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Irsogladine, an anti-ulcer drug, suppresses superoxide production by inhibiting phosphodiesterase type 4 in human neutrophils

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Abstract

Neutrophil superoxide production is implicated in the pathogenesis of gastric mucosal damage induced by various ulcerative agents and *Helicobacter pylori* infection. We investigated here the effects of an anti-ulcer drug irsogladine [2, 4-diamino-6-(2, 5-dichlorophenyl)-*s*-triazine maleate] on cAMP formation in isolated human neutrophils. The cAMP level in human neutrophils was elevated by a phosphodiesterase (PDE) type 4 selective inhibitor rolipram, but not by any inhibitors of PDE1, PDE2 and PDE3. Irsogladine also increased cAMP formation in a concentration-dependent manner in neutrophils. A non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) alone significantly increased cAMP level, whereas irsogladine was unable to further increase cAMP level in the presence of IBMX. Irsogladine inhibited concentration-dependently the superoxide (O_2^-) production induced by various stimuli including formyl-methionyl-leucyl-phenylalanine, opsonized zymosan, guanosine 5′ -[gamma-thio] triphosphate, A23187 and phorbol 12-myristate 13-acetate. These effects of irsogladine were mimicked by rolipram, IBMX and dibutyryl cAMP. The inhibitory effects of irsogladine and rolipram on the O_2^- production were reversed by a protein kinase A inhibitor H-89. These results indicate that irsogladine inhibits the superoxide production in human neutrophils by the increase of cAMP content by PDE 4 inhibition, which in turn contributing to the anti-ulcer effects of irsogladine on gastric mucosal lesions associated with oxidative stress.

Keywords: Neutrophil; Superoxide; cAMP; Phosphodiesterase; Irsogladine

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Introduction

It has been widely recognized that neutrophils are involved in the pathogenesis of gastric mucosal damage induced by non-steroidal anti-inflammatory drugs (Wallace et al., 1990; Pohle et al., 2001) or *Helicobactor pylori* (Morris, 1989; Bagchi et al., 1996; Yoshida et al., 1993). Furthermore, neutrophils have also been implicated in the experimental gastric mucosal lesions induced by hemorrhagic shock or ischemia-reperfusion (Smith et al., 1987; Wada et al., 1996). Activation of neutrophils is accompanied by the release of superoxide (O_2^-), which is generated by the NADPH oxidase and subsequently converted to reactive oxygen species such as hydrogen peroxide, singlet oxygen and hydroxyl radicals and cause injury of surrounding cells (Robinson and Badway, 1995; Babior, 2000) including gastric mucosal cells (Kozol et al., 1994).

The O_2^- production by neutrophils is modulated by the intracellular cAMP (Moore and Willoughby, 1995) which is regulated by cyclic nucleotide degrading enzyme phosphodiesterase (PDE). There are eleven structurally and pharmacologically distinct PDE families, PDE 1–11, which are differently expressed among various tissues and cells (Soderling and Beavo, 2000). These isoenzymes can be discriminated based on substrate specificity and/or affinity, and their regulation by specific inhibitors. Among them, it is well established that PDE type 4, a cAMP specific PDE, is the predominant PDE isoenzyme in various leukocytes including neutrophils and monocytes and plays a key role in the activation of inflammatory cells (Dent et al., 1994; Wang et al., 1999). Furthermore, in various animal models (e.g., for asthma and other allergic diseases, rheumatoid arthritis, multiple sclerosis, and so on), PDE4 inhibitors show pronounced anti-inflammatory effects (Teixeira et al., 1997).

Irsogladine [2, 4-diamino-6-(2, 5-dichlorophenyl)-*s*-triazine maleate], an anti-ulcer drug, has been reported to prevent the gastric mucosal damage in several experimental animal models without inhibiting gastric secretion (Ueda et al., 1984; Okabe et al., 1984). Although mechanisms for the mucosal protective effect of irsogladine have not been fully elucidated, irsogladine was recently found to have a marked protective effect against the gastric mucosal lesion elicited by monochloramine, a highly toxic substance on mucosal tissue, and also recovered the monochloramine-induced decrease in gastric mucosal blood flow, in a manner dependent on nitric oxide synthesis in rats (Kyoi et al., 2003). It has been also demonstrated that irsogladine activates gap junctional intercellular communication through the enhancement of cAMP formation, which in turn enhances gastric mucosal barrier functions by potentiating cellular integrity (Ueda et al., 1991; Iwata et al., 1998; Takahashi et al., 2000). The enhancement of cAMP formation in gastric mucosa might play a crucial role in gastroprotective actions of irsogladine.

