JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

5-Caffeoylquinic Acid and Caffeic Acid Down-Regulate the Oxidative Stress- and TNF-α-Induced Secretion of Interleukin-8 from Caco-2 Cells

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Although chlorogenic acid (CHA) easily reaches a millimolar level in the gastrointestinal tract because of its high concentration in coffee and fruits, its effects on intestinal epithelial cells have been little reported. We investigated in this study the down-regulative effects of 5-caffeoylquinic acid (CQA), the predominant isomer of CHA, on the H_2O_2- or TNF-α-induced secretion of interleukin (IL)-8, a central pro-inflammatory chemokine involved in the pathogenesis of inflammatory bowel diseases, in human intestinal epithelial Caco-2 cells. After the cells had been pre- and simultaneously treated with CQA, the oversecretion of IL-8 and overexpression of its mRNA induced by H_2O_2 were significantly suppressed in a dose-dependent manner in the range of 0.25–2.00 mmol/L. We further found that a metabolite of CQA, caffeic acid (CA), but not quinic acid, significantly inhibited the H_2O_2-induced IL-8 secretion and its mRNA expression in the same dose-dependent manner. Both CQA and CA suppressed the TNF-α-induced IL-8 secretion as well. Caffeic acid at 2.00 mmol/l was able to absolutely block the H_2O_2- or TNF-α-induced oversecretion of IL-8 in Caco-2 cells. However, CQA and CA did not suppress the TNF-α-induced increase in the IL-8 mRNA expression, indicating that the suppressive mechanisms are different between TNF-α-induced and H_2O_2-induced IL-8 production models. These results suggest that the habit of drinking coffee and/or eating fruits with a high CHA content may be beneficial to humans in preventing the genesis of inflammatory bowel diseases.

KEYWORDS: Chlorogenic acid; caffeic acid; IL-8; anti-inflammatory; Caco-2; intestinal epithelium

INTRODUCTION

Rich amounts of chlorogenic acid (CHA) are present in coffee beverages, blueberries, apples, cider, and some vegetables (*1*). Those people habitually drinking coffee may consume 300–700 mg of CHA daily (calculated from ref *1*). Its polyphenol structure endows CHA with potent antioxidative and free radical scavenging abilities (*2*). These abilities enable CHA to decrease DNA damage (*3*) and to inhibit the oxidation and peroxidation of low-density lipoproteins (*4–6*) in vitro, suggesting its benefits in preventing cardiovascular disease. In vivo, dietary CHA decreased the incidence of chemical carcinogenesis in the colon, liver, and tongue in animal models of cancer (*7–9*). CHA also shows suppressive effects on tumor promotion in mouse skin induced by chemicals (*10*). These protective effects of CHA against chemical carcinogenesis may be ascribed to its antioxidative and anti-inflammatory properties. A recent study on the A549 human lung cancer cell line has revealed that CHA up-regulated the cellular antioxidative enzymes and suppressed ROS-mediated NF-κB, AP-1, and MAPK activation (*11*).

Dietary CHA and its bacterial metabolites in the gut must reach target cells to execute their physiological functions. In other words, the biological properties of dietary CHA in animals and humans depend on its pharmacokinetics and particularly absorption, metabolism, distribution, and excretion. One hour after humans had ingested 200 mL of brewed commercial coffee containing 1.4 mmol/L CHA, about 0.56 μmol/L caffeic acid was detected in their plasma (*12*). In ileostomy humans, 33% of the ingested 5-caffeoylquinic acid (CQA) was absorbed in the foregut, and the rest reached the hindgut (*13*), where it was metabolized by microflora into caffeic acid, quinic acid, and other organic acids (*14, 15*). Intestinal epithelial cells absorb dietary nutrients and form a monolayer for separating luminal contents and systems under the intestinal epithelium. Both absorbed and retained CHA in the lumen must therefore make contact with the intestinal epithelial cells. People who drink coffee often expose their intestinal epithelial cells to a millimolar level of CHA, although the effects of CHA on intestinal epithelial cells or the mucosa are little known.

