Highlights

- Proton pump inhibitors increase the risk of developing iron deficiency
- Omeprazole enhances the expression of hepcidin via aryl hydrocarbon receptor activation in HepG2 cells
- Levels of hepcidin increase in the blood and liver of mice treated with omeprazole
- Levels of duodenal and splenic ferroportin decrease in mice treated with omeprazole
- Omeprazole suppresses iron absorption through hepcidin-ferroportin-dependent axis in addition to elevated gastric pH levels, causing iron deficiency

Proton pump inhibitors block iron absorption through direct regulation of hepcidin via the aryl hydrocarbon receptor-mediated pathway

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Abstract

Proton pump inhibitors (PPIs) have been used worldwide to treat gastrointestinal disorders. A recent study showed that long-term use of PPIs caused iron deficiency; however, it is unclear whether PPIs affect iron metabolism directly. We investigated the effect of PPIs on the peptide hepcidin, an important iron regulatory hormone. First, we used the FDA Adverse Event Reporting System database and analyzed the influence of PPIs. We found that PPIs, as well as H2 blockers, increased the odds ratio of iron-deficient anemia. Next, HepG2 cells were used to examine the action of PPIs and H2 blockers on hepcidin. PPIs augmented hepcidin expression, while H2 blockers did not. In fact, the PPI omeprazole increased hepcidin secretion, and omeprazole-induced hepcidin upregulation was inhibited by gene silencing or the pharmacological inhibition of the aryl hydrocarbon receptor. In mouse experiments, omeprazole also increased hepatic hepcidin mRNA expression and blood hepcidin levels. In mice treated with omeprazole, protein levels of duodenal and splenic ferroportin decreased. Taken together, PPIs directly affect iron metabolism by suppressing iron absorption through the inhibition of duodenal ferroportin via hepcidin upregulation. These findings provide a new insight into the molecular mechanism of PPI-induced iron deficiency.

Keywords: proton pump inhibitor; hepcidin; iron deficiency

Abbreviations: AhR, aryl hydrocarbon receptor; DMT1, divalent metal transporter 1; FAERS, FDA Adverse Event Reporting System; FPN, ferroportin; FTH, ferritin heavy chain; FTL, ferritin light chain; OME, omeprazole; PPIs, protein pump inhibitors; ROR, reporting odds ratio; TfR1, transferrin receptor 1

1. Introduction

Iron is an essential trace metal element in the body, and its deficiency causes anemia of microcytic and hypochromic corpuscles by interfering with hemoglobin synthesis. Almost all iron is recycled by the degradation of hemoglobin derived from old erythrocytes, however, it is also supplied by food. Iron has two forms, heme and non-heme. Whether non-heme iron is absorbed by the duodenum or the jejunum depends on a number of factors, including the presence of gastric acid (Zhang and Enns, 2009). The acidic environment of the stomach promotes iron absorption by reducing insoluble iron ($Fe3^{+}$) to soluble iron ($Fe2^{+}$) ions and permitting the formation of soluble chelates (Jacobs and Miles, 1969).

Worldwide, proton pump inhibitors (PPIs) and H2 blockers have been used for decades to treat gastric acid-related disorders (Scarpignato et al., 2006). Long-term use of PPIs or H2 blockers increases pH levels in the stomach by reducing the secretion of gastric acid, which decreases the digestion of proteins and the absorption of vitamins and minerals, including iron (Ito and Jensen, 2010). Several clinical studies have demonstrated that iron deficiency is induced in patients with long term use of PPIs and H2 blockers (Aymard et al., 1988; Lam et al., 2017). PPI- and H2 blocker-induced iron deficiency reduces iron absorption by altering the acidic environment of the gastrointestinal tract.

Iron is normally absorbed from the small intestine via the action of ferroportin (FPN), a cellular iron exporter. Absorbed iron is delivered into the cytosol

and mitochondria of erythroblast, where it is used for heme synthesis, including the synthesis of hemoglobin. Hemoglobin is synthesized through an eight-step enzymatic cascade, and ferrous iron (Fe^{2+}) is inserted into protoporphyrin IX to form a heme group at the last step (Severance and Hamza, 2009). Hepcidin, plays a crucial role in the regulation of systemic iron metabolism (Park et al., 2001), regulates the efflux of intracellular iron by inducing the internalization and degradation of FPN (Nemeth et al., 2004). Therefore, iron absorption by the small intestine decreases as hepcidin levels increase. In fact, transgenic mice with hepatic hepcidin overexpression have severe iron deficiency anemia due to FPN downregulation, which reduces iron absorption (Nicolas et al., 2002). In humans, hepcidin is the key mediator of inflammation associated anemia. (Ganz, 2003).