In the present study, we found that irsogladine increased intracellular cAMP content by PDE inhibition in the isolated human neutrophils. And so, the effect of irsogladine on O_2^- production in the human neutrophils was compared with those of cAMP-elevating agents including PDE inhibitors. In this instance, to activate NADPH oxidase in human neutrophil, five stimuli which utilize different signal transduction mechanisms were used. That is, besides two membrane receptor-mediated neutrophil activators such as potent chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Becker, 1976) and the opsonized zymosan (OZ) as a model for opsonized pathogens, G protein activating agent guanosine 5′-[gamma-thio] triphosphate (GTP-γS), Ca^{2+} ionophore A23187 and protein kinase C activating agent 4-phorbol 12-myristate 13-acetate (PMA) were used.

Materials and methods

Chemicals

Irsogladine was synthesized at Nippon Shinyaku Co., Ltd (Kyoto, Japan). The following chemicals and drugs were obtained from commercial sources: vinpocetine, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), cilostamide and rolipram (Tocris Cookson Ltd., Bristol, UK), 3-isobutyl-1-methylxanthine (IBMX), fMLP, PMA, zymosan A, A23187, dibutyryl cAMP (db-cAMP), GTP-γS and streptolysin O (Sigma-RBI, Natick, MA, USA), cAMP enzyme immunoassay system (Amersham Co., Buckinghamshire, U.K.), H-89 (Seikagaku Kogyo, Tokyo, Japan), lucigenin (Nacalai, Kyoto, Japan), xanthine monosodium salt (ICN Biomedicals Inc. Ohio, USA), xanthine oxidase from Butter milk (Oriental Yeast, Co., Tokyo, Japan). Other chemicals were all of guaranteed grade.

Human neutrophils

Human neutrophils were obtained from heparin-treated (5 units of preservative free heparin/ml) venous blood of healthy adult volunteers. Neutrophils were separated by standard laboratory procedures. After the centrifugation on Polymorphprep (AXIS-SHIELD, Oslo, Norway) at 500 × g for 30 min at 20 °C, two leukocyte bands (mononuclear cells in the top band and neutrophils in the lower one) were obtained, and neutrophils were harvested, mixed, and centrifuged (500 × g, 10min). The cells were finally suspended in Hank's balanced saline solution (HBSS), and held on ice under continuous bubbling with a gas mixture of 95% O_2 and 5% CO_2 until use. The neutrophils were routinely of high purity (>90%) and viability (>95%) determined by trypan blue exclusion.

cAMP assay

The content of cAMP in cells was measured according to the modified method of Oka et al. (1997). Preparations of isolated neutrophils (10^6 cells/assay) were incubated at 37 °C in HBSS under the stream of gas mixture of 95% O_2/5% CO_2 for 10 min. Irsogladine or a variety of PDE inhibitors were added and incubated for 30 min at 37 °C. The reaction was terminated by the addition of perchloric acid (final concentration was 0.2 M) and cells were homogenized. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 °C and the supernatant was neutralized by the addition of 10% K_2CO_3, then centrifuged at 10,000 *g* for 15 min at 4 °C. The cAMP content in the supernatant was determined using a cAMP enzyme immunoassay kit (Amersham, Buckinghamshire, U.K.).