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10.1021/jf073168d CCC: $40.75
Published on Web 04/30/2008

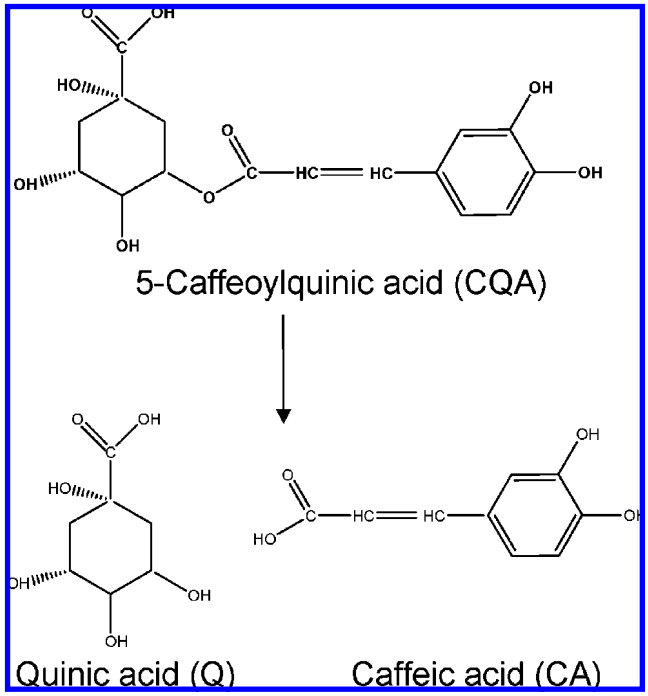

Figure 1. Chemical structure of CQA and its metabolites.

The intestinal epithelium is an organ for absorbing nutrients, as well as playing a key role in the gut immune system for defense against pathogens and xenobiotics (*16*). It acts as a physical barrier separating the host from the external environment and also as a sensor monitoring any invasion by xenobiotics. In response to bacteria, hydrogen peroxide, and cytokines such as TNF-α, the epithelial cells increase their secretion of interleukin (IL)-8, a potent neutrophil and T cell chemo-attractant (*17–19*), thereby initiating an inflammatory response. An appropriate inflammatory response is crucial to eradicate infection and repair damaged tissue (*20*). However, an excessive inflammatory response will perturb the homeostasis between gut antigens and host immunity, which represents a critical determinant in the development of inflammatory and allergic diseases (*21, 22*). Modulation of the secretion of IL-8 from epithelial cells is therefore very important to maintain the intestines and body system in healthy condition.

The modulating roles of nutrients in IL-8 secretion from intestinal epithelial cells have recently received increasing attention (*17, 23, 24*). Amino acids such as histidine and glutamine have been shown to have an anti-inflammatory effect on human intestinal epithelial cell lines (*17, 24*). We investigated in this work the down-regulative effects of CHA at a natural dosage on the H_2O_2- and TNF-α-induced IL-8 secretion in human intestinal epithelial Caco-2 cells.

MATERIALS AND METHODS

Materials. The human Caco-2 colon adenocarcinoma cell line was obtained from the American-type Culture Collection (Rockville, MD), and Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Wako Pure Chemicals (Osaka, Japan). Fetal bovine serum, penicillin-streptomycin, and nonessential amino acids (NEAA) were purchased from Gibco (Gaithersburg, MD). CQA, CA, and quinic acid were purchased from Sigma (St. Louis, MO). The monoclonal antihuman IL-8 antibody and biotinylated antihuman IL-8 antibody were purchased from Genzyme Techne (Minneapolis, MN), and recombinant human IL-8 was purchased from R&D Systems (Minneapolis, MN). The QuantaBlu fluorogenic peroxidase substrate kit was purchased from Pierce (Rockford, IL), and the ExScript RT reagent kit and SYBR Premix Ex Taq for the real-time polymerase chain reaction (PCR) were from Takara Bio (Otsu, Japan).

Cell Culture. Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% NEAA, and 200 IU/mL penicillin-streptomycin. The pH value of the culture medium was around 7.4, which did not change significantly after the supplementation of CQA or caffeic acid. The cells were incubated at 37 °C in humidified air containing 5% CO_2. The cells were maintained in a 100 mm dish and became confluent 6–7 days after seeding at 2×10^5 cells/dish. The confluent cells were passaged by being treated with 0.1% trypsin/0.5 mM EDTA in PBS. The cells were seeded at a density of 2×10^5 cells/well on a 24-well plate and then cultured for 2 weeks, before their use in the subsequent experiments. All cells were used between passages 40 and 60.

