Phosphodiesterase Type IV Inhibitors Prevent Ischemia-Reperfusion-Induced Gastric Injury in Rats

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Abstract. The effects of selective inhibitors of phosphodiesterase type IV (PDE4) on ischemia-reperfusion-induced gastric injuries were investigated in rats. Gastric ischemia was induced by applying a small clamp to the celiac artery, and reoxygenation was performed by removal of the clamp. Ischemia-reperfusion produced gastric hemorrhagic injuries and increased the content of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO) activity in gastric mucosa. Rolipram (0.03 – 0.3 mg/kg, s.c.) and Ro-20-1724 (0.3 – 3 mg/kg, s.c.) prevented the development of gastric injury in a dose-dependent manner, and it also increased the content in mucosal TNF-α content and MPO activity induced by ischemia-reperfusion. The anti-ulcer drug 1rsogladine (1 – 10 mg/kg, p.o.), which is known to possess a PDE4 inhibitory action, also inhibited the gastric injury produced by ischemia-reperfusion, as well as the increase in TNF-α levels and MPO activity. It is concluded that the ability of PDE4 inhibitors to inhibit cytokine TNF-α synthesis and the infiltration of polymorphonuclear leukocytes underlies their gastroprotective effects in ischemia-reperfusion-induced gastric injury. Our experiments suggest that drugs that inhibit PDE4 isoenzyme, such as the anti-ulcer drug irsogladine, may be a useful adjunct therapy for the treatment of the gastric damage that follows ischemia-reperfusion.

Keywords: ischemia-reperfusion, gastric injury, phosphodiesterase type IV inhibitor, rolipram, irsogladine

Introduction

Many studies have demonstrated that reactive oxygen species, such as superoxide radical and hydroxyl radical, are involved in the pathogenesis of gastrointestinal mucosal injuries induced by ischemia-reperfusion (1 – 5). The major sources of reactive oxygen species are thought to be the xanthine-xanthine oxidase system and activated polymorphonuclear leukocytes (PMNs) (6 – 8). The importance of activated PMNs in the development and aggravation of gastric injury induced by ischemia-reperfusion has been reported (4, 5).

Drugs that inhibit the phosphodiesterase type IV (PDE4) isoenzyme have received a great deal of attention in the last few years because of their inhibitory effects in various models of acute and chronic inflammation (9 – 11). PDE4 inhibitors work via the elevation of intracellular cyclic AMP, which then activates protein kinase A with subsequent phosphorylation of protein kinase A-specific substrates (11). In vivo, several mechanisms appear to be involved in the anti-inflammatory action of PDE4 inhibitors, including direct inhibition of PMN recruitment, inhibition of PMN activation, and inhibition of the production of the cytokine tumor necrosis factor-α (TNF-α) (9, 11).

Recently, the potential usefulness of the PDE4-selective inhibitor rolipram in gastrointestinal experimental animal models has been demonstrated. Rolipram prevents experimentally induced colonic mucosal lesions (12) and gastrointestinal mucosal damage induced by nonsteroidal anti-inflammatory drugs (13, 14). It has also been reported that rolipram prevents intestinal ischemia-reperfusion injury (15). However,
the effects of PDE4-selective inhibitors such as rolipram on the gastric injury induced by ischemia-reperfusion remain unknown. In this study, we have assessed for the first time the effects of PDE4-selective inhibitors on the ischemia-reperfusion-induced gastric injuries in rats. We also investigated the effects of PDE4 inhibitors on TNF-α levels in the gastric mucosa because inhibition of TNF-α production underlies some of the anti-inflammatory actions of PDE4 inhibitors in vivo. In this study, structurally unrelated compounds rolipram and Ro-20-1724 as PDE-selective inhibitors were examined. Rolipram and Ro-20-1724 selectively inhibit the PDE4 with IC₅₀ = 1.0 μM and 2.0 μM, respectively (16, 17).

Irsogladine [2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate], an anti-ulcer drug, has been reported to prevent gastric mucosal damage in several experimental animal models without inhibiting gastric secretion (18, 19). In rats, irsogladine was recently found to have a marked protective effect against the gastric mucosal damage elicited by monochloramine, which is highly toxic to mucosal tissue, and it also reversed the monochloramine-induced decrease in gastric mucosal blood flow, in a manner dependent on nitric oxide synthesis (20). It has also been demonstrated that irsogladine activates gap-junctional intercellular communication through the enhancement of cAMP formation, which in turn enhances the function of the gastric mucosal barrier by potentiating cellular integrity (21–23). More recently, it has been shown that irsogladine inhibits superoxide production in human neutrophils by increasing the cAMP content through the inhibition of PDE4 (24). Therefore, we also investigated whether irsogladine prevents ischemia-reperfusion-induced gastric injury by PDE4 inhibition in rats.