O_2^- production by neutrophils

O_2^- production was measured by use of lucigenin-enhanced chemiluminescence (LECL) method (Minkenberg and Ferber, 1984). Neutrophils (10^6 cells/assay) suspended in HBSS were preincubated for 30 min at 37 °C in the presence or absence of the test agents. O_2^- production by neutrophils was started by adding fMLP, OZ, GTP-γS, A23187 and PMA. OZ was prepared as described previously (Hasegawa

et al., 1997). Briefly, zymosan A was suspended in HBSS at a concentration of 1 mg/ml and incubated with human pooled serum at final concentration of 50% at 37 °C for 30 min to opsonize the zymosan, followed by centrifugation at 500 × g for 10 min at 4 °C. In the case of stimulation by GTP-γS, neutrophils were permeabilized as described previously (Rosales and Ernst, 1997). Briefly, cells were permeabilized with 0.5 i.u./ml streptolysin O for 10 min at 37 °C in permeabilization buffer (50 mM Hepes, pH 7.0, with 100 mM KCl, 20 mM NaCl, 1 mM EGTA, and 0.1% dextrose). LECL response was measured with Wallac ARVO-SX 1 (PerkinElmer Life Sci. Tokyo, Japan), and LECL reading was integrated the area under the curve after subtraction of the background values for unstimulated cells and values were normalized to protein content. Data are expressed as the percentage of the control value.

Scavenging of O_2^-

O_2^- scavenging activity of irsogladine and rolipram were investigated using a cell- free xanthine/xanthine oxidase O_2^-generating system (Storch and Ferber, 1988). This reaction was carried out in a mixture containing 0.2 mM lucigenin, 0.1 u/ml xanthine oxidase, varying concentrations of test drugs and 0.01 mM EDTA in 50 mM potassiumphosphate buffer (pH 7.8). The reaction was commenced by the treatment of xanthine (120 μM) and LECL was measured.

Statistical analysis

All statistical analyses were performed by using SAS program (SAS/STAT, Ver. 6, fourth edition, 1990, SAS Institute Ins., 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

Characterization of PDE isoenzyme in human neutrophils

In normal neutrophils untreated with PDE inhibitor, cAMP content was 0.94 ± 0.03 pmol/10^6 cells (N = 5), whereas in the presence of the non-selective PDE inhibitor IBMX (1 mM), cAMP content was 4.28 ± 0.60 pmol/10^6 cells (N = 5). As shown in Fig. 1a, vinpocetine (5×10^{-5} M), EHNA (5×10^{-5} M) and cilostamide (5×10^{-5} M) failed to augment cAMP formation. On the other hand, rolipram showed a marked increase of cAMP content to the level not different from those found with IBMX treatment. The increase of cAMP content by rolipram (10^{-9}–10^{-5} M) was concentration-dependent, and the significant inhibition was observed in rolipram at more than 10^{-8} M-treated group (Fig. 1b).

Effects of irsogladine on cAMP content in human neutrophils

Irsogladine (10^{-9}–10^{-5} M) caused a concentration-dependent increase of cAMP content (Fig. 2a). Significant inhibition was observed in irsogladine at more than 10^{-7} M-treated group. However, irsogladine even at 10^{-5} M did not significantly affect further the cAMP level found in the presence of IBMX (10^{-3} M) (Fig. 2b).

