heme degradation. Previous report showed that HO-1 transfected mast cells had decreased degranulation, suggesting that HO-1 has anti-allergic activity in mast cells [8].

Among flavonoids, kaempferol has been demonstrated to have potent anti-allergic and anti-inflammatory, anti-carcinogenic, and anti-oxidative activity [9, 10]. Kaempferol inhibits mast cell degranulation and the expression level of inducible nitric oxide synthase, cyclooxygenase-2 and C-reactive protein [9, 10]. Kaempferol induced the expression levels of HO-1 in macrophages [11], leading us to hypothesize that kaempferol would exert significant anti-allergic effects through the induction of HO-1 in the mast cells.

In the present study, we provide evidence that kaempferol inhibits mast cell degranulation by induction of HO-1 expression.

MATERIALS AND METHODS

Reagents

Kaempferol, A23187, hemin, polyclonal antibodies against β -actin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH were obtained from Sigma (St. Louis, MO, USA). Tin protoporphyrin IX (SnPP) was from Frontier Scientific (Logan, UT, USA). Antibodies against HO-1 were from Stressgen Biotechnologies (Ann Arbor, MI, USA). Anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bioscience (Buckinghamshire, UK).

Cell Culture

The rat basophilic leukemia (RBL-2H3) cells were purchased from American Type Culture Collection. RBL-2H3 cells were maintained in MEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Cell Activation

RBL-2H3 cells (2×10^5 cells/well) were plated on a 24-well plate and treated with different concentrations of kaempferol for the indicated times. Then, RBL-2H3 cells were stimulated with ionomycin, A23187 ($5 \times$

10^{-7} M). For IgE-mediated stimulation, RBL-2H3 cells were incubated with 0.1 μ g/ml of mouse anti-dinitrophenyl-IgE (DNP-IgE) for 2 h. After washing cells to remove excess DNP-IgE, RBL-2H3 cells were stimulated with 0.1 μ g/ml of DNP-bovine serum albumin (BSA) for 30 min. In some experiments, RBL-2H3 cells were treated with hemin or SnPP for indicated times before stimulation. After centrifugation of RBL-2H3 cells, supernatants and pellets were used for each experiment.

Western Blotting

RBL-2H3 cells were incubated with or without 30 μ M of kaempferol for 0–24 h. Then, RBL-2H3 cells were collected and resuspended in the lysis buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA-2Na, and 1% Triton-X. The total protein content was determined by the bicinchoninic acid (BCA) technique. Then, 20 μ g of protein was subjected to SDS-PAGE using a 15% separation gel and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane was incubated with 4% skim milk for 1 h at room temperature. The membrane was then incubated with primary antibodies overnight at 4°C. Subsequently, the membrane was incubated with either anti-rabbit IgG or anti-mouse IgG antibodies conjugated with horse radish peroxidase for 1 h at room temperature. The specific protein bands on the membrane were visualized using the Enhanced Chemiluminescence Kit (GE Healthcare Bioscience, Buckinghamshire, UK) according to the recommendations of the manufacturer.

Histamine Release Assay

Histamine content in the supernatants was determined as described previously, using a high-performance liquid chromatography (HPLC) [12]. The apparatus for HPLC was a Shimadzu LC-10A system equipped with a fluorescence detector (RF10A XL, Shimadzu; Em: 450 nm, Ex: 330 nm) and an LC-10A liquid pump. The conditions were as follows: column COSMOSIL PACKED COLUMN for HPLC (150 $\times$ 4.6 mm); mobile phase 50% methanol in 0.02 M acetate buffer (pH 4.0), flow rate 0.8 ml/min; oven 40°C. To quantify the total histamine content, RBL-2H3 cells were treated with 1% Triton X-100, and the extract was analyzed for histamine release. Histamine release was expressed as a percentage of the total histamine content in RBL-2H3 cells, and the spontaneous release was subtracted from stimulated cell release.

β -Hexosaminidase Release Assay

β -hexosaminidase release in supernatants was spectrophotometrically determined as previously described [13]. Briefly, 50 μ l of supernatant and 50 μ l of 1 mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) were added and incubated for 1 h at 37°C. Enzyme reaction was terminated by adding 200 μ l of 0.1 M carbonate buffer, pH 10.5. The absorbance at 405 nm was measured in a microplate reader. RBL-2H3 cells were treated with 1% Triton X-100, and total β -hexosaminidase content was measured. β -hexosaminidase release was expressed as a percentage of the total β -hexosaminidase activity in RBL-2H3 cells, and the spontaneous release was subtracted from stimulated cell release.

Measurement of Heme Oxygenase Activity

HO activity was measured as previously described [14]. Briefly, RBL-2H3 cells were homogenized and centrifuged at 10,000 $\times g$ for 30 min at 4°C, after which the resultant supernatant was ultracentrifuged at 105,000 $\times g$ for 1 h at 4°C. 1 mg of microsomal fraction was suspended in 0.1 M potassium phosphate buffer (pH 7.4), followed by sonication for 2 s at 4°C. Microsomes were added to a reaction mixture, containing 2.5 mM glucose-6-phosphate, 0.25 U glucose-6-phosphate dehydrogenase, 333 μ M NADPH, 33 μ M hemin, 1 mg protein of rat liver cytosol as source of biliverdin reductase, and 0.1 M potassium phosphate buffer, pH 7.4. The reaction was conducted for 20 min at 37°C in the dark and terminated by addition of 33 μ l of 0.01 M HCl. The bilirubin formed in the above reaction was extracted with 1 ml of chloroform, and the bilirubin concentration was calculated by the difference in absorbance between 450 and 540 nm with a molar extinction coefficient of 40 mM⁻¹cm⁻¹.

