Acute improvement of endothelial functions after oral ingestion of isohumulones, bitter components of beer

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A B S T R A C T

Isohumulones, principal components of the bitter taste of beers, have antioxidant capacity. We studied i) the effects of oral ingestion of isomerized hop extract (IHE) on the endothelial functions in smokers as well as non-smokers and ii) the effects of IHE on cultured endothelial cells in high oxidative stress state. Twelve cigarette smokers and eleven non-smokers ingested IHE and placebo in a randomized crossover design. Flow-mediated vasodilatation (FMD) was measured using ultrasonography. We also studied the effects of isohumulones on i) the cell viability under hypoxia and ii) the levels of angiotensin II (AT-II)-induced reactive oxygen species (ROS) in the cultured human aortic endothelial cells (HAECs). At baseline, the FMDs of the smokers were significantly lower than those of the non-smokers. The FMDs increased significantly after 30 min and 120 min of IHE ingestion in both the smokers and the non-smokers. IHE protected the HAECs from hypoxia-induced cell death as assessed by cell viability. IHE also reduced the AT-II-induced intracellular ROS level. Oral ingestion of IHE appears to exert acute beneficial effects on the endothelial functions in both the smokers and non-smokers, and the in vitro experiments using HAECs suggested that the effect be through reducing intracellular oxidative stress.

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1. Introduction

The vascular endothelium plays important roles in the regulation of vasomotor tone, platelet activation and inflammation [1]. Endothelial dysfunction (EDF) including the suppressed synthesis and/or the reduced bioavailability of nitric oxide (NO) occurs when various diseases and smoking injure the endothelium [2,3].

Beer hops, the female inflorescences of the hop plant (Humulus lupulus L.), are known as good preservatives and flavoring in beers. Isohumulones, the main compounds of the bitter flavor of beer, are converted from humulones by isomerization during a brewing process. Various biological effects of isohumulones have been reported [4,5]. Namikoshi et al. reported that isohumulones reduced the reactive oxygen species (ROS) production in the rat kidney and led to the restoration of bioavailable NO [6]. Accordingly, we speculated that isohumulones may ameliorate EDF through the reduction ROS under high oxidative stress conditions.

The purpose of this study was thus to assess the effects of isohumulones on the endothelial functions in smokers and non-smokers as well as in cultured endothelial cells under high oxidative stress.
2. Materials and methods

2.1. Ethical issues and approval

This study was approved by the Kasaoka City Hospital Ethics Committee and was confirmed to the Declaration of Helsinki. All the participants received the oral and written explanations about the details of this study before signing the agreement forms.

2.2. Subjects

Twelve smoking men (smokers; smoking period: 8.2 ± 4.2 years) and eleven non-smoking men (non-smokers) were enrolled. All the subjects were asymptomatic, normotensive and non-diabetic. They were instructed not to change their dietary habits during this study. Food intake and smoking were allowed before 19:00 on the day before each study. When needed, they drank only ion-exchanged water containing neither nitrite nor nitrate (NOx), both of which are oxidative products of NO.

2.3. Hop extract

Commercially available isomerized hop extract (IHE) was used (ISOHOPCON2; English Hop Ltd., Tonbridge, Kent, UK). IHE contains three kinds of water-soluble isohumulones: isohumulone, isocohumulone and isoadhumulone at a ratio of 37:48:15. IHE contains isohumulones of a purity of 79%, and the chemical structures of these isohumulones were already reported [7]. One IHE capsule contains 10.8 mg of IHE. Participants were instructed to take 1 capsule (10.8 mg IHE)/10 kg-body weight.

2.4. Protocols

We performed a randomized double-blind, cross-over study. All the subjects proceeded to the second study within one week after the first study. Each study was scheduled to start before 8 a.m. After 15-min resting, hemodynamics parameters were measured, and venous blood samples of 10 mL were obtained for the analyses of biochemical makers. Flow-mediated dilatation (FMD) and nitroglycerin-mediated dilatation (NTGMD) were assessed by ultrasonography. After 60-min resting, all the volunteers took IHE capsules or placebos with NOx-free water. Blood sampling and measuring FMD were performed after 30 min and 120 min. NTGMD was measured after NTG application.