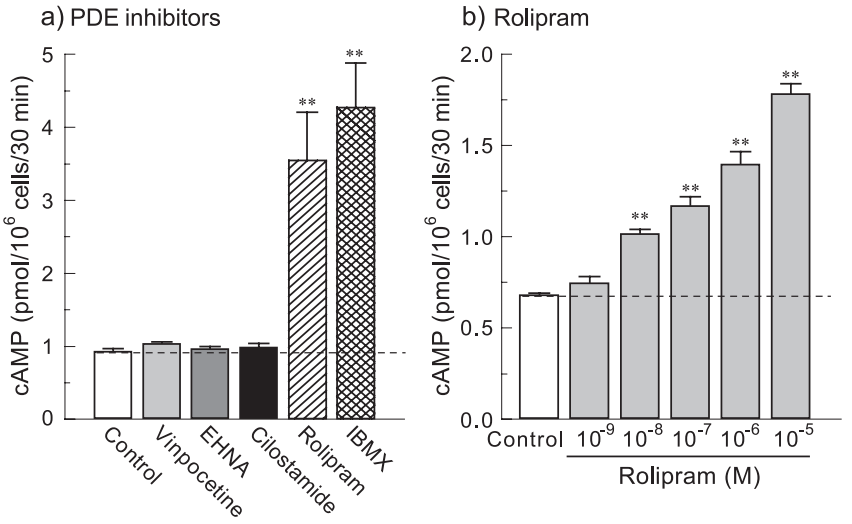

Fig. 1. Effects of PDE inhibitors on cAMP content in human neutrophils. Cells (1×10^6 cells) were incubated for 30 min at 37°C with a variety of PDE inhibitors, including vinpocetine (5×10^{-5} M), EHNA (5×10^{-5} M), cilostamide (5×10^{-5} M), rolipram (5×10^{-5} M) and IBMX (10^{-3} M) (a) or various concentrations of rolipram (b). Each column represents the mean ± S.E. of 5 experiments. **P < 0.01 as compared with control (Dunnett's test).

Effects of irsogladine and cAMP-elevating agents on the various stimuli-induced O_2^- production in human neutrophils

In this study, fMLP, OZ, GTP-γS, A23187 and PMA were used to activate human neutrophils. Stimulation of human neutrophils with 10^{-6} M fMLP caused rapid production of O_2^-. Treatment of the

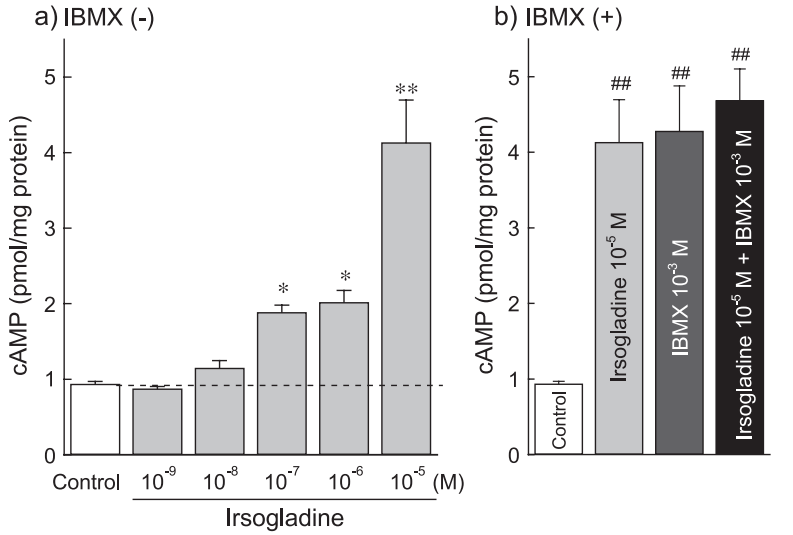

Fig. 2. Effects of irsogladine on cAMP content in human neutrophils. Cells (1×10^6 cells) were incubated for 30 min at 37°C with various concentrations of irsogladine (a) or irsogladine (10^{-5} M) with or without IBMX (10^{-3} M) (b). Each column represents the mean ± S.E. of 5 experiments. *P < 0.05, **P < 0.01 as compared with control (Dunnett's test). ##P < 0.01 (Tukey's test).

cells with irsogladine (10^{-8}–10^{-6} M) suppressed the fMLP-induced O_2^- production in a concentration-dependent manner (Fig. 3a and Table 1). In a similar manner, rolipram (10^{-8}–10^{-6} M) caused concentration-dependent inhibition of fMLP-induced O_2^- production, as shown in Fig. 3b. Significant inhibition was observed both in irsogladine (more than 10^{-7} M)-treated group and rolipram (more than 10^{-7} M)-treated groups.

Treatment with irsogladine significantly suppressed the OZ (1 mg/ml), GTP-γS (10^{-5} M), A23187 (10^{-5} M) and PMA (3×10^{-7} M)-induced O_2^- production in human neutrophils. In a similar manner, rolipram suppressed these various stimuli-induced O_2^- production at approximately same concentrations as irsogladine. Reference drug IBMX (10^{-3} M) and db-cAMP also caused remarkable inhibition of the O_2^- production induced by fMLP, OZ, GTP-γS, A23187 and PMA. Above-mentioned results were put together in Table 1.