Immunocytochemical Staining

RBL-2H3 cells were cultured in LabTek chamber slides and then incubated with kaempferol for 0–8 h. Slides were fixed in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, Osaka, Japan) for 1 h and rinsed with PBS. Immunocytochemical studies were performed with rabbit polyclonal antibodies against HO-1. Primary polyclonal antibodies against HO-1 were diluted in PBS, containing 10% FCS and incubated overnight at room temperature. Thereafter, slides were rinsed with PBS and incubated with an

anti-rabbit IgG antibody conjugated with horse radish peroxidase for 1 h at room temperature. Slides were revealed with diaminobenzidine as substrate and counterstained with methyl green. Negative controls were treated each slide using a nonspecific antibody (a rabbit IgG antibody).

Statistical Analysis

Statistical comparisons among the groups were assessed by one-way analysis of variance (ANOVA). When F ratios were significant ($p < 0.05$), Scheffe's post-hoc tests between two groups were done, and $p < 0.05$ were considered statistically significant post-hoc differences. Statistical analyses were performed with StatView (Abacus Concept Inc., Gloucestershire, UK).

RESULTS

Kaempferol Inhibited Degranulation from RBL-2H3 Cells

First, we confirmed the effect of kaempferol on degranulation in RBL-2H3 cells. As shown in Fig. 1a, b, exposure to kaempferol for 15 min resulted in a dose-dependent decrease in histamine content and β -hexosaminidase activity stimulated with A23187 in RBL-2H3 cells. We next examined the time-dependent effects of kaempferol on degranulation from RBL-2H3 cells. As shown in Fig. 1c, d, the inhibitory effect of kaempferol on mast cell degranulation was observed from 15 min and this effect continued until 24 h.

Kaempferol Induced HO-1 Expression and Activity in RBL-2H3 Cells

HO-1 induced by kaempferol is known to inhibit LPS-induced NO production in macrophages [11]. Since induction of the HO-1 protein has been reported to protect against various inflammation, we assessed expression levels of HO-1 protein in RBL-2H3 cells cultured with kaempferol by Western blot analysis. A small amount of HO-1 protein was expressed constitutively in RBL-2H3 cells, and HO-1 protein apparently increased after 8 h and continued for 24 h (Fig. 2a). Significant upregulation of HO-1 expression by kaempferol was also confirmed by immunocytochemical staining in RBL-2H3 cells (Fig. 2b). Furthermore, we measured the enzymatic activity of HO in RBL-2H3 cells exposed to kaempferol. An HO activity was

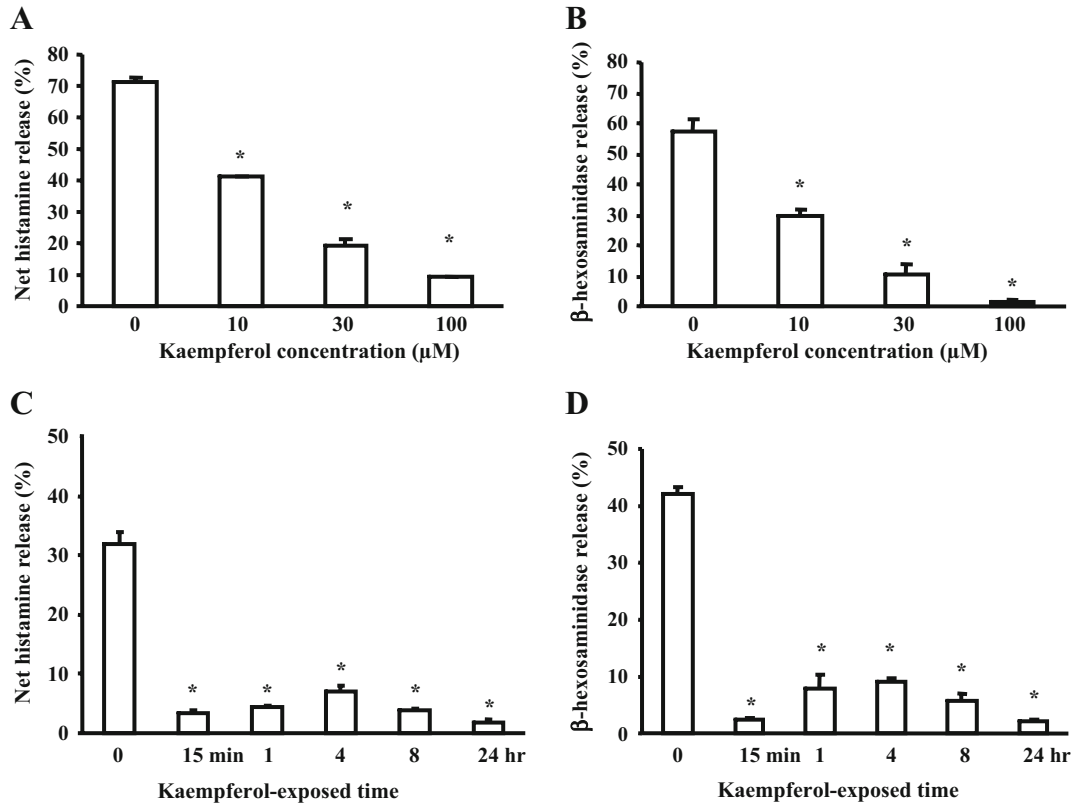


Fig. 1. Effect of kaempferol on degranulation in RBL-2H3 cells. Cells were exposed to various concentrations (0, 10, 30, 100 μM) of kaempferol for 15 min (**a**, **b**). Cells were exposed to kaempferol (30 μM) for the indicated times (0, 15 min, 1, 4, 8, 24 h) (**c**, **d**). At the end of the incubation, cells were stimulated with A23187 (5×10^{-7} M) for 15 min and the release of histamine (**a**, **c**) and β-hexosaminidase (**b**, **d**) was determined as described in “Materials and Methods”. Values are expressed as mean ± SD of a representative result in triplicate of three independent experiments. asterisk Significantly different from the data at concentration and time zero of kaempferol treated, respectively ($P < 0.05$).

increased approximately twofold and fivefold by incubation with kaempferol for 15 min and for 8 h (Fig. 2c).