2.5. Measurement of brachial artery reactivity

FMD measurement was performed in accordance with the JACC guidelines [8]. After a 15-min rest in a temperature-controlled room, the diameter of the right brachial artery and brachial blood flow were measured with a 15 MHz linear-array vascular ultrasound transducer and a HDI 5500 ultrasound system (Hitachi Medico, Chiba, Japan). Hyperemic stress was applied by inflating the cuff of a tonometer that was set on the proximal portion of the forearm to occlude arterial flow (>200 mmHg) for 5 min, and then the cuff was rapidly deflated. Pulse-Doppler blood flow velocity was measured at a 60° angle to the vessel, and the range gate adjusted to each vessel diameter and the position in the center of the artery. The maximum dilatation appeared about 1 min after cuff deflation. The FMD was calculated as the ratio of the change in brachial artery diameter before and after hyperemic stress over the baseline diameter and expressed as a percent change. After 20-min resting, to determine the maximal dilatation of the brachial artery, scanning was started 3 min after the sublingual administration of nitroglycerin (NTG; 300 μg) and was kept for another 3 min. NTGMD was calculated in the same way as FMD, i.e., the percent change before and after NTG.

2.6. Biochemical analyses

Serum levels of total cholesterol, HDL-cholesterol, triglycerides, insulin, glucose, free fatty acid, and uric acid were measured by standard blood test methods. Malondialdehyde-modified low-density lipoprotein (MDA-LDL), an oxidative stress marker, was measured by an ELISA kit (Sekisui Medical Co., Ltd., Tokyo, Japan). To measure the plasma levels of NOx, blood samples of 2 mL during hyperemia were collected from the radial vein at the maximal vasodilatation time point. After centrifugation of blood samples, plasma was mixed with an equivalent volume of ethanol for deproteinization. After another centrifugation, the NOx concentrations in each sample (supernatant plasma) were measured by a NOx analyzer (ENO-20; Eicom, Kyoto, Japan) as reported previously [9]. Since NO is stable mostly as nitrate in blood, we used nitrate as an index of NO level.

We calculated the NO production rate based on the following equations:

\[ \text{Flow volume (cm}^2/\text{beat)} = \text{CSA (cm}^2) \times \text{TVI (cm/beat)} = (D/2)^2 \times \pi \times \text{TVI} \]  
(1)

where CSA (cross section area) was calculated from the diameter of the brachial artery (D), and TVI (time velocity integral) was measured automatically by pulse-Doppler echosonography.

\[ \text{NO production rate (mol/sec)} = C (\text{mol/mL}) \times \text{Flow volume (ml/beat)} \times HR/60 \text{(beat/sec)} \]  
(2)

where C is the plasma nitrate concentration and heart rates (HR) was obtained by echography.

The ratio of the NO production rates between baseline and hyperemia (NO ratio; shown as eq. (3)), was used as an index of changes in NO production and bioavailability.

\[ \text{NO ratio} (\%) = \frac{\text{NO}_{\text{HE}} (\text{mol/sec})}{\text{NO}_{\text{BASE}} (\text{mol/sec})} \times 100 \]  
(3)

where NO\text{HE} was the NO production rate of hyperemia, and NO\text{BASE} was the baseline NO production rate.

2.7. Cell culture

Human aortic endothelial cells (HAEC; passage 3) were purchased from Clonetics Corp. (San Diego, CA, USA) and cultured in EBM-2 SingleQuots medium (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA) supplemented with 5% fetal calf serum, hydrocortisone, human fibroblast growth factor-B, human vascular endothelial growth factor (VEGF), recombinant long R insulin-like growth factor-1 (R-L-IGF-1), ascorbic acid, human epidermal growth factor (rhEGF), gentamicin sulfate, amphotericin-B, and heparin. The cells were incubated at 37 °C in the humidified atmosphere of 95% air/5% CO2 in the medium that was replaced every 2 days. Morphological observation demonstrated that these cells exhibited the specific characteristics of the endothelial cells. All the cells used in the experiments were within passage 6–8.