Effect of H-89 on inhibitory effects of irsogladine and rolipram on fMLP-induced O_2^- production in human neutrophils

Effect of protein kinase A inhibitor H-89 on inhibitory effects of irsogladine and rolipram on fMLP-induced O_2^- production was examined. H-89 (3×10^{-7} M) alone had no effect on fMLP-induced O_2^- generation (104 ± 12 as percentage of the control value, n = 6). Although irsogladine (10^{-6} M) and rolipram (10^{-6} M) reduced fMLP-induced O_2^- generation, pretreatment with H-89 remarkably reversed the inhibitory effects of these compounds (Fig. 4).

Effects of irsogladine and rolipram on O_2^- scavenging activity were observed using the cell-free xanthine/xanthine oxidase system. Irsogladine and rolipram at the concentrations used in the present study showed no O_2^-scavenging activity, precluding the possibility that these drugs act as an O_2^- scavengers (data not shown).

Discussion

There are eleven structurally and pharmacologically distinct PDE isoenzymes, PDE1-11, which are differently expressed among various tissues and cells (Soderling and Beavo, 2000). We have attempted

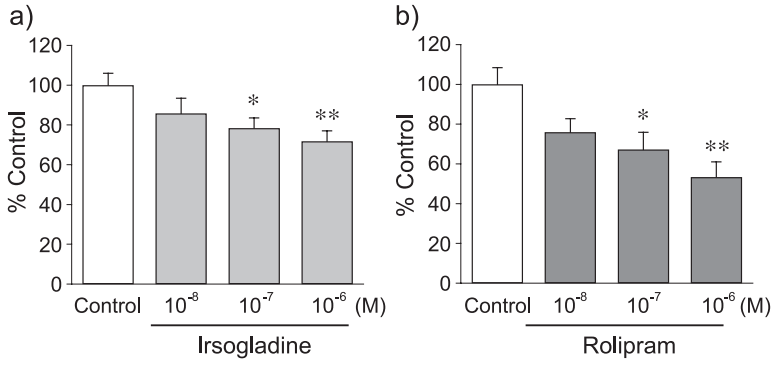

Fig. 3. Effects of irsogladine (a) and rolipram (b) on the fMLP (10^{-6} M)-induced O_2^- production in human neutrophils. O_2^- was measured by the lucigenin-enhanced chemiluminescence method. O_2^- production was expressed as percentage of the control value. Each column represents the mean ± S.E of 10 experiments. *P < 0.05, **P < 0.01 as compared with control (Dunnett's test).

Table 1
Effects of irsogladine and cAMP-elevating agents on the various stimuli-induced O_2^- production in human neutrophils

Drugs	Dose (M)	fMLP (10^{-6} M)	OZ (1 mg/ml)	GTP-γS (10^{-5} M)	A23187 (10^{-5} M)	PMA (3×10^{-7} M)
Control	–	100.0 ± 11.7 (10)	100.0 ± 8.7 (10)	100.0 ± 15.5 (8)	100.0 ± 6.5 (10)	100.0 ± 8.5 (10)
Irsogladine	10^{-8}	85.8 ± 7.7 (10)	ND	ND	80.0 ± 16.1 (10)	89.5 ± 8.9 (10)
	10^{-7}	78.4 ± 5.2* (10)	ND	ND	80.6 ± 5.8 (10)	68.2 ± 9.4 (10)
	10^{-6}	71.7 ± 5.3**	71.6 ± 10.3* (10)	59.0 ± 8.7* (8)	63.5 ± 8.6* (10)	68.0 ± 10.1** (10)
Control	–	100.0 ± 8.4 (10)	100.0 ± 8.7 (10)	100.0 ± 15.5 (8)	100.0 ± 5.1 (10)	100.0 ± 1.5 (10)
Rolipram	10^{-8}	75.9 ± 6.8 (10)	ND	ND	86.8 ± 9.3 (10)	88.4 ± 7.9 (10)
	10^{-7}	67.2 ± 8.7* (10)	ND	ND	70.6 ± 7.8* (10)	83.2 ± 5.9 (10)
	10^{-6}	53.3 ± 7.7** (10)	42.7 ± 7.5** (10)	50.6 ± 6.2** (8)	62.6 ± 3.6** (10)	76.4 ± 4.3* (10)
Control	–	100.0 ± 11.7 (10)	100.0 ± 13.0 (10)	100.0 ± 12.2 (8)	100.0 ± 3.2 (10)	100.0 ± 12.0 (10)
IBMX	10^{-3}	3.6 ± 1.7** (10)	6.8 ± 8.0** (10)	0.6 ± 4.5** (8)	3.4 ± 2.3**0 (10)	29.5 ± 9.5** (10)
Control	–	100.0 ± 6.8 (10)	100.0 ± 8.7 (10)	100.0 ± 15.5 (8)	100.0 ± 7.9 (10)	100.0 ± 8.1 (10)
db-cAMP	10^{-3}	39.3 ± 5.5** (10)	4.1 ± 3.6** (10)	6.4 ± 3.0** (8)	35.7 ± 6.2** (10)	68.9 ± 3.9* (10)