Effect of the HO-1 on Degranulation in RBL-2H3 Cells after Exposure to Kaempferol

We next investigated whether HO-1 induced by kaempferol affects mast cell activation. We used SnPP, which is widely used as a potent inhibitor of HO activity, for inhibiting HO activity. For induction of HO-1, RBL-2H3 cells were preincubated with kaempferol for 15 min or 8 h prior to stimulation with A23187 for degranulation. Figure 3 clearly showed that the inhibitory effect of kaempferol was effectively recovered by the 1 h exposure of SnPP on mast cell degranulation. Western blot analysis indicated that SnPP slightly inhibited

baseline and kaempferol-induced HO-1 expression (Fig. 3e). These results suggest that kaempferol inhibited mast cell degranulation through HO-1 activity.

Effect of Exposure to Hemin on Degranulation in RBL-2H3 Cells

Hemin is a strong inducer of HO-1 expression in a variety of cell types both *in vitro* and *in vivo*. Furthermore, we examined the effect of HO-1 overexpression on degranulation to investigate the inhibitory activity of HO-1. Pretreatment with hemin for 4 h significantly increased the expression of HO-1 level (Fig. 4e), resulting in the inhibition of A23187-induced histamine and β-hexosaminidase release from RBL-2H3 cells (Fig. 4a, b). Although pretreatment with hemin for 15 min didn't change in the expression of HO-1 level

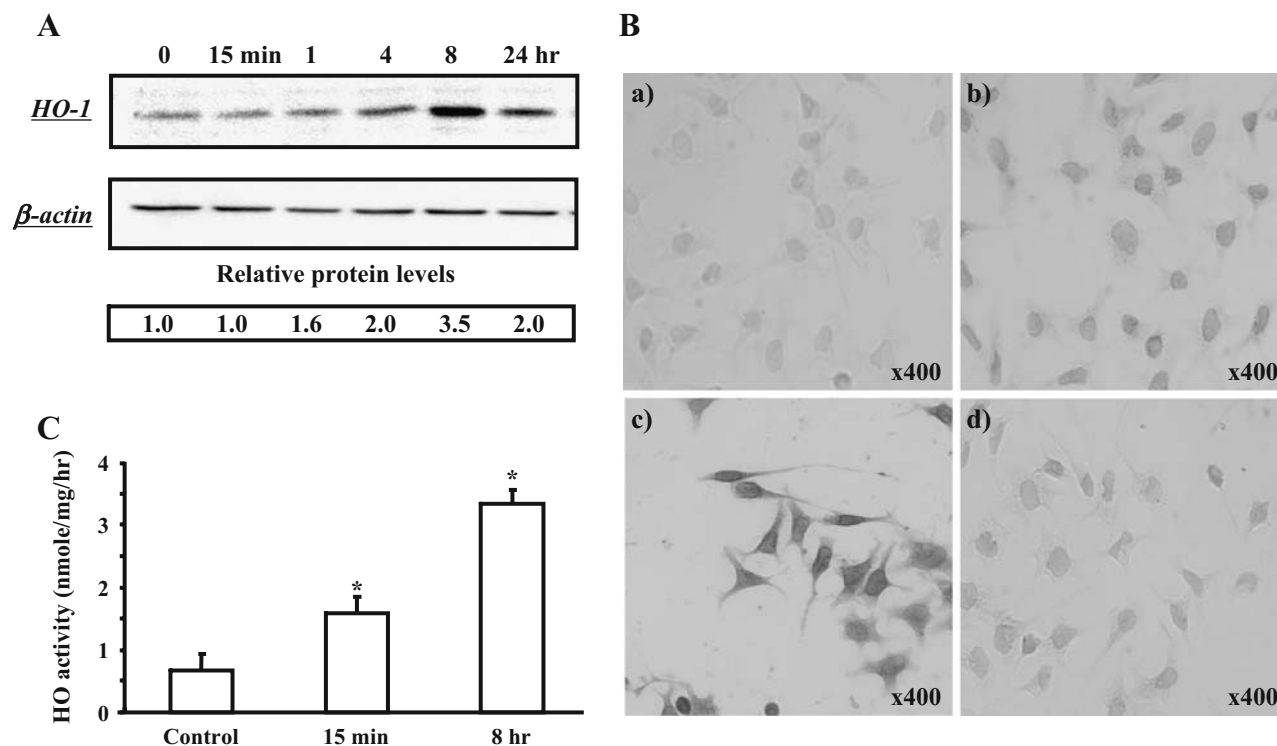


Fig. 2. Expression levels of HO-1 protein and HO activity in RBL-2H3 cells. Cells were treated with kaempferol (30 μ M) for the indicated times (0, 15 min, 1, 4, 8, 24 h). The expression levels of HO-1 protein were determined by Western blotting (a) and immunocytochemistry (a 0 min, b 15 min, c 8 h, d negative control) (b). Microsomal fraction of treated cells was performed for measurement of HO activity (C). Values are expressed as mean $\pm$ SD of a representative result in triplicate of three independent experiments. *asterisk* Significantly different from the data of control ($P < 0.05$).

(Fig. 4e), the inhibition of histamine and β -hexosaminidase release were observed (Fig. 4c, d). These results were similar to those observed in pretreatment with kaempferol. These results demonstrated that HO-1 had a potent inhibitory activity in mast cell degranulation.

