Fortified Antioxidative Potential by Chrysoeriol through the Regulation of the Nrf2/MAPK-mediated HO-1 Signaling Pathway in RAW 264.7 Cells

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Chrysoeriol is a widespread flavone, and it is usually found in alfalfa, which has been used as a traditional medicine to treat dyspepsia, asthma, and urinary system disorders. Recently, analysis has been conducted on the anti-inflammatory activity of chrysoeriol, but information on its antioxidative capacity is limited. In this study, the antioxidative potential of chrysoeriol against oxidative damage and its molecular mechanisms were evaluated by analysis of the cell viability, reactive oxygen species (ROS) formation, and Western blots in the RAW 264.7 cell line. Chrysoeriol significantly scavenged lipopolysaccharide (LPS)-induced intracellular ROS formation in a dose-dependent manner, without any cytotoxicity. Heme oxygenase-1 (HO-1), a phase II enzyme that exerts antioxidative activity, was also potently induced by chrysoeriol treatment, which corresponded to the translocation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) into the nucleus. Moreover, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) were analyzed due to their important role in maintaining cellular redox homeostasis against oxidative stress. As a result, chrysoeriol-induced HO-1 upregulation was mediated by extracellular signal‐regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 phosphorylation. To identify the antioxidative potential exerted by HO-1, tert-butyl hydroperoxide (t-BHP)-induced oxidative damage was applied and mitigated by chrysoeriol treatment, which was confirmed by the HO-1 selective inhibitor and inducer, respectively. Consequently, chrysoeriol strongly strengthened the HO-1-mediated antioxidative potential through the regulation of the Nrf2/MAPK signaling pathways.

Key words : Chrysoeriol, heme oxygenase-1, mitogen activated protein kinase, nuclear factor-erythroid 2 p45-related factor 2

Introduction

Excessively produced reactive oxygen species (ROS) can damage various cellular components, which contributes to the pathogenesis of various disease including cancer, aging and atherosclerosis [19]. In order to survive under the circumstance of oxidative stress, cells should have evolved their own antioxidative mechanisms to eliminate the ROS production through the induction of intracellular phase II enzymes [15]. Among these phase II enzymes, heme oxygenase (HO)-1 has exhibited a critical role in maintaining a cellular redox homeostasis against the oxidative stress [18, 24]. In advance of the HO-1 protein induction, transcription is regulated by an inducible transcription factor, nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). Nrf2 is activated in response to various extracellular stimuli, including the oxidative stress [11, 15]. Activated Nrf2 translocates into the nucleus and binds the antioxidant response element (ARE), which induces the HO-1 overexpression. In addition, the nuclear translocation of Nrf2 requires the activation of the upstream signaling molecules, such as the mitogen activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) [7]. MAPKs are made up of three subfamilies, including extracellular signal-regulated protein kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK [9]. Upon the oxidative stress, the phosphorylated signaling kinases mediate the Nrf2 activation, and the antioxidative/cytoprotective cascades are initiated [26]. MAPKs and PI3K are activated by the extracellular oxidative responses, whose signaling cascade is manifested by a transcription factor, Nrf2 [16, 26]. Therefore, Nrf2 is a pivotal transcriptional regulator for the antioxidative genes induction and MAPKs; PI3K are key molecules for the Nrf2-mediated signaling transduction [9].

So far, a lot of natural resources which have radical scav-

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enging capacity have been identified, and they are proposed as therapeutic agents to attenuate the progress of the oxidataive stress induced various disorders [13, 23]. Chrysoeriol (4′, 5, 7-Trihydroxy-3′-methoxyflavone) is abundant in the aerial parts of alfalfa (*Medicago sativa* L.) and has been used in folk medicine to treat dyspepsia, asthma and urinary system disorders [21]. Chrysoeriol also has been reported to exert inhibitory activities against inflammation obesity and oxidation [4]. Among them, several reports about antioxidative activity of chrysoeriol were usually focused on radical scavenging activity. Therefore, the present study aimed to analyze both the antioxidative potential of chrysoeriol against the oxidative damage and its underlying molecular mechanism in RAW 264.7 cells.

