Antagonism of the Human Thromboxane A₂ Receptor by an Anti-asthmatic Agent AA-2414

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The human thromboxane A₂ receptor (TXA₂-R)-coding gene was introduced into Chinese hamster ovary cells and a cell line (TCHO-25) stably expressing TXA₂-R, at a level of 3×10^5 /cell, was obtained. An anti-asthmatic agent AA-2414 [(±)-7-(3,5,6-trimethyl-1,4-benzoquinon-2-yl)-7-phenylheptanoic acid] competitively inhibited the specific binding of a TXA₂ mimic ([³H]U-46619) to the TCHO-25 cells, with an IC₅₀ of 6.0×10^{-8} M, indicating that the drug is an antagonist of human TXA₂-R. The TCHO-25 cells offer a tool for the screening and characterization of human TXA₂-R antagonists.

Keywords thromboxane A₂ receptor antagonist; anti-asthmatic agent; AA-2414; (±)-7-(3,5,6-trimethyl-1,4-benzoquinon-2-yl)-7-phenylheptanoic acid

Thromboxane A₂ (TXA₂), derived from prostaglandin endoperoxide (PGH₂), exerts potent biological effects on platelet aggregation and constriction of vascular and respiratory smooth muscles.^{1–4} Because of its potent biological activity, TXA₂ is believed to be involved not only in cardiovascular diseases, including myocardial infarction and stroke, but also in respiratory diseases like bronchial asthma.^{5,6} A number of TXA₂/PGH₂ receptor antagonists have been examined as potential treatments for these diseases.^{7–9}

(±)-7-(3,5,6-Trimethyl-1,4-benzoquinon-2-yl)-7-phenylheptanoic acid (AA-2414), synthesized as a derivative of the 5'-lipoxygenase inhibitor, AA-861,^{10–12} inhibits platelet aggregation and bronchoconstriction induced in guinea pigs by the TXA₂ mimic, 9,11-dideoxy-9α,11α-methanoepoxy-PGF₂α (U-46619).^{13,14} Oral administration of AA-2414 inhibits experimental allergic asthma much more potently than AA-861, although the former compound shows much less inhibitory activity towards 5'-lipoxygenase (IC₅₀: 9.2 μM) than the latter (IC₅₀: 0.8 μM).¹⁵ AA-2414 competitively inhibits contractions of guinea pig tracheal strips in response to U-46619, PGD₂ and 9α,11β-PGF₂α, but does not inhibit contractions induced by leukotriene D₄ (LTD₄), platelet activating factor (PAF), or histamine.¹⁰ The compound also competitively inhibits the binding of [³H]U-46619 to washed platelets from guinea pigs.¹¹ These results strongly suggest that the pharmacological effects of AA-2414 are due to its antagonism of the TXA₂ receptor (TXA₂-R).

Recently Hirata *et al.* cloned a human TXA₂-R-coding cDNA from a human placenta cDNA library and expressed it in COS-7 cells.¹⁶ They demonstrated that the membrane preparation from the transfected COS cells bound a selective TXA₂-R antagonist ([³H]S-145)⁹ in a saturable manner, with a dissociation constant (*K_d*) of 1.2×10^{-9} M. Here we describe the stable expression of human TXA₂-R in Chinese hamster ovary (CHO) cells and investigate the inhibitory activity of AA-2414 on the specific binding of [³H]U-46619 to TXA₂-R.

MATERIALS AND METHODS

Materials A cDNA clone (HPL), coding human TXA₂-R, was the kind gift of Prof. S. Narumiya of Kyoto University.¹⁶ Dihydrofolate reductase negative (DHFR[−]) CHO cells¹⁷ were obtained from Dr. P. Berg of Stanford University, U.S.A. AA-2414 was synthesized as described elsewhere.^{12,18} U-46619 (Cayman Chemical Company, MI, U.S.A.), [³H]U-46619 (24.5 mCi/mmol; Du Pont/NEN Research Products, MA, U.S.A.), and other reagents were commercial products. Male Hartley guinea pigs weighing approximately 350 g were purchased from SLC (Japan).

Preparation of the Expression Plasmid The cDNA encoding human TXA₂-R was obtained by digestion of the plasmid HPL¹⁶ with *Bam*HI, and an *Eco*RI site was introduced at the 5' position of the fragment. This 2.0-kilobase (kb) DNA fragment was inserted into the *Eco*RI site of the plasmid, pcDL-SRα296, containing an SRα promoter and a poly-adenylation/termination signal¹⁹ to give the expression plasmid pTB1477.

Preparation of TXA₂-R-Expressing CHO Cells The plasmid pTB1477 was mixed with plasmid pTB348,²⁰ containing DHFR cDNA at a weight ratio of 10:1, and introduced into (DHFR[−])CHO cells by the conventional calcium phosphate co-precipitation method.^{21, 22} After growth for 2 d in non-selective medium, the cells were trypsinized and seeded into 96-well microplates with Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 0.3 mM L-proline and 0.1 mM methotrexate. Colonies grown in the selection medium were transferred to Linbro 24-well plates (Flow Laboratories, Inc., VA, U.S.A.) and TXA₂-R-expressing cells were selected by a [³H]U-46619 binding assay, as described below. The transformants thus obtained were cloned by a limiting dilution method, giving clone TCHO-25, a CHO cell line stably expressing TXA₂-R.