HO-1 Induced by Kaempferol Inhibited IgE-Mediated Degranulation

We also evaluated the anti-allergic action of HO-1 induced by kaempferol on IgE-mediated degranulation in RBL-2H3 cells. Figure 5a, b showed that kaempferol inhibited IgE-mediated degranulation in a dose-dependent manner. Kaempferol inhibited IgE-induced degranulation more strongly than A23187 in RBL-2H3 cells. To investigate the involvement of HO-1 in the inhibitory activity of kaempferol in IgE-mediated degranulation, RBL-2H3 cells were exposed to SnPP for 1 h and kaempferol for 15 min before IgE stimulation. Inhibitory activity of kaempferol on degranulation was recovered by SnPP, indicating that HO-1 was involved in kaemp-

ferol-induced inhibition on IgE-mediated degranulation (Fig. 5c, d).

DISCUSSION

In this study, we examined the potential involvement of HO-1 in the anti-allergic activity induced by kaempferol in RBL-2H3 cells. We found that kaempferol potentially increased the HO activity and the level of HO-1 expression in RBL-2H3 cells. Inhibition of HO activity by SnPP recovered mast cell degranulation from kaempferol-induced inhibition, suggesting that the anti-allergic properties of kaempferol may involve in HO-1.

Kaempferol is polyphenolic compounds found in a wide variety of fruits and vegetables. Kaempferol has been demonstrated to have potent anti-allergic and anti-inflammatory, anti-carcinogenic, and anti-oxidative activities [9, 15, 16]. It has been reported that kaempferol induced the expression levels of HO-1 in macrophages, and HO-1 transfected mast cells decreased degranulation

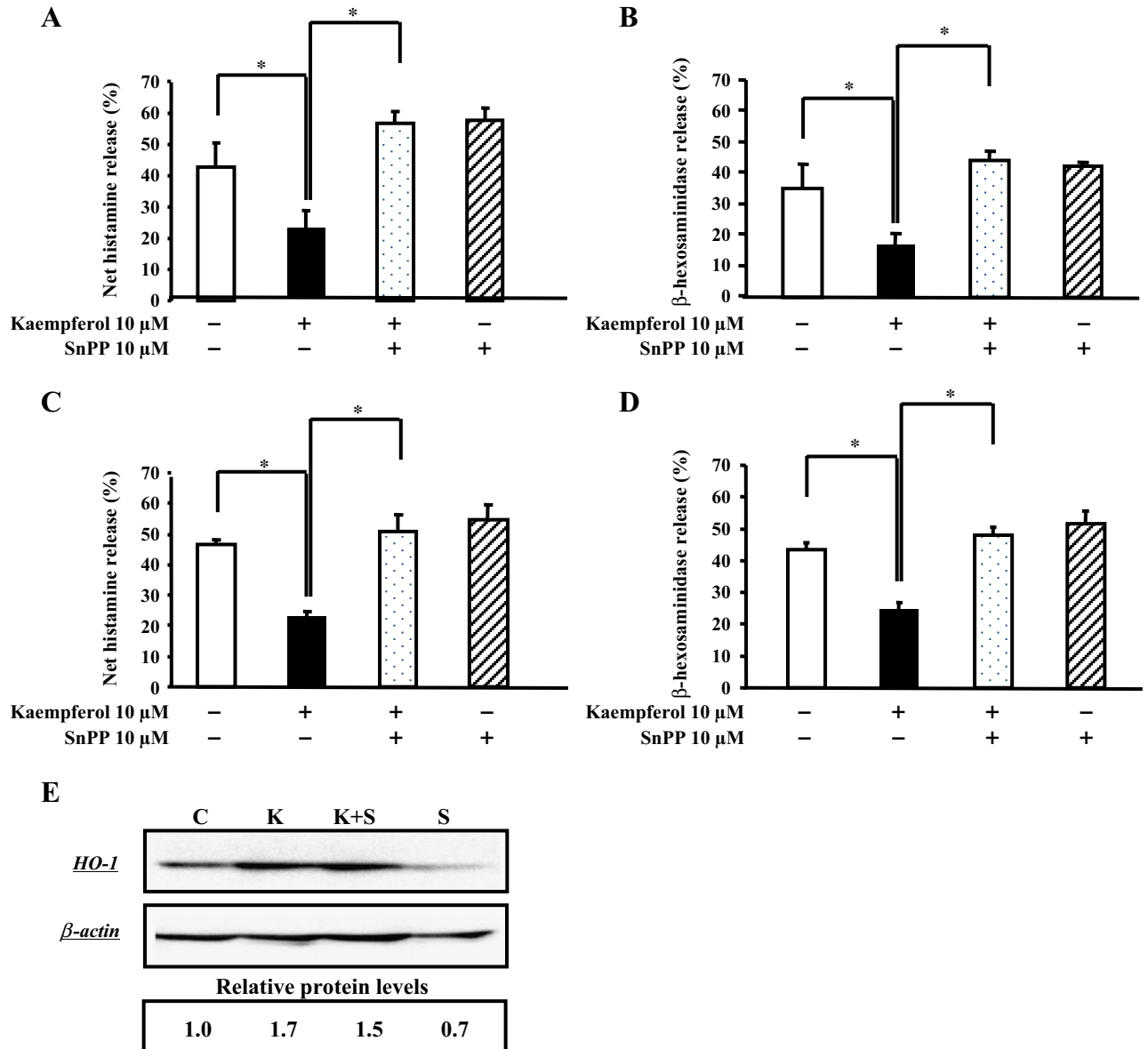


Fig. 3. Effect of SnPP on degranulation and HO-1 protein levels after exposure to kaempferol. Cells were treated with or without SnPP [35] (10 μ M) for 1 h, following treatment with kaempferol (10 μ M) for 15 min (a, b) or 8 h (c, d). At the end of the incubation, cells were stimulated with A23187 (5×10^{-7} M) for 15 min. The release of histamine (a, c) and β -hexosaminidase (b, d) was determined. *asterisk* Significantly different from the data at concentration 0 μ M of kaempferol and SnPP or 10 μ M of kaempferol ($P < 0.05$). The expression levels of HO-1 protein were determined by Western blotting (C control, K kaempferol, K+S kaempferol + SnPP, S SnPP) (e). Values are expressed as mean $\pm$ SD of a representative result in triplicate of three independent experiments.