Treatment with H_2O_2. Consistent with our results from preliminary experiments, we treated the Caco-2 cells with 2 mmol/L H_2O_2 for 30 min to activate the secretion of IL-8. The 24-well plates of Caco-2 cells were preincubated for 3 h with a freshly prepared medium containing the dietary agents before being treated with H_2O_2 for 30 min. The cells were then post-treated with the agent-containing medium for a further 3 h (mRNA determination) or 24 h (protein determination). To prove that CQA inhibited the secretion of IL-8 by its anti-inflammatory action inside cells and not by neutralizing the oxidative effect of H_2O_2 in the medium, the cells were washed with PBS after being preincubated with the CQA-containing medium. They were then treated with H_2O_2 for 30 min and post-treated for 24 h with the normal medium in the absence of CQA. To investigate as to whether isomers and/or metabolites of CQA, which are probably produced during the treatment of Caco-2 cells with CQA, contribute to the inhibitive effects of CQA on the H_2O_2-induced IL-8 secretion, a medium containing 2 mM CQA was first used to incubate Caco-2 cells for 48 h. Then, the preincubated CQA medium instead of the freshly prepared CQA medium was used to treat a new plate of Caco-2 cells to test inhibitive effects on H_2O_2-induced IL-8 secretion as described previously.

Treatment with TNF-α. Caco-2 cells were preincubated for 3 h with a freshly prepared medium containing the dietary agents and then for a further 3 h together with 10 ng/mL TNF-α (mRNA determination) or for 24 h (protein determination). After each treatment, mRNA of IL-8 in the cells was extracted and quantified by real-time PCR. The IL-8 concentration in the supernatant was determined by an enzyme-linked immunosorbent assay (ELISA). We selected the two time points (3 h for mRNA determination and 24 h for protein determination) according to the results of the time course studies that showed that H_2O_2 or TNF-α treatment led to a peak in the overexpression of the mRNA 3 h after treatment and a peak in the secretion of the IL-8 protein 24 h after treatment. The damage caused to the treated cells was evaluated by microscopic observation and by measuring the amount of lactate dehydrogenase (LDH) released from the cells.

LDH Assay. The LDH assay was performed according to the manufacturer's protocol. Briefly, the Caco-2 monolayers that had been cocultured were rinsed twice with Hank's balanced salt solution (HBSS) and then incubated with 0.5 mL/well HBSS at 37 °C for 1 h. Then, the supernatant was collected, and 0.5 mL of 0.1% Triton X-100 was added to each well to solubilize the cells. Last, the LDH activity was measured in both supernatants and cell lysate fractions using an LDH-cytotoxic test kit (Wako, Osaka, Japan). All the samples were assayed in triplicate in each test. The percent of LDH release from the cells was determined using the following formula: LDH release = (amount of LDH in the supernatant)/(total amount of LDH in both supernatant and cell lysate) × 100.

ELISA for Measuring Il-8 Secretion. The concentration of IL-8 in the culture medium of each collected supernatant was determined by using an ELISA kit according to the manufacturer's instructions. Standard human IL-8/CXCL8 was diluted with the same medium as the one used in the cell culture. All the standards and test samples were assayed in duplicate in each test, and quantification was accomplished by calibrating against the standards.

Real-Time PCR for IL-8 mRNA. Total RNA was extracted from the cell lysate by a guanidinium thiocyanate−phenol (Isogen, Nippon Gene Co., Japan)−chloroform method according to the manufacturer's instructions. The steady-state levels of IL-8 and β-actin were determined by real-

