Aggravation by Clopidogrel, an Antiplatelet Drug, of Antral Lesions Induced by NSAIDs in Rats

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Abstract

Background/Aim: Recent studies suggested a risk of gastric adverse reaction on the concomitant use of antiplatelet drugs with NSAIDs. The present study was performed to examine the adverse effects of anti-platelet drug clopidogrel, a P2Y12 receptor antagonist, on gastric antral lesions induced by conventional NSAIDs in refed rats.

Methods: Rats fasted for 24 h were refed for 1 h, and subsequently administered NSAIDs such as indomethacin (30 mg/kg), loxoprofen (100 mg/kg), or flurbiprofen (30 mg/kg) s.c. 1 h after refeeding. The animals were killed 6 h after NSAID treatment, and the stomachs were examined for non-hemorrhagic and hemorrhagic lesions, separately. Clopidogrel (30 mg/kg) was administered p.o. 48, 24, and 0.5 h before NSAID treatment. Antisecretory drugs (atropine, omeprazole or famotidine) and mucosal protective drugs (rebamipide or teprenone) were given p.o. 1 h before indomethacin.

Results: NSAIDs used alone produced non-hemorrhagic lesions in the antrum of refed rats. Clopidogrel, despite causing no damage by itself, aggravated the severity of antral lesions in response to NSAIDs, with an increase of MPO activity; the lesions induced by NSAIDs alone were mostly non-hemorrhagic, while they became hemorrhagic by the co-administration with clopidogrel. The aggravation by clopidogrel of indomethacin-induced antral lesions was significantly prevented by antisecretory drugs or mucosal protective drugs, with the concomitant suppression of MPO activity.

Conclusion: Clopidogrel, an antiplatelet drug, aggravates the severity of NSAID-induced antral lesions in refed rats, especially converting from non-hemorrhagic damages into hemorrhagic ones. Both antisecretory and mucosal protective drugs are useful for preventing the development of antral lesions induced by NSAIDs plus clopidogrel.

INTRODUCTION

Antiplatelet therapy has been shown to be effective in reducing the incidence of cerebrovascular events, myocardial infarction, and death from vascular causes in individuals with symptomatic atherothrombic diseases [1]. However, recent studies show a risk of adverse gastric reactions in the upper gastrointestinal (GI) tract in patients taking multiple antiplatelet drugs and a markedly increased risk of bleeding on the concomitant use of antiplatelet drugs with nonsteroidal anti-inflammatory drugs (NSAIDs) or low-dose aspirin [2-5]. Antisecretory drugs such as proton pump inhibitors (PPIs) or histamine H2 receptor antagonists (H2RAs) are effective in preventing the upper GI bleeding induced by the concomitant use of antiplatelet drugs with NSAIDs [6,7]. However, there has been no direct evidence of the effectiveness of PPIs and H2RAs on GI bleeding caused by the co-administration of NSAIDs and clopidogrel in animal models.

Clopidogrel, one of antiplatelet drugs, is a prodrug, and its actions may be related to an adenosine diphosphate (ADP)
receptor on platelet cell membranes [8]. The drug specifically and irreversibly inhibits the P2Y$_{12}$ subtype of the ADP receptor, which is important to the aggregation of platelets and cross-linking by the protein fibrin. The blockade of this receptor inhibits platelet aggregation by blocking activation of the glycoprotein IIb/IIa pathway. Recently, we found using a rat model that gastric lesions induced by acidified ASA were markedly aggravated by pretreatment of the animals with clopidogrel [9,10]. It remains, however, unknown whether this drug affects the gastric ulcerogenic response induced by other conventional NSAIDs such as indomethacin. In general, NSAID-induced gastric ulcers are known to predominantly occur in the antrum of the stomach [11,12]. Since Satoh et al., [13,14] reported the development of antral ulcers by indomethacin in re-fed rats, this model may be suitable for evaluating the influences of antiplatelet drugs on NSAID-induced gastric ulcers. By the way, the gastric ulcerogenic effects of NSAIDs are known to require inhibition of both cyclooxygenase (COX)-1 and COX-2 [15,16]. However, it remains unexplored whether NSAID-induced antral lesions can be reproduced by the combined administration of selective COX-1 and COX-2 inhibitors.

