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The pulmonary pharmacology of [4-methoxy-N1-(4-trans-nitrooxycyclohexyl)-N3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide] (2NTX-99), an anti-atherotrombotic compound with therapeutic potential in pathological conditions that target lung vasculature

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ABSTRACT

The pharmacological activity of 2NTX-99 ([4-methoxy-N1-(4-trans-nitrooxycyclohexyl)-N3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide]) was investigated *in vitro* in the intact, rat pulmonary vasculature and in guinea pig airways. Rat lungs were perfused at constant flow and changes in vascular tone recorded. Challenge with the TXA_2 analogue 9,11-dideoxy-9α11α-methanoepoxy $ProstaglandinF_2$ (U46619, 0.5 μM) increased vessel tone (32.48 ± 1.5 *vs* 13.13 ± 0.56 mmHg; $n = 12$). 2NTX-99 (0.1–100 μM; $n = 5$), caused a concentration-dependent relaxation, prevented by 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 10 μM, $n = 4$), an inhibitor of soluble guanylate cyclase. Acetylcholine (0.1–10 μM; $n = 3$) and a reference NO-donor, isosorbide-5-mononitrate (5–100 μM; $n = 4$), were ineffective. Intraluminal perfusion of washed human platelets (2×10^8 cells/ml) increased intravascular pressure after challenge with arachidonic acid (AA, 2 μM; $n = 5$), an increase abolished by acetylsalicylic acid and significantly reduced by 2NTX-99 (40 μM; $n = 5$). TXB_2 in the lung perfusate was detected after platelet activation, 2NTX-99 inhibited TXA_2 synthesis (6.45 ± 0.6 and 1.10 ± 0.2 ng/ml, respectively). 2NTX-99 did not alter central or peripheral airway responsiveness to Histamine (0.001–300 μM; $n = 6$), U46619 (0.001–3 μM, $n = 3$) or LTD_4 (1 pM–1 μM; $n = 6$). 2NTX-99 vasodilates the pulmonary vasculature *via* the release of nitric oxide (NO) and reduces intraluminal, AA-induced, TXA_2 formation. The combined activity of 2NTX-99 as an NO-donor and a TXA_2-synthesis inhibitor provides strong support for its potential therapeutic use in pathologies of the pulmonary vascular bed (*e.g.* pulmonary hypertension).

1. Introduction

Thromboxane A_2 (TXA_2) is an eicosanoid generated from arachidonic acid (AA) by cyclooxygenase (COX) and thromboxane synthase (TXs) which exerts its prothrombotic and smooth muscle constrictor effects *via* the activation of specific G-protein coupled receptors (TP) [1]. Two isoforms of TP receptors have been described, TPα and TPβ, and their activation lead to increased cytoplasmatic production of IP_3 and subsequent increase in intracellular concentrations of Ca^{2+}, stimulating smooth muscle contraction and platelet activity. Due to its effects, this eicosanoid is the physiological antagonist of prostacyclin (PGI_2), which is produced predominantly by the endothelium, causing vasodilation and inhibition of platelet aggregation.

TXA_2, which is mainly produced by platelets, macrophages and lung parenchyma [2], is a potent agonist of platelet aggregation, smooth muscle constriction and bronchial hyper-responsiveness [3]. TXA_2 has been linked to the pathogenesis of asthma since it is a potent constrictor of bronchial smooth muscles and an activator of airway smooth muscle cells proliferation [4,5]. Moreover, TXA_2 synthesis is increased in the airways of patients suffering from asthma after antigen challenge and increased concentrations of the stable end-product of this eicosanoid (*e.g.* TXB_2) and its metabolites have been detected in bronchoalveolar lavage fluid and urine from asthmatic and COPD (Chronic Obstructive Pulmonary Disease) patients [6]. TP receptor activation also contributes to bronchial smooth muscle hyperplasia and airway remodelling which may occur in response to chronic airway inflammation, a hallmark of asthma.

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TXA_2 may also play an important role in the thromboembolic complications of COPD, including pulmonary hypertension. In fact, an increased release of the vasoconstrictor TXA_2 has been documented in these pathological conditions, suggesting that activation of platelets occurs in both the primary and secondary forms of pulmonary hypertension; by contrast, the release of prostacyclin (PGI_2) is depressed in these patients [7]. Moreover, the pathogenesis of mild to moderate pulmonary hypertension has been attributed to vascular and parenchymal remodeling effects and indeed activation of the thromboxane prostanoid (TP) receptor causes human airway smooth muscle cells proliferation, suggesting a role in airway remodeling [8].

Altogether, the important roles played by TXA_2 in cardiopulmonary diseases, make this bioactive lipid a potential therapeutic target for such pathologies.

Nitric oxide (NO) is a vasodilator and inhibitor of platelet aggregation and a lack of NO production by endothelium is considered one of the predisposing factors for thrombotic events [9]. Moreover NO has been shown to inhibit release of histamine in mast-cells [10] as well as to suppress synthesis of inflammatory mediators in airway macrophages [11,12] supporting the notion that endogenous NO may act as a protective anti-inflammatory autacoid in the airways.

2NTX-99 ([4-methoxy-N1-(4-trans-nitrooxycyclohexyl)-N3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide]) is a new antiatherothrombotic agent [13] combining, within a single molecular entity, NO-donor properties with the inhibition of TXA_2 synthesis. 2NTX-99 is a powerful vasorelaxant as well as an inhibitor of human platelet aggregation, showing a TXA_2 synthetase inhibitory activity equipotent with ozagrel.