2.8. Hypoxic treatment

After reaching confluence, the cell culture medium was replaced with the fresh serum free medium. HAECs of about 2 × 10⁴ were seeded in each well of 24-wells (Corning Inc., Corning, NY, USA) and further cultured for 24 h. HAECs were kept under the hypoxic
condition for 24 h with or without IHE. A hypoxic condition was achieved using a CO2 incubator (99% CO2/1% air; Panasonic Healthcare Co., Ltd., Tokyo, Japan).

2.9. Cell counting assay

An index of cell proliferation was obtained by the method using a CellTitre-Blue™ Cell Viability Assay kit (Promega Corporation, Madison, WI, USA). An increase in cell number is correlated with an increase in the absorbance at 570 nm. In our experimental condition, an increase of 0.2 in absorbance reflects an increase of 20,000 cells/well in cell culture.

2.10. Oxidative stress treatment

HAECs cultured for 24 h in serum free medium were exposed to either vehicle or 1 μg/ml IHE for 12 h, and then 1 μmol/L angiotensin II (AT-II; WAKO Pure Chemical Industries, Tokyo, Japan) was added to the cell culture medium and incubated for 3 h. After 40 min, intracellular level of ROS was measured by using the 2′, 7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) method. HAECs from each group were incubated with 10 μmol/L DCFH-DA (Invitrogen, Tokyo, Japan) for 1 h. After permeating the cells, DCFH-DA is hydrolyzed to DCFH by esterase. Intracellular ROS if any oxidize DCFH to fluorescent DCFH, which is detected by confocal laser microscopy (Leica-Microsystems, Tokyo, Japan; excitation at 495 nm, emission at 530 nm). The mean ROS fluorescence intensity was analyzed by Leica TCS-NT system software (Leica-Microsystems).

2.11. Statistical analysis

Comparisons of the baseline characteristics between the smokers and the non-smokers were performed by one-way ANOVA. The data for pre- and post-ingestions of IHE were analyzed by paired t tests. A repeated measure ANOVA was performed to evaluate for significant time-course changes of the composite effect of the IHE versus placebo in each group. All in vitro data were from at least three independent experiments. Continuous variables were described by means ± SD. P values < 0.05 were considered statistically significant. Data analysis was performed using SPSS 23 (IBM SPSS Advanced Statics; IBM Japan, Tokyo, Japan).

3. Results

3.1. Subjects characteristics

Twenty six volunteers were enrolled. Three volunteers were excluded because their ultrasound images were inadequate for analysis. The results of the baseline characteristics of the subjects for the placebo studies are summarized in Table 1. The averages of total cholesterol, LDL-cholesterol, triglyceride, and MDA-LDL of the smokers were significantly higher than those of the non-smokers. The HDL level of the smokers was lower than that of the non-smokers.

3.2. Hemodynamic parameters

As shown in Table 1, at baseline, blood pressure, heart rate and brachial artery diameter under the resting condition were nearly equivalent in both the groups. These parameters were not significantly changed even after ingesting either IHE or placebo.

3.3. Biochemical parameters

As shown in Table 1, triglyceride levels decreased significantly from the baseline after ingesting IHE in both the groups. In the smokers, triglyceride decreased remarkably even after taking placebo. Glucose levels decreased significantly compared with the baseline at each time point in both the groups after IHE intake. Insulin levels decreased significantly after ingesting IHE and placebo at 120 min compared with the baseline. In the non-smokers, glucose levels did not change significantly after taking placebo, although insulin levels decreased significantly at 120 min compared with other time points. However, there was no statistical difference in the triglyceride, glucose and insulin levels between IHE and placebo at each time point in both the smokers and non-smokers. The other parameters did not show any significant changes between IHE and placebo in both the groups.