In the present study, we examined the effects of clopidogrel on the antral mucosa in rat stomachs when administered concomitantly with conventional NSAIDs (indomethacin, loxoprofen or flurbiprofen) as well as selective COX-1 inhibitor (SC-560) and/or selective COX-2 inhibitor (rofecoxib), and investigated whether antral lesions induced by NSAIDs or COX-1/COX-2 inhibitors are aggravated by the co-administration of clopidogrel. In addition, we also examined the effects of various antiulcer drugs, such as antisecretory and mucosal protective drugs, on the antral lesions produced by the co-administration of indomethacin and clopidogrel.

**MATERIAL AND METHODS**

**Animals**

Male Sprague-Dawley rats (220-260 g; Nippon Charles River, Shizuoka, Japan) were acclimatized to standard laboratory conditions (12:12-h light–dark cycle, temperature 22 ± 1°C). Experiments were carried out using 4–6 rats per group under unanesthetized conditions, unless otherwise specified. All experimental procedures involving animals were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

**Induction of antral lesions**

For the induction of antral lesions, the animals were first deprived of food for 24 h, and re-fed for 1 h, then given conventional NSAIDs such as indomethacin (10 and 30 mg/kg), loxoprofen (30 and 100 mg/kg), and flurbiprofen (30 mg/kg) s.c. 1 h after the re-feeding, and killed 6 h later (Figure 1). In some cases, the ulcerogenic effects of SC-560 (a selective COX-1 inhibitor: 10 mg/kg) and rofecoxib (a selective COX-2 inhibitor: 10 mg/kg) in the antral mucosa were also examined; they were administered p.o., either alone or in combination, in place of NSAIDs, 1 h after the re-feeding, and killed 6 h later. Clopidogrel (3-30 mg/kg) was administered p.o. three times such as 48 h, 24 h and 0.5 h before the administration of NSAIDs, or SC-560 and/or rofecoxib. In addition, the effects of various antiulcer drugs on the antral lesions produced by indomethacin plus clopidogrel (30 mg/kg) were also examined; antisecretory drugs such as atropine (an anticholinergic agent: 3 mg/kg), omeprazole (a PPI: 30 mg/kg), and famotidine (a H$_2$RA: 1-10 mg/kg), and mucosal protective drugs such as rebamipide (1-10 mg/kg), and teprenone (30-300 mg/kg). These drugs were administered p.o. 30 min before the administration of indomethacin. The doses of these antiulcer drugs were selected in order to induce the respective pharmacological actions according to the findings of previous studies [9,10,16].

**Macroscopic evaluation of gastric lesions**

Animals with various treatments were killed for examination of the gastric mucosa by deep ether anesthesia 6 h after the treatment with indomethacin, loxoprofen, flurbiprofen, or SC-560 and/or rofecoxib. The stomach was excised, treated with 2% formalin for fixation of the tissue walls, and then opened along the greater curvature or the anti-mesenteric attachment, respectively, and the mucosa was examined for damage under a dissecting microscope (x10). The area (mm$^2$) of macroscopically visible lesions was measured separately for hemorrhagic, and non-hemorrhagic damage, summed for each tissue, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals.

**Measurement of myeloperoxidase activity**

Myeloperoxidase (MPO) activity in the antral mucosa was measured as described by Krawisz et al., [17] with some modifications. The animals were killed 6 h after the administration of indomethacin (30 mg/kg, s.c.) with or without clopidogrel treatment (30 mg/kg, p.o.) given 48, 24 and 0.5 h before. In some cases, atropine (3 mg/kg), omeprazole (30 mg/kg), famotidine (10 mg/kg), teprenone (300 mg/kg), and rebamipide (10 mg/kg) was administered p.o. 30 min before the administration of indomethacin. All blood was withdrawn from the heart by perfusing with saline, and the stomach was excised and opened along the greater curvature. After the tissue was rinsed with cold