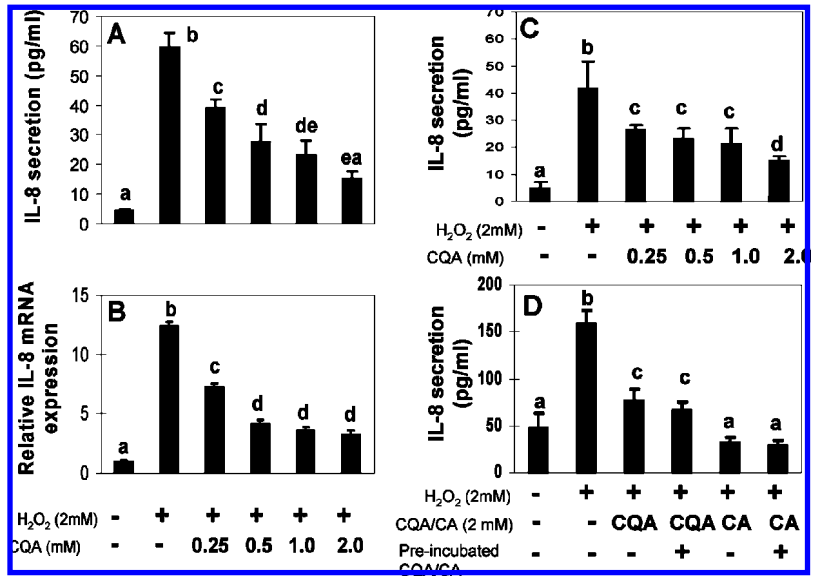

Figure 2. Simultaneous treatment with CQA and H_2O_2 inhibited IL-8 secretion (**A**) and its mRNA expression (**B**) induced by H_2O_2 in Caco-2 cells. A pretreatment with only CQA also inhibited the H_2O_2-induced IL-8 secretion (**C**). A preincubation (48 h) of CQA or CA in the medium of Caco-2 cells did not change their inhibitive effects on H_2O_2-induced IL-8 secretion (**D**). Each value is the mean ± SEM ($n = 3$). Means without common letters differ by Tukey's test, $p < 0.05$.

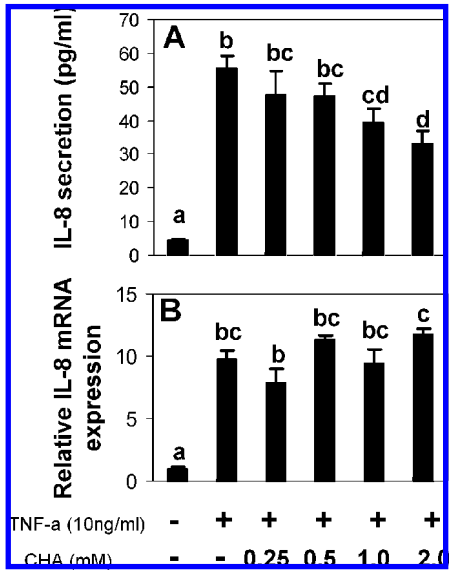

Figure 3. CQA inhibited IL-8 secretion (**A**) but not its mRNA expression (**B**) induced by TNF-α in Caco-2 cells. Each value is the mean ± SEM ($n = 3$). Means without common letters differ by Tukey's test, $p < 0.05$.

time PCR. Total mRNA (1 μg) was reverse-transcribed with an ExScript reverse transcription reagent kit. The obtained cDNA (1 μL) was amplified by a SYBR Green real-time PCR kit, using the Lightcycler system (Roche Diagnostics). The reaction mixture was incubated for 15 min at 95 °C and then subjected to 40 amplification cycles consisting of denaturing at 95 °C for 15 s, annealing at 57 °C for 15 s, and extension at 72 °C for 15 s. The primer sequences were as follows: human IL-8, 5′-AGA GTG ATT GAG AGT GGA CC-3′ (forward) and 5′-ACT TCT CCA CAA CCC TCT G-3′ (reverse) and β-actin, 5′-CCA CGA AAC TAC CTT CAA C-3′ (forward) and 5′-GAT CTT CAT TGT GCT GGG-3′ (reverse). Their specificity was verified by analyzing the melting curve for each product and by agarose gel electrophoresis. mRNA was quantified by the $\Delta\Delta C_T$ comparative method (*25*). The gene expression levels of IL-8 were finally normalized by using β-actin as a housekeeping gene. To detect any contamination by genomic DNA and/or by the preparation of real-time PCR, RT minus the control and nontemplate control were included in all quantitative analyses.

Statistical Analysis. Each data value is presented as the mean ± SEM ($n = 3$). The data were tested with Tukey's multiple-range test when significant differences ($p < 0.05$) were obtained by one-way ANOVA.

