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RESEARCH ARTICLE

Heme induces DNA damage and hyperproliferation of colonic epithelial cells *via* hydrogen peroxide produced by heme oxygenase: A possible mechanism of heme-induced colon cancer

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Epidemiological and animal model studies have suggested that high intake of heme, present in red meat, is associated with an increased risk of colon cancer. However, the mechanisms underlying this association are not clear. This study aimed to investigate whether heme induces DNA damage and cell proliferation of colonic epithelial cells *via* hydrogen peroxide produced by heme oxygenase (HO). We examined the effects of zinc protoporphyrin (ZnPP; a HO inhibitor) and catalase on DNA damage, cell proliferation, and IL-8 production induced by the addition of hemin (1–10 μM) to human colonic epithelial Caco-2 cells. DNA damage was determined with a comet assay, and cell proliferation was evaluated with 5-bromo-2′-deoxyuridine incorporation assay. Both ZnPP and exogenous catalase inhibited the hemin-induced DNA damage and cell hyperproliferation dose-dependently. IL-8 messenger RNA expression and IL-8 production in the epithelial cells increased following the hemin treatment, but the production was inhibited by ZnPP and catalase. These results indicate that hemin has genotoxic and hyperproliferative effects on Caco-2 cells by HO and hydrogen peroxide. The mechanism might explain why a high intake of heme is associated with increased risk of colon cancer.

Keywords:
Cell proliferation / Colon cancer / DNA damage / Heme oxygenase / Hydrogen peroxide

1 Introduction

Colorectal cancer is one of the major causes of cancer-related death in affluent societies [1]. About one million new colorectal cancer cases are diagnosed worldwide each year, and the mortality is approximately half that of the incidence (~529 000 deaths in 2002 for both sexes) [2]. Colorectal cancer most commonly occurs sporadically and is inherited in only 5% of the cases. Diet is the most important exogenous factor identified thus far in the etiology of this cancer [3]. High consumption of red meat (beef, pork, or lamb), in contrast to white meat (poultry or fish), has been associated with increased risk of colorectal cancer in many epidemiological studies [4–6]. Several biological mechanisms have been investigated to explain the possible effect of red meat consumption on colorectal carcinogenesis. Sesink *et al.* [7] hypothesized that heme, the iron porphyrin pigment of red meat, is an important dietary risk factor. They argued that heme might better explain the differential effects of red *versus* white meat on the colon cancer risk, instead of the earlier proposed meat-associated mutagens such as heterocyclic amines [7]. The heme content of red meat is 10-fold higher than that of white meat [8]. Heme

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Abbreviations: **ACF,** aberrant crypt foci; **BrdU,** 5-bromo-2′-deoxyuridine; **CO,** carbon monoxide; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **HO,** heme oxygenase; **H_2O_2,** hydrogen peroxide; **mRNA,** messenger RNA; **ROS,** reactive oxygen species; **ZnPP,** zinc protoporphyrin

absorption is low, and most ingested heme is therefore delivered to the colon and rectum. Heme has been recovered from feces after consumption of red meat but not white meat [8].

In the colon, iron possibly increases the production of reactive oxygen species (ROS), possibly causing cellular toxicity and promutagenic lesions [9–11]. Intracellular reactions with ROS can result in the initiation and progression of carcinogenesis by induction of gene mutations, chromosomal damage and other cytotoxic effects [12–14]. Furthermore, ROS regulate the expression of genes active during cell differentiation and proliferation [15–17] and therefore probably play an important role in the promotion phase of tumor generation. Previous studies have shown that heme has cytotoxic and hyperproliferative effects on rat colonic epithelium [7, 18, 19]. Feeding heme but not nonheme iron to rats resulted in hyperproliferation and apoptosis in rat colon [7, 19]. In addition, the incidence of aberrant crypt foci (ACF) and mucin-depleted foci increased as the heme content of the diet increased, suggesting that heme is carcinogenic [20, 21]. In fact, heme is genotoxic to human colonic epithelial cell line HT-29 cells [9]. Heme metabolic degradation products rather than heme or iron *per se* are responsible for the inflammation and ACF formation [7, 18]. Although it has been suggested that heme is converted to a cytotoxic factor, the factor has not been fully characterized [7, 22].

Heme is the prosthetic group of heme proteins, which are responsible for oxygen transport and storage (*via* hemoglobin or myoglobin). Red meat is abundant in myoglobin, comprising a single globin chain holding one heme. As it is not recycled, most of the heme is enzymatically degraded by the heme degradation system, consisting of heme oxygenase (HO) and microsomal cytochrome P450 reductase [23, 24]. HO is the rate-limiting enzyme in the cellular catabolism of heme, in which heme is degraded to biliverdin, carbon monoxide (CO), and a ferrous ion. To date, three HO isoforms have been characterized, each encoded by a different gene. HO-1 and HO-2 are catalytically very active, whereas HO-3 activity appears to be less than that of the other isozymes. Noguchi *et al.* [25] have reported that a considerable amount of hydrogen peroxide (H_2O_2) is produced during heme degradation, catalyzed by the reconstituted HO system. In addition, a free ferrous iron is yielded when dietary heme is resolved by HO-1 in intestinal mucosa epithelium [26]. Ferrous irons and H_2O_2 reportedly cleave DNA by Fenton reaction at sequence-specific sites often found in telomere repeats [27]. Moreover, H_2O_2 may also have an important physiological role in the stimulation of cell proliferation [17, 28, 29].

