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Comparison of inhibition of ovalbumin-induced bronchoconstriction in guinea pigs and *in vitro* inhibition of tumor necrosis factor-α formation with phosphodiesterase 4 (PDE4) selective inhibitors

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Abstract

Phosphodiesterase 4 (PDE4) inhibitors elevate cyclic adenosine 5′-monophosphate (cAMP), and this elevation has been shown to inhibit inflammatory cytokines such as tumor necrosis factor-α (TNF-α). Using TNF-α as a biomarker, we have developed transcription-based assays to examine inhibition of PDE4 activity in human and guinea pig whole blood. *In vitro* inhibition by PDE4 inhibitors was measured using quantitative PCR (qPCR) analysis of TNF-α mRNA levels in whole blood stimulated with lipopolysaccharide (LPS). The kinetics of human TNF-α mRNA production were analyzed and shown to be highest 4 hr following LPS stimulation. The guinea pig displayed kinetics of TNF-α transcription similar to those of the human. Analysis of inhibition of human TNF-α protein production was performed by immunoassay and shown to correlate with inhibition of transcription for three of the four compounds tested. Roflumilast was found to be 9-fold more potent for TNF-α inhibition in the qPCR assay than in the protein assay. The potencies of L-826,141 and roflumilast were determined in human and guinea pig whole blood by qPCR, with IC_{50} values of 270 and 20 nM, respectively, in humans and 100 and 10 nM, respectively, in guinea pigs. These results show that the potency of PDE4 inhibitors can be monitored in whole blood using a transcription-based assay, and that this type of assay can be adapted to various species provided the TNF-α nucleotide sequence is known. The *in vitro* whole blood IC_{50} for TNF-α inhibition was compared to inhibition in the ovalbumin-challenged guinea pig model of bronchoconstriction. Obtaining plasma levels at the IC_{50} determined *in vitro* for L-826,141 and roflumilast provides significant inhibition of bronchoconstriction. This suggests that TNF-α can be used as a whole blood biomarker in the guinea pig for PDE4 inhibition in this inflammatory model. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Phosphodiesterase 4; TNF; qPCR; Asthma; Guinea pig; Bronchoconstriction

1. Introduction

Levels of intracellular cAMP are regulated by the activity of PDEs, a family of enzymes with at least eleven distinct members, which catalyze the hydrolysis of cAMP and/or cGMP [1]. The cAMP-specific PDE4 is the PDE subfamily that is expressed predominantly in inflammatory cells, including mast cells, macrophages, monocytes, eosinophils, and neutrophils [2]. Specific inhibition of PDE4 activity leads to increased intracellular cAMP levels resulting in decreased expression and release of several pro-inflammatory mediators by activated macrophages/monocytes [3,4]. Other effects of increased intracellular cAMP include prevention of superoxide anion formation in neutrophils, decreased chemotaxis of immune cells, inhibition of eosinophil degranulation, and down-regulation of lymphocyte proliferation [5–8]. The main focus in generating PDE4 inhibitors has been to down-regulate the production by activated monocytes of cytokines shown to be of key importance to the inflammatory process, most notably TNF-α, IL-1β, IL-6, and IL-8 [9,10].

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Abbreviations: cAMP, cyclic adenosine 5′-monophosphate; LPS, lipopolysaccharide; PDE4, phosphodiesterase 4; TNF, tumor necrosis factor; IL, interleukin; RT–PCR, reverse transcription–polymerase chain reaction; qPCR, quantitative PCR.

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The importance of TNF-α in promoting inflammation has resulted in a large effort to understand the role of this pleiotropic cytokine in the pathogenesis of a number of illnesses. TNF-α has been implicated in acute syndromes associated with endotoxic shock and bacterial sepsis, as well as in chronic inflammatory conditions such as rheumatoid arthritis, multiple sclerosis, and allergy-induced asthma [11,12]. TNF-α is produced predominantly by activated macrophages and monocytic cell lines, but other cell populations shown to be capable of secreting this cytokine include natural killer (NK) cells, $CD4^+$ T-lymphocyte populations, and, in some cases, tumor cell lines [13]. Induction of TNF-α production can result from many external stimuli, including a very strong response to LPS [14]. TNF-α contributes to the immune response against infection by activating neutrophils and platelets, enhancing the killing activity of macrophages and NK cells, and stimulating the immune system. The central role of TNF-α as a pro-inflammatory mediator makes it a suitable marker for the efficacy of novel anti-inflammatory agents.

Evidence that increased intracellular cAMP inhibits the production of TNF-α following LPS stimulation has been obtained for several monocytic cell types [15–19]. Observed decreases in TNF-α mRNA indicate that transcription is being inhibited [20], while other studies have shown that the 3′-untranslated region of the TNF-α mRNA is important in translational control [21,22]. Increases in intracellular cAMP resulting from PDE inhibition have been shown to inhibit production of TNF-α by the murine monocytic cell line RAW 264.7, primarily at the level of transcription [22]. Others have shown that post-transcriptional events are relatively more important in determining the quantity of TNF-α produced by macrophages from different mouse strains [23]. A later report showed that LPS-stimulated induction of TNF-α expression in the RAW 264.7 cell line occurs primarily at the level of transcription, with relatively minor effects on translation of the mRNA [24]. Quantitative measurement of endogenous levels of TNF-α mRNA could, therefore, provide a method of analyzing the relative inhibition of PDE4 activity in LPS-stimulated cells. The emergence of real-time PCR technology has provided a means of quantifying endogenous levels of specific mRNA messages more efficiently than by traditional northern blotting, making it possible to develop rapid and quantitative transcription-based assays.