RESULTS

Damaging Effects of Treatments on Caco-2 Cells. First, the damaging effects of all the treatments on the cells were evaluated by microscopic observation. No significant change in cell morphology was apparent by microscopic observation after the cells had been treated with H_2O_2, TNF-α, and/or a dietary agent. The damage to the cells caused by these treatments also was evaluated by an LDH assay. The normal cells released 0.5–1% LDH into HBBS when incubated for 1 h. None of the treatments led the cells to release a significant amount of LDH into HBBS. These results show that the cells used in our studies maintained their normal state.

Effects of CQA on H_2O_2-Induced IL-8 Secretion in Caco-2 Cells. As shown in **Figure 2A**, normal Caco-2 cells spontaneously secreted IL-8. However, a 30 min treatment with 2 mmol/L H_2O_2 increased the secretion of IL-8 by about 10-fold. CQA suppressed the secretion of IL-8 in a dose-dependent manner. The promotion of IL-8 secretion induced by oxidative stress was suppressed by 65% with 1.0 mmol/L CQA, the natural concentration in commercial brewed coffee, (**Figure 2A**). To determine as to whether CQA inhibited the secretion of IL-8 by neutralizing the oxidative effect of H_2O_2 in the medium, the cells were only pretreated with CQA and washed with PBS before being treated with H_2O_2 and post-treated with the normal medium in the absence of CQA. Such a treatment also significantly inhibited the oversecretion of IL-8 induced by H_2O_2 (**Figure 2C**), suggesting that CQA inhibited IL-8 oversecretion inside the cells. To determine as to whether isomers of CQA, caffeic acid, and/or its metabolites possibly transforming from CQA in the medium during the incubation contributed to the inhibitive effect of CQA, the cells were treated with a 48 h preincubated CQA medium instead of the fresh CQA-supplemented medium to test the inhibitive effect on the secretion of IL-8 induced by H_2O_2. The result did not differ from that of the treatment with fresh CQA medium (**Figure 2D**).

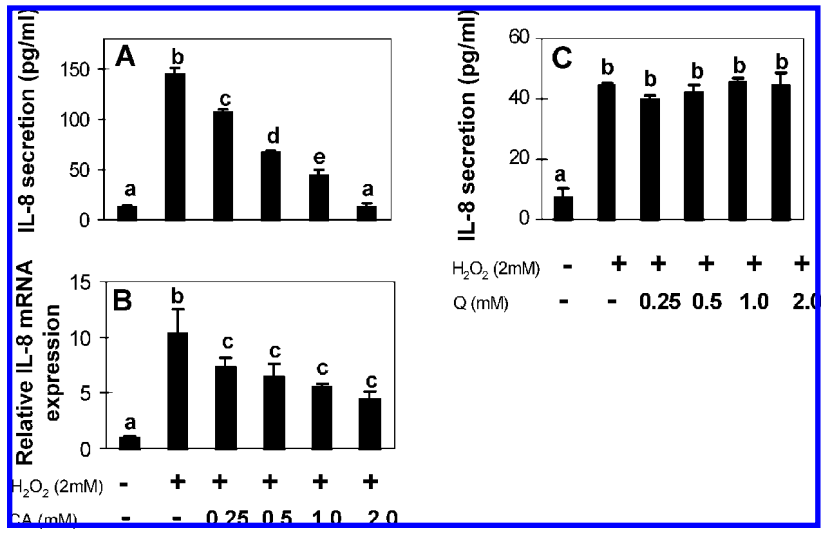

Figure 4. H_2O_2-induced IL-8 secretion and its mRNA expression were inhibited (**A** and **B**) by CA but not (**C**) by quinic acid (Q). Each value is the mean ± SEM ($n = 3$). Means without common letters differ by Tukey's test, $p < 0.05$.

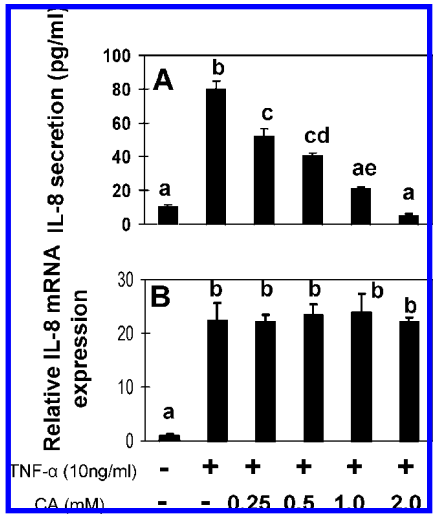

Figure 5. CA inhibited IL-8 secretion (**A**) but not its mRNA expression (**B**) induced by TNF-α in Caco-2 cells.