Therefore, we hypothesized that H_2O_2 is produced when heme is resolved by HO in cells, and affects the mutation and proliferation of the colonic epithelium. The present study was designed with an *in vitro* cell system to investigate whether heme can initiate and promote carcinogenesis. We used human colonic epithelial-like Caco-2 cells as the model system. Three hypotheses were tested: (i) addition of the HO inhibitor zinc protoporphyrin (ZnPP) and catalase dose-dependently inhibit hemin-induced DNA damage in the cells; (ii) ZnPP and catalase dose-dependently inhibit hemin-induced cell proliferation; (iii) H_2O_2 is produced in the cells during heme degradation by HO. Oxidative stress such as H_2O_2 promotes the production of several cytokines, including IL-8, IL-6, IL-1β and tumor necrosis factor-α in epithelial cells [30–32]. In this study, we indirectly determined whether IL-8 production is an indicator of oxidative stress induced by hemin.

2 Materials and methods

2.1 Materials

Unless otherwise stated, all the biological reagents used in this study were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan); cell culture media and molecular biology reagents were from Invitrogen Japan K.K. (Tokyo, Japan); and the flasks for growing cells and cell culture plates were from Techno Plastic Products AG (Trasadingen, Switzerland).

2.2 Cell culture

Human colon carcinoma Caco-2 cells (RCB0988) from the Riken Cell Band (Tsukuba, Japan) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, and 1% nonessential amino acids. The cells were cultured in a humidified incubator at 37°C with 95% atmospheric air/5% CO_2. Caco-2 cells were used between the 15th and 30th passages. For all experiments, the cells were seeded at a density of 3×10^4 cells/cm^2 in cell culture plates. The cells were carefully treated to avoid as much light exposure as possible.

2.3 Cell treatments

After 24-h growth in the appropriate culture plates, the growth media was replaced with a medium containing reduced 5% fetal bovine serum. Hemin (Acros Organics, Geel, Belgium) and ZnPP (ZnPP IX; Frontier Scientific, Logan, UT, USA) were prepared on a stock solution in 1 M sodium hydroxide and further diluted in the nonsupplemented DMEM. Caco-2 cells were exposed to different concentrations of hemin for 0–24 h in the absence and presence of various concentrations of ZnPP (0.1–10 μM) or catalase (0.01–1 U/mL). Control cells were maintained in nonsupplemented DMEM. Each experiment was performed independently at least three times.

2.4 Cell viability

The cell viability was assessed from the mitochondrial activity in reducing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan. Briey, after treatment with hemin for 6 h, Caco-2 cells were washed with PBS twice. Then, the cells were incubated with 100 μL serum-free medium and 10 μL WST-8 assay solution (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) for 1 h at 37°C. The amount of formazan formed was measured from the absorbance at 450 nm with a reference wavelength of 655 nm by using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). This absorbance was expressed as a percentage of that in the control cells.

2.5 Single-cell gel electrophoresis assay (comet assay)

To detect DNA damage in individual cells, we adopted a comet assay [33]. Briefly, cells (2×10^4) were collected after the treatments and resuspended in 0.2 mL of PBS containing 0.5% low-melting-point agarose. Eighty-five microliters of the mixture was applied to the slides and submerged in cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100 (pH 10)). Electrophoresis was performed at 4°C using a field strength of 0.7 V/cm and 300 A. After electrophoresis, the slides were neutralized with 0.4 M cold Tris-HCl buffer (pH 7.4) and then stained with ethidium bromide. Comets were visualized by using a fluorescence microscope (1×70; Olympus, Tokyo, Japan). The proportion and extent of DNA migration were determined for at least 300 cells *per* slide using Quantity One 1-D analysis software (Bio-Rad Laboratories). The evaluation criterion used was the intensity of fluorescence in the comet tail, expressed as ''percent tail intensity.''

2.6 Cell proliferation

Cell proliferation was measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. This assay was performed with a cell proliferation ELISA kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, Caco-2 cells were labeled with BrdU after the treatments and incubated for an additional 2 h. The labeling medium was removed, and the cells were dried. Thereafter, cellular DNA was denaturated by adding a fixative solution for 30 min at room temperature. After removal of the fixative solution, anti-BrdU antibody conjugated with peroxidase solution was added to each well, and the cells were incubated for 90 min at room temperature. After three washes with PBS, 3,3′,5,5′-tetramethylbenzidine solution was added to each well, and the cells were incubated for 15 min at room temperature. The reaction was terminated with 1 M H_2SO_4, and the absorbance at 450 nm (reference wavelength = 655 nm) was read using the microplate reader.