Assessment of the biological activity in whole blood can provide invaluable information on the behavior of PDE4 inhibitors *in vivo* [1]. Murine *in vivo* and canine *ex vivo* models for determining the therapeutic index of PDE4 inhibitors through measurement of TNF-α protein have been developed [25,26]. A human whole blood assay for determining PDE4 inhibition using TNF-α protein as a marker has also been described [27]; however, the difficulties inherent in quantifying specific proteins from whole blood of different species underlie the need for alternative methods of measuring the expression of cytokines at the mRNA level. The development of a protein-based whole blood assay for cytokine detection also depends heavily on the availability of specific antibodies to the protein of interest or the ability to generate them. Low amounts of a given protein in the blood of various species make detection difficult due to limitations in the sensitivity of immunoassays. The high sensitivity of RT–PCR techniques allows the detection of very low levels of mRNA, and the recognition of small variations in these levels. It is imperative, however, that inhibition of transcription accurately represents the ultimate expression of the gene to be analyzed. In this paper, we report the development of a transcription-based assay for measuring PDE4-mediated inhibition of LPS-stimulated TNF-α production in human and guinea pig whole blood. The inhibition of TNF-α mRNA formation in the guinea pig is evaluated as a biomarker for PDE4 inhibition by comparison with *in vivo* efficacy in a guinea pig model of airway resistance.

2. Materials and methods

2.1. Human whole blood assay

2.1.1. Effect of L-826,141 on expression of various cytokine genes induced by LPS

Human whole blood was collected in vacutainers containing heparin. Blood was then incubated for 15 min at 37° with either 10 μM L-826,141 or 0.4% DMSO. LPS (serotype at 10 μg/mL in 0.1% BSA-PBS; Sigma) was added to the appropriate samples, and all samples were incubated for 4 hr at 37° in a humidified incubator supplemented with 5% CO_2. Total RNA was isolated using the RNeasy Blood Mini kit (Qiagen Inc.) as per the recommendations of the manufacturer. To perform the RT–PCR reactions, the GeneAmp RNA PCR kit (Roche Molecular Systems Inc.), 1 μg total RNA, and random hexamers supplied from the kit were used as per the recommendations of the manufacturer. Control reactions were carried out in the absence of RT. The GeneAmp PCR System 9600 (Roche) was used, and incubations were carried out as specified by the manufacturer, with the exception that the initial incubation at 42° proceeded for 1 hr.

The resultant cDNAs were utilized as templates to amplify various cytokines using the CytoXpress Multiplex-PCR kit (human sepsis-related cytokines, set 2) from BioSource International according to the instructions of the manufacturer. The reactions were analyzed by gel electrophoresis using a 2% agarose gel.

2.2. Blood collection and treatment

Fresh blood was collected in heparinized tubes by venipuncture from both male and female human volunteers

with consent. Fresh blood was collected in tubes containing either 0.13 M sodium citrate or EDTA (dipotassium EDTA, 13.8% (w/v), approximately 10 μL/5 mL of whole blood) and by cardiac puncture from isofluorane-anaesthetized male Hartley guinea pigs. Human subjects had no apparent inflammatory conditions and had not taken any nonsteroidal anti-inflammatory drugs (NSAIDs) for at least 4 days prior to blood collection. Blood was treated as previously described [27]. Briefly, 200- to 500-μL aliquots of blood were preincubated with 0.5 to 2 μL vehicle (DMSO or 50% ethanol) or PDE4 inhibitor at 37° for 15 or 30 min. This was followed by incubation of the blood for 0.5 to 7 hr (or as indicated) at 37° with 5–10 μL LPS (Sigma; from *Escherichia coli* serotype 0111:B4) at 1 or 100 μg/mL final concentration, dissolved in a 0.1% bovine serum albumin fraction V (BSA) solution (Sigma), diluted in PBS. The PDE4 inhibitors tested were synthesized by the Medicinal Chemistry Department at Merck Frosst.

2.3. Human plasma TNF-α protein quantification

After the appropriate incubation period, the blood was centrifuged at 1500 *g* for 10 min at 4°, and the plasma TNF-α protein was quantified by ELISA (Cistron Biotechnology) according to the instructions of the manufacturer.

2.4. Isolation of white blood cells and RNA extraction

The RNeasy Blood Mini kit and RNeasy 96 kit from Qiagen Inc. were used to isolate human total RNA from isolated white blood cells, according to the instructions of the manufacturer. For heparinized-treated guinea pig blood, 150 μL of 6% Dextran T500 (Pharmacia Biotech AB) diluted in PBS was added to the samples, which were then vortexed, inverted, and incubated for 15 min at room temperature. Supernatants and a small amount of buffy coat interface were collected in 1.5-mL microfuge tubes and centrifuged at 400 *g* for 6 min at 4°. Pellets (still containing some RBC) were resuspended in 400–500 μL of EL buffer (erythrocyte lysis buffer from RNeasy Blood Mini kit, Qiagen Inc.) and incubated on ice for 10 min. For EDTA-treated guinea pig blood, samples were centrifuged at 400 *g* for 6 min at room temperature. Approximately 200 μL of the supernatant was removed, while the remaining supernatant with buffy coat was transferred to a 1.5-mL microfuge tube. Five hundred microliters of EL buffer was added to each tube, and the contents of the tubes were mixed and incubated on ice for 10–20 min (or until the solution was translucent or RBC visibly lysed). The samples from both heparin and EDTA treatments were then spun at 400 *g* for 6 min at 4°. WBC pellets were resuspended in 350 μL of RLT buffer (RNeasy Blood Mini kit) containing β-mercaptoethanol at a concentration of 10 μL/mL of RLT. Total RNA was then isolated as above using the RNeasy 96 kit.

