Natural Product Sciences
18(4) : 221-226 (2012)

Hesperidin Improves the IL-6-Mediated Hepatic Insulin Resistance in Hepa-1c1c7 Cells

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Abstract – Hesperidin (HES) is a bioflavonoid with antioxidant, anti-inflammatory and anti-diabetic properties. IL-6 is well known as a primary proinflammatory cytokine that contributes to impaired insulin signaling in liver. This study was to investigate whether HES improves IL-6-mediated impairment of insulin sensitivity in liver. Hepa-1c1c7 cells were pre-treated with 50 and 100 μM HES in complete media for 1 h and then cultured in the presence or absence of IL-6 (20 ng/ml). These results demonstrated that HES restored IL-6-suppressed expression of IRS-1 protein, downregulated IL-6-increased expression of CRP and SOCS-3 mRNA, and inhibited LPS-induced production of IL-6 in Hepa-1c1c7 cells. These findings indicate that HES may ameliorate hepatic insulin resistance via improvement of IL-6-mediated impaired insulin signaling in hepatocytes.
Keywords – Hesperidin, IL-6, Hepatic insulin resistance, SOCS-3, CRP, IRS-1.

Introduction

Obesity is well-known to play a causal role in the complex disease state of metabolic syndrome, as well as being a significant risk factor for cardiovascular disorders and type 2 diabetes (Berg and Scherer, 2005; Bastard *et al*., 2006). Obesity is characterized by chronic low-grade inflammation that can lead to insulin resistance and type 2 diabetes (Xu *et al*., 2003). Hepatic insulin resistance is a major contributor to hyperglycemia in metabolic syndrome and type 2 diabetes (Meshkani and Adeli, 2009). IL-6 is one of the potential mediators that link obesity-derived chronic low-grade inflammation with hepatic insulin resistance (Klover *et al*., 2003; Klover *et al*., 2005; Kim *et al*., 2009). IL-6 is produced much more in visceral adipose tissue than in subcutaneous adipose tissue, which visceral adipocyte IL-6 may be closely associated with systemic inflammation and insulin resistance in obesity (Fain *et al*., 2004; Fontana *et al*., 2007).

Liver is a target organ of IL-6 secreted by immune cells and adipose tissues, in the development of hepatic insulin resistance (Klover *et al*., 2003; Klover *et al*., 2005). Several studies demonstrated that IL-6 can be a primary marker of subclinical systemic inflammation that contributes to impaired hepatic insulin signaling *in vivo* and *in vitro* (Senn *et al*., 2002; Senn *et al*., 2003). IL-6 has been shown to inhibit insulin-dependent tyrosine phosphorylation of insulin receptor substrate (IRS)-1 in hepatocytes (Senn *et al*., 2002). IL-6 impairs signaling transduction and insulin action in hepatocytes through activation of STAT3, a transcription factor that plays a key role in downregulation of insulin receptor signaling in liver (Kaptein *et al*., 1996). IL-6 also increases suppressor of cytokine signaling (SOCS)-3 gene expression, a player on the development of IL-6-dependent insulin resistance, in liver (Emanuelli *et al*., 2000; Senn *et al*., 2003). C-reactive protein (CRP), which is strongly induced by IL-6 in liver, has been reported to impair hepatic insulin sensitivity and insulin signaling (Pradhan *et al*., 2001; Xi *et al*., 2010). Therefore, the IL-6-mediated impaired insulin signaling in liver is thought to be an important target for prevention/ treatment of metabolic syndrome and type 2 diabetes.