2.7 IL-8 messenger RNA expression

To detect IL-8 messenger RNA (mRNA) expression, RT-PCR assay was performed according to a previous report [32]. Total RNA was isolated from Caco-2 cells by using TRIzol reagent (Invitrogen Japan K.K.) after the treatments, according to the manufacturer's instruction. The complementary DNA product obtained by reverse transcription using random primers and reverse transcriptase was amplified by PCR. The nucleotide sequences of the oligonucleotide primers used for PCR were as follows: IL-8 sense primer, 5′-AGGAACCATCTCACTGTGTG-3′; IL-8 antisense primer, 5′-ATTTGGGGTGGAAAGGTT-3′. After an initial denaturing step at 94°C, 30 PCR cycles were performed as follows: denaturing for 1 min at 94°C, primer annealing for 1 min at 62°C, and DNA synthesis for 45 s at 72°C. The mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was examined as a standard mRNA expressed in the cells in the same manner as for IL-8. The nucleotide sequences of the primer for RT-PCR with GAPDH were as follows: GAPDH sense primer, 5′-TGAAGGTCG-GAGTCAACGGATTTGGT-3′; GAPDH antisense primer, 5′-CATGTGGGCCATGAGGTCCACCAC-3′. The RT-PCR products were loaded onto 2% agarose gel in Tris-acetate-EDTA buffer and electrophoresed. The products were visualized using ethidium bromide and an ultraviolet illuminator (ChemiDoc XRS; Bio-Rad Laboratories). The intensity of the DNA bands was calculated using Quantity One 1-D analysis software (Bio-Rad Laboratories).

2.8 Measurement of IL-8 production

Caco-2 cells were incubated for 24 h with various concentrations of hemin in the absence and presence of various concentrations of ZnPP or catalase. After the treatments, the culture supernatants were collected and stored at −80°C. IL-8 production was measured by sandwich ELISA assay according to the recommended protocol of the Biotrak human IL-8 ELISA system (GE Healthcare UK). Briefly, an antibody specific for IL-8 has been coated on the microtiter plate provided in the ELISA kit. Samples were added into the wells, followed by incubation with biotinylated antibody reagent. After washing away any unbound samples and biotinylated antibody, a streptavidin–horse radish peroxidase conjugate was added to the wells. Following a wash to remove unbound conjugate, a substrate 3,3′,5,5′-tetramethylbenzidine solution was added to the wells, and the absorbance was read at 450 nm by using the microplate reader.

2.9 Statistical analyses

All data are presented as the mean ± SD for the number of experiments. One-factor analysis of variance and Dunnett's test were performed to compare differences between the groups.

3 Results

3.1 Effect of hemin on viability of Caco-2 cells

The effect of hemin on the cell viability is shown in Fig. 1. Caco-2 cells were treated with various concentrations of hemin for 6 h. Hemin did not significantly affect the cell viability at the concentrations up to 100 μM. At 1000 μM, hemin significantly decreased the cell viability. Therefore, hemin was cytotoxic toward the cells at the 1000 μM concentration but not in the range from 1 to 100 μM.

3.2 Effect of hemin on DNA damage in Caco-2 cells

We examined the effect of hemin on DNA damage in Caco-2 cells by a comet assay. Because the DNA damage (expressed as percent tail intensity) was detected as early as 3 h and peaked at 6 h (data not shown), the cells were treated for 6 h with various concentrations of hemin. As shown in Fig. 2, hemin significantly increased the DNA damage in the cells by 50, 75, and 159% at the respective concentrations of 1, 10, and 100 μM. Hemin could therefore be defined as genotoxic at the concentrations from 1 to 100 μM.

3.3 Effect of ZnPP and catalase on hemin-induced DNA damage in Caco-2 cells

To determine whether HO influences the hemin-induced DNA damage in the cells, we treated the colonic epithelial cells with 1 μM hemin alone or together with various concentrations of ZnPP for 6 h. Figure 3A shows that increasing the ZnPP concentration decreased the hemin-induced DNA damage in the cells, with the inhibition rate reaching more than 80% at the concentration of 0.1 μM. The

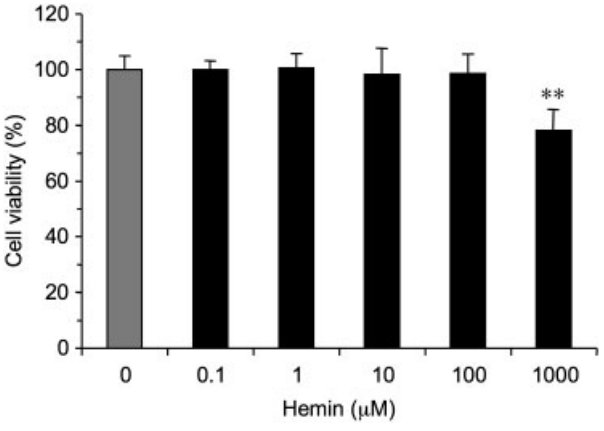

Figure 1. Effect of the concentration of hemin on the viability of the human colonic epithelial Caco-2 cells. The cells were treated for 6 h with various concentrations of hemin. $^{**}p<0.01$ *versus* the control (without hemin treatment).