In this study, we indicated that PDE4 inhibitors, including irsogladine, prevented ischemia-reperfusion-induced gastric injury by inhibiting TNF-α release, subsequently inhibiting gastric PMN infiltration.

Materials and Methods

Animals
Male Sprague-Dawley rats weighing 200 – 250 g (Charles River, Shizuoka) were used in all experiments. The animals were housed under standardized conditions of light and temperature and were fasted for 18 h before the experiments, but with free access to tap water. All experimental procedures were approved by the Committee for the Institutional Care and Use of Animals at Nippon Shinyaku Co.

Induction of gastric injuries induced by ischemia-reperfusion
Ischemia-reperfusion was carried out according to a previously described method with a slight modification (3). Briefly, under urethane anaesthesia (1.2 g/kg, i.p.), the trachea was cannulated to ensure a patent airway and laparotomy was performed. After the pylorus ligation, 0.15 N HCl (10 ml/kg) was instilled into the stomach. Immediately thereafter, the celiac artery was isolated and ischemia was induced by clamping the artery with a small clip (BEAR Medic, Chiba) for 30 min. Reperfusion was initiated by removal of the clamp. Sixty minutes after reperfusion, the rats were killed by exsanguination via the abdominal aorta, and the stomachs were removed. In the sham-operated group, 90 min after pylorus- ligation and intragastric acid instillation, the rats were killed without ischemia-reperfusion procedures. The stomachs were inflated by injection with 10 ml of 2% formalin. They were immersed in 2% formalin for 15 min to fix the gastric walls and opened along the greater curvature. A digital picture of the entire gastric mucosa was taken and all injured areas were measured using imaging-analysis software (WinRoof Ver3.14; Mitani Corporation, Tokyo), and the total area of the injuries calculated in mm². Rolipram and Ro-20-1724 were administered s.c. and irsogladine and omeprazole were administered p.o. 60 min before ischemia was induced.

Measurement of TNF-α concentration in gastric mucosa
The TNF-α concentration in gastric mucosa was measured using ELISA techniques. The stomachs of sham-operated and ischemia-reperfused animals were removed and gastric mucosa were homogenized in 10 vol of 0.1% (v/v) Tween 20 in phosphate-buffered saline, pH 7.2. The samples were then quickly frozen in liquid nitrogen, thawed in a 37°C water bath, sonicated for 15 s, and centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were used for the determination of TNF-α concentration with an ELISA kit (Diaclone Research, Besançon, France) according to the manufacturer’s instructions. The TNF-α concentration was expressed as pg per 100 mg wet weight of tissue.

Measurement of myeloperoxidase (MPO) activity in gastric mucosa
Myeloperoxidase activity was measured in the gastric mucosa according to a slight modification of the method of Krawisz et al. (25). The animals were exsanguinated via the abdominal aorta. The stomachs of sham-operated and ischemia-reperfused animals were excised, weighed, and homogenized with a homogenizer.
Gastroprotection by PDE4 Inhibitor

(Polytron® PT3000; Kinematica, Littau, Switzerland) in ice-cold 50 mM phosphate buffer, pH 6.0, containing 1% hexadecyltrimethylammonium bromide. The homogenate was sonicated on ice for 30 s (Sonifire 250; Branson Ultrasonics Corp., Danbury, CT, USA), freeze-thawed three times, and centrifuged at 10,000 × g for 15 min at 4°C. After the addition of reaction buffer (50 mM phosphate buffer containing 0.125 mg/ml o-dianisidine hydrochloride and 0.0005% hydrogen peroxide) to the supernatant, the change in absorbance at 450 nm was measured with a microplate reader (Benchmark; Bio-Rad, Tokyo). One unit of MPO activity was defined with reference to human neutrophil MPO purchased from Sigma (St. Louis, MO, USA). MPO activity was expressed as units per mg wet weight of tissue.

Drugs

Irsogladine [2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate] was synthesized at Nippon Shinyaku Co., Ltd. (Kyoto). Rolipram [4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2-pyrrolidinone] and Hexadecyltrimethylammonium bromide was purchased from Nacalai Tesque (Kyoto). Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], omeprazole, and o-dianisidine hydrochloride were from Sigma Chemicals. Other chemicals used were of guaranteed grade. Irsogladine, rolipram, Ro-20-1724, and omeprazole were suspended in 0.5% methylcellulose.

Statistical analyses

Statistical analyses were performed with the SAS program (SAS/STAT, version 8.02; SAS Institute, Cary, NC, USA). Data were analyzed for statistical significance by Dunnett’s or Tukey’s test for multiple comparison or by Student’s t-test for comparison between two groups.