Hesperidin (HES) is a flavanone glycoside with no toxicity abundantly found in citrus fruits including lemons and oranges (Garg *et al*., 2001). HES exhibits biological and pharmacological properties, such as antioxidant, immunomodulatory, anti-inflammatory, and anticancer effects (Yeh *et al*., 2007; Kamaraj *et al*., 2009; Jain and Parmar, 2010). Recently, HES has been shown to have hypoglycemic and hypolipidemic effects in diabetic rats (Akiyama *et al*., 2009; Akiyama *et al*., 2010). HES has also been reported to play important roles in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen concentration and/or by

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lowering hepatic gluconeogenesis (Jung *et al*., 2004). However, the mechanism by which HES has anti-diabetic effect remains unclear. Accordingly, the present study has been examined effect of HES on the IL-6-mediated impaired hepatic insulin signaling in Hepa-1c1c7 cells.

Experimental

Materials – Hepa-1c1c7 cells were purchased from the Korean Cell Bank (Seoul, Korea). Anti-insulin receptor substrate (IRS)-1 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dexamethasone, insulin, isobutylmethyxanthine, recombinant IL-6 and IL-1β were purchased from Sigma (St. Louis, MO) except where indicated differently. Hesperidin (HES) was obtained from Dae Keun Kim professor in college of pharmacy, Woosuk University and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiment.

Extraction and isolation of hesperidin – The fruit peels of *Citrus aurantium* were collected and air-dried in December 2009 at Wanju, Chonbuk, Korea. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-09-018). The ^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. The TLC was carried out on pre-coated silica gel F254 plates (Merck, Darmstadt, Germany), and the silica gel for column chromatography was Kiesel gel 60 (230 - 400 mesh, Merck). The column used for LPLC was the Lobar A (Merck Lichroprep Si 60, 240 - 10 mm). The shade dried plant material (500 g) was extracted three times with MeOH at room temperature and filtered. The extracts were combined and evaporated in vacuo at 40 ºC. The resultant methanolic extract (95 g) was partitioned with ethyl acetate three times to afford an ethyl acetate-soluble fraction on drying (24 g). The ethyl acetate fraction (5 g) was chromatographed on silica gel column (CH_2Cl_2-MeOH, 10 : 1 → 1 : 1) to give six fractions (MC1-MC6). The fraction MC3 (1.2 g) was purified by Lobar-A column (CH_2Cl_2-MeOH, 5 : 1 1) to give hesperidin (HES; Fig. 1, 210 mg).

Cell culture – Hepa-1c1c7 cells were maintained in complete DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma) and 1 × antibiotic/antimycotic (Invitrogen). The cells were pretreated with various concentrations of HES in complete media for 1 h and then cultured for 24 h for IL-6 production in the presence or absence of LPS 1 μg/ml (Sigma Chemical Co., St., Louse, MO) at 37 ºC in a humidified atmosphere containing 5% CO_2. The cell supernatants were stored at −70 ºC for cytokine assay.

Cytokine assay – The concentrations of cytokine in the supernatants from Hepa-1c1c7 cells were determined by using cytokine monoclonal antibodies (BD Biosciences Pharmingen, U.S.A.). All measurements were carried out in triplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA microplate reader (Molecular Devices Co., Ltd., U.S.A.). The lower limit of sensitivity for each of the ELISA was equal to or smaller than 5 pg/ml.

Western blot analysis – Following preincubation with hesperidin and treatment of IL-6 or insulin, Hepa-1c1c7 cells were washed with ice-cold PBS and then scraped from the plate in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). After 30 min at 4 ºC, the lysates were centrifuged (15,000 × g for 15 min) and stored at −80 ºC until use. Proteins were separated on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF). Nonspecific binding was blocked with TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h. Primary antibodies were diluted and incubated with membranes overnight at 4 ºC with agitation. After washing three times with TBS-T, secondary antibodies were incubated for 1 h. After 5 additional washes with TBS-T, the bands were visualized with chemiluminescence according to the manufacturer's instructions.