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**Figure 1** Experimental time schedule for induction of antral lesions in rats by NSAIDs or selective COX-1/COX-2 inhibitors, with or without pretreatment of clopidogrel. The animals were first deprived of food for 24 h, and re-fed for 1 h, then given conventional NSAIDs (indomethacin, loxoprofen or flurbiprofen) s.c. or selective COX-1 inhibitor (SC-560) and/or selective COX-2 inhibitor (rofecoxib) p.o., and killed 6 h later. In some cases, clopidogrel was administered p.o. three times, i.e., 48 h, 24 h and 0.5 h, before the administration of NSAIDs, or SC-560 and/or rofecoxib. Antiulcer drugs were administered p.o. 1 h before the administration of NSAIDs.
saline, the antral mucosa was scraped with glass slides, weighed, and homogenized in a 50 mmol phosphate buffer containing 0.5% hexadecyl-trimethyl-ammonium bromide (Sigma Chemicals, St. Louis, MO) and centrifuged at 2,000 rpm for 10 min at 4°C. MPO activity in the supernatant was determined using o-dianisidine dihydro-chloride (Sigma-Aldrich). The changes in absorbance at 450 nm were recorded on a microplate reader (VERSA max; Molecular Device, Sunnyvale, CA). Sample protein content was estimated by spectrophotometric assay (Pierce Protein Assay Kit, IL). The MPO activity was obtained from the slope of the reaction curve, based on the following equation: Specific activity (µmol H₂O₂/min/mg protein) = (OD/min)/OD/µmol H₂O₂ × mg protein).

Preparation of drugs

The drugs used were indomethacin, loxoprofen, flurbiprofen, atropine (Sigma Chemicals, St. Louis, MO), SC-560 (Cayman Chemicals, Ann Arbor, MI), rofecoxib (synthesized in our laboratory), clopidogrel (Sanofi-Aventis, Tokyo, Japan), teprenone (Eisai, Tokyo, Japan), rebamipide (Otsuka, Tokyo, Japan), famotidine (Nacalai Tesque, Kyoto, Japan), and omeprazole (Astra Zeneca, Mönndal, Sweden). All NSAIDs and COX inhibitors were suspended in a hydroxypropylcellulose solution (Wako). Omeprazole was suspended in a 0.5% carboxymethylcellulose solution. Other agents were dissolved in saline. All drugs were prepared immediately before use and administered s.c. or p.o. in a volume of 0.5 ml/100 g body weight. Control animals received the vehicle alone.

Statistics

Data are presented as the mean±SE for 7 to 9 rats per group. Statistical analyses were performed using a two-tailed unpaired t-test and Dunnett’s multiple comparison test, and values of P<0.05 were regarded as significant.

RESULTS

Gastric Ulcerogenic Effects of NSAIDs in Re-fed Rats

Subcutaneously administered Indomethacin (10 and 30 mg/kg) dose-dependently produced lesions, mostly non-hemorrhagic damage, in the antrum within 6 h in the re-fed rats, without damage in the corpus of the stomach (Figure 2). Loxoprofen at 30 mg/kg did not cause damage in the stomach but at 100 mg/kg produced non-hemorrhagic lesions only in the antrum, similar to indomethacin. Likewise, another conventional NSAID, flurbiprofen at 30 mg/kg also caused damage, mostly non-hemorrhagic lesions, only in the antrum but not corpus of the stomach.

Effect of Clopidogrel on Antral Lesions Caused by NSAIDs

Pretreatment of the animals with clopidogrel (3-30 mg/kg, p.o.) dose-dependently aggravated the antral lesions caused by indomethacin, the lesion score at 10 mg/kg and 30 mg/kg being significantly greater than the control value (Figure 3). Notably, indomethacin alone produced non-hemorrhagic lesions, but additional pretreatment with clopidogrel converted non-hemorrhagic damage into hemorrhagic lesions in the mucosa (Figure 4). Clopidogrel alone did not cause any injury in the stomach (data not shown). Other NSAID such as loxoprofen (100 mg/kg) or flurbiprofen (30 mg/kg) also produced antral lesions in the re-fed rats. Pretreatment with clopidogrel significantly aggravated the antral damage in response to loxoprofen or flurbiprofen, resulting in deep, hemorrhagic lesions (Figure 5).