Materials and Methods

Reagents

Chrysoeriol was obtained from Chromadex (Irvine, CA, USA). Dimethyl sulfoxide (DMSO), U0126, SP600125, SB 202190, LY294002, DCFH-DA (2′, 7′-dichlorofluorescin diacetate) and sodium dodecyl sulfate (SDS) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Tin protoporphyrin (SnPP) and cobalt protoporphyrin (CoPP) were obtained from Frontier scientific (Logan, UT, USA). Antibodies against HO-1, phospho-extracellular signal-regulated kinase (ERK), ERK, phospho-c-Jun NH2-terminal kinase (JNK), JNK, phospho-p38, p38, Nrf2, β-actin and poly (ADP-ribose) polymerase (PARP) as well as the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell culture and treatment

The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection (TIB-71; Rockville, MD, USA) and cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

Cell viability

The cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)‐5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay purchased from Promega (Madison, WI, USA), which measures the mitochondrial activity in living cells as previously described [17]. The cells were seeded in 96-well plates and attached for 24 hr. Then the cells were treated with chrysoeriol and selective inhibitors at an indicated concentration and time. The cells were then treated with MTS for 1 hr at 37℃. The absorbance of the formazan product was measured at 490 nm with a multi-detection reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Intracellular reactive oxygen species formation assay

The ROS scavenging activity was measured by the cell permeable fluorescent dye that is based on the ROS-dependent oxidation of DCFH-DA to DCF described previously [25, 27]. RAW 264.7 cells were stained with 50 μM of DCFH-DA for 2 hr. The cells were then pre-incubated with chrysoeriol for 2 hr and subsequently incubated with t-BHP (0.5 mM) for 30 min to induce the ROS generation. The dye fluorescence was measured in a multi-detection reader (Bio-Tek Instruments Inc.) at an excitation and emission wavelength of 485 nm and 530 nm, respectively.

Isolation of cytoplasmic and nucleic protein

The nuclear and the cytoplasmic extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Western blot analysis

The cells ($5×10^6$ cells/dish) in 100-mm dishes were incubated with or without indicated concentrations for an indicated duration of time. The cells were washed twice with PBS, scraped into 0.5 ml of ice-cold protein extraction solution (M-PER, Thermo Fisher Scientific) for 10 min on the ice. The lysis buffer containing the disrupted cells was centrifuged at 13,000× g for 5 min. The protein samples (50 μg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electrotransferred to polyvinylidene fluoride (PVDF) membrane (Bio-rad, Hercules, CA, USA). The membranes were blocked for 1 hr at room temperature with 5% nonfat dry milk in TBST solution. The reactions were then incubated at 4℃ overnight with a 1:1,000 dilution of each primary antibody. After the overnight incubation, the membranes were washed and then further incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG for 2 hr at room temperature. The blots were developed with an ECL developing solution (Santa Cruz Biotechnology), and the data were quantified using the Gel Doc EQ System (Bio-Rad).

Statistical analysis

Oneway ANOVA with Duncan's multiple range test was performed using the SPSS program (version.10.0, SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± the standard deviation of three independent experiments. $p<0.05$ was considered as statistically significant.

Results and Discussion

Chrysoeriol induced HO-1 expression in accordance with the nuclear Nrf2 accumulation in RAW 264.7 cells

This study aimed to investigate the antioxidative potential of chrysoeriol through the HO-1 induction and its molecular mechanism in RAW 264.7 cells.