2.5. Quantitative PCR

RT was performed using the GeneAmp RNA PCR kit (Perkin Elmer Biosystems, Roche Biochemicals), according to the instructions of the manufacturer, using approximately 25–100 ng total RNA per 20 μL reaction, and increasing the 42° incubation time to 25 min. Quantitative PCR was performed using pre-developed assay reagents for the human GAPDH, human TNF-α, and ribosomal 18S genes, and the ABI 7700 Sequence Detection System (PE Applied Biosystems), according to the instructions of the manufacturer. Quantitative PCR also was performed with custom-made primers and probes for the human GAPDH, human TNF-α, guinea pig GAPDH, guinea pig TNF-α, and the LightCycler Instrument (Roche Diagnostics). The primers and probes used were [5′ to 3′, forward primer, reverse primer, LightCycler probe 1 (3′ Fluorescein), LightCycler probe 2 (5′ LC-Red640, 3′ phosphate)] as follows:

human GAPDH:	GCGCCTGGTCACCAGGGCTGC, TACCAAAGTTGTCATGGATGACCTT, GTCCACTGGGTCTTCACCACC, TGGAGAAGGCTGGGGTCATTTG;
human TNF-α:	CACGCTCTTCTGCCTGCTGCAC, TTGGCCAGGAGGGCATTGGC, GTCACTCGGGGTTCGAGAAGATGA, CTGACTGCCTGGGCCAGAGGGCTG;
guinea pig GAPDH:	CGTGGAAGGACTCATGACCACA, GGGTGTCGCTGTTGAAGTCACA, GTATGGCCTTCCGTGTACCCACACCT, TGTGTCGGTTGTGGATCTGACCTG;
guinea pig TNF-α:	TCAGCCTCTTCTCCTTCCTGCTGGTGGCA or CACGCTCTTCTGCCTGCTGCAC, GGACCTGGGAGTAGATGAGGTACAGCCCAT, TGCTAACGCCCTCCTGGCCAATGGC, TGGGCCTGAGCGACAACCAGCTGGTG.

LightCycler probes were synthesized by GensetOligos (Genset). The LightCycler amplification reactions were performed using the LightCycler DNA Master Hybridization Probes kit (Roche Diagnostics) according to the instructions of the manufacturer with 5 mM $MgCl_2$, 0.5 μM primers, 0.2 μM LightCycler probe 1, and 0.4 μM LightCycler probe 2 as final concentrations. Amplification conditions were 30 s at 95° followed by cycling for 1 s at 95°, 15 s at 60°, and 20 s at 72°. Data analysis was performed using the LightCycler Software version 3 (Roche Diagnostics) using threshold crossing point (Ct) values determined by fit point analysis.

2.6. Measurement of bronchoconstriction in ovalbumin-challenged guinea pigs

All procedures used in the *in vivo* assays were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research according to guidelines established by the Canadian Council on Animal Care.

Male Hartley guinea pigs were sensitized by i.p. injection of ovalbumin [0.5 mL of a solution of 100 μg/mL in 10% $Al(OH)_3$]. An additional 0.5 mL of the ovalbumin solution was injected s.c. in the proximity of the lymph nodes (neck, axilla, and inguinal regions). Both injections were administered on the same day, 4–6 weeks prior to the experiment. On the day of the experiment, animals were treated with mepyramine (5 mg/kg, i.p., to protect them from anaphylactic shock) and the PDE4 inhibitor under study or the vehicle (PEG200/saline, 1:1). Each guinea pig was then placed individually in a whole body plethysmographic chamber that allowed the animal to move freely without stress. Thirty minutes later, the animals were exposed to an aerosol of ovalbumin (10 mg/mL in saline) for 1 min. Changes in pulmonary parameters (Penh, enhanced pause) were recorded for 1 hr using a Buxco pulmonary mechanics analyzer model XA. Results were calculated as percent inhibition, comparing the area under the curve (AUC) of the treated animal to the average AUC of the control group. The results represent the means of at least 3 animals at each of the doses tested.

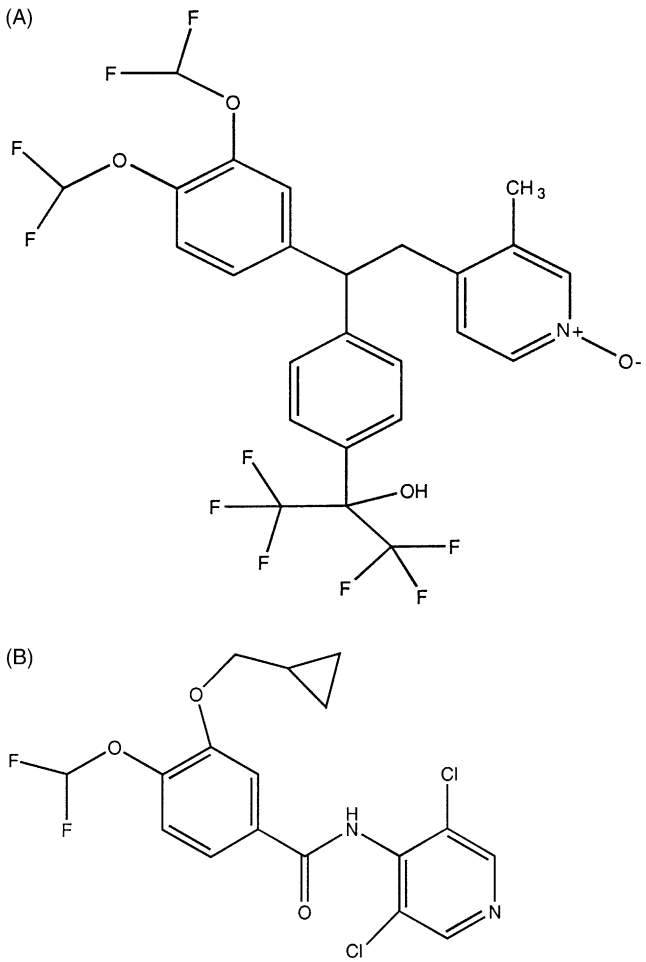


Fig. 1. (A) Chemical structures of L-826,141 (4-{2-(3,4-bis-difluoromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-ethyl}-3-methylpyridine-1-oxide), and (B) roflumilast (3-(cyclopropylmethoxy)-*N*-(3,5-dichloropyridin-4-yl)-4-difluoromethoxy benzamide).