3.4. Brachial artery responses

As shown in Fig. 1, the FMDs of the smokers at the baseline before taking IHE were significantly lower than those of the non-smokers (3.1 ± 2.1% and 6.3 ± 2.2%, p < 0.001). At 30 min and 120 min after ingestion of IHE, the FMDs of the smokers increased significantly up to 6.2 ± 1.2% (p < 0.0001 vs baseline) and 7.7 ± 1.0% (p < 0.001 vs baseline; p < 0.01 vs 30 min). The FMDs of the non-smokers increased further to 8.4 ± 2.5% (p < 0.01 vs baseline) and 10.7 ± 2.1% (p < 0.001 vs baseline; p < 0.05 vs 30 min). The FMDs of the non-smokers were significantly higher than those of the smokers at every time point (p < 0.05). No significant changes in the FMDs were observed after ingestion of placebo in both the groups. The effect of IHE almost disappeared after 4 h in both the groups (data not shown). NTGMDs were equivalent at baseline in the smokers and non-smokers. (20.2 ± 6.7% and 18.9 ± 4.9%; p = NS). NTGMDs did not change significantly 120 min after treatment with IHE or placebo in the smokers (20.0 ± 6.7% and 18.7 ± 7.6%; p = NS vs baseline) and non-smokers (19.3 ± 5.9% and 15.5 ± 6.3%; p = NS vs baseline). There were no significant differences of NTGMDs between the two groups.

3.5. Effect of IHE on NO production

Blood samples for NO production analysis were obtained from ten volunteers (7 smokers and 3 non-smokers). As in Fig. 2A, after IHE intake, the NO ratios (NOHENOBASE × 100) of the smokers increased from baseline of 179 ± 94% to 235 ± 106% at 30 min (p < 0.05), while after 120 min to 289 ± 170% (p = NS vs baseline or 30 min). In contrast, the NO ratios after placebo did not change significantly. The NO ratios at 30 min after IHE intake significantly increased compared with placebo (235 ± 106% vs 169 ± 69%; p < 0.05), while no significant difference between the two groups was observed after 120 min.

Fig. 2B shows the results for 7 smokers and 3 non-smokers. Including non-smokers, the NO ratios of IHE differed significantly from those of placebo. After taking IHE, the NO ratios increased from the baseline values of 183 ± 81% to 281 ± 197% (30 min; p < 0.05) and 351 ± 273% (120 min; p < 0.05 vs baseline, NS vs 30 min). In addition, the NO ratios of IHE were significantly higher than those of placebo at 30 min and 120 min (p < 0.05).

3.6. Effect of IHE on hypoxia-induced endothelial cell death

Since IHE is dissolved in ethanol for avoiding crystallization at the pH for cell culture, we checked if there was any effect of ethanol in this experimental model. The exposure to IHE or ethanol did not
Table 1
Hemodynamic and biochemical parameters of the smokers and non-smokers. IHE: isomerized hop extract, BMI: body mass index, BP: blood pressure, MDA-LDL: Malondialdehyde-modified low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein. *p < 0.05 and **p < 0.01 vs baseline. p < 0.05 for 120 min vs 30 min **p < 0.05 for IHE vs placebo.

<table>
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<tr>
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<th>Smokers (n=12)</th>
<th>Non-smokers (n=11)</th>
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<tr>
<td>Age (year)</td>
<td>29 ± 3</td>
<td>28 ± 2</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>21.7 ± 1.7</td>
<td>20.9 ± 1.3</td>
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<tr>
<td></td>
<td>Before intake</td>
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<td></td>
<td>120 min</td>
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<td></td>
<td>30 min</td>
<td>120 min</td>
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<tr>
<td>Systolic BP (mmHg)</td>
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<td>104.9 ± 7.4</td>
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<tr>
<td>Diastolic BP (mmHg)</td>
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<td>Heart rate (beats/min)</td>
<td>60.7 ± 10.9</td>
<td>58.2 ± 8.7</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
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<td>172 ± 20</td>
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<td>LDL-Cholesterol (mg/dL)</td>
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<td>46 ± 9</td>
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<td>Insulin (μU/mL)</td>
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<td>Free fatty acid (μEq/L)</td>
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<td>0.3 ± 0.1</td>
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<td>MDA-LDL (μU/L)</td>
<td>78 ± 35</td>
<td>80 ± 29</td>
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**Placebo**