Total RNA isolation and RT-PCR – Following preincubation with HES for 1 h, Hepa-1c1c7 cells were incubated for indicated hours in the presence or absence of IL-6 20 ng/ml or IL-1β 1 ng/ml at 37 ºC, and 5% CO_2. Total RNA was extracted from the cells using an RNA purification kit (QIAGEN) according to the manufacturer's instructions and quantitated spectrophotometrically at 260 nm. cDNA synthesis from total RNA (2 μg) was performed with QuantiTect® Reverse Transcription kit (QIAGEN). PCR was performed in a 20 μl final volume containing 2 μl of the first strand cDNA, 1 μM of sense and antisense primers (BIONEER, Kor.), and 10 μl of 400 nM of QuantiTect® SYBR Green PCR Master Mix (QIAGEN) using a MultiGene PCR (Labnet International Inc.). With a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, was amplified by PCR at the same time. Amplification was performed for 15 min at 95 ºC, followed by 40 cycles of denaturation at 95 ºC for 15 s, annealing at 55 ºC for 30 s, and extension at 72 °C for 30 s in a thermocycler (GeneAmp

9600-R, Perkin-Elmer, Wellesley, MA). The primers were as follows: SOCS-3 (sense 5'-CAGCTCCAAAAGCGAG TACCA-3' and antisense 5'-CGGTCACGGGCGCTCCA GTAGA-3'); CRP (sense 5'-AGATTCCTGCTCCAACAC-3' and antisense 5'-TCAAAGTCACCGCCATACGA-3'); GAPDH (sense 5'-GCCAAGGTCATCCATGACAAC-3' and antisense 5'-AGTGTAGCCCAAGATGCCCTT-3') was used as positive control. The amplified PCR products were analyzed by electrophoresis on a 1.2% agarose gels and visualized by ethidium bromide staining. Quantification of the band intensity on the Hyperfilm was performed using the public domain NIH image software.

Statistical analysis – All data were expressed as means ± standard error (S.E.). Experiments were always run in triplicate and repeated at least twice. Analysis of variation and Student's *t*-test were used to determine statistical significance, and $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Hesperidin increased IL-6-suppressed expression of IRS-1 in Hepa-1c1c7 cells – Hesperidin (HES) has been shown to significantly protect hepatocytes against t-BuOOH-induced cell cytotoxicity almost 100% in 100 μM HES about 80% in 200 ~ 1,600 μM HES treatment in human hepatic L02 cells (Chen *et al*., 2010). Moreover, we observed that 50 and 100 μM HES but not 5 μM remarkably inhibited LPS-induced production of IL-6, TNF-α, and NO by RAW 264.7 cells in a dose-dependent manner (data not shown). Therefore, in the present study we used 50 and 100 μM HES. Downregulated expression of hepatic IRS-1 and IRS-2 is associated with abnormalities in hepatic metabolism including glucose homeostasis and lipid metabolism. Insulin-dependent tyrosine phosphorylation of hepatic IRS-1 and IRS-2 has been shown to be inhibited in chronic IL-6-treated animals (Senn *et al.,* 2002). It has been reported that HES is beneficial for improving hyperlipidemia and hyperglycemia in type-2 diabetic animals (Jung *et al.,* 2006; Akiyama *et al.,* 2009). However, the mechanism by which HES has anti-diabetic effect remains unclear. To examine effect of HES on the IL-6-dependent reduction of insulin receptor signaling such as IRS-1 in liver, Hepa-1c1c7 cells were pretreated with 50 and 100 μM HES in complete media for 1 h and cultured in the presence or absence of IL-6 20 ng/ml at 37 °C, 5% CO_2 incubation for 2 h. Thereafter, the cells were stimulated with 100 nM human insulin for last 3 min at 37 °C. The cells were harvested and total proteins were isolated from the cells for western blot analysis. In

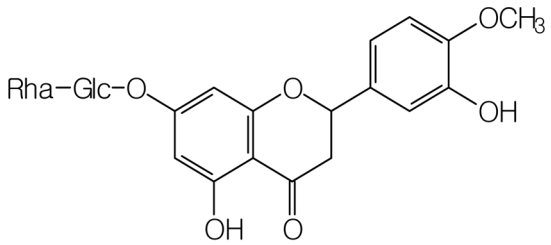

Fig. 1. Chemical structure of hesperidin.