Effects of CQA on H_2O_2-Induced IL-8 mRNA Expression in Caco-2 Cells. We next determined the effect of CQA on the H_2O_2-induced overexpression of IL-8 mRNA in Caco-2 cells. A 30 min treatment with 2 mmol/L H_2O_2 led to a peak in the overexpression of IL-8 mRNA 3 h after treatment. At 3 h after treatment, the H_2O_2-induced expression of IL-8 mRNA had increased by about 12-fold as compared to the control (**Figure 2B**). CQA in a dose range of 0.25–2.0 mmol/L significantly suppressed any overexpression.

Effects of CQA on TNF-α-Induced IL-8 Secretion and Its mRNA Expression in Caco-2 Cells. CQA showed a similar suppressive effect on TNF-α-induced IL-8 secretion to the H_2O_2-induced secretion of IL-8, although 2 mmol/L CQA decreased the promoted IL-8 secretion by about 60% (**Figure 3A**). However, CQA did not significantly inhibit the overexpression of IL-8 mRNA induced by TNF-α (**Figure 3B**).

Effect of CA and Quinic Acid on H_2O_2-Induced IL-8 Secretion and Its mRNA Expression in Caco-2 Cells. Since part of ingested CQA was transformed into CA and quinic acid in the gut and/or in the intestinal epithelial cells, we subsequently determined the effects of CA and quinic acid on the H_2O_2-induced IL-8 secretion in Caco-2 cells. CA inhibited both the secretion of IL-8 and its mRNA expression induced by H_2O_2 in a dose-dependent manner in the range of 0.25–2.0 mmol/L (**Figure 4A**,**B**). Quinic acid did not show any significant effect on the secretion of IL-8 (**Figure 4C**).

Effects of CA on TNF-α-Induced IL-8 Secretion and Its mRNA Expression in Caco-2 Cells. We also determined the effect of CA on the TNF-α-induced IL-8 secretion and its mRNA expression in Caco-2 cells. The results were similar to those found in the CQA experiments. CA inhibited IL-8 secretion but not IL-8 mRNA expression induced by TNF-α (**Figure 5A**,**B**). The inhibitory effect of CA on TNF-α-induced IL-8 secretion was dependent on the dose of CA in the range of 0.25–2.0 mmol/L. Furthermore, the inhibitive effect of CA was stronger than that of CQA. CA at a dose of 2.0 mmol/L was able to absolutely inhibit the secretion of IL-8 (**Figure 5A**).

DISCUSSION

Among several cytokines/chemokines secreted by intestinal epithelial cells, IL-8 plays a central role in inducing the infiltration of neutrophils and T cells into the intestinal mucosa that is involved in the pathology of inflammatory bowel diseases (*18, 26, 27*). Some amino acids are expected to down-regulate the oversecretion of IL-8 and prevent inflammatory bowel disease genesis (*17, 23, 24, 28*). Interestingly, we found that CQA, with a different chemical structure from that of those amino acids, also significantly inhibited the oversecretion of IL-8 induced by H_2O_2 and TNF-α in human intestinal Caco-2 cells. Further studies showed that CA, one of the metabolites of CQA, presented stronger inhibitory effects on IL-8 secretion than did CQA. CQA and CA also suppressed the expression of IL-8 mRNA induced by H_2O_2.

Both CQA and CA are absorbed in the gastrointestinal tract of rats and humans (*13, 14, 29*). Almost all ingested CA, but only 33% of CQA, can be absorbed in the foregut (*13*). The remaining CQA reaches the hindgut where it is absorbed, after being metabolized by colonic microflora into CA, quinic acid, 3-hydroxyphenylpropionic acid, and other compounds (*14, 15*). The different absorptivity between CQA and CA in the small intestine may be ascribed to different absorption mechanisms in the intestinal epithelial cells. An absorption study with Caco-2 monolayers showed that CQA was absorbed mainly via paracellular diffusion but that CA was absorbed via both paracellular diffusion and by a monocarboxylic acid transporter (MCT) (*30*). For this reason, a little more CA than CQA accumulated in

Caco-2 cells after the cells had been cultured with a medium containing the same concentration of CA or CQA (*30*). This may be one of the reasons as to why CA seemed to show a little stronger anti-inflammatory effect than CQA in this present study. Another possible reason might be that CA can more easily access the target and block the pathway for H_2O_2- and TNF-α-induced IL-8 secretion. Nevertheless, CQA may inhibit the oversecretion of IL-8 with the same mechanism as that by CA because the CA moiety, but not quinic acid moiety, of CQA is likely to have been responsible for the anti-inflammatory effect of CQA (**Figure 4A** vs **B**).