Here, we evaluated the effect of PPIs on iron absorption via hepcidin regulation.

2. Materials and Methods

2.1. Large-scale database analysis

We used the World Medical & Drug Information Service to define drug names and the Medical Dictionary for Regulatory Activities (MedDRA/J) version 18D to define “iron deficiency anaemia”. We analyzed data recorded from January 2007 to January 2017 (among a total of 6,992,882 reports) in the FDA Adverse Event

Reporting System (FAERS) database, using reporting odds ratio (ROR), a signal detection method. The reports were divided into the following four groups: (a) individuals who received PPIs and exhibited iron deficiency anemia, (b) individuals who received PPIs but did not exhibit iron deficiency anemia, (c) individuals who did not receive PPIs and exhibited iron deficiency anemia, and (d) individuals who did not receive PPIs and did not exhibit iron deficiency anemia. ROR was calculated for each group using the following equation: ROR = (a/b)/(c/d), 95% CI= exp [log (ROR) ± 1.96 √(1/a+1/b+1/c+1/d)]. We assumed that there was a signal when the calculated lower limit value of the 95% confidence interval of ROR was > 1 (van Puijenbroek et al., 2002).

2.2 Chemicals and reagents

Omeprazole (OME) was purchased from the Fujifilm Wako Pure Chemical Corporation, (Osaka, Japan); lansoprazole, rabeprazole, pantoprazole, and famotidine were purchased from the Tokyo Chemical Industry (Tokyo, Japan); and CH-223191, an aryl hydrocarbon receptor (AhR) inhibitor, was obtained from Sigma-Aldrich (St. Louis, MO, USA). The following commercially available antibodies were used in this study: anti-NRAMP2/divalent metal transporter 1 (DMT1), anti-ferritin heavy chain (FTH), anti-ferritin light chain (FTL), and anti-AhR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-transferrin receptor 1 (TfR1) antibody (Zymed Technologies; Carlsbad, CA, USA); anti-FPN antibody (Alpha

Diagnostics; San Antonio, TX, USA); anti-α-tubulin (Merck KGaA, Darmstadt, Germany) was used as protein loading control; and anti-histone H3 antibody (Abcam, Cambridge, UK) was used as a loading control for nuclear proteins.

2.3. Cell culture

HepG2, a human hepatoma cell line, was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). The methods of cell culture have been described previously (Hamano et al., 2017). In brief, when the cells reached sub-confluency, the cells were placed in serum-free media overnight. Subsequently, the cells were treated with PPIs and an H2 blocker for 24 hours. In another experiment, cells were pre-treated for 1 hour with CH-223191 (AhR inhibitor) before stimulation with OME.

2.4. Small interfering RNA experiments

Small interfering RNA (siRNA) targeting human AhR, and a non-targeting siRNA control sequence, were obtained from Sigma Aldrich (Mission siRNA; Tokyo, Japan), and used as previously described (Hamano et al., 2017).

2.5. Experimental animals and treatment

All experimental animal procedures were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University Graduate School (Permit Number: T30-125). Eight-week-old male C57BL6/J mice were purchased from Nippon CLEA (Tokyo, Japan). The mice were maintained under conventional conditions, with a regular 12-hour light/dark cycle. They were given free access to food (Type NMF; Oriental Yeast, Tokyo, Japan) and water during the study. OME (20 mg/kg/day) was orally administered to mice (Hess et al., 2015; Wang et al., 2015) for 1 or 2 weeks. OME was suspended in 0.5 % carboxymethylcellulose. Control mice received orally-administered carboxymethylcellulose alone. The mice were euthanized by intraperitoneal over-dose injection of anesthetic. Tissues and blood samples were collected and stored at -80 °C until use.