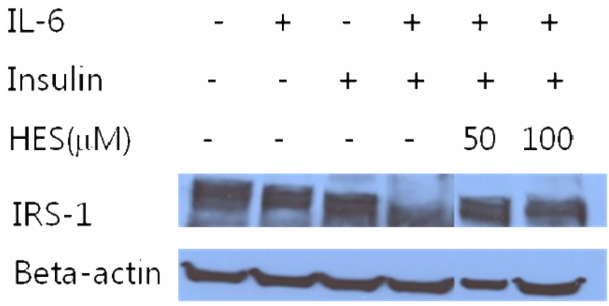

Fig. 2. Hesperidin upregulates the IL-6-suppressed expression of IRS-1 protein in Hepa-1c1c7 cells - Hepa-1c1c7 cells were pretreated with 50 and 100 μM hesperidin (HES) for 1 h and then cultured for 2 h in the presence or absence of IL-6 (20 ng/ml). Thereafter, the cells were stimulated with 100 nM human insulin for 3 min at 37 °C. Protein expression was determined by western blot.

the present study, insulin-dependent expression of IRS-1 protein was remarkably suppressed by IL-6 treatment in Hepa-1c1c7 cells (Fig. 2). And HES reversed IL-6-suppressed expression of IRS-1 protein in the presence of insulin in Hepa-1c1c7 cells. Therefore, these results suggest that HES may ameliorate hepatic insulin resistance through upregulation of IL-6-suppressed expression of IRS-1 in liver.

Hesperidin attenuated IL-6-induced expression of SOCS-3 mRNA in Hepa-1c1c7 cells – SOCS-3 is known as a player in the development of IL-6-induced insulin resistance in liver (Emanuelli *et al.,* 2000; Senn *et al.,* 2003). IL-6-induced expression of hepatic SOCS-3 mRNA is associated with inhibition of hepatic insulin-dependent receptor autophosphorylation and IRS-1 tyrosine phosphorylation (Ueki *et al.,* 2004). Therefore, SOCS-3 gene expression in liver may be an important target for treatment of IL-6-mediated hepatic insulin resistance. In this study, to investigate whether HES attenuate IL-6-dependent expression of SOCS-3 gene in liver, Hepa-1c1c7 cells were pre-treated with 50 and 100 μM HES in complete media for 1 h and then cultured for 1 h in the presence or absence of IL-6 20 ng/ml at 37 °C, 5% CO_2 incubation. The cells were harvested and total RNA was isolated, and the presence of SOCS-3 mRNA

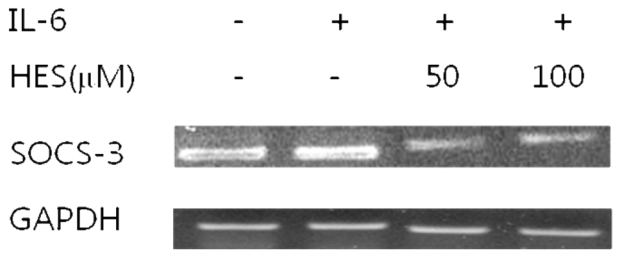

Fig. 3. Hesperidin suppresses the IL-6-induced expression of SOCS-3 mRNA in Hepa-1c1c7 cells - Hepa-1c1c7 cells were pretreated with HES and then cultured for 1 h for SOCS-3 mRNA in the presence or absence of IL-6. Total RNA was then isolated from the cells and gene expression was determined by RT-PCR. Other legends and methods are the same as in Fig. 2.

was examined by RT-PCR analysis. Our results demonstrated that HES significantly attenuated IL-6-induced expression of SOCS-3 mRNA in Hepa-1c1c7 cells (Fig. 3), suggesting that HES may in part ameliorate impaired hepatic insulin sensitivity via downregulation of IL-6-induced expression of SOCS-3 mRNA in hepatocytes.

