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A new $CysLT_1$ and $CysLT_2$ receptors-mediated anaphylaxis guinea pig model

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ABSTRACT

Although the effectiveness of $CysLT_1$ receptor antagonists on asthma has been clinically established, the effects of $CysLT_2$ receptor antagonists are still unclear. The purpose of this study was to develop a new $CysLT_1$ and $CysLT_2$ receptors-mediated anaphylaxis guinea pig model using S-hexyl GSH, a γ-glutamyl transpeptidase (GTP) inhibitor, to suppress conversion of LTC_4 to LTD_4. Actively sensitized guinea pigs were challenged with OVA in the absence or presence of S-hexyl GSH, and survival rate following anaphylactic response was monitored. OVA-induced fatal anaphylaxis in the absence of S-hexyl GSH was almost completely inhibited by montelukast, a $CysLT_1$ receptor antagonist, but not by the $CysLT_2$ receptor antagonist $BayCysLT_2RA$. However, under treatment with S-hexyl-GSH, the inhibitory effect of motelukast was dramatically diminished, whereas that of $BayCysLT_2RA$ was markedly increased. The dual $CysLT_{1/2}$ receptor antagonist ONO-6950 effectively inhibited anaphylactic response in both S-hexyl GSH-treated and non-treated animals. LC/MS/MS analysis revealed that S-hexyl GSH treatment actually inhibited LTC_4 metabolism in the blood and lung tissues. Using S-hexyl GSH, we developed a novel $CysLT_1$ and $CysLT_2$ receptors-mediated anaphylaxis guinea pig model that can be useful for not only screening both $CysLT_2$ and $CysLT_{1/2}$ receptors antagonists, but also for functional analysis of $CysLT_2$ receptors.

1. Introduction

Cysteinyl leukotrienes (CysLTs, such as LTC_4, LTD_4, and LTE_4) are inflammatory lipid mediators involved, via activation of $CysLT_1$ receptors, in various pathophysiological conditions, including airway smooth muscle constriction, vascular hyperpermeability, mucus secretion, and inflammatory cell migration [1–5]. $CysLT_1$ receptor antagonists, such as pranlukast, montelukast and zafirlukast are widely used as therapeutic agents for the treatment of bronchial asthma [6]. However, the efficacy of these agent is limited in almost half of asthma patients [7,8].

Previous studies have shown that beside $CysLT_1$ receptors, $CysLT_2$ receptors are also expressed on the bronchial epithelium, smooth muscle and inflammatory leukocytes [9–11]. We have also reported that not only $CysLT_1$ receptors, but also $CysLT_2$ receptors are expressed in airway tissue isolated from bronchial asthma subjects [12]. Although the exact role of $CysLT_2$ receptors in asthma pathogenesis is still unclear, evidence has shown that $CysLT_2$ receptors are involved in the induction of asthma [13,14]. It is therefore expected that $CysLT_2$ / $CysLT_{1/2}$ receptor antagonists provide beneficial effects in the treatment of asthma.

Guinea pigs are widely used as asthma experimental models, because their airway smooth muscle responds well to CysLTs stimulation. Guinea pig $CysLT_2$ receptors are activated mainly by LTC_4, but have only weak binding affinity for LTD_4 [15]. In contrast, human $CysLT_2$ receptors are equally stimulated by both LTC_4 and LTD_4 [16]. Because LTC_4 is rapidly metabolized to LTD_4 by γ-glutamyl transpeptidase (GTP) [17,18] in any species, LTC_4-induced bronchoconstriction in guinea pigs is preferentially mediated via LTD_4, which activates $CysLT_1$ receptors. Thus, in order to elicit asthmatic response via $CysLT_2$ receptors both *in vitro* and *in vivo*, it is necessary to inhibit LTC_4 metabolism to LTD_4 [19]. We have previously reported that guinea pigs pre-treated with S-hexyl GSH, a synthetic substrate of γ-GTP, exhibit LTC_4- or OVA-induced bronchoconstriction, airway vascular hyperpermeability and/or lung air-trapping via not only $CysLT_1$ but also $CysLT_2$ receptors activation [20–22]. However, it is still unclear whether treatment with S-hexyl GSH in guinea pigs alters antigen-induced systemic anaphylaxis to $CysLT_2$ receptor-mediated

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response. In addition, the decline in $CysLT_2$ receptor-mediated asthmatic response has not been pharmacologically validated.