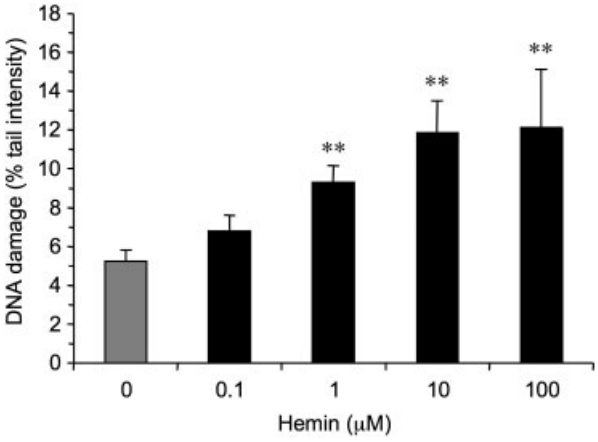

Figure 2. Effect of the concentration of hemin on DNA damage in the human colonic epithelial Caco-2 cells. The cells were treated for 6 h with various concentrations of hemin. The extent of DNA damage was expressed as ''percent tail intensity.'' $^{**}p<0.01$ *versus* the control (without hemin treatment).

statistically significant inhibition of the hemin-induced DNA damage by ZnPP was observed at 0.1 μM and higher concentrations. The treatments with ZnPP alone did not affect the DNA damage in unstimulated cells.

Because we expected that H_2O_2 is produced by HO and participates in the genotoxicity of hemin, Caco-2 cells were treated with various concentrations of catalase in the absence and presence of 1 μM hemin for 6 h. The effect of catalase on the hemin-induced DNA damage is shown in Fig. 3B. Endogenous catalase inhibited the hemin-induced DNA damage in a dose-dependent manner, this being statistically significant at 0.01 U/mL and higher catalase concentrations.

3.4 Effect of hemin on the proliferation of Caco-2 cells

We next examined the effect of hemin on the proliferation of the Caco-2 cells. The cells were treated for 24 h with various concentrations of hemin, and cell proliferation was determined by the BrdU incorporation assay. As shown in Fig. 4, the cell proliferation was significantly increased by the treatments with 0.1–100 μM hemin and peaked at the concentration of 1 μM, the increase being 45%.

3.5 Effect of ZnPP and catalase on hemin-induced proliferation of Caco-2 cells

Figure 5A shows the effect of ZnPP on the hemin-induced proliferation of the Caco-2 cells. Dose-dependent inhibition of cell proliferation by ZnPP was observed, which was statistically significant at the concentrations of 1 μM and higher.

Although substantial ROS formation is associated with cell damage and apoptosis [34, 35], recent evidence suggests

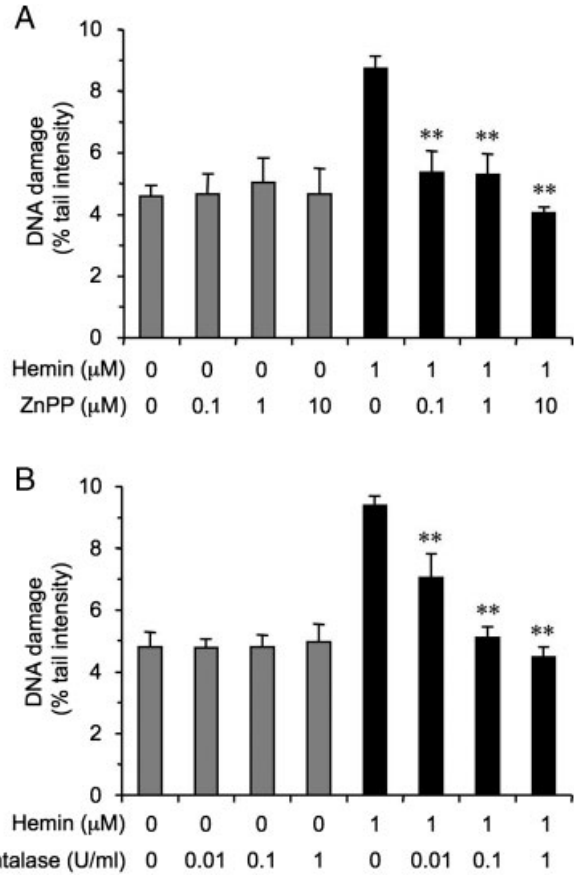

Figure 3. Effects of ZnPP (A) and catalase (B) on the hemin-induced DNA damage in the human colonic epithelial Caco-2 cells. The cells were treated for 6 h with various concentrations of ZnPP either alone or in combination with 1 μM hemin. Caco-2 cells were also treated with various concentrations of catalase in the absence and presence of 1 μM hemin for 6 h. The extent of DNA damage was expressed as ''percent tail intensity.'' $^{**}p<0.01$ *versus* the treatments with 1 μM hemin only.

that low concentrations of endogenous H_2O_2 play an important role in cell proliferation as an intracellular messenger [15–17, 36]. We therefore examined the effect of exogenously added catalase on the hemin-induced proliferation of the Caco-2 cells. Figure 5B shows that exposure to catalase dose-dependently inhibited the hemin-induced cell proliferation, with the inhibition rate reaching more than 50% at the concentration of 0.1 U/mL and over 75% inhibition at the concentration of 1 U/mL.