Hesperidin suppressed expression of CRP mRNA induced by IL-6 and IL-1β in Hepa-1c1c7 cells – CRP is an inflammatory marker associated with insulin resistance, metabolic syndrome, and cardiovascular disease (Pradhan *et al.,* 2001; Ridker and Morrow, 2003). CRP has also been reported to impair hepatic insulin sensitivity and insulin signaling in rats, at least in part by activating extracellular signal-regulated kinase (ERK)1/2 (Xi *et al.,* 2011). CRP has been shown to be mainly mediated by the transcription factor STAT3 (Zhang *et al.,* 1996), which leads to development of hepatic insulin resistance and type 2 diabetes (Pradhan *et al.,* 2001; Xi *et al.,* 2011). IL-6 is well known as an excellent inducer of CRP synthesis in liver. Kramer *et al*. (2008) has reported that IL-6 and IL-1β synergistically interacted in inducing CRP gene transcription in the hepatoma cell lines. Herein, we used both of IL-6 20 ng/ml and IL-1β 1 ng/ml to strongly induce CRP mRNA in Hepa-1c1c7 cells (Ganapathi *et al.,* 1991). In the present study, Hepa-1c1c7 cells were pre-treated with 50 and 100 μM HES in complete media for 1 h and then cultured for 18 h in the presence or absence of IL-6 20 ng/ml and IL-1β 1 ng/ml at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were harvested and total RNA was then isolated from the cells, and then CRP mRNA expression was examined using RT-PCR. Our observation showed that 100 μM HES remarkably attenuated CRP mRNA expression induced by stimulation of IL-6 and IL-1β in Hepa-1c1c7 cells (Fig. 4 A and B). Exogenous IL-6 has been reported to play a more crucial role in induction of CRP mRNA

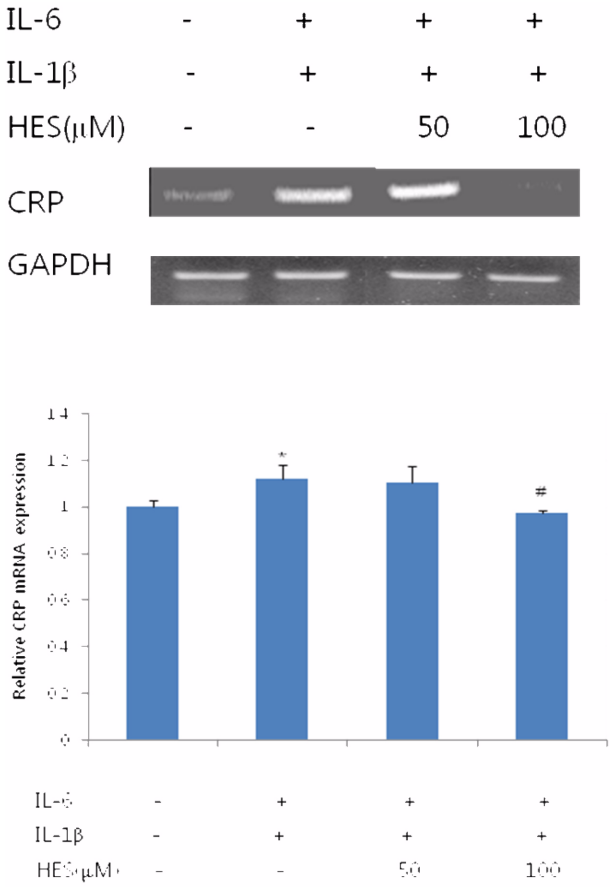

Fig. 4. Hesperidin attenuates CRP mRNA expression induced by IL-6 and IL-1β in Hepa-1c1c7 cells - Hepa-1c1c7 cells were pretreated with HES for 1 h and then cultured for 18 h in the presence or absence of IL-6 and IL-1β for CRP mRNA expression. Other legends and methods are the same as in Fig. 3. $^{*}(p < 0.05)$: Significantly different from the value in negative control. $^{\#}(p < 0.05)$: Significantly different from the value in positive controls.

rather than IL-1β in liver (Kramer *et al.,* 2008). Therefore, these data indicate that HES may inhibit IL-6-induced expression of CRP mRNA in hepatocytes, resulting in improvement of impaired insulin sensitivity in liver.