In this study, we describe the development of a new animal model that can be used to pharmacologically evaluate the efficacy of $CysLT_1$ and $CysLT_2$ receptors antagonists on anaphylactic response *in vivo*. In our experiments, we examined whether antigen-induced anaphylaxis in sensitized guinea pigs is altered to a partially $CysLT_2$ receptor-mediated response by treatment with S-hexyl GSH. We also examined whether S-hexyl GSH actually inhibits the conversion of LTC_4 to LTD_4 in guinea pig peripheral blood and lung tissue. Finally, we pharmacologically validated antigen-induced anaphylactic response using two anti-asthma drugs, i.e. dexamethasone, a corticosteroid, and salmeterol, an adrenergic β_2 receptor agonist.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Japan SLC, Shizuoka, Japan) aged 6–8 weeks were used in this study. The animals were housed in air-conditioned room kept at 24 ± 2 °C and 55 ± 5% relative humidity with alternating 12 h light/dark cycles and provided with food (LRC4, Oriental Yeast Co., Ltd., Japan) and tap water *ad libitum*. All animal experiments were approved by the Animal Ethical Committee of Ono Pharmaceutical Co., Ltd., and were performed in accordance with the institutional animal care guidelines.

2.2. Materials and methods

The $CysLT_1$ receptor antagonist montelukast was purchased from Sequoia Research Products, Ltd. (Pangbourne, United Kingdom). The $CysLT_2$ receptor antagonist $BayCysLT_2RA$; 1-(5-carboxy-2-{3-[4-(3-cyclohexylpropoxy)phenyl]propoxy}benzoyl)-4- piperidinecarboxylic acid [23], and the dual $CysLT_{1/2}$ receptor antagonist ONO-6950 4,4'-[4-Fluoro-7-(2-{4-[4-(3-fluoro-2-methylphenyl)butoxy]phenyl}ethynyl)-2-methyl-1*H*-indole-1,3-diyl]dibutanoic acid [24] were synthesized in our laboratories. LTC_4, LTD_4, LTE_4, LTC_4-d5, LTD_4-d5, and LTE_4-d5 were purchased from Cayman Chem. (Ann Arbor, MI, USA). Ovalbumin (OVA, grade V), S-hexylglutathione (S-hexyl GSH), indomethacin, pyrilamine maleate, dexamethasone 21-phosphate disodium salt, and salmeterol xinafoate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dead *Bordetella pertussis* suspension was obtained from Cusabio Co. (Wuhan, China). Montelukast and ONO-6950 were suspended in 0.5 (w/v)% methylcellulose solution (Wako Pure Chem., Osaka, Japan). $BayCysLT_2RA$ was dissolved in physiological saline containing 1 (v/v)% Wellsolve (Celeste Co., Tokyo, Japan). Dexamethasone was dissolved in distilled water. Salmeterol was dissolved in physiological saline containing 2 (v/v)% N,N-dimethylformamide.

The dose of montelukast was set at 0.1 mg/kg (p.o.) due to the following reasons: 1) In our previous study, montelukast at 0.1 and 0.3 mg/kg, p.o. inhibited LTD_4 (0.3 μg/kg, i.v.)-induced bronchoconstriction in guinea pigs by 81% and 97%, respectively (unpublished data). 2) Montelukast, given orally to guinea pigs at 1 mg/kg, reached a maximum blood concentration of 1100–2450 ng/ml (1800–4000 nM), a level at which montelukast antagonizes not only $CysLT_1$ receptors, but also $CysLT_2$ receptors (IC_{50} =1800 nM) [20].

Regarding the dose of $BayCysLT_2RA$, we have previously reported that this $CysLT_2$ receptor antagonist at 1 mg/kg (i.v.) effectively suppresses LTC_4 (15 μg/kg, i.v.)-induced bronchoconstriction by 53% in S-hexyl GSH-treated guinea pigs [20]. In addition, we found in an acute toxicity study that $BayCysLT_2RA$, given at 3 mg/kg, i.v. induced hemolysis in one out of 8 guinea pigs (unpublished data). Based on these findings, we selected to use $BayCysLT_2RA$ at 1 mg/kg in this study. It is noteworthy that $BayCysLT_2RA$ at 1 mg/kg (i.v.) affected neither LTD_4 (0.3 μg/kg, i.v.)-induced bronchoconstriction (unpublished data) nor LTC_4 (15 μg/kg, i.v.)-induced bronchoconstriction in S-hexyl GSH non-treated guinea pigs [20].