3.6 Effect of hemin on IL-8 mRNA expression and IL-8 production in Caco-2 cells

IL-8 production in intestinal epithelial cells increases in response to pro-inflammatory cytokines such as tumor necrosis factor-α [37, 38] and IL-1 [32], or oxidative stress such as that by H_2O_2 [32, 39, 40]. To determine whether

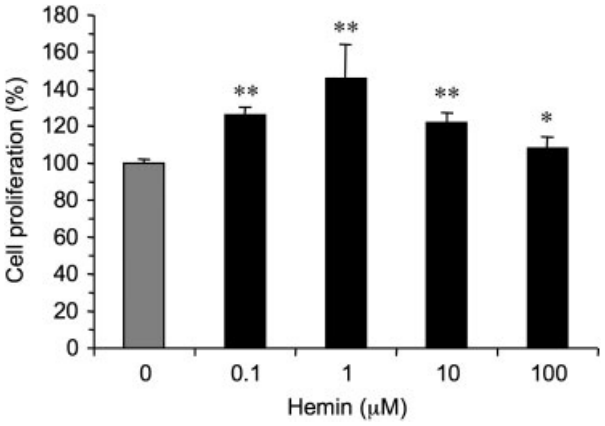

Figure 4. Effect of the concentration of hemin on the proliferation of human colonic epithelial Caco-2 cells. The cells were treated for 24 h with various concentrations of hemin, and cell proliferation was determined by the BrdU incorporation assay. $^{*}p<0.05$ or $^{**}p<0.01$ *versus* the control (without hemin treatment).

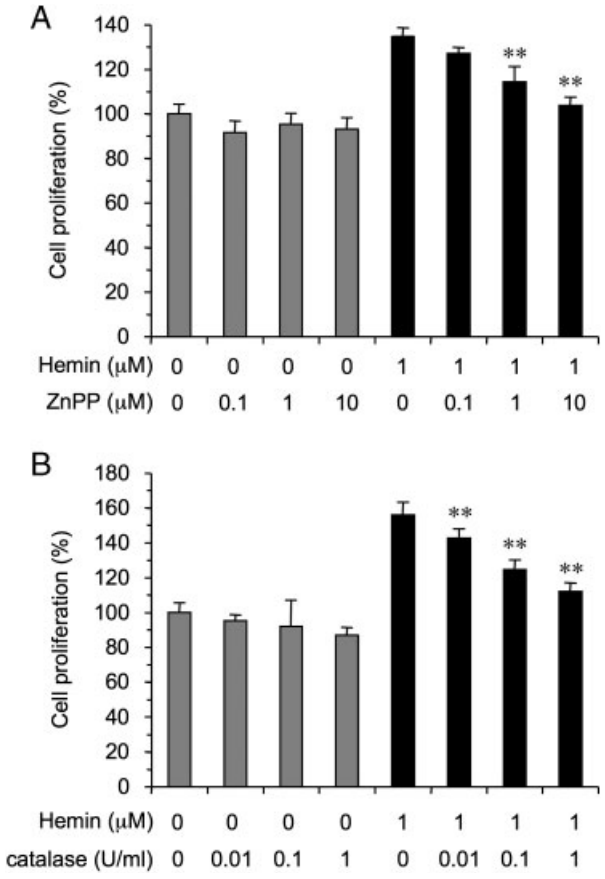

Figure 5. Effects of ZnPP (A) and catalase (B) on the hemin-induced proliferation of human colonic epithelial Caco-2 cells. The cells were treated for 24 h with various concentrations of ZnPP either alone or in combination with 1 μM hemin. Caco-2 cells were also treated with various concentrations of catalase in the absence and presence of 1 μM hemin for 24 h. $^{**}p<0.01$ *versus* the treatments with 1 μM hemin only.

heme induces oxidative stress in the epithelial cells, we examined the effect of hemin on IL-8 mRNA expression and IL-8 production in Caco-2 cells. Total RNA from the cells was extracted for RT-PCR at 0, 1, 2, 4, 6, and 10 h after the addition of 10 μM hemin. As shown in Figs. 6A and B, IL-8 mRNA expression transiently increased and peaked at 4 h after the hemin treatment, suggesting that hemin enhanced IL-8 induction at the transcriptional level.