2.6. Measurement of plasma iron levels, plasma ferritin levels, tissue iron concentration, and peripheral blood

Serum iron levels and tissue iron concentrations were measured using an iron assay kit (Metallo Assay) according to the manufacturer's instructions (Metallogenics Co. Ltd., Chiba, Japan), and plasma ferritin levels were determined using a Mouse Ferritin ELISA Kit (Immunology Consultants Laboratory; Newberg, OR, USA) according to the manufacturer's instructions (Hamano et al., 2017). Complete blood count was evaluated using Microsemi LC-662 (HORIBA, Ltd., Kyoto, Japan).

2.7. RNA extraction and evaluation of mRNA expression levels

The methods of RNA extraction, cDNA synthesis, and quantitative RT-PCR have been described previously (Ikeda et al., 2016). The primer sets used were as follows: 5′-CTGCCTGTCTCCTGCTTCTC-3′ and 5′-AGATGCAGATGGGGAAGTTG-3′ for mouse *hepcidin-1*, and 5′-GCTCCAAGCAGATGCAGCA-3′ and 5′-CCGGATGTGAGGCAGCAG-3′ for *36B4* (an internal control). The expression levels of all target genes were normalized to *36B4*. Values were compared to the control group, and expressed as relative fold changes.

2.8. Protein extraction and western blot analysis

The methods of protein preparation and western blotting have been previously described (Ikeda et al., 2016). In brief, prepared protein samples were separated using SDS-PAGE and transferred onto a PVDF membrane. A chemiluminescent reagent was used to detect immunoreactive bands. Immunoblot bands were visualized by exposure to X-ray film or by a C-DiGit chemiluminescent scanner (LI-COR C-DiGit Blot Scanner, Lincoln, Nebraska, USA). Densitometry of the visualized bands was quantified using Image J 1.38x software (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.9. Measurement of hepcidin concentrations

Hepcidin concentrations in mouse plasma and in cell culture media were measured using surface-enhanced laser desorption ionization time of flight mass spectrometry as described previously (Tomosugi et al., 2006). The assays were performed by the Medical Care Proteomics Biotechnology Co. Ltd. (Kanazawa, Japan).

2.10. Statistical analysis

Data are presented as the mean ± standard deviation (SD). An unpaired, 2-tailed, Student's *t*-test was used to compare two groups. To compare among more than two groups, the statistical significance of each difference was evaluated using a post-hoc test (either Dunnett's method or Tukey-Kramer's method). P values < 0.05 indicated statistical significance.

3. Results

3.1. Analysis of the effects of PPIs and H2 blocker on iron deficiency anemia using the FAERS database

A larger analysis to evaluate the influence of PPIs in iron deficiency anemia using data from many hospitals was conducted using the FAERS database (Table 1). Treatment with PPIs was positively associated with the incidence of iron deficiency

anemia. Increasing RORs were observed in combination therapy that included OME (ROR = 3.90, 95% CI = 3.43–4.43), lansoprazole (ROR = 5.02, 95% CI = 4.27–5.89), rabeprazole (ROR = 7.29, 95% CI = 5.77–9.21), and pantoprazole (ROR = 4.75, 95% CI = 4.15–5.45). Similarly, the increase was observed for famotidine (ROR = 5.06, 95% CI = 4.01–6.38). Consistent with a previous report (Lam et al., 2017), PPIs, as well as the H2 blocker, were significantly associated with the incidence of iron deficiency anemia by database analysis.

3.2. Effect of PPIs and H2 blocker on hepcidin mRNA and secretion in HepG2 cells

We checked the action of PPIs and the H2 blocker on hepcidin expression in HepG2 cells. All PPIs, including OME, lansoprazole, rabeprazole, and pantoprazole enhanced hepcidin mRNA expression, but the H2 blocker, famotidine, did not (Fig. 1A). In addition, secreted hepcidin-25 protein levels also increased in the culture medium with OME treatment (Fig. 1B).

3.3. Effect of OME on hepcidin expression via an AhR-mediated pathway

We previously reported that uremic toxin indoxyl sulfate-induced hepcidin upregulation is mediated through activation of the AhR (Diaz et al., 1990), and OME can activate AhR in human and rat hepatocytes (Diaz et al., 1990; Kashfi et al., 1995).

Consistent with these data, we found that OME promoted translocation of AhR from the cytosol to the nucleus in HepG2 cells (Fig. 1C). To examine whether OME-induced hepcidin upregulation was mediated through AhR, we used AhR-specific siRNA and the AhR inhibitor CH-223191. Both AhR silencing and CH-223191 reduced OME-induced hepcidin expression (Fig. 1D and E). These findings suggest that OME induces hepcidin upregulation via an AhR-mediated pathway.