Results

Macroscopic mucosal injury induced by ischemia-reperfusion

The ischemia-reperfusion itself caused slight damage in the gastric mucosa after 60 min of reperfusion following 30 min of ischemia (data not shown). However, ischemia-reperfusion combined with the instillation of 0.15 N HCl into the stomach produced marked hemorrhagic mucosal injuries (Fig. 1A). The total area of the injuries induced by ischemia-reperfusion in the presence of intragastric 0.15 N HCl was decreased in a dose-dependent manner by pretreatment with the PDE4-selective inhibitors rolipram (0.03 – 0.3 mg/kg) (Fig. 2) and Ro-20-1724 (0.3 – 3 mg/kg).

Fig. 1. Macroscopic findings of the gastric injury induced by ischemia (30 min)-reperfusion (60 min) in the presence of intragastric acid with or without pretreatment with irsogladine in rats. A: Control: Linear hemorrhagic injuries in the glandular stomach. B: Pretreatment with irsogladine at a dose of 10 mg/kg markedly reduced these injuries.

Fig. 2. Effect of rolipram on gastric injuries induced by ischemia-reperfusion in rats. Rats were subjected to ischemia for 30 min, followed by reperfusion for 60 min. Rolipram (0.03 – 0.3 mg/kg) was given s.c. 60 min prior to ischemia. Each column represents the mean ± S.E.M. of 6 rats. *P<0.05, compared with the control-group (Dunnett’s test).
Irsogladine (1 – 10 mg /kg), which is reported to possess PDE4-inhibitory activity (24), also showed a dose-dependent protective effect against gastric injury (Fig. 1B) (4).

**TNF-α concentration in gastric mucosa**

The procedures of ischemia-reperfusion caused a significant increase in mucosal TNF-α concentrations compared with sham-operated rats. This increase was inhibited in a dose-dependent manner by pretreatment with rolipram (Fig. 5). Irsogladine also inhibited the increase in TNF-α levels induced by gastric ischemia-reperfusion (Fig. 6). For comparison, the concentration of plasma TNF-α was not changed by the procedures of ischemia-reperfusion, the concentration being 9.2 ± 4.1 pg/ml in sham-operated rats and 7.0 ± 4.2 pg/ml in ischemia-reperfusion-treated rats.
MPO activity in gastric mucosa

The procedures of ischemia-reperfusion caused a significant increase in mucosal MPO activity compared with sham-operated rats (Fig. 7). This increase was significantly reduced by pretreatment with rolipram (0.3 mg/kg) at a dose expected to inhibit the progression of the gastric mucosal injury and the increase in TNF-α levels induced by ischemia-reperfusion. Irsogladine (10 mg/kg) also decreased the gastric MPO activity.

Effect of omeprazole on rolipram-induced gastroprotection

Pretreatment with the proton-pump inhibitor omeprazole (30 mg/kg) had no effect on the gastric injury elicited by ischemia-reperfusion (Fig. 8). Furthermore, this acid-inhibitory agent had absolutely no effect on the prevention of ischemia-reperfusion-induced gastric injury by rolipram.

Discussion

The potential usefulness of PDE4-selective inhibitors, including rolipram, as novel anti-inflammatory agents has recently been reported (9–11). PDE4-selective inhibitors increase the intracellular cAMP concentration by suppressing the degradation of cAMP and suppress the production of superoxide in PMNs and the TNF-α-mediated adherence of PMNs to the vascular endothelium (26, 27). In the present study, both rolipram and Ro-20-1724 inhibited in a dose-dependent manner the gastric injury induced by ischemia-reperfusion. To our knowledge, our study demonstrates for the first time that PDE4-selective inhibitors can prevent the gastric injury induced by ischemia-reperfusion.

In several diseases, the proinflammatory cytokine TNF-α is a necessary element in the chain of pathophysiological events leading to inflammation. Success-
ful treatment with anti-TNF-α antibodies of patients with Crohn’s disease (28–30), rheumatoid arthritis (31), or Jarisch-Herxheimer reaction (32) exemplifies anti-inflammatory strategies based on the specific blockade of TNF-α (33). Among agents known to inhibit TNF-α production rather than block its function, cAMP-elevating PDE inhibitors have attracted the most attention. Because PDE4 is the predominant PDE isoenzyme in monocytes, the main source of TNF-α (34, 35), PDE4 inhibitors such as rolipram have high potency in suppressing TNF-α synthesis. The potential therapeutic use of rolipram in TNF-α-dependent inflammatory disease has recently been demonstrated in several animal models (36–38).