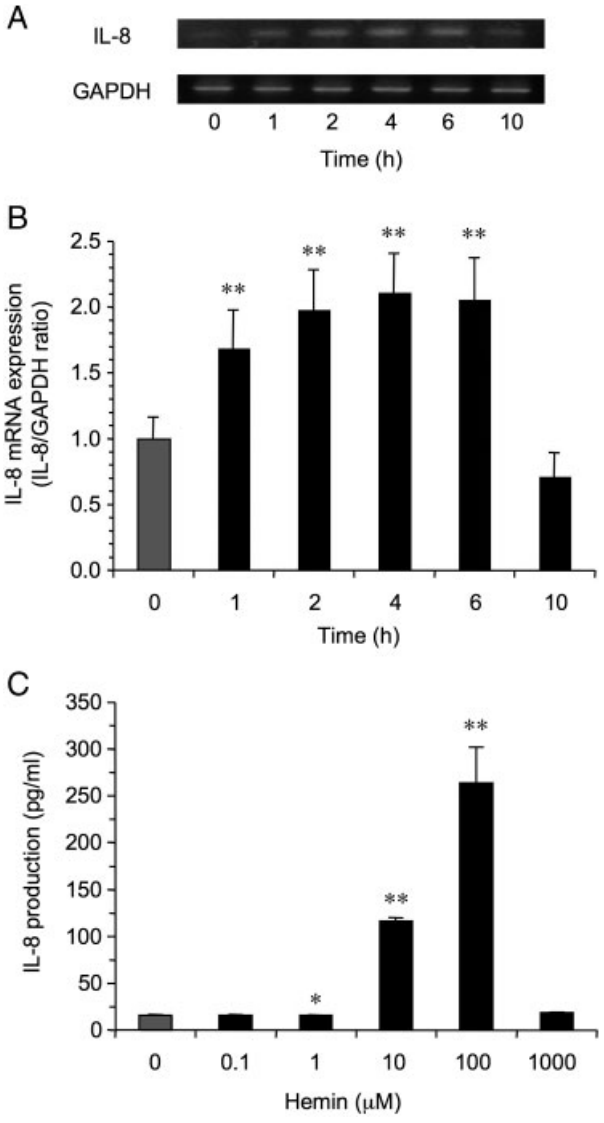

Figure 6. Effects of time on IL-8 mRNA expression (A, B) and effect of the concentration of hemin on IL-8 production (C). Total RNA from the human colonic epithelial Caco-2 cells was extracted for RT-PCR at 0, 1, 2, 4, 6, and 10 h after the addition of 10 μM hemin to determine IL-8 mRNA expression. Further, Caco-2 cells were treated with hemin at various concentrations for 24 h, and the culture medium was collected for determining IL-8 production by using ELISA. $^{*}p<0.05$ or $^{**}p<0.01$ *versus* the control (without hemin treatment).

We next treated Caco-2 cells with hemin at various concentrations for 24 h, and the culture medium was collected for IL-8 determination by ELISA. Figure 6C shows that IL-8 production increased dose-dependently. Intestinal epithelial cell lines such as Caco-2 and HT-29 are known to spontaneously secrete IL-8 [38, 40]. Nontreated control Caco-2 cells released 16.2 ± 0.3 pg/mL of IL-8. The level of IL-8 production by the cells significantly increased to 116.4 ± 3.4 and 264.2 ± 37.9 pg/mL on the addition of 10 and 100 μM hemin, respectively.

3.7 Effect of ZnPP and catalase on hemin-induced IL-8 production in Caco-2 cells

Figure 7 shows the effect of ZnPP and catalase on the hemin-induced IL-8 production in the Caco-2 cells. Increasing the ZnPP and catalase concentrations decreased the IL-8 production induced by hemin. Statistically significant

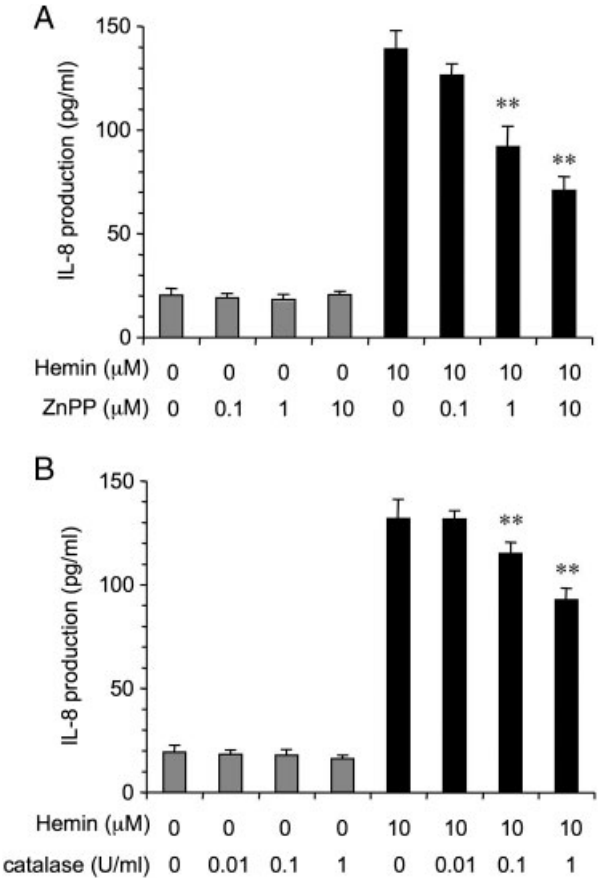

Figure 7. Effect of ZnPP (A) and catalase (B) on the hemin-induced IL-8 production. The cells were treated for 24 h with various concentrations of ZnPP either alone or in combination with 10 μM hemin. Caco-2 cells were also treated with various concentrations of catalase in the absence and presence of 10 μM hemin for 24 h. $^{**}p<0.01$ *versus* the treatments with 10 μM hemin only.

inhibition of IL-8 production by ZnPP and catalase was observed at the respective concentrations of 1 μM or above and 0.1 U/mL or above. In the presence of 10 μM ZnPP, the hemin-induced IL-8 production in the cells was reduced from 139.3 ± 8.8 to 70.9 ± 6.7 pg/mL. In the presence of 1 U/mL catalase, the hemin-induced IL-8 production was reduced from 132.1 ± 9.0 to 92.9 ± 5.4 pg/mL.