[8, 11]. Based on these observations, we examined the possibility that HO-1 was involved in kaempferol-induced inhibition of mast cell degranulation. First, we examined induction of HO-1 protein expression after kaempferol treatment in mast cells. A small amount of HO-1 was observed at the basal level, and the strongest induction of

HO-1 protein by kaempferol appeared at 8 h and it continued for 24 h. Despite protein levels of HO-1 were not induced in 15 min exposure to kaempferol, the inhibitory effect of degranulation was observed 15 min after treatment with kaempferol. Our results showed that treatment by kaempferol for 15 min upregulated HO

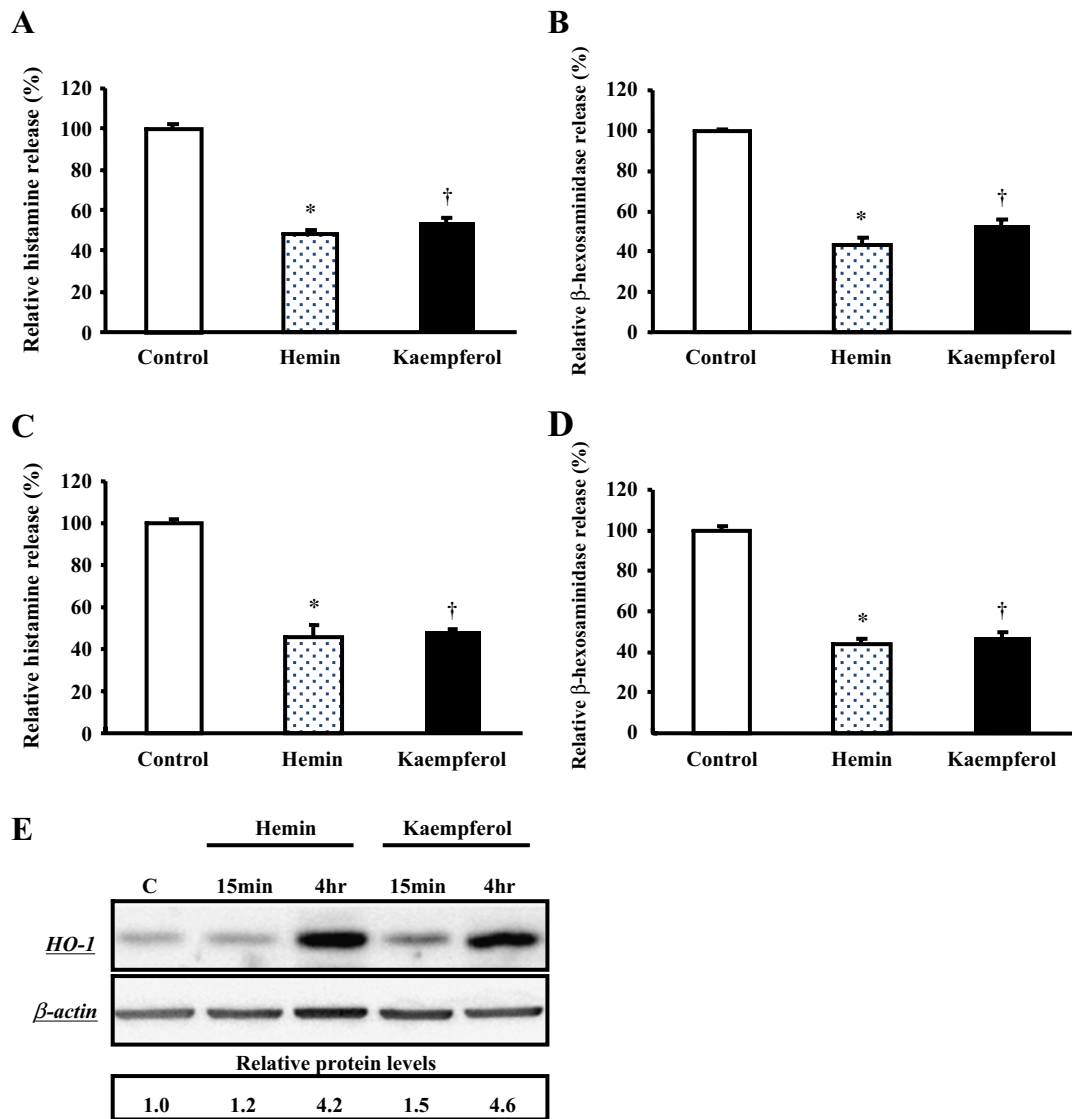


Fig. 4. Effect of hemin on degranulation and HO-1 protein levels in RBL-2H3 cells. Cells were treated with hemin (100 μ M) or kaempferol (10 μ M) for 15 min (**a**, **b**) or 4 h (**c**, **d**). At the end of incubation, cells were stimulated with A23187 (5×10^{-7} M) for 15 min. Release of histamine (**a**, **c**) and β -hexosaminidase (**b**, **d**) was determined. The expression levels of HO-1 protein were determined by Western blotting (**e**). Values are expressed as mean $\pm$ SD of a representative result in triplicate of three independent experiments. *asterisk*, *dagger* Significantly different from the data of control ($P < 0.05$).

activity, suggesting that the inhibitory effect of degranulation by kaempferol before induction of HO-1 protein expression was due to the increase in enzymatic activity of HO. We confirmed that SnPP recovered mast cell degranulation from kaempferol-induced inhibition [8].