3. Results

3.1. Effect of L-826,141 on LPS-stimulated human cytokine gene induction

PDE4 inhibitors elevate cAMP, and this elevation in cAMP has been shown to inhibit the production of TNF-α in whole blood. This study has focused on the use of qPCR analysis to determine if the inhibition of TNF-α can be monitored at the level of transcription for PDE4 inhibitors. To investigate the validity of using TNF-α transcription as a biomarker for PDE4 activity, we tested the effects of a known specific PDE4 inhibitor, L-826,141 (Fig. 1), on the transcription of a number of cytokines. L-826,141 has been shown to inhibit purified PDE 4A, B, C, and D with IC_{50} values of 0.2 to 2.5 nM (Huang *et al.*, unpublished data; cited with permission). When these compounds are tested in whole blood which contains a significant amount of plasma protein, inhibition of PDE4 is decreased, and compounds are shifted 50- to 500-fold in potency. We have incubated human whole blood in the presence or absence of L-826,141 for 15 min prior to challenge with or without 1 μg/mL of LPS. Four hours post-LPS challenge, cells were harvested from the whole blood samples for RNA analysis. Human GAPDH (quantitative control), TNF-α, IL-1β, IL-12, IL-6, IL-8, and IL-10 sequences were amplified from human total RNA by Multiplex PCR and subsequently visualized by agarose gel electrophoresis (Fig. 2). Compared with unstimulated samples, LPS-treated samples showed significant increases in all of the cytokines tested. The GAPDH levels remained constant in all three treatments, indicating that similar amounts of PCR-amplified products were loaded onto the gel. In the presence of L-826,141, the levels of TNF-α and IL-12 were the only cytokines that displayed a significant decrease in signal, indicating that these would be the most suitable markers of PDE4 activity. Since human TNF-α is easily detectable by immunoassay, we chose to follow this cytokine by qPCR and validate the assay by correlation to the inhibition of TNF-α formation at the protein level.

3.2. Time–course of TNF-α formation in human whole blood

In this experiment, we analyzed the production of TNF-α mRNA and protein following LPS stimulation of human whole blood *in vitro* as well as the effects of preincubating

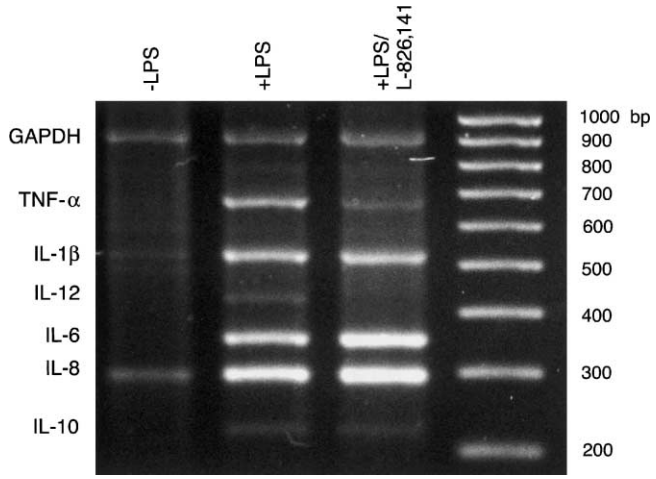


Fig. 2. Human whole blood assay. Effect of L-826,141 on the expression of various cytokine genes induced by LPS. PCR fragments were obtained using the human-specific Multiplex PCR primer mix (sepsis set 2), and cDNAs generated from human whole blood were subjected to various treatments; 15 μL of each 100-μL reaction was separated on a 2% agarose gel. PCR fragments of the expected sizes were obtained and are as follows: GAPDH, 921 bp; TNF-α, 680 bp; IL-1β, 556 bp; IL-12, 432 bp; IL-6, 359 bp; IL-8, 300 bp; and IL-10, 223 bp.

the blood with the PDE4 inhibitor compound L-826,141. The relative amount of human TNF-α mRNA increased approximately 7- to 8-fold in blood treated with 1 μg/mL of LPS at 2 to 4.5 hr post-LPS challenge when compared with 0.5 hr (Fig. 3A). By 6.5 hr, the relative TNF-α mRNA level dropped down to approximately 3-fold when compared with 0.5 hr post-LPS challenge. In the absence of LPS stimulation, no significant induction of TNF-α mRNA was detected. When blood was preincubated with 10 μM L-826,141 and then challenged with 1 μg/mL of LPS, the human TNF-α mRNA induction was inhibited significantly (Fig. 3A). A similar experiment was performed except that human TNF-α protein in the plasma was measured. As demonstrated for the TNF-α mRNA, TNF-α protein was induced in blood treated with 1 μg/mL of LPS but was undetectable in blood treated with the 0.1% BSA vehicle (Fig. 3B). Induction of the TNF-α protein occurred beginning at 2 hr post-LPS challenge and reached 9.3 ng/mL at 6.5 hr post-LPS challenge. If the LPS challenge was performed in the presence of 10 μM L-826,141, significant inhibition of TNF-α formation was obtained (Fig. 3B). Based on these results and previously published methods [3,5], a 4-hr incubation at 37° following LPS stimulation was used in subsequent human blood assays prior to isolation of total RNA from the samples.