3.4. Changes of hepatic hepcidin mRNA and plasma hepcidin levels in OME-treated mice

To assess the effect of OME on iron metabolism *in vivo*, we administered OME orally to mice. Similar to the findings in HepG2 cells, hepatic hepcidin expression significantly increased in mice after both 1 and 2 weeks of OME treatment (Fig. 2A). Additionally, plasma hepcidin concentration increased after 1 week of OME treatment (Fig. 2B).

3.5. Changes in ferroportin expression in OME-treated mice

As expected, FPN expression decreased in the duodenum and the spleen of mice treated with OME (Fig. 2C and D); however, 1 week of OME treatment did not change TfR1 and DMT1 expression in the duodenum and spleen (Fig. 3). These

findings suggest that in mice treated with OME, reduced FPN expression is due to increased hepcidin production.

3.6. Alteration of tissue and plasma iron content by OME administration

We examined the iron content of the liver and iron concentration in the plasma of mice treated with OME. As shown in Table 2, OME administration for 1 or 2 weeks did not change the iron content of the liver. Meanwhile, the incidence of anemia increased with reduced plasma iron levels. There were no differences in the protein expression of liver and spleen ferritin (Fig. 4), or in plasma ferritin levels at 2 weeks when comparing vehicle-treated and OME-treated mice (OME 399.9 ± 48.1 ng/mL, vehicle 389.8 ± 51.3 ng/mL).

4. Discussion

Using the FAERS database, we demonstrated that there is an increased risk of iron deficiency anemia in patients treated with PPIs or an H2 blocker. *In vitro*, we found that PPIs, but not the H2 blocker, upregulated hepcidin through the AhR pathway. Mice treated with OME had increased expression of hepatic hepcidin mRNA and higher plasma hepcidin levels, leading to a decrease in FPN expression in the duodenum and spleen. These findings indicate that PPI-induced iron deficiency involves a hepcidin-FPN dependent pathway in addition to elevated gastric pH levels.

The FAERS is a database of self-reported drug-related adverse events from multiple treatment centers that reflects the realities of clinical practice. Patients treated with PPIs or an H2 blocker had a significantly higher rate of iron deficiency anemia compared to patients who had not taken PPIs or an H2 blocker. These data are consistent with (Lam et al., 2017) who found that patients who had taken PPIs long-term (i.e. for more than two years) were at increased risk of iron deficiency. This case-control study also showed that the risk of iron deficiency was higher in the patients using PPIs (adjusted odds ratio, 2.49) than in patients using H2 blockers (odds ratio, 1.58) (Lam et al., 2017). The subsequent risk for iron deficiency due to PPI use may be attributed to elevation of pH levels in the stomach and other factors.

Hepcidin is an important iron regulator that controls cellular iron efflux via FPN (Nemeth et al., 2004). We found that hepcidin mRNA expression increased with

PPI treatment; however, famotidine, a H2 blocker, did not augment hepcidin expression in HepG2 cells. Hepcidin expression is regulated by many factors including iron, anemia, and inflammation (Ganz, 2011). In addition, indoxyl sulfate regulates hepcidin expression through an AhR-mediated pathway (Hamano et al., 2017), suggesting the involvement of AhR in hepcidin regulation. Previous studies have shown that OME activates AhR in hepatocytes (Diaz et al., 1990; Kashfi et al., 1995). Consistent with these data, we confirmed that OME promoted AhR translocation from the cytosol to the nucleus, suggesting that OME may be an activating ligand for AhR. Silencing or inhibiting AhR suppressed OME-induced hepcidin upregulation. Taken together, these data indicate that OME upregulates hepcidin through an AhR-mediated pathway.

Similar to the effect of PPIs on hepcidin *in vitro*, mice treated with OME for 1 week showed increased hepatic hepcidin mRNA expression and increased plasma hepcidin levels. The mice also displayed reduced FPN expression in the duodenum and spleen. Moreover, the increase in hepatic hepcidin mRNA lasted for 2 weeks, and the mice developed anemia, suggesting that iron absorption and utilization were impaired. Therefore, in addition to increasing gastrointestinal pH levels, PPIs may also inhibit iron absorption but regulating the hepcidin-FPN pathway. We did not find a decrease in hepatic iron content or ferritin expression, therefore, we propose that OME-induced hepcidin upregulation by was not due to iron accumulation. Further studies are

necessary to further clarify the effect of PPIs on hepcidin, and on the induction of iron deficiency anemia.