Radical scavenging assay was conducted to identify whether chrysoeriol has the antioxidative activity or not. Fig. 1 indicates that chrysoeriol significantly attenuated the ROS production in a dose dependent manner in RAW 264.7 cells, which suggests chrysoeriol could be a promising candidate for an antioxidant. HO-1 is the rate limiting the enzyme which catalyzes heme to biliverdin, carbon monoxide and free iron. As the by-products of HO-1 catabolism, CO and biliverdin/bilirubin, have shown to provide protection against the oxidative and the inflammatory stimuli [7]. CO, a gaseous by-product of heme metabolism, contributes to the attenuation of proinflammatory processes. Bilirubin po-
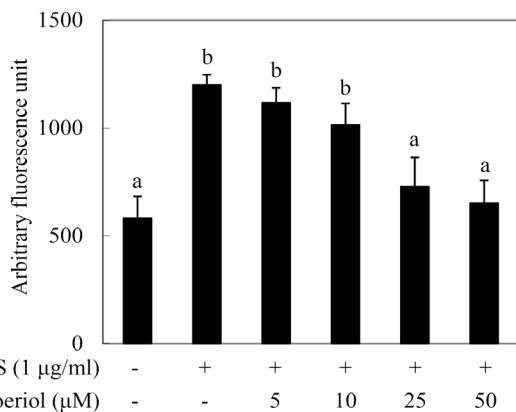
tently scavenged peroxyl radicals in vitro as strong as α-tocopherol and protected cells from the hydrogen peroxide-induced oxidative damage [2, 22]. In addition, the ferritin induction provides an outstanding antioxidative capacity by a rapid segregation of free cytosolic iron, an important catalyst of oxygen-oriented radical production [6]. Fig. 2A and Fig. 2B indicate that chrysoeriol significantly induced the HO-1 expression at 50 μM for 12 hr treatment without any cytotoxicity (data not shown).

Nrf2, one of transcription factors, has known to play an important role in the induction of the antioxidant response element (ARE)-dependent genes expression, including the phase II enzymes [8]. Nrf2 is localized as an inactive form in the cytoplasm, which is anchored by the cytoskeleton-associated protein, Kelch-like ECH-associated protein 1 (Keap1). Extracellular stimuli (i.e., ROS and electrophiles) provide signals for the dissociation of Nrf2-Keap1 complex leading to a nuclear translocation of Nrf2 and the induction ARE-related genes, including HO-1 [14]. To investigate whether Nrf2 translocates into the nucleus, a nuclear protein was extracted and determined by Western blot analysis. Fig. 3 shows that the nuclear translocation of Nrf2 was initiated when RAW 264.7 cells were exposed against 50 μM of chrysoeriol and the Nrf2 activation was constantly increased by an uninterrupted exposure, which was in accordance with the accelerated HO-1 expression.

ERK, JNK and p38 MAPKs regulated HO-1 expression in RAW 264.7 cells

MAPKs or PI3K/Akt could be a candidate among various upstream signaling pathways for Nrf2 related HO-1 regulation in RAW 264.7 cells [12]. Chrysoeriol phosphorylates ERK, JNK and p38 MAPKs, while PI3K/Akt signaling molecule did not give any visible effect (Fig. 4). It has been reported that the modulation of intracellular kinase cascades was involved in the regulation of the HO-1 expression. Quercetin was an effective inducer of HO-1 gene through an activation of ERK but not JNK and p38 in macrophages [5]. In addition, the bark extract from *Lannea coromandelica* induced HO-1 expression through upregulation of Nrf2-mediated pathway via the phosphorylation of p38 and JNK [1, 10]. This study further investigated whether the activation of the p38 and JNK MAPKs signaling pathways was an inevitable event for the HO-1 expression through a pharmacological study applied to three well-known selective inhibitors, including U0126 (for ERK), SP600125 (for JNK) and

Fig. 1. Chrysoeriol scavenged LPS-induced ROS generation in RAW 264.7 cells. The cells were stained with 50 μM of DCFH-DA for 2 hr. Then, the cells were pre-incubated with either chrysoeriol for 2 hr and subsequently incubated with LPS (1 μg/ml) for 30 min to induce the ROS generation. Data represent the mean ± standard deviation of triplicate experiments. Values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test.
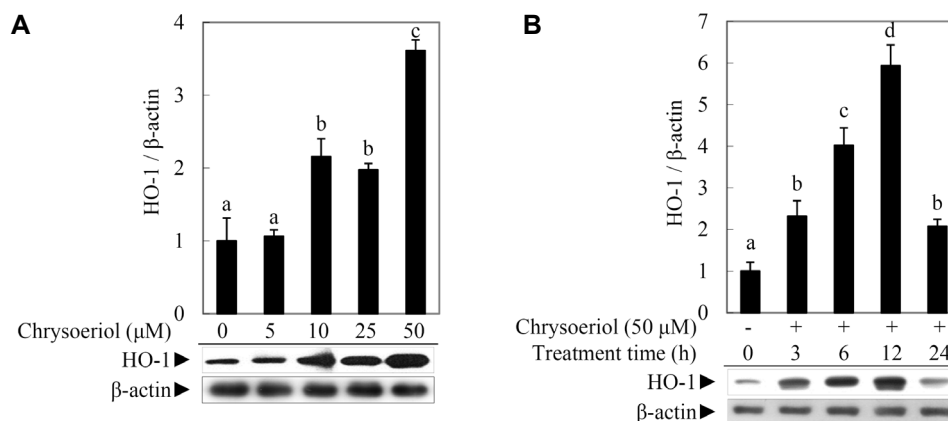
A