3.3. Potency of inhibition of PDE4 inhibitors on TNF-α mRNA and protein formation

Human whole blood samples were preincubated with different concentrations of the PDE4 inhibitors L-826,141 and roflumilast [28] prior to LPS stimulation to investigate the potency of these compounds for inhibition of TNF-α

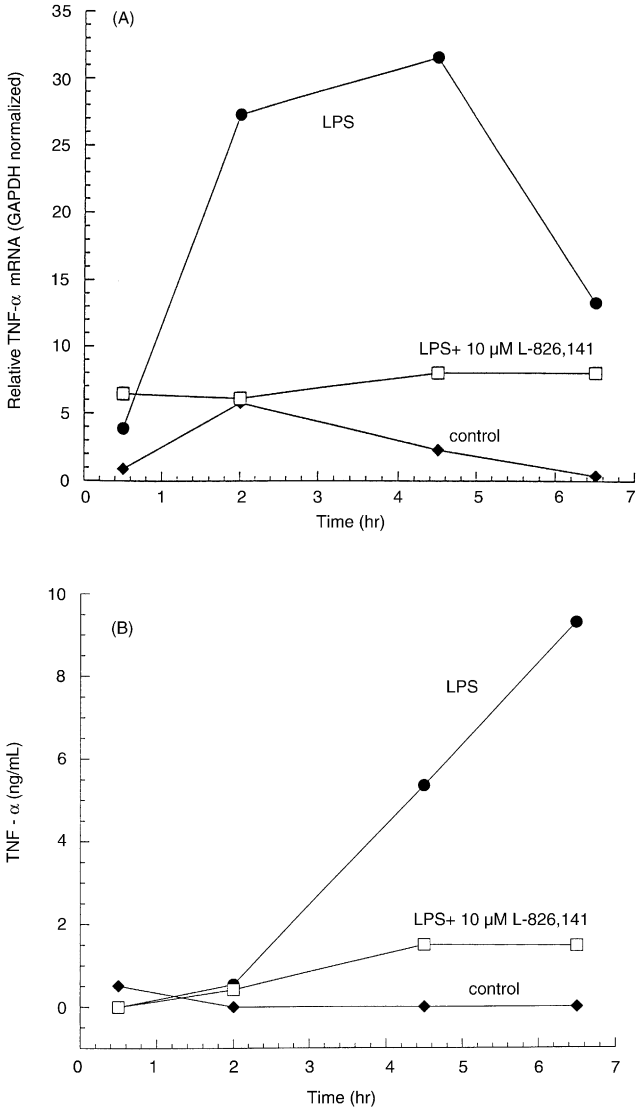


Fig. 3. Effect of L-826,141 on LPS-induced human TNF-α production: RNA vs. protein. (A) Aliquots (500 μL) of whole blood were preincubated with 10 μM L-826,141 or vehicle (DMSO) for 30 min (see Section 2). Samples were then treated with either 1 μg/mL of LPS or 0.1% BSA from 0.5 to 6.5 hr, and the relative human TNF-α mRNA (human GAPDH normalized) was determined. The graph illustrates representative results from experiments done at least 3 times in duplicate. (B) Secreted human TNF-α protein was measured in the plasma of the same blood samples as in panel A.

formation. After incubation for 4 hr at 37°, the amounts of TNF-α mRNA were determined and used to construct inhibition curves. L-826,141 had an IC_{50} of 270 ± 80 nM ($N = 4$) (Fig. 4A) and roflumilast (Fig. 1) an IC_{50} of 20 ± 10 nM ($N = 4$) (Fig. 4B) for inhibition of TNF-α formation. Percent inhibition values less than zero simply indicate that these samples showed a slightly higher signal (more TNF-α message) than vehicle-pretreated controls.

The inhibition of human TNF-α protein production was performed as previously described [27]. Roflumilast inhibited TNF-α formation with an IC_{50} of 183 ± 33 nM ($N = 15$) and L-826,141 with an IC_{50} of 280 ± 33 nM

(N = 21) (Fig. 4C). These results demonstrate that the inhibition of PDE4 by L-826,141 can be measured by monitoring the expression of TNF-α at either the mRNA or the protein level since the results obtained by both methods correlate well. The inhibition by roflumilast was 9-fold more potent at the mRNA level than that measured at the protein level. *R*-Rolipram and CT-2450 [29] also were tested and found to inhibit TNF-α mRNA with IC_{50} values of 0.85 ± 0.23 (N = 3) and 2.0 ± 0.2 μM (N = 4), respectively, and TNF-α protein with IC_{50} values of 2.2 ± 0.8 (N = 15) and 3.2 ± 0.2 μM (N = 97), respectively (Table 1). Based on these results, qPCR analysis of TNF-α mRNA levels provides an alternative to protein-based determination of TNF-α production as a marker for inhibition of PDE4 activity in human whole blood.

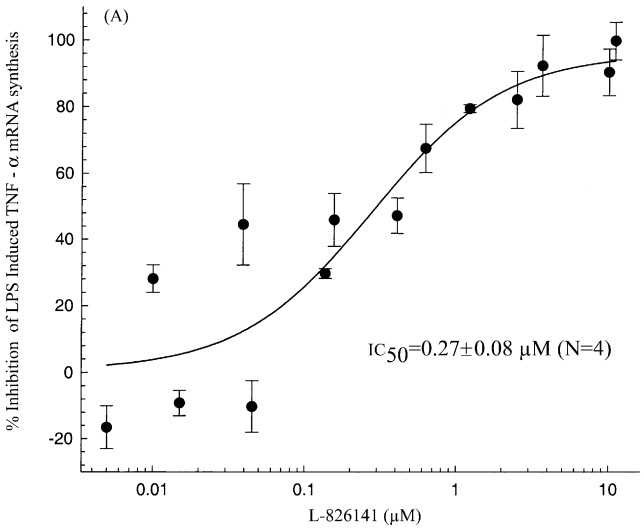
3.4. Development of qPCR assays for measurement of TNF-α in guinea pig whole blood