Binding of [³H]U-46619 to TCHO-25 Cells TCHO-25 cells were seeded at 3×10^5 cells/well in Linbro 24-well plates, followed by overnight cultivation at 37 °C. After

one wash with Hank's balanced salt solution (pH 6.2) (HBSS; Gibco/BRL Life Technologies, Inc., MD, U.S.A.), various amounts of [3 H]U-46619 dissolved in 0.4 ml HBSS containing 3% ethanol were added to the cells and the plates were incubated at room temperature for 30 min. After three washes with 1.0 ml of HBSS, the cells were solubilized in 0.6 ml 5% sodium dodecyl sulfate solution. The cell lysate was mixed with 4 ml liquid scintillator A (Wako Pure Chemicals, Japan) and the bound [3 H]U-46619 radioactivity was measured with a liquid scintillation counter (Aloka Inc., Japan). Non-specific binding of [3 H]U-46619 to the cells was estimated from the binding in the presence of a 1000-fold molar excess of unlabeled U-46619. The inhibitory activity of AA-2414 towards the specific binding of [3 H]U-46619 to TXA₂-R was evaluated as follows. Various amounts of AA-2414 and 1.25×10^{-8} M [3 H]U-46619 in 0.4 ml HBSS containing 3% ethanol were added to TCHO-25 cells and the plates incubated at room temperature for 30 min. After three washes with HBSS, the radioactivity remaining in each well was measured as described above.

Contractile Response of Smooth Muscle Tracheal strips were prepared from guinea pigs as described previously.¹⁰⁾ Strips were placed in a 10-ml organ bath containing aerated Tyrode's solution at 37 °C, and an initial tension of 1.0 g was applied to the strips. After a 1-h equilibration period, a contractile response was induced by U-46619 and the resulting tension was measured isotonicity by a conventional method. When the magnitude of the contractions had reached a plateau, AA-2414 was added at one minute intervals to the organ bath until maximum relaxation of the strip was obtained.

RESULTS AND DISCUSSION

A 2.0-kb DNA fragment coding TXA₂-R was subcloned into the eucaryotic expression vector pcDL-SRα296 to give pTB1477 and pTB1477 was co-transfected, together with the DHFR gene-coding plasmid pTB348, into (DHFR⁻)-CHO cells. Transformant cells stably expressing TXA₂-R were selected by a ligand binding assay using the TXA₂ mimic, [3 H]U-46619. The binding of [3 H]U-46619 to parent CHO cells was negligible (data not shown) but the TCHO-25 cells thus obtained bound the labeled compound in a saturable manner, with a K_d value of 3.1×10^{-8} M and a receptor number of approximately 3×10^5 mol/cell (Fig. 1A). A novel antagonist of spasmogenic prostanoids, AA-2414, competed for the specific binding of [3 H]U-46619 (1.25×10^{-8} M) to the TCHO-25 cells, exhibiting an IC₅₀ of 6.0×10^{-8} M (Fig. 1B). This suggests an antagonist activity of the compound to human TXA₂-R. AA-2414 inhibited the contraction of guinea pig tracheal strips induced by U-46619 (1.0×10^{-8} M). The IC₅₀ for the inhibition (4.0×10^{-8} M) was of the same order as that for the inhibition of [3 H]U-46619 binding. There seems to be a good correlation between the inhibition of specific binding to TCHO-25 cells and the inhibitory activity towards contraction of tracheal strips.

The TXA₂-R expressed in TCHO-25 cells is assumed to be identical to that in platelets and vascular tissues¹⁶⁾ although there has been some controversy over the

Fig. 1. A. Saturation of Isotherms of the Specific Binding of [3 H]U-46619 to Transformed CHO Cells, Expressing Human TXA₂-R

The results shown are representative of two experiments, each conducted in duplicate. Inset: Scatchard plot of the same data.

B. Competition of Specific [3 H]U-46619 Binding Carried out with Various Concentrations of AA-2414

A representative curve of two experiments each, conducted in duplicate, is shown.

presence of receptor subtypes.^{23,24)} Kattelman *et al.* investigated the binding of U-46619 to intact human platelets and its inhibition by some TXA₂-R antagonists²⁵⁾ while Imura *et al.* reported the inhibitory effect of AA-2414 on the specific binding of [3 H]U-46619 to washed guinea pig platelets.¹¹⁾ Platelets, however, are not ideal for such receptor-binding assays because of their fragility, tendency to aggregate and the complicated changes in the state of their TXA₂ receptors. For this reason, the TCHO-25 cells described here may be preferable for screening and characterizing selective TXA₂-R antagonists with accuracy and reproducibility.

In conclusion, the anti-asthmatic agent AA-2414 might be a potent antagonist of human TXA₂-R.

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