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<th>Non-smokers (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>29 ± 3</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.7 ± 1.7</td>
<td>20.9 ± 1.3</td>
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<td></td>
<td>Before intake</td>
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<td>120 min</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>109.5 ± 8.4</td>
<td>102.5 ± 8.3</td>
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<td>Diastolic BP (mmHg)</td>
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<td>Total cholesterol (mg/dL)</td>
<td>171 ± 26</td>
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<tr>
<td>HDL-Cholesterol (mg/dL)</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
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<td>87 ± 51</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>102 ± 7</td>
<td>100 ± 6</td>
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<tr>
<td>Insulin (μU/mL)</td>
<td>8.8 ± 4.0</td>
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<tr>
<td>Free fatty acid (μEq/L)</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>MDA-LDL (μU/L)</td>
<td>82 ± 20</td>
<td>90 ± 31</td>
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</table>

Fig. 1. Effects of IHE on FMDs. FMDs were shown for (A) smokers and (B) non-smokers treated with IHE (black bar) and placebo (white bar). *p < 0.01 vs baseline. *p < 0.05 and **p < 0.01 for IHE vs placebo. * p < 0.05 for 120 min vs 30 min.

affect the HAEC viability significantly in our study. As in Fig. 3A, exposure to IHE resulted in an attenuation of the hypoxia-induced HAEC death in a dose-dependent manner, although a statistically significant difference in cell number was observed only for 2 μg/mL IHE.

3.7. Effect of IHE on AT-II-induced intracellular generation of ROS

Fig. 3B shows the effect of IHE on the level of ROS in HAECs. HAECs exposed to either vehicle or IHE for 12 h were further treated with AT-II for 3 h. AT-II enhanced the level of ROS (2.4 ± 0.6 fold, p < 0.01 vs control), and it was significantly reduced by IHE (1.7 ± 0.4 fold, p < 0.01 vs AT-II).

4. Discussion

In this study, we demonstrated that ingestion of IHE, the bitter compounds extracted from hops, acutely increased the FMDs in both the smokers and non-smokers. In addition, the in vitro experiments using HAECs demonstrated that IHE attenuated intracellular ROS levels.

EDF is one of the major risk factors for various cardiovascular diseases [10]. Especially, smoking causes EDF of healthy young people [11]. The average value of the FMDs of healthy males in their 30s was reported to be about 6.6% [12]. In our study, three of the smokers (25%) and seven of the non-smokers (70%) were over 6.6%. After IHE intake, the FMDs of eleven of the smokers (92%) were after IHE intake, the FMDs of eleven of the smokers (92%) were
from 6.3% to 10.7%. However, no significant changes were observed in blood pressures and heart rates.

Some previous studies reported the acute positive effects of beer on EDF [13]. Karatzi et al. reported that beer improved the FMDs of healthy non-smokers from 2.5% (95% CI 1.4, 3.5) to 4.2% (95% CI 2.8, 5.5) after drinking 400 mL of beer. In our study, the subjects with an average body weight (59.8 ± 6.6 kg) took IHE of about 64.8 mg, equivalent to almost 2 L of beer.

Smoking increases oxidative stress that causes EDF and atherosclerosis and interferes with the production and bioactivity of NO by various kinds of mechanisms [14,15]. Endothelium-derived NO plays a vital role in the regulation of vascular tone by opposing the effect of AT-II [16,17]. Though NO is quickly oxidized or inactivated into nitrate by oxyhemoglobin and/or ROS, the direct in vivo measurement of NO levels during reactive hyperemia is difficult [18]. Here, we measured the nitrate (an oxidative product of NO) concentration in each sample collected from the radial vein at the maximal vasodilatation time point reflecting the immediate change of NO generation induced by increasing shear stress. The baseline samples were obtained after 14-hr fasting, based on our previous study to exclude possible effects of nitrates derived from food and drinks [9]. As shown in Fig. 2, we observed a significant increase in the NO ratio during hyperemic stress from the baseline, suggesting the flow-induced NO generation from the endothelium. IHE further increased the NO ratio compared to placebo, suggesting that IHE may improve the production and/or bioavailability of endothelium-derived NO. Here, blood pressures did not change significantly, and thus isohumulones may not exert any further antihypertensive effects within the range of normal blood pressure in the young subjects.