4 Discussion

The mechanisms underlying the association between the colon cancer risk and consumption of red meat are not clear. However, recent epidemiological studies have shown that the intake of heme might explain this association [41–43]. In addition, animal model studies suggest that dietary heme has cytotoxic and hyperproliferative effects on rat colonic epithelium [7, 18, 19]. Our data demonstrate hemin-induced DNA damage in and hyperproliferation of human colonic epithelial Caco-2 cells, indicating that heme is associated with both the initiation and promotion processes of carcinogenesis. However, the carcinogenic compounds and mechanism are still unknown. Here, we present a possible mechanism for why intake of heme might be associated with increased colon cancer risk.

The initiation of carcinogenesis is linked to peroxidation, cytotoxicity, and DNA damage by ROS such as superoxide, H_2O_2 and hydroxyl radicals. Previous studies have shown that dietary heme promotes ACF formation in rats and induces peroxidation and cytotoxicity of fecal water [20, 44]. The lipid peroxyl radicals generated from simultaneous fat and heme ingestion and the resulting ROS can cleave DNA or modify DNA bases, which could increase carcinogenesis [45].

Well-known metabolites of heme in the gastrointestinal tract, namely protoporphyrin, inorganic iron, and bilirubin, do not increase the cytotoxicity [7]. The difference between heme and its metabolites in terms of the cytotoxic and hyperproliferative effects might be explained by the induction of HO. HO catalyzes the rate-limiting step in the degradation of heme to produce biliverdin, CO, and a ferrous ion. Bilirubin reportedly behaves as an antioxidant by scavenging free radicals [46]. CO has been shown to protect cells against injury of various kinds both *in vitro* and *in vivo* [47, 48]. Furthermore, iron produced by HO stimulates ferritin synthesis, which ultimately provides an iron detoxification [23]. However, ferrous ions produce very harmful hydroxyl radicals under the existence of H_2O_2 by Fenton reaction [25]. DNA is a major target in H_2O_2-mediated cell damage through such reactions [27]. HO is generally considered to protect cells from oxidative stress because HO-1 is a member (HSP-32) of the HSP family and its expression is triggered by diverse stress-including stimuli including hypoxia, heavy metals, ultraviolet irradiation and ROS [49]. However, the heme metabolites have both antioxidative and prooxidative activities [50].

We proceeded with this study under the assumption that heme induces DNA damage in and proliferation of the colonic epithelial cells *via* H_2O_2 produced by HO. As expected, the HO inhibitor ZnPP inhibited the hemin-induced DNA damage in a dose-dependent manner. Especially, at the concentration of 10 μM, the genotoxic effect was almost diminished by ZnPP. Therefore, our results indicate that HO may be strongly involved in the heme-induced initiation process of carcinogenesis.

The promotion of carcinogenesis is linked to cell proliferation, apoptosis and differentiation. Intestinal epithelial cell turnover and differentiation are functions of crypt epithelium. Similar to that seen in intestinal crypt epithelium, HO-1 activity is high in undifferentiated epithelial Caco-2 cells [51], suggesting that HO-1 and cell proliferation or apoptosis are linked [52]. In addition, in human intestinal cell line HT-29 cells, induction of HO-1 reduces expression of the proapoptotic gene caspase-3 and inhibits apoptosis [53], supporting the idea that HO-1 has antiapoptotic activity. In fact, high expression of HO-1 has been observed in solid tumors in humans and experimental animal models [54, 55]. Moreover, ZnPP or its pegylated derivative (PEG-ZnPP) significantly reduced tumor growth in an animal model [56, 57]. These findings suggest that HO-1 expressed in tumors confers cytoprotection to tumor cells, thus permitting rapid growth *in vivo*. Our results show that ZnPP inhibited the hemin-induced cell hyperproliferation in a concentration-dependent manner. HO mediates the hyperproliferative and antiapoptotic activities; however, the cell hyperproliferative compound is unknown.

H_2O_2 might be produced by HO and participate in hemin-induced DNA damage and cell hyperproliferation because H_2O_2 is produced during heme degradation catalyzed by the reconstituted HO system [25]. Noguchi *et al.* [25] have reported that production of H_2O_2 in the system do not appear to be due to dismutation of superoxide anions but rather seemed to be due to dissociation of a ''peroxo'' species formed on heme or intermediates of heme degradation. Our results show that exogenous catalase inhibited the hemin-induced DNA degradation and cell proliferation in a dose-dependent manner. Especially, at the concentration of 1 U/mL, the hemin-induced DNA damage and hyperproliferation of the Caco-2 cells were almost diminished by catalase. These results support that H_2O_2 production is relevant to carcinogenesis.