B

Fig. 2. Chrysoeriol induced HO-1 protein expression in RAW 264.7 cells. (A) Chrysoeriol induced HO-1 protein expression as a function of concentration. RAW 264.7 cells were treated for 4 hr with chrysoeriol at the indicated concentrations (0, 5, 10, 25 or 50 μM). The HO-1 protein expression was analyzed by Western blot analysis. The data are representative of three independent experiments. (B) Chrysodriol induced HO-1 protein expression as a function of time. RAW 264.7 cells were treated with 50 μM of chrysoeriol for indicated durations (0, 3, 6, 12 or 24 hr). The HO-1 protein expression was analyzed by Western blot analysis. The data are representative of three independent experiments. The relative induction of the HO-1 protein expression was quantified by densitometry and β-actin was used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test.
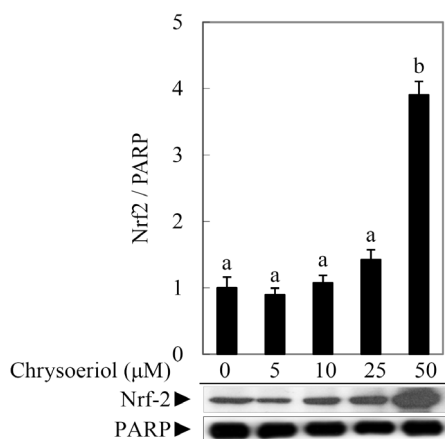

Fig. 3. Chrysoeriol induced Nrf2 activation, the nuclear translocation of Nrf2, in RAW 264.7 cells. The cells were treated for 4 hr with chrysoeriol at the indicated concentrations (0, 5, 10, 25 or 50 μM). The Nrf2 nuclear translocation was determined from a nuclear extract by Western blot analysis. The data are representative of three independent experiments. The relative induction of Nrf2 translocation was quantified by densitometry and PARP was used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p<0.05$ by Duncan's multiple range test. Nrf2, nuclear factor-erythroid 2 p45-related factor 2; PARP, poly (ADP-ribose) polymerase.

SB202190 (for p38). Fig. 5 shows that the addition of each selective inhibitor significantly abolished the HO-1 protein expression induced by chrysoeriol. These results suggest that the chrysoeriol induced HO-1 expression is regulated by the ERK, JNK and p38 signaling molecules in RAW 264.7 cells.

Pretreatment of chrysoeriol protected RAW 264.7 cells against oxidative stress induced cell death

The exposure to ROS can be led cell death, as a result of severe damage to cellular lipid, protein and DNA. In this study, t-BHP, one of the organic hydroperoxides, was applied to induce an oxidative damage in RAW 264.7 cells. The t-BHP is metabolized by cytochrome P450, which leads