In both short and long exposure, kaempferol undoubtedly increase HO-1 activity directly. However, in long exposure, there are two possible pathways for enhancement of the HO-1 expression in response to

kaempferol; one is direct pathway induced by kaempferol and the other is an indirect (positive feedback) pathway induced by the products from the degeneration of heme, which is promoted by kaempferol-mediated upregulation of HO-1 activity. Further study is needed to investigate the role of these two possible mechanisms for kaempferol-mediated upregulation of HO-1 protein expression.

Recently, the various biological/pharmacological activities of flavonoids, naturally occurring polyphenols,

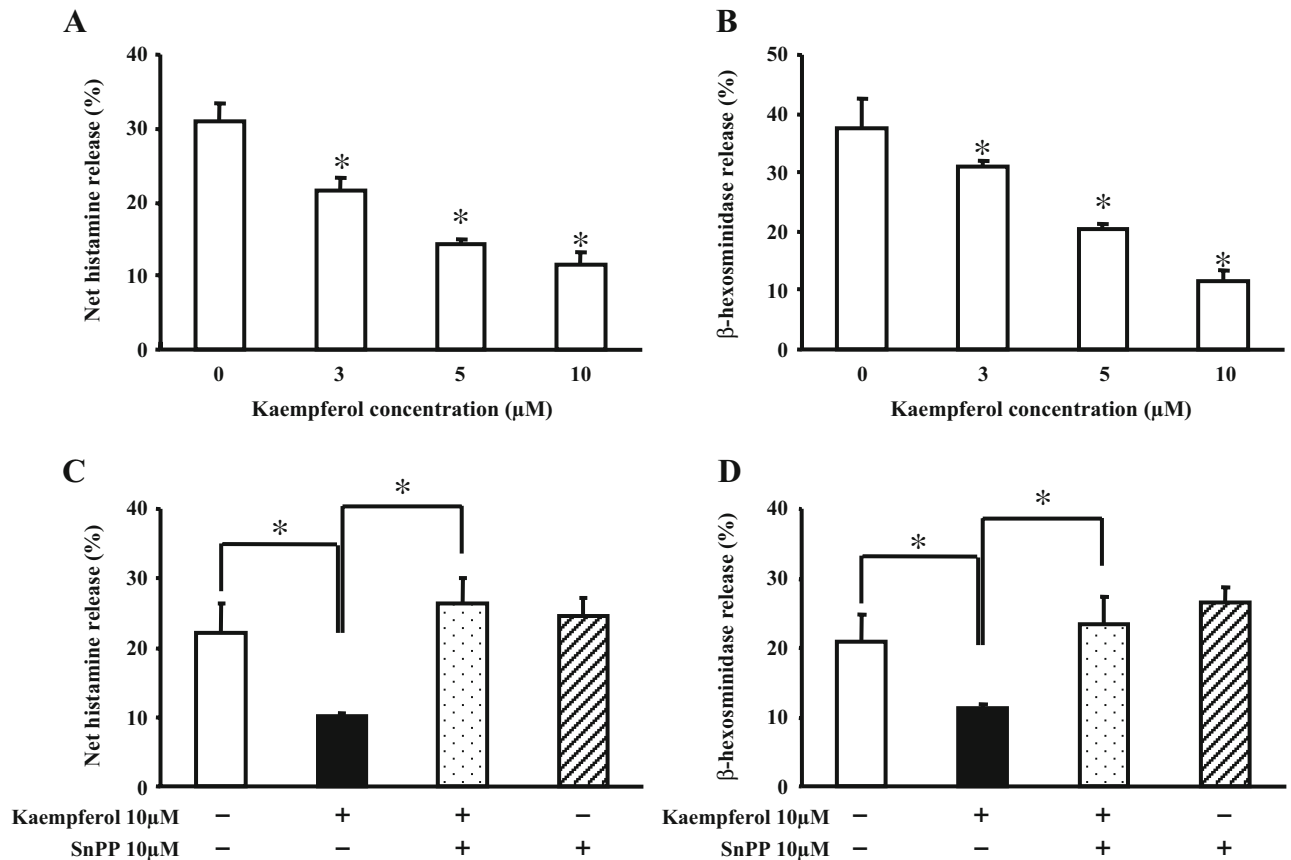


Fig. 5. Effect of HO-1 induced by kaempferol on IgE-mediated degranulation in RBL-2H3 cells. Cells were exposed to various concentrations (0, 3, 5, 10 μM) of kaempferol for 15 min. Cells were treated with or without SnPP (10 μM) for 1 h, following treatment with kaempferol (5 μM) for 15 min. At the end of incubation, cells were stimulated with DNP-IgE and DNP-BSA. Release of histamine (a, c) and β-hexosaminidase (b, d) was determined. Values are expressed as mean ± SD of a representative result in triplicate of three independent experiments. *asterisk* Significantly different from the data at concentration zero of kaempferol and SnPP ($P < 0.05$).

and their related structure-activity relationships have also been investigated [17–21]. Flavonoids are composed of a three-ring structure (A, B and C rings) with various substitutions; they can be subdivided according to the presence of an oxy group at position 4, a double bond between carbon atoms 2 and 3 or a hydroxyl group in position 3 of the C (middle) ring [3]. The position and the degree of hydroxylation have been demonstrated to be the most important for their biochemical and pharmacological actions [3]. It has been suggested that flavonols, which have 3-OH, are the strongest antioxidants among flavonoids [19]. Moreover, the presence of a dihydroxyl group or three adjacent hydroxyl groups on the B ring of the flavonoid molecule, the number of free hydroxyl groups, and a C2-C3 double bond in the C ring are related to the degree of anti-oxidative effect of flavonoids [20, 21]. To investigate wide range of

flavonoids may found whether structure activities or other functions of flavonoids can be related to anti-allergic, anti-inflammatory and other effects.