In conclusion, PPIs upregulate hepcidin expression by activating AhR. Increased hepcidin production by PPIs leads to a reduction in FPN expression, which inhibits iron absorption and utilization, and promotes the development of iron deficiency anemia. The effect of PPIs on iron metabolism suggests that the risk of iron deficiency anemia should be carefully monitorized in patients who receive long-term PPI treatment. Although future clinical study is needed, our study would caution healthcare providers to consider PPIs' potential effects on body iron dysmetabolism.

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Figure legends

Fig. 1. (A) Effect of proton pump inhibitors and a histamine H2-receptor antagonist on hepcidin mRNA expression in HepG2 cells. Cells were treated with 200 μM omeprazole (OME), 50 μM lansoprazole, 100 μM pantoprazole, 12.5 μM rabeprazole, 200 μM famotidine, or vehicle. Values are expressed as the mean ± SD. *P < 0.05, **P < 0.01; n = 5–10 in each group. (B) Concentration of hepcidin, secreted by HepG2 cells, in the culture media. Cells were treated with either 200 μM OME or vehicle. Values are expressed as the mean ± SD. *P < 0.05 (vs. vehicle treatment); n = 3 in each group. (C) OME action on AhR translocation from the cytoplasm to the nucleus of HepG2 cells. Cells were treated with either 200 μM OME or vehicle. Values are expressed as the mean ± SD. *P < 0.05 (vs. vehicle treatment); n = 3 in each group. (D) Treatment with AhR siRNA inhibits OME-induced hepcidin upregulation in HepG2 cells. Forty-eight hours after siRNA transfection, cells were treated with either 200 μM OME or vehicle. Values are expressed as the mean ± SD. **P < 0.01; n = 6–9 in each group. (E) Treatment with CH-223191 inhibits OME-induced hepcidin upregulation in

HepG2 cells. Cells were treated with either 200 μM OME or vehicle 1 hour after 10 μM CH-223191 pretreatment. Values are expressed as the mean ± SD. **P < 0.01; n = 5–10 in each group.

Fig. 2. (A) Hepcidin mRNA expression in the liver of mice treated for 1 and 2 weeks with vehicle or OME. Values are expressed as the mean ± SD. **P < 0.01 (vs. vehicle treatment); n = 9–13 in each group. (B) Plasma hepcidin concentration. Values are expressed as the mean ± SD. *P < 0.05 (vs. vehicle treatment); n = 3–6 in each group. Effects of 1 and 2 weeks of treatment with vehicle or OME on the expression of FPN and tubulin in murine (C) duodenum and (D) spleen. Upper panels: Representative immunoblots. Lower panels: Semi-quantitative densitometric analyses of FPN protein levels normalized to tubulin. Values are expressed as the mean ± SD. *P < 0.05; n = 6–16 in each group.

Fig. 3. Effects of 1 week of treatment with vehicle or OME on protein expression in murine duodenum and spleen. Upper panels: Representative immunoblots for tubulin, (A) duodenal DMT1, (B) splenic DMT1, and (C) splenic TfR1. Lower panels: Semi-quantitative densitometric analyses of DMT1 and TfR1 protein levels normalized to tubulin. Values are expressed as means ± SD. n = 7–9 in each group.

Fig. 4. (A, B) Effects of 1 week of treatment with vehicle or OME on protein expression in murine liver. Upper panels: Representative immunoblots for tubulin, (A) FTH, and (B) FTL. Lower panels: Semi-quantitative densitometric analyses of FTH

and FTL protein levels normalized to tubulin. Values are expressed as the mean ± SD. *P < 0.05; n = 4–6 in each group. (C, D) Effects of 1 week of treatment with vehicle or OME on protein expression in murine spleen. Upper panels: Representative immunoblots for tubulin, (C) FTH, and (D) FTL. Lower panels: Semi-quantitative densitometric analyses of FTH and FTL protein levels normalized to tubulin. Values are expressed as the mean ± SD.n = 4–9 in each group.