Effect of Clopidogrel on Antral Lesions Caused by Selective COX-1 and/or COX-2 Inhibitors

The gastric ulcerogenic effects of NSAIDs require the inhibition of both COX-1 and COX-2 [15,16]. However, it remains unknown whether NSAID-induced antral lesions are reproduced by the combined administration of selective COX-1 and COX-2 inhibitors. We examined the ulcerogenic effects of selective COX-1/COX-2 inhibitors in the antrum in the absence or presence of clopidogrel pretreatment.
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The occurrence of antral lesions in refed rats was produced by the combined p.o. administration of a selective COX-1 inhibitor, SC-560 (10 mg/kg), and a selective COX-2 inhibitor, rofecoxib (10 mg/kg), although neither of these agents alone damaged the antrum (Figure 6). In addition, clopidogrel did not damage the antral mucosa when given together with rofecoxib, but did provoke few lesions when given together with SC-560, although the damage was mostly non-hemorrhagic. However, the concurrent administration of clopidogrel together with SC-560 plus rofecoxib produced severe hemorrhagic lesions in the antrum, the lesion score being equivalent to that produced by indomethacin plus clopidogrel (see Figure 3).

Effect of Various Agents on Antral Lesions Produced by Indomethacin Plus Clopidogrel

Antisecretory drugs: An anticholinergic agent, atropine (3 mg/kg, p.o.), and a PPI, omeprazole (30 mg/kg, p.o.), showed a significant reduction in the severity of antral damage induced by indomethacin (30 mg/kg, s.c.) alone (data not shown), and potently inhibited the aggravation by clopidogrel (30 mg/kg, p.o.) of these lesions; both hemorrhagic and total lesions were

![Figure 4 Gross appearance of antral lesions induced by indomethacin in refed rats, in the absence or presence of clopidogrel. Animals fasted for 24 h were refed for 1 h, and subsequently administered indomethacin (30 mg/kg, s.c.) 1 h after refeeding, and killed 6 h later. Clopidogrel (30 mg/kg, p.o.) was administered 48, 24 and 0.5 h before indomethacin. Figures show; A and C (high magnification): indomethacin (30 mg/kg) alone; B and D (high magnification): indomethacin plus clopidogrel (30 mg/kg) Arrows indicate lesions.

![Figure 5 Effects of clopidogrel on antral lesions induced by loxoprofen or flurbiprofen in refed rats. The animals fasted for 24 h were refed for 1 h, and subsequently administered loxoprofen (100 mg/kg, s.c.) or flurbiprofen (30 mg/kg, s.c.) 1 h after refeeding, and killed 6 h later. Clopidogrel (3-30 mg/kg, p.o.) was administered 48, 24 and 0.5 h before loxoprofen or flurbiprofen. Data are presented as the mean ± SE from 4-6 rats. *Significant difference from vehicle at p<0.05.

![Figure 6 Effects of clopidogrel on antral lesions induced by SC-560 and rofecoxib, either alone or in combination, in refed rats. The animals fasted for 24 h were refed for 1 h, and subsequently administered SC-560 (10 mg/kg, p.o.) and rofecoxib (10 mg/kg, p.o.), either alone or in combination, 1 h after refeeding, and killed 6 h later. Clopidogrel (30 mg/kg, p.o.) was administered 48, 24 and 0.5 h before SC-560 or rofecoxib or both. Data are presented as the mean ± SE from 4-6 rats. *Significant difference from vehicle at p<0.05.

![Figure 7 Effects of antisecretory drugs on antral lesions induced by indomethacin plus clopidogrel in refed rats. The animals fasted for 24 h were refed for 1 h, and subsequently administered indomethacin (30 mg/kg, s.c.) 1 h after refeeding, and killed 6 h later. Clopidogrel (30 mg/kg, p.o.) was administered 48, 24 and 0.5 h before indomethacin. Atropine (3 mg/kg), omeprazole (30 mg/kg) or famotidine (1-10 mg/kg) was administered p.o. 1 h before the administration of indomethacin. Data are presented as the mean ± SE from 4-6 rats. *Significant difference from vehicle at p<0.05.
significantly decreased in area compared to those produced by indomethacin plus clopidogrel (Figure 7). Likewise, the aggravation of indomethacin-induced antral damage was significantly and dose-dependently attenuated by famotidine, a H2RA (1-10 mg/kg, p.o.). Macroscopically, the severity of indomethacin-induced hemorrhagic lesions in the presence of clopidogrel was apparently reduced by the prior administration of famotidine (Figure 8). Furthermore, these antisecretory agents also decreased the severity of antral lesions caused by indomethacin alone (data not shown).