We also studied the effects of isohumulones on the cultured HAECs in vitro. Previously, it was reported that hypoxia induced the apoptosis of endothelial cells through increasing oxidative stress [19]. The activation of angiotensin I receptor by AT-II also promoted

![Fig. 2. Changes in total NO ratio. NO ratio (the ratio of NO production rate between reactive hyperemia and baseline) for (A) smokers only and (B) seven smokers and three non-smokers at 0, 30 and 120 min after IHE (black bar) or placebo (white bar) intake. * p < 0.05 vs baseline. # p < 0.05 IHE vs placebo.](image)

![Fig. 3. Effects of IHE on HAECs. (A) Hypoxia: endothelial cells cultured under hypoxic condition for 24 h; IHE: IHE-treated endothelial cells cultured under hypoxic condition for 24 h. ** p < 0.001 vs control (hypoxia-untreated cells). * p < 0.01 vs control. # p < 0.01 vs hypoxia without IHE. (B) AT-II-induced intracellular production of ROS visualized by DCF: HEACs were preincubated for 12 h (control) and then followed by a 3-hr incubation with 1 µmol/L angiotensin II (AT-II) or by a 3-hr incubation with 1 µmol/L AT-II and 1 µg/mL IHE (AT-II + IHE). (C) Summary of DCF fluorescence intensities. * p < 0.01 vs control. # p < 0.01 vs AT-II.](image)
the increase of ROS in vascular smooth muscle cells and induced the apoptosis of endothelial cells [20]. In our study, isohumulones decreased the hypoxia-induced ROS as well as AT-II-induced ROS, resulting in the protection of HAECS from the cell injury. These findings suggest that isohumulones protect endothelial cells from the cytotoxic effects of ROS, maintaining the cell viability under the conditions of high oxidative stress.

Leikart et al. showed that alcohol-free red wine polyphenol (a well-known antioxidant) extract of 400 µg/mL increased the endothelial NO synthase expression and the endothelium-derived NO release in cultured human umbilical vein endothelial cells [21]. Here, the polyphenol concentration was almost at the same level as the plasma concentration as reported by Nigdikar et al. [22]. Rodda et al. reported that the plasma levels of trans-isoisohumulones reached ~0.1 µg/mL after consumption of 600–800 mL of beer containing isohumulones of ~40 mg/L (50 mg IHE) [23]. In our study, the effective concentration of isohumulones was 2.0 µg/mL, and this is much higher than 0.2 µg/mL which was estimated from the results by Rodda et al. Although the cis-isomers are more abundant in beer, Rodda et al. measured only trans-isomers of isohumulones, and thus the overall plasma concentration of isohumulones in vivo may be higher than their estimations. Rodda et al. also reported the trans-isoisohumulones concentrations peaked at 30 min and decreased to baseline by 120 min after consumption of about 600–800 mL of beer. In our study, capsulized isohumulones may need longer time to reach the peak plasma concentration along with concomitant peak of the FMDs. Further study is necessary to determine the metabolism and the bioavailability of isohumulones.

It was reported that isohumulones have agonistic effects on the activities of peroxisome proliferator-activated receptors (PPARs) [24]. The activators of PPARγ such as pioglitazone protected the vascular functions in both the diabetic and non-diabetic patients with cardiovascular risk factors [25,26]. In our study, there were no statistical time-course differences in glucose, insulin, and triglyceride levels between IHE and placebo in both the groups (Table 1). The prolonged fasting periods may affect those values. Since we did not measure the activities of PPARs simultaneously, it is still unclear whether the PPARs activation by isohumulones ameliorates the FMDs in an acute phase.

In conclusion, the FMD measurements and HAECS studies suggested that even a single ingestion of isohumulones acutely ameliorate the endothelial functions of smokers and non-smokers by reducing ROS.

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