Higher levels of endogenous H_2O_2 are associated with disease, whereas lower levels are essential for normal physiological functioning and signaling [58]. Within diseased tissues, concentrations in excess of 100 μM have been measured, although 1–15 μM appears to be the upper limit of the healthy physiological range [58]. Burdon *et al.* [59] have indicated that low concentrations of H_2O_2 (10 nM to 1 μM) are effective in stimulating the *in vitro* growth of hamster and rat fibroblasts when added to the culture medium. H_2O_2 at submicromolar levels appears to act as intracellular ''messenger'' capable of promoting growth

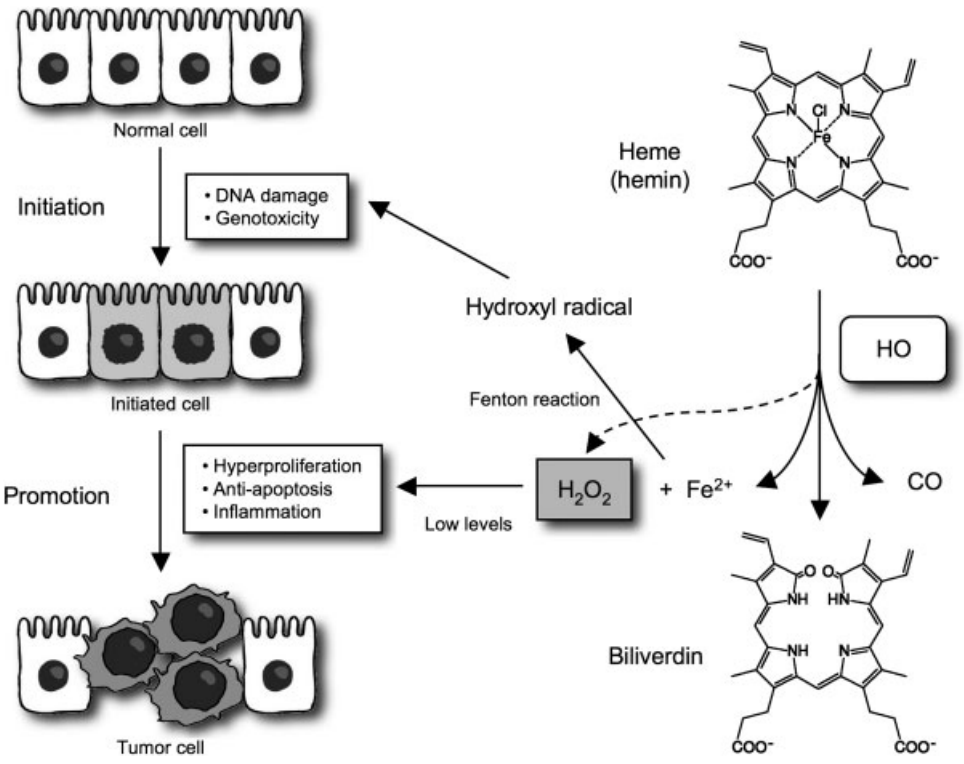

Figure 8. The proposed mechanism for heme-induced colon carcinogenesis.

responses in culture [15–17, 36]. The mechanisms may involve direct interaction with specific receptors or oxidation of growth signal transduction molecules such as protein kinases, protein phosphatases, transcription factors, or transcription factor inhibitors [15]. It is also possible that H_2O_2 may modulate the redox state and activity of these important signal transduction proteins indirectly through changes in cellular levels of reduced glutathione and oxidized glutathione [15].

Therefore, it is important to detect H_2O_2 in cells or culture media. Many analytical techniques have been reported for H_2O_2 determination, such as fluorescent spectroscopy, spectrophotometry, electrochemistry and chemiluminescence [60]. When using biological samples, the majority of these methods have some drawbacks, including large sample requirements, low sensitivity, and interference by several coexistences. In this study, we examined the production of IL-8 as an indirect indicator of oxidative stress because it was difficult to determine low levels of H_2O_2 in cells or culture media directly. The results suggest that the hemin-induced IL-8 mRNA expression and IL-8 secretion were partly abolished by the addition of ZnPP or catalase, indicating that H_2O_2 is generated in the process of hemin degradation and leads to the production of the inflammatory cytokine. Therefore, long-term intake of heme is possibly involved in carcinogenesis by inducing chronic inflammation. At molecular level, ROS such as H_2O_2 activate nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) transcription factors, and enhance the expression of both pro-inflammatory and protective antioxidant genes [61]. NF-κB and AP-1 are in turn regulated by complex signal transduction pathways mediated by mitogen-activated protein kinases, including c-Jun amino-terminal kinases, extracellular signal-regulated kinases (ERK), and p38 proteins [62].

The absorption of hemin into the cells was not significantly inhibited by the treatments with ZnPP or catalase at the concentrations used in this study (data not shown). Therefore, the inhibitory effects of ZnPP and catalase on the hemin-induced DNA damage, cell hyperproliferation, and IL-8 production were not due to inhibition of the absorption of hemin.

To summarize, our proposed mechanism for heme-induced colon carcinogenesis is shown in Fig. 8. Heme appears to induce the production of H_2O_2 by HO in colonic epithelial cells, and the produced H_2O_2 participates in DNA damage, cell proliferation, apoptosis, and the production of inflammatory cytokines. Heme is associated with both the initiation and promotion processes of carcinogenesis, and H_2O_2 might be a key metabolite catalyzed by HO for heme-induced colon cancer. The molecular mechanism indicates that people who consume diet rich in red meat are especially at risk of developing colon cancer.

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