**Mucosal protective drugs:** Similar to antisecretory drugs, the mucosal protective drugs, such as rebamipide (1-10 mg/kg, p.o.) and teprenone (30-300 mg/kg, p.o.), significantly and dose-dependently inhibited the aggravation by clopidogrel of indomethacin-induced antral lesions; in particular, the areas of hemorrhagic and total lesions were markedly decreased as compared to the vehicle-treated group (Figure 9).

**Effects of Various Agents on Changes in Antral MPO Activity Induced by Indomethacin Plus Clopidogrel**

The MPO activity in the normal antral mucosa was around 0.01 µmol H2O2/min/mg tissue and increased slightly in response to indomethacin (30 mg/kg, s.c.), loxoprofen (100 mg/kg, s.c.) or flurbiprofen (30 mg/kg, s.c.), reaching 0.036 ± 0.007, 0.029±0.004 or 0.034 ± 0.006 µmol H2O2/min/mg tissue, respectively (Figure 10). The increase in MPO activity caused by indomethacin was markedly potentiated by additional treatment with clopidogrel (30 mg/kg, p.o.), the value being 0.139 ± 0.018 µmol H2O2/min/mg tissue, which is about 5 times greater than the control level. The increased MPO activity induced by indomethacin plus clopidogrel was significantly suppressed by pretreatment with teprenon (300 mg/kg, p.o.) and rebamipide (10 mg/kg, p.o.), the degree of inhibition being 67.6% and 62.6%, respectively. Likewise, the antisecretory agents atropine (3 mg/kg, p.o.), omeprazole (30 mg/kg, p.o.) and famotidine (10 mg/kg, p.o.) also significantly attenuated the increase in MPO activity in response to indomethacin plus clopidogrel, and the values in all cases were equivalent to that in the control, the degree of inhibition being 72.7%, 89.9% and 85.6%, respectively.

**DISCUSSION**

Antiplatelet therapy has been shown to be effective in
reducing the incidence of cerebrovascular events, myocardial infarction, and death from vascular causes in individuals with symptomatic atherothrombic diseases [1]. However, the risk of bleeding in the upper gastrointestinal tract is increased with the concomitant use of antiplatelet drugs with NSAIDs or low-dose aspirin [2-5]. We previously reported that gastric bleeding caused by the luminal perfusion of ASA was markedly increased by pretreatment with clopidogrel, a P2Y12 receptor antagonist [9,10]. The present study further showed that clopidogrel aggravated the severity of NSAID-generated antral lesions in reed rats, changing superficial non-hemorrhagic lesions into deep hemorrhagic lesions. Other NSAIDs, such as lopropafen or flurbiprofen, similarly produced antral lesions in the reed rats, and this property of NSAIDs was reproduced by the combined administration of selective COX-1 and COX-2 inhibitors. In addition, we also found that the generation of antral lesions by indomethacin plus clopidogrel was suppressed by antisecretory and mucosal protective drugs.

The present study confirmed in experimental animals the clinical findings that the risk of gastric bleeding in patients taking NSAIDs was increased by the co-administration of clopidogrel [2-5]. In this study, we used an antral lesion model caused by NSAIDs in reed rats, since in patients the incidence of NSAID-induced injury in the antrum is reportedly high [11,12]. Satoh et al., [13,14] reported for the first time that indomethacin selectively produced antral lesions in reed rats without damaging the corpus mucosa. We further confirmed that this action of indomethacin was mimicked by other conventional NSAIDs, such as lopropafen or flurbiprofen, and also reproduced by the combined administration of SC-560 and rofecoxib, suggesting the inhibition of both COX-1/COX-2 to be part of the pathogenic mechanism. These results are consistent with the previous findings that the gastric ulcerogenic properties of NSAIDs are not accounted for solely by inhibition of COX-1 and requires inhibition of COX-2 as well [15,16]. As expected, pretreatment of the animals with clopidogrel, an antiplatelet drug, aggravated the gastric ulcerogenic response to NSAIDs, and eventually increased the severity of the antral lesions; as clearly shown in this study, conventional NSAIDs alone produced non-hemorrhagic lesions consisting of mostly superficial damage, but clopidogrel aggravated these lesions, converting into deep hemorrhagic damage. Unfortunately, the present antral lesion model did not allow us to measure the amount of bleeding in the stomach. However, since we previously reported that clopidogrel significantly increased gastric bleeding in response to the luminal perfusion of aspirin [9,10] it is assumed that clopidogrel increases gastric bleeding associated with the aggregation of NSAID-induced antral lesions. Anyhow, these results support clinical reports suggesting an increased risk of gastric bleeding with the concomitant use of antiplatelet drugs and NSAIDs [2,3,5].