the formation of toxic peroxyl and alkoxyl radicals and initiates a lipid peroxidation leading to cell death. The condition for cytotoxic damage issued from t-BHP on RAW 264.7 cells was described in previous report [20]. Cells were pre-incubated with 40 μM of chrysoeriol for 12 hr in order to induce the HO-1 expression in the presence or the absence of each selective inhibitor or inducer. The selective inhibitors, as well as SnPP and CoPP (HO-1 inhibitor and inducer, respectively), were treated as 20 μM [3]. The untreated and the chrysoeriol treated cells with or without the inhibitors or the inducers were exposed to 0.5 mM of t-BHP for 3 hr in order to induce a cytotoxic damage. A sharp increase in cytotoxicity was shown in Fig. 6 as a result of the
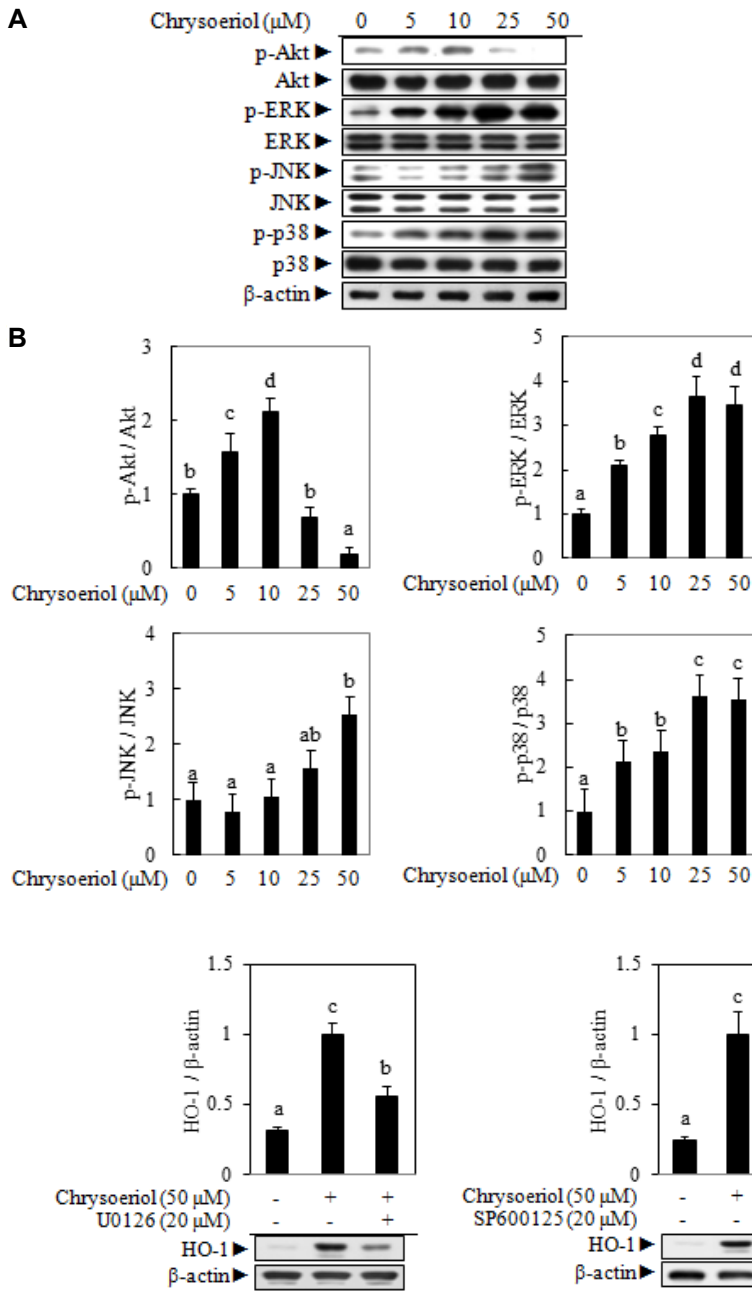
Fig. 4. Chrysoeriol activated ERK, JNK and p38 MAPKs signaling pathways in RAW 264.7 cells. (A) Chrysoeriol induced phosphorylation of ERK, JNK and p38 in RAW 264.7 cells, respectively. The cells were treated with 50 μM of chrysoeriol for 4 hr. The phosphorylated status of PI3K and MAPKs were analyzed by Western blot analysis. The data are representative of three independent experiments. (B) The relative induction of PI3K and MAPKs phosphorylation was quantified by densitometry and unphosphorylated forms of each signaling molecule were used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p < 0.05$ by Duncan's multiple range test.

Fig. 5. Chrysoeriol induced HO-1 expression depends on the ERK, JNK and p38 MAPK signaling pathways. Chrysoeriol was co-treated with selective inhibitors of each MAPK signaling molecule in RAW 264.7 cells. Fifty microliter of chrysoeriol was co-treated with 20 μM of selective inhibitors for ERK, JNK and p38. The HO-1 protein expression was analyzed by Western blot analysis. The data are representative of three independent experiments. The relative induction of HO-1 protein expression was quantified by densitometry and β-actin was used as an internal control. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p < 0.05$ by Duncan's multiple range test.