Bilirubin, one of the catabolites of heme by HO, has been reported to serve as an anti-inflammatory substance [8, 22]. CO, another HO-1 product, has numerous biological functions, including anti-inflammatory properties [6, 23, 24]. It has also reported that CO selectively inhibited the expression of the pro-inflammatory cytokines; TNF-α, IL-1β and MIP-1β, and increased the production of the anti-inflammatory cytokine, IL-10 [23]. Further studies are needed to elucidate which of these products are more important for HO-1-dependent suppression of allergic inflammation.

HO-1 activity has recently attracted significant attention due to its apparent involvement in modulation of the inflammatory response [7, 25]. HO is induced in

airways during asthma [26]. Experimental models of allergic asthma in guinea pigs revealed that upregulation of HO pathway had a significant protective effect against airway inflammation and hypersensitivity [27]. Upregulation of HO-1 genes are induced coordinately by the activation of the antioxidant response element (ARE) in its promoter regions [28]. Induction of HO-1 involves the transcription factor, NF-E2-related factor-2 (Nrf2), which forms heterodimers with the small Maf proteins and binds to the ARE [29, 30]. Consistent with previous reports, quercetin, one of the flavonoids with anti-allergic activity, has been shown to cause nuclear localization of Nrf2 in a rat hepatocyte-derived cell line, H4IIE cells [31] and adult human retinal pigment epithelial (ARPE-19) cells [32]. Nrf1 and Nrf2 are constitutively expressed in mast cells [33]. Thus, HO-1 induction mediated by kaempferol seems to occur via the Nrf2-mediated pathway in mast cells, and resolution of this issue is the focus of our ongoing experiments.

In conclusion, the present study showed that kaempferol mediated anti-inflammatory effects via HO-1-dependent pathway in mast cells. Considering that HO-1 is one of several molecules emerging as a central player in diseases [34], kaempferol, or its derivatives, which could induce HO-1 more effectively, will lead to new therapeutic strategies for promoting not only anti-allergic but also a wide range of anti-inflammatory therapy.

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REFERENCES

1. Cho, S., S. Park, M. Kwon, T. Jeong, S. Bok, W. Choi, W. Jeong, S. Ryu, S. Do, C. Lee, J. Song, and K. Jeong. 2003. Quercetin suppresses proinflammatory cytokines production through map kinases and NF-kappaB pathway in lipopolysaccharide-stimulated macrophage. *Mol. Cell. Biochem.* **243**:153–160. doi:10.1023/A:1021624520740.
2. Huxley, R., M. Lean, A. Crozier, J. John, and H. Neil. 2004. Effect of dietary advice to increase fruit and vegetable consumption on plasma flavonol concentrations: Results from a randomised controlled intervention trial. *J. Epidemiol. Community Health* **58**:288–289. doi:10.1136/jech.2003.014274.
3. Middleton, E. J., C. Kandaswami, and T. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52**:673–751.
4. Wedemeyer, J., and S. Galli. 2000. Mast cells and basophils in acquired immunity. *Br. Med. Bull.* **56**:936–955. doi:10.1258/0007142001903616.
5. Theoharides, T. 1996. The mast cell: A neuroimmunoendocrine master player. *Int. J. Tissue React.* **18**:1–21.
6. Morse, D., S. Pischke, Z. Zhou, R. Davis, R. Flavell, T. Loop, S. Otterbein, L. Otterbein, and A. Choi. 2003. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J. Biol. Chem.* **278**:36993–36998. doi:10.1074/jbc.M302942200.
7. Wagener, F., H. Volk, D. Willis, N. Abraham, M. Soares, G. Adema, and C. Figdor. 2003. Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol. Rev.* **55**:551–571. doi:10.1124/pr.55.3.5.
8. Takamiya, R., M. Murakami, M. Kajimura, N. Goda, N. Makino, Y. Takamiya, T. Yamaguchi, Y. Ishimura, N. Hozumi, and M. Suematsu. 2002. Stabilization of mast cells by heme oxygenase-1: An anti-inflammatory role. *Am. J. Physiol. Heart Circ. Physiol.* **283**:H861–H870.
9. García-Mediavilla, V., I. Crespo, P. Collado, A. Esteller, S. Sánchez-Campos, M. Tuñón, and J. González-Gallego. 2007. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive c-protein, and down-regulation of the nuclear factor kappaB pathway in chang liver cells. *Eur. J. Pharmacol.* **557**:221–229. doi:10.1016/j.ejphar.2006.11.014.
10. Kempuraj, D., B. Madhappan, S. Christodoulou, W. Boucher, J. Cao, N. Papadopolou, C. Cetrulo, and T. Theoharides. 2005. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol.* **145**:934–944. doi:10.1038/sj.bjp.0706246.
11. Lin, H., S. Juan, S. Shen, F. Hsu, and Y. Chen. 2003. Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1. *Biochem. Pharmacol.* **66**:1821–1832. doi:10.1016/S0006-2952(03)00422-2.
12. Suzuki, R., T. Kimura, K. Kitaichi, Y. Tatsumi, M. Matsushima, Y. Zhao, E. Shibata, K. Baba, T. Hasegawa, and K. Takagi. 2002. Platelet factor 4 fragment induces histamine release from rat peritoneal mast cells. *Peptides* **23**:1713–1717. doi:10.1016/S0196-9781(02)00143-2.
13. Gomi, K., F. Zhu, and J. Marshall. 2000. Prostaglandin E2 selectively enhances the IgE-mediated production of IL-6 and granulocyte-macrophage colony-stimulating factor by mast cells through an EP1/EP3-dependent mechanism. *J. Immunol.* **165**:6545–6552.
14. Foresti, R., J. Clark, C. Green, and R. Motterlini. 1997. Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J. Biol. Chem.* **272**:18411–18417. doi:10.1074/jbc.272.29.18411.
15. Blonska, M., Z. Czuba, and W. Krol. 2003. Effect of flavone derivatives on interleukin-1beta (IL-1beta) mRNA expression and IL-1beta protein synthesis in stimulated RAW 264.7 macrophages. *Scand. J. Immunol.* **57**:162–166. doi:10.1046/j.1365-3083.2003.01213.x.
16. Yokomizo, A., and M. Moriwaki. 2006. Effects of uptake of flavonoids on oxidative stress induced by hydrogen peroxide in human intestinal Caco-2 cells. *Biosci. Biotechnol. Biochem.* **70**:1317–1324. doi:10.1271/bbb.50604.
17. Comalada, M., I. Ballester, E. Bailón, S. Sierra, J. Xaus, J. Gálvez, F. de Medina, and A. Zarzuelo. 2006. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity