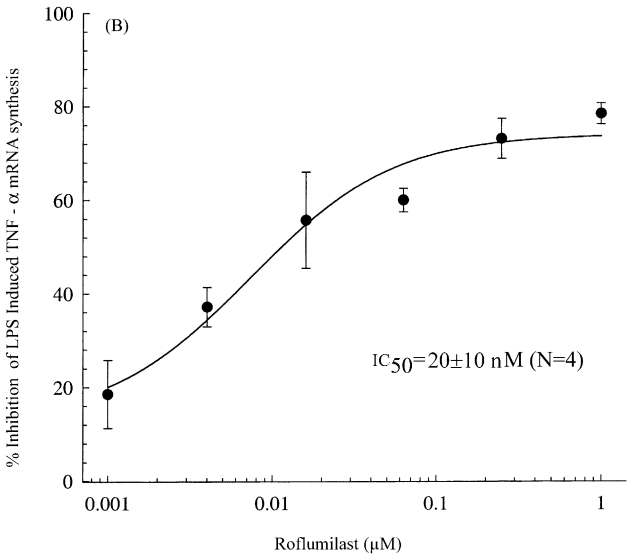
The rationale for developing transcription-based assays is based primarily upon their role as an alternative method in animal models for which development of a protein-based assay is inconvenient and/or impractical. In the case of PDE4 inhibition, one animal model used extensively for *in vivo* studies is the guinea pig. A time–course study was performed for the analysis of guinea pig TNF-α mRNA production in guinea pig whole blood. The guinea pig displayed kinetics of TNF-α induction following LPS stimulation similar to those of the human. Following LPS stimulation, guinea pig TNF-α mRNA levels remained fairly low for the first 2 hr before showing a

Table 1
Inhibition of TNF-α in LPS-treated human whole blood

Compound	IC_{50} (μM)	
	qPCR	Protein-ELISA
Roflumilast	0.02 ± 0.01 (N = 4)	0.18 ± 0.03 (N = 15)
L-826,141	0.27 ± 0.08 (N = 4)	0.28 ± 0.03 (N = 21)
Rolipram	0.85 ± 0.23 (N = 3)	2.2 ± 0.8 (N = 15)
CT-2450	2.0 ± 0.2 (N = 4)	3.2 ± 0.2 (N = 97)

Aliquots 200 μL of human blood, preincubated for 15–30 min with various concentrations of the compounds listed, were treated with 1 μg/mL of LPS (see Section 2) for 4 hr. Relative human TNF-α mRNA levels (18S normalized) were determined, and percent inhibition was calcu lated using vehicle preincubated blood treated with either 1 μg/mL of LPS or 0.1% BSA as maximum and minimum values, respectively. The data in the table represents means ± SEM (N represents the number of experiments performed).





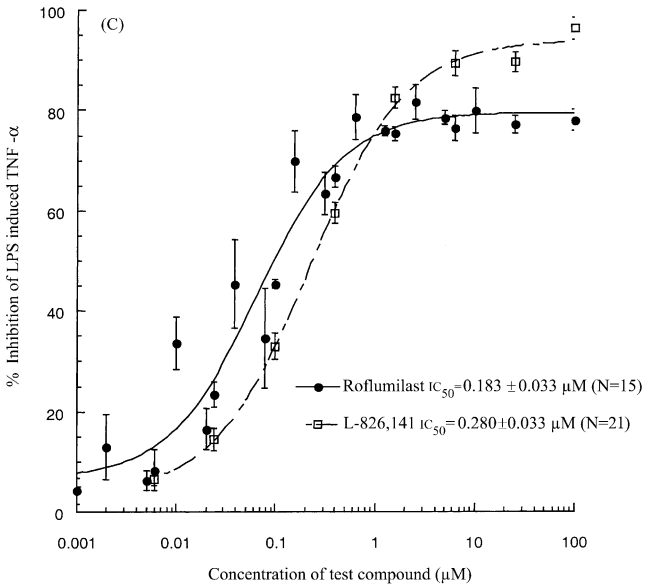


Fig. 4. Concentration-dependent inhibition of TNF-α at the mRNA and protein level in human whole blood. Aliquots (200 μL) of human blood, preincubated with various concentrations of L-826,141 (A) or roflumilast (B) for 15–30 min, were treated with 1 μg/mL of LPS (see Section 2) for 4 hr. Relative human TNF-α mRNA levels (18S normalized) were determined, and percent inhibition was calculated using vehicle-preincubated blood treated with either 1 μg/mL of LPS or 0.1% BSA to obtain maximum and minimum values, respectively. Values are means ± SEM. (C) Assay to determine inhibition of TNF-α formation at the protein level, performed as described in Brideau *et al.* [27].

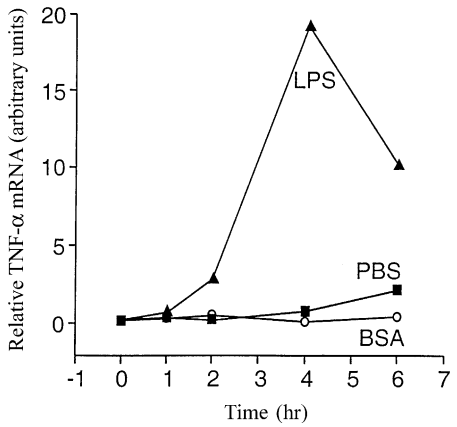


Fig. 5. Time–course of TNF-α mRNA formation in guinea pig whole blood. Aliquots (500 μL) of whole blood were treated with either 1 μg/mL of LPS or 0.1% BSA from 0 to 6 hr, and the relative guinea pig TNF-α mRNA (18S normalized) was determined. Values are the averages of two determinations.

sharp increase in transcription and a peak at 4 hr (Fig. 5). After reaching a maximum level of approximately 20-fold at the 4-hr mark, TNF-α mRNA levels began to fall off sharply, dropping to 10-fold induction at 6 hr post-stimulation.