O_2^- was measured by the lucigenin-enhanced chemiluminescence method. O_2^- production was expressed as percentage of the control value.
The number in parentheses indicate the number of experiments. ND: not determined.

* $P<0.05$ as compared with control (Dunnett's test or Student's t-test).

** $P<0.01$ as compared with control (Dunnett's test or Student's t-test).

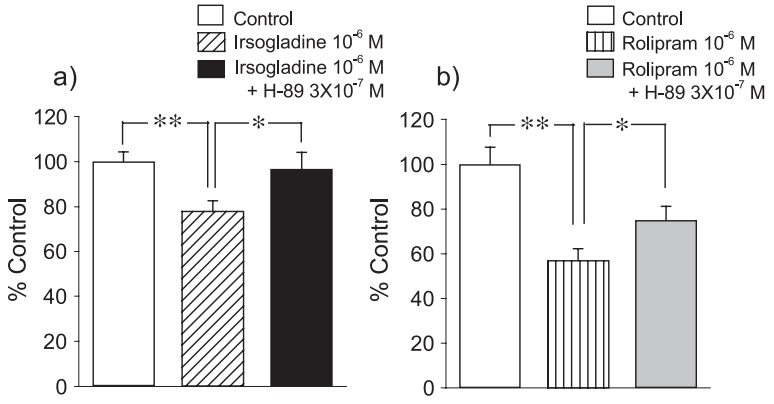

Fig. 4. Effects of irsogladine (a) or rolipram (b) with and without H-89 on O_2^- production by fMLP (10^{-6} M)-activated human neutrophils. O_2^- was measured by the lucigenin-enhanced chemiluminescence method. O_2^- production was expressed as percentage of the control value. Each column represents the mean ± S.E of 10 experiments. **P < 0.05, **P < 0.01 as compared with irsogladine or rolipram alone (Student's t-test).

to identify the PDEs responsible for degrading cAMP in human neutrophils by using now available selective inhibitors for each PDE isoenzyme: vinpocetine (PDE1 comparative selective) (Hagiwara et al., 1984), EHNA (PDE2 selective) (Michie et al., 1996), cilostamide (PDE3 selective) (Sudo et al., 2000), rolipram (PDE4 selective) (Souness and Rao, 1997). The present study demonstrated that human neutrophils contained PDE4 as the predominant PDE isoenzymes, because rolipram, but not vinpocetine, EHNA and cilostamide, augmented cAMP content. It is unlikely that other PDE isozymes such as PDE7, 8, 10, 11 participate for cAMP hydrolysis in human neutrophils, because both a PDE4 selective inhibitor rolipram and a non-selective PDE inhibitor IBMX showed the augmentation of cAMP contents up to the almost same levels. This consideration was supported by the study with polymerase chain reaction analysis that PDE activity of human neutrophil was consisted of PDE4B (Wang et al., 1999; Jacob et al., 2002). Irsogladine also increased cAMP content in the absence of IBMX but not in the presence of IBMX, suggesting that irsogladine potently inhibit PDE4, although the precise mechanisms underlying PDE inhibition by irsogladine have not been clarified.