- relationship. *Biochem. Pharmacol.* **72**:1010–1021. doi:10.1016/j.bcp.2006.07.016.
18. Ichimatsu, D., M. Nomura, S. Nakamura, S. Moritani, K. Yokogawa, S. Kobayashi, T. Nishioka, and K. Miyamoto. 2007. Structure-activity relationship of flavonoids for inhibition of epidermal growth factor-induced transformation of JB6 Cl 41 cells. *Mol. Carcinog.* **46**:436–445. doi:10.1002/mc.20292.
 19. Kawai, M., T. Hirano, S. Higa, J. Arimitsu, M. Maruta, Y. Kuwahara, T. Ohkawara, K. Hagihara, T. Yamadori, Y. Shima, A. Ogata, I. Kawase, and T. Tanaka. 2007. Flavonoids and related compounds as anti-allergic substances. *Allergol. Int.* **56**:113–123. doi:10.2332/allergolint.R-06-135.
 20. Sadeghipour, M., R. Terreux, and J. Phipps. 2005. Flavonoids and tyrosine nitration: Structure-activity relationship correlation with enthalpy of formation. *Toxicol. In Vitro* **19**:155–165. doi:10.1016/j.tiv.2004.06.009.
 21. Wang, L., Y. Tu, T. Lian, J. Hung, J. Yen, and M. Wu. 2006. Distinctive antioxidant and antiinflammatory effects of flavonols. *J. Agric. Food Chem.* **54**:9798–9804. doi:10.1021/jf0620719.
 22. Overhaus, M., B. Moore, J. Barbato, F. Behrendt, J. Doering, and A. Bauer. 2006. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290**:G695–G703. doi:10.1152/ajpgi.00152.2005.
 23. Otterbein, L., F. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R. Davis, R. Flavell, and A. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat. Med.* **6**:422–428. doi:10.1038/74680.
 24. Ryter, S., L. Otterbein, D. Morse, and A. Choi. 2002. Heme oxygenase/carbon monoxide signaling pathways: Regulation and functional significance. *Mol. Cell Biochem.* **234–235**:249–263. doi:10.1023/A:1015957026924.
 25. Belcher, J., H. Mahaseth, T. Welch, L. Otterbein, R. Hebbel, and G. Vercellotti. 2006. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. *J. Clin. Invest.* **116**:808–816. doi:10.1172/JCI26857.
 26. Kitada, O., T. Kodama, K. Kuribayashi, D. Ihaku, M. Fujita, T. Matsuyama, and M. Sugita. 2001. Heme oxygenase-1 (HO-1) protein induction in a mouse model of asthma. *Clin. Exp. Allergy* **31**:1470–1477. doi:10.1046/j.1365-2222.2001.01179.x.
 27. Almolki, A., C. Taillé, G. Martin, P. Jose, C. Zedda, M. Conti, J. Megret, D. Henin, M. Aubier, and J. Boczkowski. 2004. Heme oxygenase attenuates allergen-induced airway inflammation and hyperreactivity in guinea pigs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**:L26–L34. doi:10.1152/ajplung.00237.2003.
 28. Prestera, T., W. Holtzclaw, Y. Zhang, and P. Talalay. 1993. Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. USA* **90**:2965–2969. doi:10.1073/pnas.90.7.2965.
 29. Dhakshinamoorthy, S., and A. Jaiswal. 2001. Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H: Quinone oxidoreductase1 gene. *Oncogene* **20**:3906–3917. doi:10.1038/sj.onc.1204506.
 30. Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. Engel, and M. Yamamoto. 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13**:76–86. doi:10.1101/gad.13.1.76.
 31. Kim, M., J. Lee, H. Lee, D. Aryal, Y. Kim, S. Kim, E. Woo, and K. Kang. 2006. Antioxidative effects of quercetin-glycosides isolated from the flower buds of *Tussilago Farfara* L. *Food Chem. Toxicol.* **44**:1299–1307. doi:10.1016/j.fct.2006.02.007.
 32. Hanneken, A., F. Lin, J. Johnson, and P. Maher. 2006. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Invest. Ophthalmol. Vis. Sci.* **47**:3164–3177. doi:10.1167/iovs.04-1369.
 33. Novotny, V., E. Prieschl, R. Csonga, G. Fabjani, and T. Baumruker. 1998. Nrf1 in a complex with fosB, c-jun, junD and ATF2 forms the AP1 component at the TNF alpha promoter in stimulated mast cells. *Nucleic. Acids Res.* **26**:5480–5485. doi:10.1093/nar/26.23.5480.
 34. Morse, D., and A. Choi. 2005. Heme oxygenase-1: From bench to bedside. *Am. J. Respir. Crit. Care Med.* **172**:660–670. doi:10.1164/rccm.200404-465SO.
 35. Alexandrakis, M., R. Letourneau, D. Kempuraj, K. Kandere-Grzybowska, M. Huang, S. Christodoulou, W. Boucher, D. Seretakis, and T. Theoharides. 2003. Flavones inhibit proliferation and increase mediator content in human leukemic mast cells (HMC-1). *Eur. J. Haematol.* **71**:448–454. doi:10.1046/j.0902-4441.2003.00167.x.