2.3. Evaluation of survival rate in antigen-induced anaphylaxis in sensitized guinea pigs

On Day 0, guinea pigs were actively sensitized by intraperitoneal injection of saline containing 1 mg OVA and 5×10^9 dead *Bordetella pertussis*. On Day 9 or 12, 20 μl of a 30 mg/ml OVA solution was instilled in both eyes, and ocular sensitization symptoms were monitored for 30 min. Animals with unilateral or bilateral hyperemia, eyelid swelling, or both were considered to be sensitized, and were therefore used in the following experiments.

Evaluation of animals survival rate: On Day 13 or 16, the sensitized animals were intravenously treated with a mixture of pyrilamine (1 mg/kg), an anti-histaminic drug, and indomethacin (5 mg/kg), a cyclooxygenase inhibitor, to minimize histamine involvement and promote CysLT production. Fifteen min later, the animals were intravenously treated with S-hexyl GSH (5, 15 or 30 mg/kg) or saline, and then challenged with OVA (0.3–1.0 mg/kg, i.v.). The time until death for each animal was recorded up to 120 min after OVA challenge. Montelukast (0.1 mg/kg) or ONO-6950 (0.03, 0.3 and 3 mg/kg) was orally administered to the animals 24 h before OVA challenge. $BayCysLT_2RA$ (1 mg/kg) was intravenously administered 1 min before OVA challenge. Dexamethasone (10 mg/kg/time) was orally administered twice at 24 and 2 h before OVA challenge, and salmeterol (0.03 and 0.3 mg/kg) was intravenously administered 1 min before OVA challenge.

2.4. Determination of LTC_4 and LTD_4 in lung tissue and peripheral blood

The sensitized guinea pigs were treated with S-hexyl GSH (5, 15 or 30 mg/kg), indomethacin (5 mg/kg), pyrilamine (1 mg/kg) and OVA (0.6 mg/kg) as described above. One to 20 min after OVA challenge, the animals were anesthetized with pentobarbital sodium (i.v., bolus), and peripheral blood was drawn from the femoral artery. The lungs were then isolated, homogenized in ice-cold 99.5 (v/v)% ethanol using a polytron homogenizer, and stored at −80 °C until quantification of CysLTs. A 200 μl portion of the collected blood was added to 4 ml of ice-cold 99.5 (v/v)% ethanol (blood sample) and stored at −80 °C until quantification of CysLTs.

The lung homogenates and blood samples were centrifuged, and CysLTs in the supernatants were partially purified according to the method of Yonetomi et al. [20]. In brief, the supernatants were evaporated to dryness by vacuum centrifugation, and the resultant residues were each dissolved in 2 ml of distilled water. The obtained solution was then loaded onto a solid phase extraction column (Oasis MAX SPE cartridge, Waters Co., Milford, MA, USA) pre-conditioned with 2 ml of methanol and 2 ml of distilled water. After washing the column with 2 ml of distilled water followed by 2 ml of 1 (v/v)% aqueous ammonia and 2 ml of methanol, CysLTs were eluted with 2 ml of methanol containing 2 (v/v)% formic acid. The eluate was evaporated to dryness by vacuum centrifugation, and the residue was reconstituted in 150 μl of 0.1 (v/v)% formic acid containing acetonitrile/distilled water (1:4). CysLTs in the reconstituent were analyzed by a Shimadzu Prominence 20 A System (Kyoto, Japan) coupled with an API 5000 (Applied Biosystems, Foster City, CA) LC/MS/MS system with negative ion mode. Molar ratio (LTC_4/LTD_4) in each sample was expressed as index of LTC_4 metabolism inhibition.

2.4.1. Statistical analysis

Data in Fig. 1 express survival rate in 8 to 14 animals. Data in Figs. 2A, 2C, 2E, and 2G express survival rate in 14 to 19 animals. Data in Figs. 2B, 2D, 2F, and 2H express area under the survival rate curve (AUC) in 14 or 25 animals up to 60 min after OVA challenge. Data in