As in the human whole blood qPCR assay described above, the effects of PDE4 inhibition on TNF-α mRNA expression were investigated in guinea pigs by preincubating whole blood from this species with different concentrations of L-826,141 or roflumilast before LPS stimulation. The IC_{50} for inhibition of TNF-α mRNA formation in guinea pig whole blood was 0.08 ± 0.01 μM ($N = 18$) for L-826,141 and 0.01 ± 0.003 μM ($N = 5$) for roflumilast (Table 2). The next step was to correlate this potency with *in vivo* potency for inhibition of bronchoconstriction in the guinea pig.

3.5. In vivo inhibition of bronchoconstriction

Inhibition of TNF-α mRNA expression by PDE4 compounds in an *in vitro* whole blood experiment as described above could provide a surrogate marker for inhibition *in vivo*. Therefore, we used an *in vivo* model of bronchoconstriction in order to assess the efficacy of PDE4 inhibitors and compare this with the *in vitro* potency of these compounds. This model utilizes sensitization with ovalbumin and measurement of airway resistance using a plethysmograph and Penh measurement. A representative time–course of ovalbumin challenge is depicted; the increase in Penh detected signifies a significant increase in lung resistance (Fig. 6). The PDE4 inhibitor was given 30 min prior to ovalbumin challenge, and inhibition was determined as percent inhibition of the area under the curve, using Penh measurement. Using this model, significant inhibition of lung resistance was achieved with a single oral dose of roflumilast (0.01 mg/kg) or L-826,141 (1 mg/kg). The plasma levels of these compounds were determined, and maximal efficacy was achieved at a concentration of 0.012 μM for roflumilast and at 0.15 μM for L-826,141 (Table 2). If we compare the plasma levels and efficacy *in vivo* with the IC_{50} *in vitro* in whole blood, it is striking that by achieving plasma levels that were within 2-fold of the *in vitro* IC_{50} for these compounds, maximal efficacy was obtained in a guinea pig model of lung resistance.

4. Discussion

In this study, we describe the establishment of a transcription-based assay for determining PDE4 inhibitor potency in human and guinea pig whole blood. It has been demonstrated, mostly through mRNA analysis, that the PDE4A, B, and D isoforms are present in human white blood cells, while PDE4C is undetectable [8]. We have also been able to use RT–PCR techniques to detect: PDE4A4, PDE4B2, PDE4D1, D2, and occasionally D3 mRNAs in human whole blood and in purified white blood cells, although the isoform profile varied according to which inflammatory cell types were purified. L-826,141 is an equipotent inhibitor of phosphodiesterase activity on purified recombinant human PDE4A, B, C, and D. Therefore, at relevant doses, L-826,141 should inhibit the majority of PDE4 activity in inflammatory cells from whole blood.

LPS stimulation elicits a very strong immunologic response, inducing the expression of a number of genes,

Table 2
Comparison of *in vitro* inhibition of TNF-α mRNA formation with *in vivo* inhibition of bronchoconstriction

Compound	Dose (mg/kg)	Efficacy (% inh. broncho.)	Plasma concentration (μM)	qPCR WBA IC_{50} (μM)
Roflumilast	0.001	27	ND	0.01 ± 0.003 ($N = 5$)
	0.003	65	ND	
	0.01	91	0.012	
	0.03	81	0.042	
L-826,141	0.1	−9	0.018	0.08 ± 0.01 ($N = 18$)
	0.3	40	0.041	
	1	82	0.15	

The potency of roflumilast and L-826,141 in the ovalbumin-challenged guinea pig model is compared to the potency for inhibition in guinea pig whole blood. The compounds were pre-dosed for 30 min prior to ovalbumin challenge. Plasma levels at the various doses of compounds tested were obtained at 30 min post-dosing. The IC_{50} values in the table represent means ± SEM. ND: not detectable.