The intracellular signaling mechanisms responsible for NADPH oxidase activation in neutrophils are extremely complex and remain elusive. fMLP and OZ activate neutrophils by binding to G protein-coupled receptors on the membrane. An early event downstream of agonist binding to G protein-coupled receptors is the activation of phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate and diacylglycerol, resulting in an increase in $[Ca^{2+}]i$ and activation of protein kinase C, respectively (Downey et al., 1995). These two second messengers act synergistically in O_2^- production. It has been also reported that phospholipase D, PI3-kinase and mitogen-activated protein kinase are activated by fMLP and appear to be functionally linked to O_2^- production in neutrophils (Bonser et al., 1989; Okada et al., 1994; Zu et al., 1998). Our study indicated that irsogladine inhibited O_2^- production from fMLP or OZ-stimulated neutrophils. To investigate the underlying mechanism that irsogladine blocks the fMLP or OZ-mediated signal transduction, we further examined the ability of irsogladine to suppress O_2^- production stimulated by GTP-γS, A23187 and PMA. GTP-γS bypasses the membrane receptors and directly activates the G protein. Calcium-ionophore A23187 directly increases $[Ca^{2+}]i$, followed by a cascade of events leading to activation of NADPH oxidase. Although PMA is not

a physiological stimulus for neutrophil activation, PMA is a direct activator of protein kinase C and is one of the most potent activators of the neutrophil respiratory burst. In the present study, irsogladine showed marked inhibitory effect on GTP-γS, A23187 and PMA-induced O_2^- production. The action site of irsogladine might be at the down stream of protein kinase C in the signal transduction pathway. In contrast, rebamipide, a gastroprotective agent like irsogladine, is reported to show the competitive inhibitory action on fMLP receptor in human neutrophils, indicating a component of the signal transduction pathway upstream of G protein (Nagano et al., 2001).

The involvement of cAMP in the inhibitory effect of irsogladine on O_2^- production by neutrophil was supported by the experiments using several cAMP elevating agents including PDE inhibitors. Addition of rolipram, db-cAMP and IBMX to neutrophils largely mimicked the effects of irsogladine i.e., inhibition of O_2^- production activated by fMLP, OZ, GTP-γS, A23187 and PMA. These results indicate that the mechanisms underlying inhibitory effect of irsogladine on these stimulants-evoked O_2^- production are essentially the same as those of other cAMP elevating agents such as PDE inhibitors. The involvement of protein kinase A in the negative regulation of fMLP-stimulated O_2^- production mediated by irsogladine and rolipram was shown by the observation that the inhibitory effects of these agents were antagonized by H-89, a selective antagonist of this kinase. Noteworthy, it has been shown that the phosphorylation of an essential component of neutrophil NADPH oxidase (p47 phox) in response to fMLP is inhibited by cAMP analogue db-cAMP and that this effect is prevented by KT 5720, a selective inhibitor of protein kinase A (Bengis-Garber and Gruener, 1996).

Since NO has been reported to inhibit neutrophil adhesion and activation, cGMP, the intracellular mediator of NO action, is also thought to inhibit the O^{2-} production (Gluckman et al., 2000; Wanikiat et al., 1997). We showed that irsogladine reversed almost completely the decrease of cGMP content in monochloramine-induced damaged gastric mucosa, while the compound had no influence on the cGMP production in non-treated normal mucosa (Kyoi et al., 2003). In our another experiment using purified bovine brain PDE, we found that irsogladine up to 10^{-6} M did not inhibit cGMP hydrolysis, although we do not know the precious reasons about the substrate specificity for cAMP compared with cGMP (Kyoi et al., 2004a). Therefore, cAMP but not cGMP is suggested to be involved in the inhibitory effect of irsogladine on O^{2-} production in human neutrophils.