Table

Table 1. Number of reported cases and reporting odds ratio of iron deficiency anemia in patients who took proton pump inhibitors and an H2 blocker in FAERS analysis

Drug name	Iron deficiency anemia without drug	Iron deficiency anemia with drug	Reporting odds ratio (95% CI)
Omeprazole	3275/6856188	254/136695	3.90 (3.43–4.43)
Lansoprazole	3372/6925076	157/64277	5.02 (4.27–5.89)
Rabeprazole	3457/6972908	72/19975	7.29 (5.77–9.21)
Pantoprazole	3309/6892944	220/96409	4.75 (4.15–5.45)
Famotidine	3456/6960307	73/29046	5.06 (4.01–6.38)

Table 2. Body weight, liver iron content, plasma iron levels and hematological data in mice treated with vehicle or omeprazole

	1 wk		2 wk	
	Vehicle	**OME**	**Vehicle**	**OME**
Body weight (g)	23.2 ± 0.2	22.9 ± 0.8	23.9 ± 0.9	23.5 ± 0.5
Liver iron (*μ*g/g protein)	125.2 ± 32.0	117.9 ± 20.7	107.2 ± 44.5	120.1 ± 47.4
Plasma iron (*μ*g/dL)	114.6 ± 21.1	80.8 ± 31.7*	115.7 ± 31.4	78.6 ± 15.3*
RBC (× 10^4/*μ*l)	821 ± 31	773 ± 27	867 ± 51	777 ± 18*
Hb (g/dL)	12.0 ± 0.2	11.1 ± 0.3**	12.8 ± 0.7	11.5 ± 0.4*
Ht (%)	37.2 ± 1.5	35.0 ± 1.2	39.1 ± 2.4	35.1 ± 0.9*

Data are means ± SD; *n* = 4-10, respectively. *P < 0.05, **P < 0.01 vs. vehicle mice at the same week.

RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit

Figure 1
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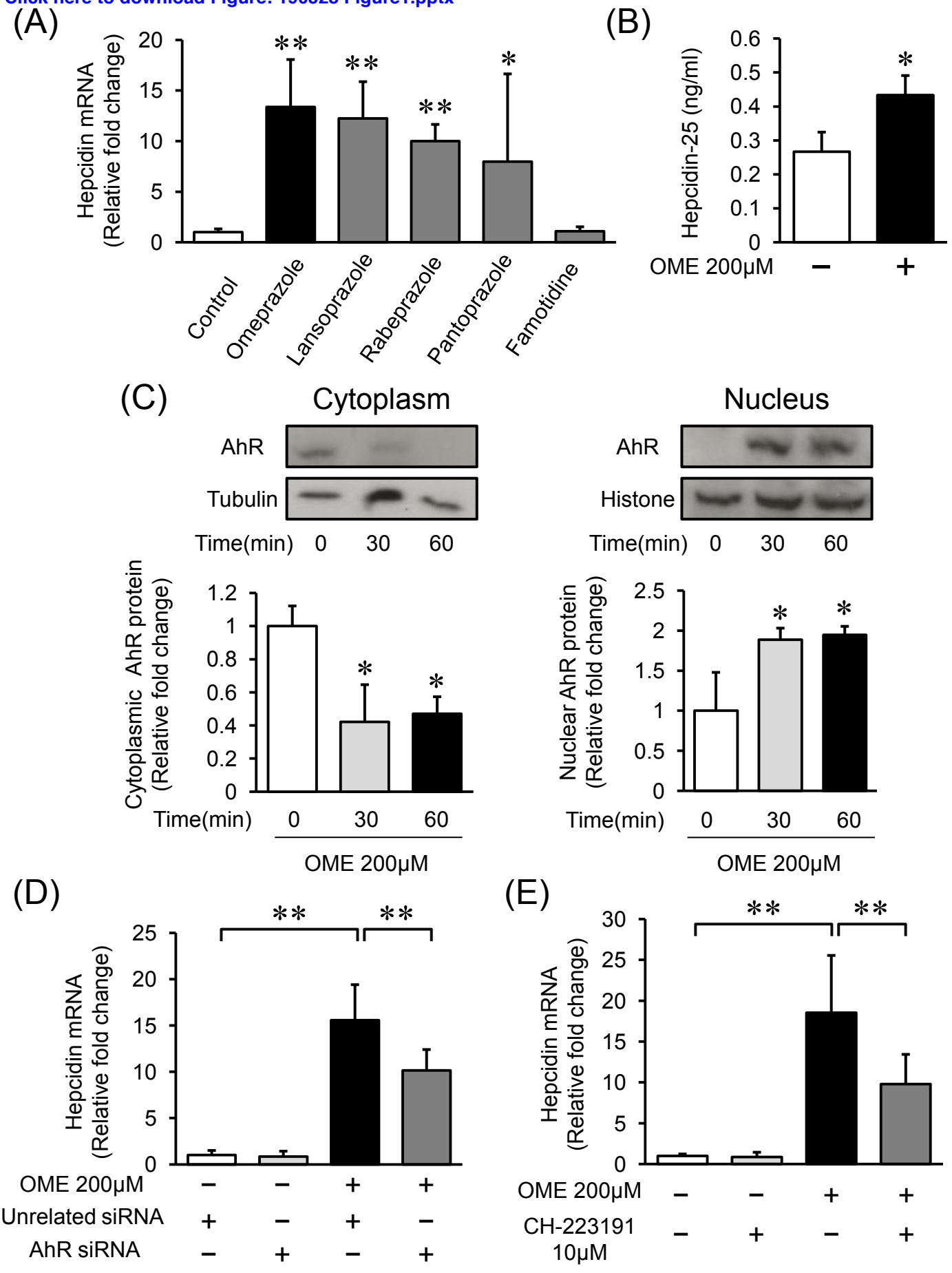