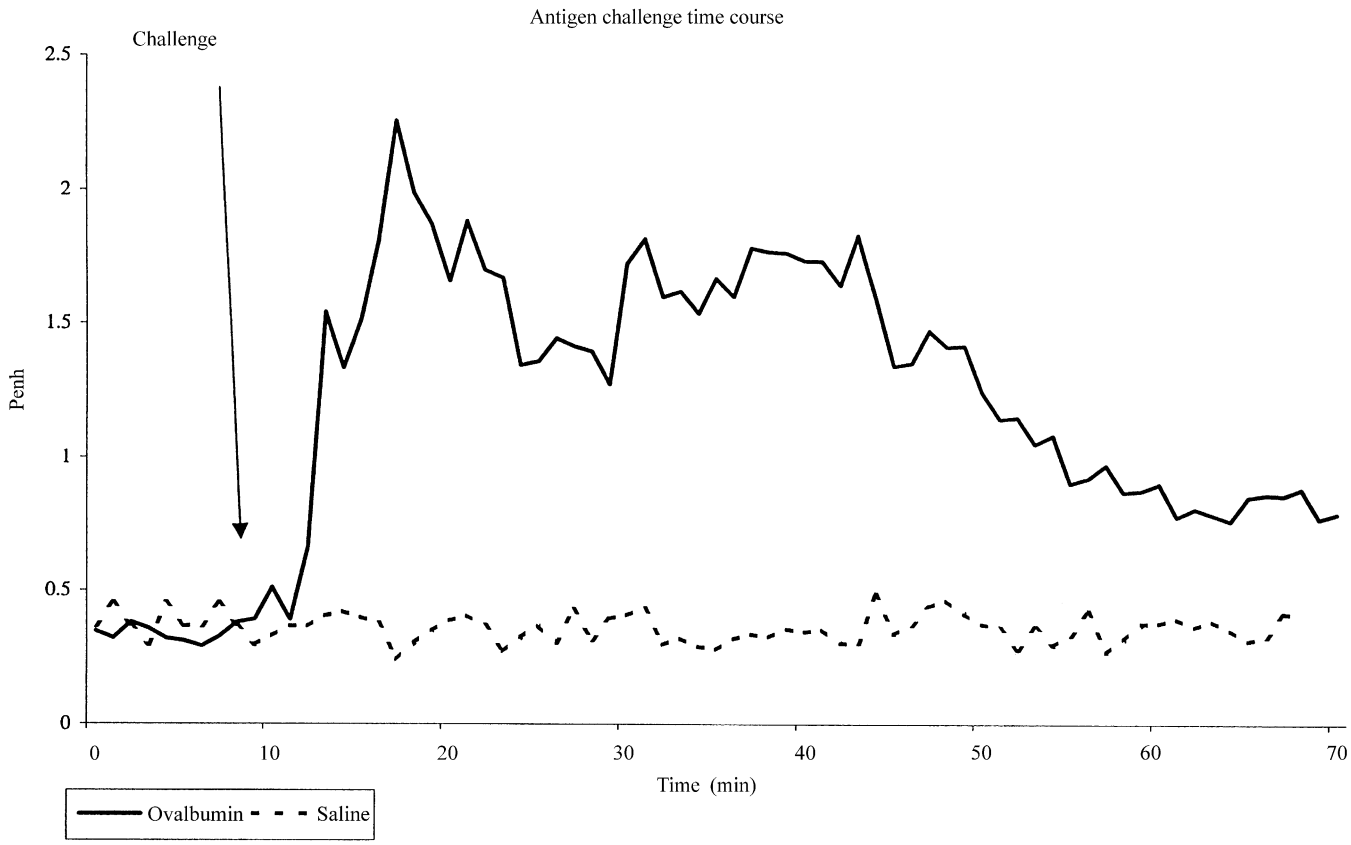


Fig. 6. Time–course of ovalbumin challenge in guinea pigs. Ovalbumin-sensitized male Hartley guinea pigs were treated with mepyramine (5 mg/kg, i.p.) and placed in a whole body plethysmograph chamber to acclimatize. Thirty minutes later, the guinea pigs were exposed to aerosolized ovalbumin (10 mg/mL in saline) or saline vehicle control for 1 min. Changes in airway resistance (Penh) were monitored for 1 hr using a Buxco pulmonary mechanics analyzer. A representative tracing of the Penh measurement is depicted.

including TNF-α [14,30]. Using a semiquantitative cytokine RT–PCR kit, we have demonstrated that although LPS induces the expression of several cytokine genes in human whole blood, preincubation with L-826,141 inhibits the induction of only TNF-α and IL-12 transcription. This demonstrates that the TNF-α and IL-12 signal transduction pathway is more sensitive than the other cytokine genes tested to modulation by intracellular cAMP, which is hydrolyzed primarily by PDE4.

We have performed time–course assays to determine the kinetics of LPS-stimulated TNF-α mRNA production and found that in humans TNF-α mRNA peaks at 4 hr post-LPS challenge. Kinetics of TNF-α transcription in the guinea pig proved to be quite similar to those of the human, with peak mRNA levels occurring 4 hr after LPS stimulation. To correlate inhibition of human TNF-α mRNA induction with the previously established PDE4-mediated inhibition of human TNF-α protein induction, we performed simultaneous concentration–response curves looking at mRNA and protein by qPCR and ELISA, respectively, using L-826,141 and three other PDE4 specific inhibitors. The IC_{50} values obtained by each method demonstrate reasonable correlation for inhibition by L-826,141, *R*-rolipram, and CT-2450, thus supporting the notion that PDE4-mediated TNF-α inhibition occurs primarily at the level of transcription. The increased potency detected for roflumilast in the qPCR assay as compared with the protein assay may be due to differences in the assay. The inhibition at the protein level was performed by a 24-hr LPS incubation, while the RNA assay was a 4-hr incubation. It is possible that roflumilast is less stable over 24 hr, and this would result in decreased activity in the protein assay because of *de novo* synthesis of TNF-α during this time period. A colleague (W. Tanaka, Merck Research Laboratories, unpublished results; cited with permission) has also tested roflumilast in a similar protein-ELISA assay that we have described, and obtained an IC_{50} of 50 nM for inhibition of TNF-α by roflumilast, suggesting that the variability seems to be approximately 3-fold with this compound. The latter IC_{50} value is closer to the one obtained by qPCR (20 nM; Fig. 4B). Therefore, several time points of inhibition may be required for analysis of TNF-α inhibition by compounds such as roflumilast.

We have titrated L-826,141 and roflumilast in guinea pig whole blood and have analyzed the TNF-α mRNA by quantitative RT–PCR techniques, as was done in human whole blood. We have shown here that qPCR analysis of TNF-α mRNA can be used to monitor potency of PDE4 inhibitors *in vitro* within a whole blood milieu. The *in vitro* data were then correlated with *in vivo* inhibition

of bronchoconstriction. Roflumilast was more potent than L-826,141 in the *in vivo* assay where only achieving the IC_{50} for this compound resulted in maximal efficacy, while 2-fold the IC_{50} was required for L-826,141 to achieve maximal efficacy.

Since cAMP signaling is involved in many pathophysiological conditions including suppression of inflammatory cytokines such as TNF-α, control of its hydrolysis through inhibition of PDE4 has become a major pharmaceutical initiative for the development of novel anti-inflammatory agents. Correlation of a biomarker such as TNF-α with an *in vivo* inhibition of airway resistance provides a rational approach for the clinical development of PDE4 inhibitors.

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