In the present work we investigate in detail the pharmacology of 2NTX-99 in the rat intact pulmonary vascular bed as well as in different models of central and peripheral airways (trachea and lung parenchyma) from male guinea-pig. Our results strengthen the therapeutic potential of 2NTX-99 in afflictions that target the pulmonary vessels.

2. Material and methods

The *in vitro* preparations of guinea-pig airways and rat lung vasculature were approved by the local Institutional Animal Care and Use Committee. Animal housing at the Department of Pharmacological Sciences complies with the Italian law, N. 116, January 27 1992, recipient of the EU directive 86/609 for the safety and protection of experimental animals.

The use of human platelets has been approved by the ethical committee of the Department of Pharmacological Sciences. Platelets, isolated from daily withdrawn buffy-coat, have been supplied by the Centro Trasfusionale (Blood Bank), Ospedale Policlinico of Milan.

2.1. In vitro preparations of guinea-pig trachea and parenchymal lung

Guinea-pig tracheas or lung parenchymal strips were isolated and set up for isometric recording of smooth muscle tone as previously described [14]. Briefly, tissues were set up in a 5 ml organ bath chamber, perfused with an oxygenated Tyrode solution (mM: NaCl 118, KCl 4.69, $MgSO_4$ 1.05, NaH_2PO_4 0.42, Glucose 5.60, $NaHCO_3$ 11.5 and $CaCl_2$ 1.80; pH 7.4) at 37 °C, and connected to isometric force transducers for tension recording (PowerLab, ADInstruments, UK).

Tracheal and parenchymal sections were stabilized 1 h at a resting tension of 1 g, then a standard reference contraction of smooth muscle was evoked using a maximally-active concentration of acetylcholine (Ach, 100 μM). After wash-out of the preparations and recovery of the basal smooth muscle tone, concentration-response curves to Histamine (H; 0.1–300 μM), compound U46619 (a stable synthetic analogue of TXA_2; 0.001–3 μM; 9,11-dideoxy-9α11α-methanoepoxy Prostaglandin$F_2\alpha$) or Leukotriene D_4 (LTD_4, only in experiments with parenchymal strips, 1 pM–0.1 μM) were evoked, in the absence or presence of different compounds or their solvents (*e.g.* DMSO, 0.1% v:v; 2NTX-99, 10 μM; Pyrilamine, 10 μM; CGP-57698 10 μM (4-[3-(7-fluoro-2-quinolinyl-methoxy)phenyl-amino]-2,2-diethyl-4-oxo-butanoic acid); Indomethacin 10 μM; IsoSorbide MonoNitrate (ISMN, 10 μM; [(3S,3aR,6R,6aS)-3-hydroxy-2,3,3a,5,6,6a-hexahydrofuro[3,2-b]furan-6-yl]nitrate).

2.2. Vascular reactivity of in vitro isolated and perfused rat lung

Intact rat lung was isolated and set up for perfusion (constant flow) of its vascular bed and recording of resistance to perfusion pressure. Briefly, animals were sacrificed with an *i.p.* injection of thiopenthal sodium (20 mg/kg), and heart and lungs were quickly removed and kept in cold Krebs–Henseleit solution (mM: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.1, $CaCl_2$ 2.5, Glucose 5.5 and $NaHCO_3$ 25; pH 7.4); the major parts of the ventricles were removed and the pulmonary artery cannulated (G25 catheter, 0.5 mm outer diameter) and connected, *via* one arm of a T shaped catheter, to a peristaltic pump, whereas the other arm was linked to a pressure transducer (Gould P23 ID). The preparation was set up in a suitable organ bath and the infusion rate of the oxygenated Krebs–Henseleit solution at 37 °C was set constant at 4 ml/min.

The mean pulmonary arterial pressure (mmHg) was measured and recorded *via* the pressure transducer connected to a computer-based data acquisition system (Power Lab, ADInstrument, UK).

During the stabilization period (30 min) the resistance opposed by the lung vasculature to perfusion pressure (defined as "resting pressure") stabilized at 13 ± 0.5 mm Hg (n = 12, mean ± S.E.M.), and kept constant throughout perfusion, in line with previously published data [15]. After equilibration, an intra-arterial infusion using a TP receptor agonist, compound U46619 (0.5 μM) was used to increase the vessel tone (32.5 ± 1.5 mm Hg, mean ± S.E.M.) which kept constant as long as U46619 was present; the increase in resistance was promptly reversible upon washout of the preparation with normal buffer. Since pulmonary arterial perfusion flow was constant, a change in arterial pressure reflected directly a change in pulmonary vascular resistance.

A concentration-response curve to 2NTX-99 (0.1–100 μM) was obtained in the presence of a stable plateau of vasoconstriction induced by U46619 0.5 μM; similarly, concentration-response curves to Isosorbide MonoNitrate (ISMN, 5–100 μM) and acetylcholine (Ach, 0.1–10 μM) were also evoked. In some experiments, concentration-response curves of 2NTX-99 were obtained after pre-treating the lung vasculature with compound ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one), an inhibitor of soluble guanylate cyclase (10 μM, 15 min).

2.3. Platelets perfusion

The isolated rat lung preparation was also utilized for intravascular perfusion with human washed platelets [16], in order to mimic functional alterations of the pulmonary vascular bed that may occur when an intravascular thrombus is formed. Before perfusion into the rat pulmonary vascular bed, an aliquot of platelets was analyzed using the Born turbidimetric technique in a dual-channel Chrono-Log aggregometer (Mascia Brunelli, Milan, Italy); platelets aggregation was induced by arachidonic acid (AA, 2 μM), in the presence and in the absence of 2NTX-99 (data not shown). Only platelet preparations that in response to AA granted complete