After 48 h of incubation, part of CQA transformed into its isomer and/or glucuronide/sulfate in Caco-2 cells (data not shown), which also possibly contributed to their inhibitive effects on the secretion of IL-8. However, the transformation did not change the total inhibitive effects of CQA (**Figure 2D**), suggesting that the isomers and metabolic conjugates of CQA may have the same potential power in the inhibition of IL-8 secretion in this experimental model.

IL-8 can be synthesized by many different cell types in response to various stimuli. Maximal IL-8 amounts are generated by a combination of three different mechanisms: depression of the gene promoter, transcriptional activation of the gene by the NF-κB and JNK pathways, and stabilization of mRNA by the p38 MPK pathway (*31*). The regulation of IL-8 is cell type-dependent and stimulus-dependent (*31, 32*). For example, H_2O_2 has induced IL-8 expression in A549, a human lung cancer epithelial cell line, but not in the human microvessel endothelial cell line (*33*). H_2O_2 activated AP-1 in A549 through the JNK pathway, but not NF-κB, whereas TNF-α activated both AP-1 and NF-κB (*33*). The regulation mechanism for the IL-8 expression induced by H_2O_2 in intestinal epithelial cells has not yet been well-studied. Our preliminary experiments (data not shown) revealed that H_2O_2-induced IL-8 mRNA expression was partly through the activation of NF-κB in Caco-2 cells. On the other hand, several studies have shown that TNF-α induced IL-8 expression and secretion in intestinal epithelial cells through a combination of NF-κB, JNK, ERK, and p38 MPK pathways (*17, 34, 35*). A study has revealed that histidine inhibits TNF-α-induced IL-8 secretion through blocking NF-kB-dependent activation of the IL-8 promoter (*17*). Taurine also showed a weaker inhibitive effect on TNF-α-induced IL-8 secretion, possibly involving NF-kB (*23*). The results of the present study indicate that CQA/CA may have suppressed H_2O_2-induced IL-8 at the transcriptional level but suppressed TNF-α-induced IL-8 at the post-transcriptional level. However, the suppression mechanisms of CQA in experimental systems using the two different stimulants remain unknown. It has been reported that CQA up-regulated the cellular antioxidative enzymes and suppressed ROS-mediated NF-κB, AP-1, and MAPK activation induced by UV in A549 human lung cancer cells (*11*). Curcumin, another phenolic antioxidant with a similar structure to that of CQA, also regulated the cellular antioxidative enzymes and increased the cellular glutathione level in the same cell line (*36, 37*). Such a mechanism would probably be involved in the inhibition by curcumin of the H_2O_2-induced activation of NF-κB and the secretion of IL-8 in the lung cells. In human intestinal epithelial cells, CQA might similarly inhibit IL-8 expression by modulating the antioxidative enzyme activity. Nevertheless, an unequivocal conclusion awaits the results of further detailed studies.

Coffee is one the most popular beverages. The concentration of CQA in common brewed coffee is 1–2 mmol/L (*18*). Our results show that such a concentration of CQA significantly suppressed H_2O_2- and TNF-α-induced IL-8 secretion in human intestinal epithelial cells. Since IL-8 is a central pro-inflammatory chemokine inducing the infiltration of neutrophils and being involved in the pathogenesis of inflammatory bowel diseases, our results suggest that habitual coffee drinking may be beneficial in preventing the genesis of inflammatory bowel diseases.

ABBREVIATIONS USED

CHA, chlorogenic acid; CQA, 5-caffeoylquinic acid, the predominant isomer of CHA in foods; CA, caffeic acid; LDH, lactate dehydrogenase; IL, interleukin.

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Received for review October 30, 2007. Revised manuscript received February 21, 2008. Accepted March 12, 2008. This work was partially supported by a grant for scientific research (S-15108002) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

JF073168D