The involvement of TNF-α in the development of gastrointestinal injury has also been reported. It has been shown that in indomethacin-treated rats, there is a correlation among the degree of gastric damage, PMN infiltration, and TNF-α release (39, 40). Souza et al. (15) have stated that the capacity of rolipram to inhibit the infiltration of PMNs and the production of TNF-α underlies its anti-inflammatory effects in ischemia-reperfusion-induced small-intestinal injury. Thus, it is of interest to investigate the involvement of TNF-α in ischemia-reperfusion-induced gastric injury and the possible effects of PDE4 inhibitors on tissue TNF-α concentrations. In our study, a pronounced increase in gastric TNF-α concentration was observed after gastric ischemia-reperfusion. This increase was inhibited by pretreatment with the PDE4-selective inhibitor rolipram at doses expected to inhibit the progression of the gastric injury. These results imply that changes in mucosal TNF-α concentration are correlated with gastric injury and gastric infiltration and activation of PMNs.

PMN infiltration via PMN-endothelial cell interactions plays a significant role in the pathogenesis of ischemia-reperfusion-induced gastrointestinal injuries (4, 5). In this study, MPO activity, which is widely used as a marker of PMN tissue infiltration, was significantly increased in the gastric mucosa by the ischemia-reperfusion procedure used. Pretreatment with PDE4-selective inhibitor such as rolipram markedly decreased its MPO activity, indicating that the inhibition of PMN infiltration by PDE4 inhibitors is one of the factors preventing the gastric injury induced by ischemia-reperfusion.

It has been suggested that superoxide radical or hydroxyl radical is the major oxygen radical contributing to ischemia-reperfusion injury in the stomach (1–3). The superoxide radical scavenger superoxide dismutase and the hydroxyl radical scavenger dimethyl sulfoxide attenuate ischemia-reperfusion injury (6, 41, 42). In this regard, neither isogladine nor the PDE4 inhibitor rolipram show the superoxide-radical-scavenging effect in a cell-free hypoxanthine-xanthine oxidase superoxide generating system (24). Furthermore, neither compound has any influence on the increase in thiobarbituric acid-reactive substances produced by NADPH-dependent lipid peroxidation of liver microsomes in vitro (T. Kyoi et al., unpublished observations). However, the direct scavenging properties of these drugs of reactive oxygen species such as hydroxyl radical remain unknown.

The PDE4-selective inhibitors rolipram and Ro-20-1724 are able to increase the secretion of gastric acid (43, 44). On the other hand, ischemia-reperfusion-induced gastric injury has been shown to be acid-dependent (45, 46). Actually, preliminary studies in our laboratory showed that ischemia-reperfusion procedures without intragastric HCl produced much less severe mucosal injury. We have now confirmed that the acid-inhibitory agent omeprazole does not affect the rolipram-induced gastroprotective effect under ischemia-reperfusion experimental conditions with acid instillation into the stomach. These findings indicate that the ability of PDE4 inhibitors to modulate acid secretion is independent of its protective effect on ischemia-reperfusion injury.

In another study (24), we have shown that the anti-ulcer agent isogladine increases cAMP formation in human neutrophils by inhibiting PDE4 and that it subsequently causes dose-related inhibition of superoxide production by neutrophils activated by various stimuli which utilize different signal-transduction mechanisms. The enhancement of cAMP formation by the inhibition of PDE4 is considered to play an important role in the gastroprotective action of isogladine. In the present study, isogladine inhibited in a dose-dependent manner the gastric injury induced by ischemia-reperfusion. In addition, isogladine inhibited TNF-α production and PMN infiltration in the gastric mucosa produced by ischemia-reperfusion. Taken together, these results suggest that the mechanisms underlying the gastroprotective effect of isogladine against ischemia-reperfusion injury demonstrated in this study are essentially the same as those of PDE4-selective inhibitors.

In this study, the usefulness of PDE4 inhibitors including isogladine administered before the ischemia-reperfusion was demonstrated. In clinical conditions, it is very important to estimate the therapeutic effect. Wada et al. (45) have demonstrated that 72 h after the ischemia (30 min)-reperfusion (60 min), gastric ulcers involving damage to the muscularis mucosae in the areas of gastric glands are observed. Administration of nonspecific PDE inhibitor pentoxifylline, which has been used for many years in the treatment of peripheral
vascular disease, after the ischemia-reperfusion has been reported to decrease significantly the total area of ulcers examined at 72 h.

In conclusion, we provide evidence that PDE4-selective inhibitors can inhibit TNF-α release and gastric PMN infiltration and activation, subsequently preventing ischemia-reperfusion-induced gastric injury. Our experiments suggest that drugs that inhibit the PDE4 isoform, such as the anti-ulcer drug irsogladine, may be a useful adjunct therapy for the treatment of the gastric injury that follows ischemia-reperfusion.

References