Satoh et al., [13] reported that the indomethacin-induced antral lesions were significantly suppressed by cimetidine, the H2RA. Consistent with their results, we found that antisecretory drugs such as atropine and omeprazole as well as famotidine significantly reduced the severity of antral lesions produced by indomethacin plus clopidogrel. These results support clinical findings that the use of acid-suppressing agents limits the increased risk of gastric bleeding associated with co-

administration of antiplatelet drugs and NSAIDs [6,7,18]. However, little is known about the effects of gastroprotective agents with the increased risk of gastric bleeding under such conditions. The present study showed that gastric protective drugs were also effective against gastric bleeding in response to indomethacin plus clopidogrel. The mucosal protective drugs employed in this study, rebamipide and teprenone are used to treat gastritis and gastric ulcers in Japan. Certainly, none of these agents are known to have any effect on acid secretion [10]. Rebamipide suppresses inflammatory cell infiltration and the generation of free radicals, exhibits radical-scavenging action, and exerts a potent anti-inflammatory effect [19-21]. On the other hand, teprenone exhibits a protective effect in various models by stimulating the secretion of mucus and the expression of heat shock proteins [22-24]. In the present study, it was found that rebamipide and teprenone significantly reduced the severity of hemorrhagic gastric lesions produced by indomethacin plus clopidogrel, together with the suppression of increased MPO activity, although the effects were less pronounced than that of famotidine or omeprazole. We recently found that these drugs also prevented gastric bleeding after aspirin plus clopidogrel treatment under conditions of acid secretion [10]. At present, the precise mechanisms by which these drugs reduced the gastric ulcerogenic response to indomethacin plus clopidogrel remain unknown. Moreover, the clinical effectiveness of gastroprotective agents on gastric bleeding associated with the co-administration of NSAIDs and clopidogrel remains still unproven. Further animal and clinical studies are needed to clarify these points.

Clopidogrel is a prodrug and requires several biotransformational steps, mediated mainly by cytochrome P-450 isoenzymes, to generate an active metabolite. It exerts its antiplatelet effect by forming an inactivating disulfide bond with the platelet ADP receptor P2Y12 [8]. The isoenzyme CYP2C19 seems to be one of the determinants of the pharmacodynamic response to clopidogrel and is also involved in the metabolism of omeprazole [25-27]. It is assumed that omeprazole reduces the biological action of clopidogrel, through competitive metabolic effects on CYP2C19. In the present study, however, since clopidogrel was administered 48, 24 and 0.5 h before indomethacin treatment, and since omeprazole was given 30 min before indomethacin treatment, it is unlikely that the activation of clopidogrel is influenced by omeprazole.

Given the present findings, it is concluded that clopidogrel aggravates the severity of NSAID-induced antral lesions in reed rats, especially converting from non-hemorrhagic damages into hemorrhagic ones, and the antisecretory drugs such as ropaxine and famotidine are highly effective in preventing gastric bleeding under such conditions. In addition, the mucosal protective drugs, such as rebamipide and teprenone are also effective against gastric bleeding and ulceration after the co-administration of NSAIDs and antiplatelet drug, although less than antisecretory drugs. Finally, the present model may be useful for the screening of drugs that protect against gastric bleeding induced by the co-administration of NSAIDs and antiplatelet drugs.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Yamada N, Kimura M, Kaneko S, and Yamanaka S, the undergraduate students at the
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