Figure 2 3 4
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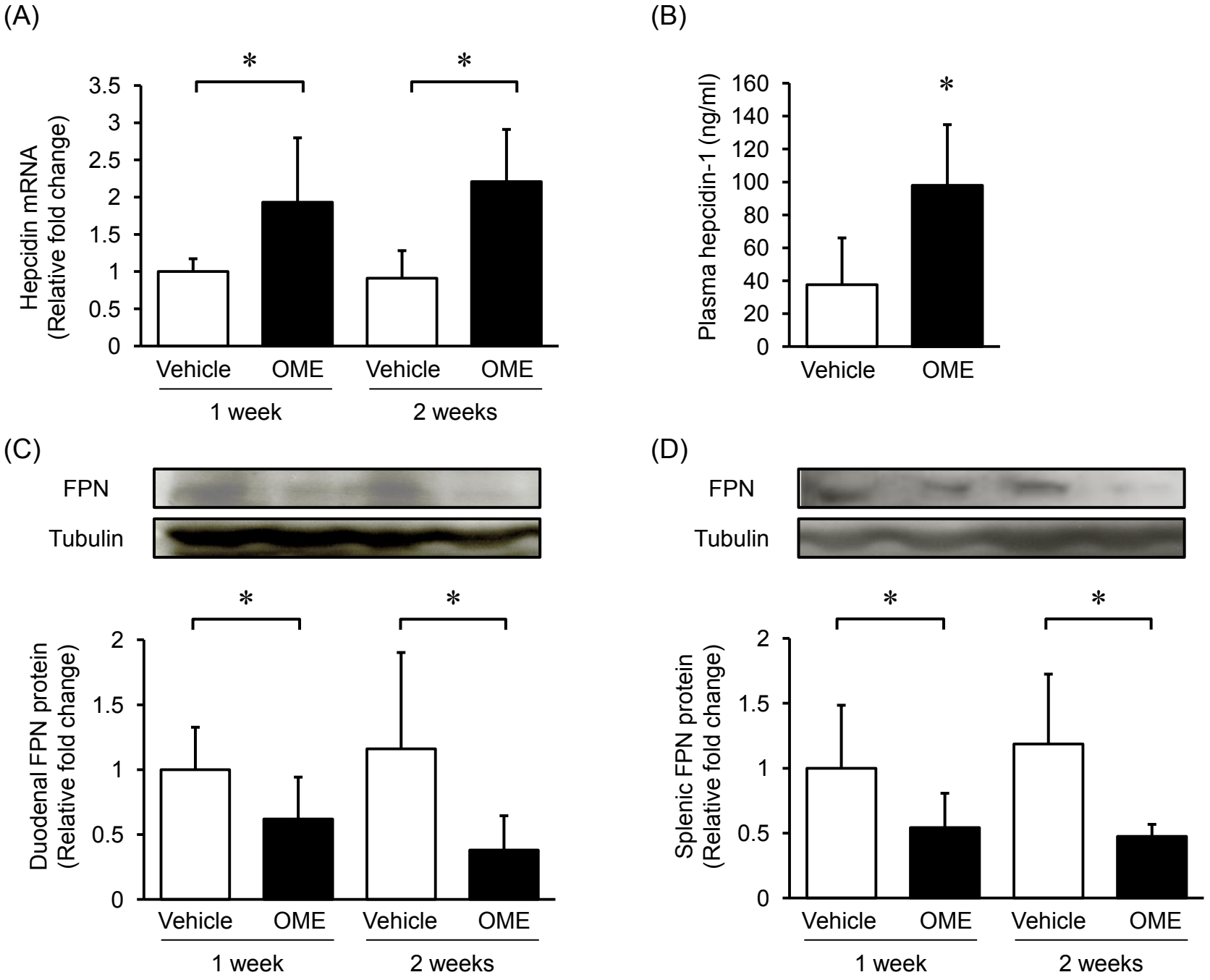