Hesperidin inhibited LPS-induced production of IL-6 in Hepa-1c1c7 cells – LPS induces hepatocyte injury and increases expression of TNF-α and IL-6 in hepatocytes (Zhou *et al.,* 2012). In the present study, we observed that LPS enhanced production of IL-6 in Hepa-1c1c7 cells. Hepatocyte-derived IL-6 also mediates acute-phase response including CRP associated with impaired hepatic insulin sensitivity in hepatocytes (Saad *et al.,* 1995). HES has been reported to have protective effect on the LPS-induced hepatotoxicity (Kaur *et al.,* 2006). Herein, to further evaluate the inhibitory effects of HES on the LPS-induced production of IL-6 in Hepa-1c1c7 cells, the cells were pretreated with 50 and 100 μM HES in complete

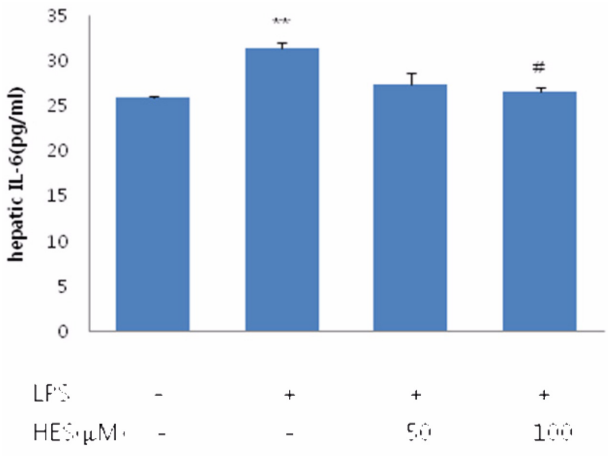

Fig. 5. Hesperidin inhibits the LPS-induced production of IL-6 in Hepa-1c1c7 cells - Hepa-1c1c7 cells were preincubated with HES for 1 h and then cultured for 24 h in the presence or absence of LPS. Concentrations of cytokine were measured using ELISA. Each value represents the mean ± S.E. $^{**}(p < 0.01)$: Significantly different from the value in negative control. $^{\#}(p < 0.05)$: Significantly different from the value in positive controls.

media for 1 h and then cultured for 24 h in the presence or absence of LPS 1 ug/ml at 37 ºC in a humidified atmosphere containing 5% CO_2. These results demonstrated that HES significantly inhibited LPS-induced production of IL-6 in Hepa-1c1c7 cells in a dose-dependent manner (Fig. 5), suggesting that HES may attenuate IL-6-induced activation of downstream inflammatory signaling including ERK1/2 and p38 via inhibition of hepatic IL-6 production, which may lead to partial amelioration of hepatic insulin resistance.

In conclusion, these results demonstrated that HES restored IL-6-suppressed expression of IRS-1, attenuated IL-6-induced expression of SOCS-3 and CRP mRNA, and inhibited LPS-induced production of IL-6 in Hepa-1c1c7 cells. These findings indicate that HES may ameliorate hepatic insulin resistance via improvement of IL-6-mediated impaired insulin signaling in hepatocytes.

Acknowledgements

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (20100043) and by Leaders in Industry University Cooperation (LINC) in Woosuk University (2012).

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Received August 24, 2012
Revised October 22, 2012
Accepted October 24, 2012