lipid peroxidation, which was significantly attenuated by chrysoeriol treatment. On the other hand, the cytoprotective potential against the oxidative stress could not be exerted on U0126, SP600125, SB202190 and SnPP (ERK, JNK, p38 inhibitors and HO-1 inhibitor, respectively) treated cells since the abolished HO-1 expression. Consequently, these results suggest that the Nrf2 mediated HO-1 expression by chrysoeriol strengthens the antioxidative potential against the t-BHP-induced oxidative damage through the modulation of ERK, JNK and p38 signaling pathways in RAW 264.7 cells.
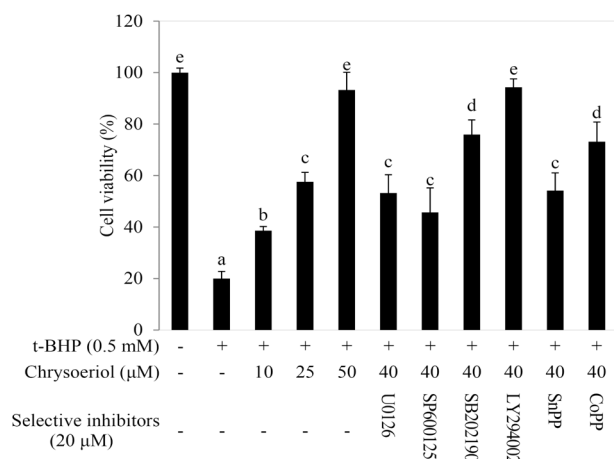
Fig. 6. The antioxidative potential of crysoeriol against the t-BHP-induced oxidative damage in RAW 264.7 cells. The cells were treated with indicated concentrations of chrysoeriol (0, 10, 25 or 50 μM) for 12 hr in the presence or absence of each selective inhibitor or inducer. The untreated and the chrysoeriol treated cells with or without inhibitor or inducer were exposed to 0.5 mM t-BHP for 3 hr. The data represent the mean ± standard deviation of triplicate experiments. The values sharing the same superscript are not significantly different at $p < 0.05$ by Duncan's multiple range test.

Acknowledgements

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초록 : 생쥐 대식세포에서 HO-1 발현 유도를 통한 chrysoeriol의 항산화 효과

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　　Chrysoeriol은 alfalfa에서 주로 발견되는, 식물계에 많이 분포하고 있는 flavone으로 전통의학에서 소화불량, 천식, 비뇨기계 이상의 치료에 사용되어 왔다. 최근의 연구에서는 항염증 효과가 있는 것으로 밝혀졌으나 항산화 효과에 대한 분석은 없었다. 본 연구에서는 chrysoeriol의 항산화 효과와 그 분자적 기전을 RAW 264.7 cell에서 세포생존율, reactive oxygen species (ROS)와 Western blot분석을 통해 알아보고자 하였다. Chrysoeriol은 lip-opolysaccharide (LPS)에 의해 발생한 ROS를 세포독성없이 농도의존적으로 제거하였다. 그리고 항산화효과를 보이는 2상 효소 중 하나인 heme oxygenase (HO)-1의 발현을 강하게 유도하였고, 그와 동시에 전사인자인 Nrf2의 핵내 이동도 촉진하는 것으로 밝혀졌다. 특히, 산화스트레스에 대한 세포내 산화환원항상성 유지에 중요한 역할을 하고 있는 것으로 알려진 mitogen activated protein kinase (MAPK)와 phosphoinositide 3-kinase (PI3K)의 분석 결과, chrysoeriol은 extracellular signal regulated kinase (ERK), c-Jun NH$_2$-terminal kinase (JNK)와 p38의 인산화를 통해 HO-1의 발현을 유도하는 것으로 나타났다. HO-1에 의한 항산화 효과를 확인하기 위하여 chrysoeriol을 전처리한 후 t-BHP에 의한 산화 스트레스에 세포를 노출시킨 결과, chrysoeriol 처리에 의해 세포사멸이 줄어드는 것을 확인하였고, HO-1의 유도제와 억제제의 처리에 따라 세포생존율 또한 조절되는 것을 확인할 수 있었다. 따라서, chrysoeriol은 HO-1의 발현을 유도하여 항산화 효과를 높이고 이것은 Nrf2/MAPK 신호전달 체계에 의한다는 것을 알 수 있었다.