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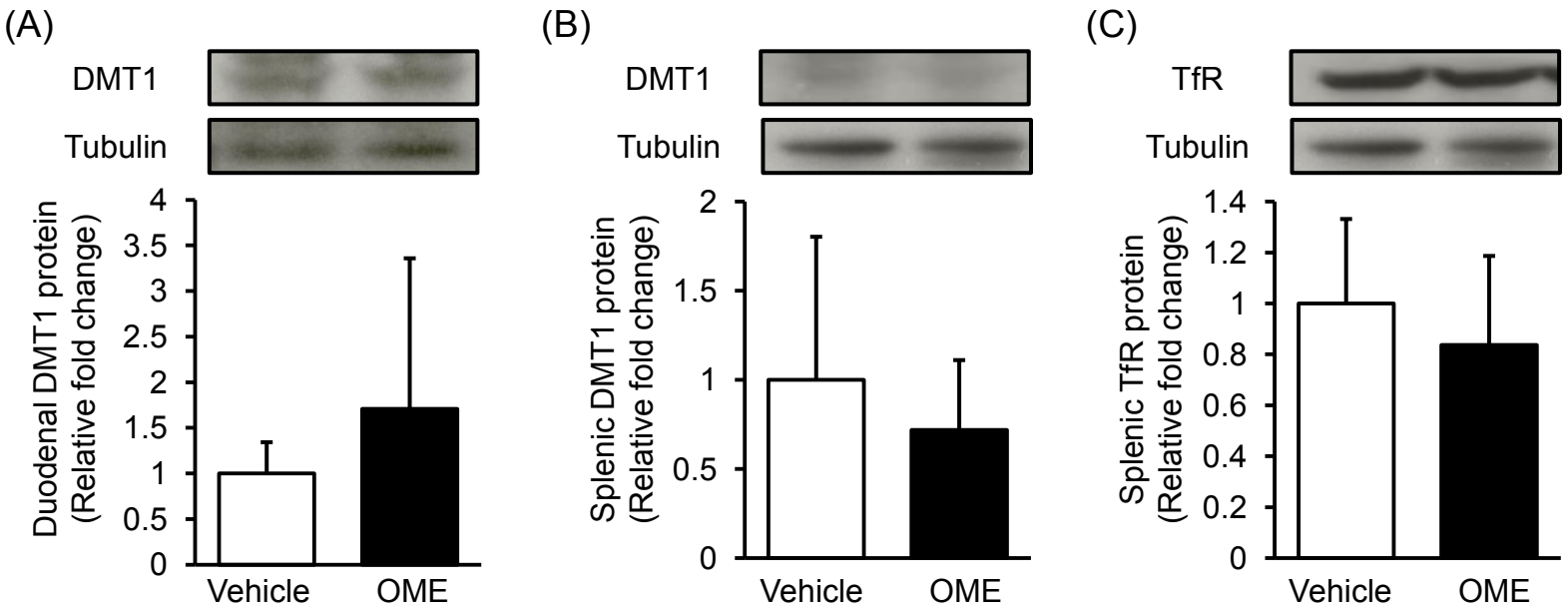

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