Although it had been well known that the activation of neutrophil NADPH oxidase induced by fMLP, OZ and A23187 was inhibited by the treatment with PDE inhibitors (Nielson et al., 1990; Anderson et al., 1998; Mahomed et al., 1998), the effect of these agents on PMA-stimulated O_2^- production was not clearly determined. The O_2^- production elicited by PMA has been reported to be unaffected by PDE inhibitors (Sedgwick et al., 1985; Mahomed et al., 1998). In contrast, nimesulide and RO 20–1724, potent PDE4 inhibitors, were able to inhibit PMA-induced O_2^- production by human neutrophils, and H-89 abolished the inhibitory effect of nimesulide on PMA-stimulated O_2^- production (Bevilacqua et al., 1994). Moreover, it was also reported that the O_2^- production by PMA and GTP-γS was obviously inhibited by cAMP and the inhibition was completely restored by H-89 in electropermeabilized human neutrophils (Mitsuyama et al., 1993). Chini et al. (1994) reported that the activation of NADPH oxidase stimulated by PMA in rat glomeruli was significantly attenuated by rolipram. In rat glomeruli, cAMP is clearly able to affect protein kinase C-related events possibly due to the inhibition of protein kinase C translocation from the cytosol to the membrane (Miyanoshita et al., 1989). Although the precise explanation for such inconsistency could not be presented, a downstream of protein kinase C might be listed as one of sites where cAMP negatively regulates the O_2^- production, as indicated by Mitsuyama et al. (1993).

Although less is known about the precise mechanism of cAMP with respect to the inhibition of NADPH oxidase, the removal of Ca^{2+} from cytosol of activated neutrophils are possibly involved. Exposure of neutrophils to cAMP-elevating agents has been shown to inhibit the release of Ca^{2+} from intracellular store (Nielson et al., 1988), or to cause accelerated efflux and/ or decreased influx of the cation (Villagrasa et al., 1996). Recently, Anderson et al. (1998) has been reported that rolipram and db-cAMP accelerate the resequestration of cytosolic Ca^{2+}, as a consequence of activation of the endo-membrane Ca^{2+}-ATPase, leading to inhibition of Ca^{2+}-dependent neutrophil functions.

In the present study, we used irsogladine at the concentration of 10^{-6} M in the case of the experiments of O_2^- production , since plasma concentration of irsogladine achieved about 10^{-6} M during clinical medication for gastric ulcers. Irsogladine at 10^{-6} M was effective in both increase of cAMP and suppression of O_2^- production. Therefore, it is likely that the inhibitory effect on activated neutrophils, namely anti-inflammatory effect of irsogladine observed in the present study is physiologically relevant. IBMX (10^{-3} M) was invariably more potent in inhibiting the O_2^- production than irsogladine (10^{-6} M). However, we might obtain the similar potency of irsogladine at 10^{-5} M and IBMX, because irsogladine at 10^{-5} M showed a marked increase of cAMP content in human neutrophils to the level not different from those found with IBMX.

In conclusion, it has been shown that an anti-ulcer agent irsogladine inhibits O_2^- production in human neutrophils by increasing the cAMP content through the inhibition of PDE4. Therefore, it is expected that irsogladine is effective on the gastric damages induced by non-steroidal anti-inflammatory drugs, *Helicobacter pylori* infection and ischemia-reperfusion and so on which activated neutrophils are involved in the pathogenesis (Pohle et al., 2001; Bagchi et al., 1996; Wada et al., 1996). Recently, we have reported that irsogladine inhibited the gastric injury produced by ischemia-reperfusion, as well as the increases in proinflammatory cytokine TNF-α levels and myeloperoxidase activity (Kyoi et al., 2004b). Typically, the inhibition of activated neutrophils could result in a variety of pharmacological actions through its anti-inflammatory property. Irsogladine has been reported to prevent the induction of experimental acute hepatic failure and pancreatitis in rodents (Mizoguchi et al., 1991; Ito et al., 1997). In addition, irsogladine is clinically effective for the treatment of aphthous stomatitis (Yoshida and Hirakata, 2003; Hara et al., 1999). The anti-inflammatory property elucidated in the present study participates at least in part in the protective effects of irsogladine against the tissue damages associated with oxidative stress.

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