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Anti-inflammatory effects of exogenous uridine in an animal model of lung inflammation

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Abstract

Nucleosides like adenosine, uridine and their nucleotide derivatives (e.g. ATP and UTP) play important roles in many cellular functions, sometimes by acting as signalling molecules through binding to specific P2 nucleotide receptors. P2 receptors are subdivided into P2X and P2Y subfamilies, the latter of which are G-protein coupled receptors. P2Y receptors and nucleoside transporters have been detected in human and rat lungs, where they mediate effects of interest in airway diseases. The aim of this study was to investigate whether uridine has any anti-inflammatory properties in an asthma-like animal model of lung inflammation.

The Sephadex-induced lung inflammation model in Sprague–Dawley rats was chosen mainly due to its localised inflammatory response and uridine's limited oral bioavailability. The dextran beads, with or without the addition of uridine, were instilled intratracheally into the lungs, which were excised and examined after 24 h.

Sephadex alone led to massive oedema and infiltration of macrophages, neutrophils and eosinophils. Microgranulomas with giant cell formations were clearly visible around the partially degraded beads. Uridine reduced both the oedema and the infiltration of leukocytes significantly, measured as lung wet weight and leukocyte counts in bronchoalveolar lavage fluid, respectively. Uridine appeared to affect the tumour necrosis factor (TNF) levels, although this could not be statistically confirmed due to large variations within the Sephadex control group.

We conclude that uridine has anti-inflammatory effects, and that the exact mechanism(s) of action requires further study.
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Keywords: Asthma; Inflammation; Leukocytes; Sephadex; Tumour necrosis factor; Uridine

1. Introduction

Nucleobases like adenine and uracil are important in the everyday life of a cell. They and their nucleoside and nucleotide derivatives are essential building blocks of biomembranes, deoxyribonucleic and ribonucleic acid structures. They also combine with, or activate enzymes, act synergistically with other signals to potentiate cell migration [1], and transport chemical energy in the form of phosphate groups. In addition to these and many other functions, a growing body of literature shows that they can act as signalling molecules in their own right. Most well-studied is perhaps adenosine and its

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derivatives (e.g. cyclic adenosine monophosphate (cAMP) or adenosine triphosphate (ATP)), but uridine derivatives have increasingly received more attention after they were shown to activate several G-protein coupled P2Y nucleotide receptors with equal or greater efficacy than adenine nucleotides [2].

We have previously shown [3] that uridine seems to affect *in vitro* adhesion between granulocytes and cultured endothelial cells without a clear dose–response relationship. Leukocyte influx plays an important role in the inflammatory response. Strategies aimed at reduction of leukocyte adhesion and extravasation are therefore being considered as interesting therapeutic alternatives to regular anti-inflammatory therapies in many chronic diseases. Reports indicating that nucleosides and nucleotides can act directly on receptors present on inflammatory cells (Ref. [4], for instance), as well as affect mucus production, ion transport and other mechanisms involved in pulmonary inflammatory conditions, like e.g. asthma, led us to search for an appropriate lung inflammation model where potential effects of uridine could be investigated. We used the Sephadex (SDX)-induced inflammation model, involving intratracheal instillation of dextran polymer beads. The microscopic dextran beads, trapped in the lungs of rats, cause an initially eosinophil-dominated pulmonary inflammation within 24 h. The SDX-induced inflammation also has a strong neutrophil component, usually accompanied by a pronounced increase in tumour necrosis factor (TNF, formerly known as TNF-alpha) levels. The model, thus, displays several major hallmarks of inflammation. In addition, uridine has a very low bioavailability when administered orally, which necessitated a model where the substance could be directly administered to the inflamed tissue. The direct intratracheal instillation of SDX and other agents allows a manageable and localised study, avoiding many of the bioavailability problems associated with oral or intravenous administration routes. This study was primarily aimed to probe the possibility of general anti-inflammatory effects of uridine in a model of local inflammation and, secondly, to further document these effects.

2. Materials and methods

2.1. Study design

Animals where instilled with either SDX or SDX and uridine or saline (vehicle) under anaesthesia. After 24 h the animals were euthanised. The lungs were excised for weighing and lavage, and were subsequently preserved in formalin before being studied microscopically. Bronchoalveolar lavage fluid (BALF) was used for counting cell numbers, differentiation and ELISA analysis for TNF. The primary variables of interest were macroscopic and microscopic lung tissue appearance, relative changes in BALF total leukocyte number as well as of specific cell types and expression of TNF protein. Blood smears were made to confirm that the inflammation was local and that no systemic effects could be seen on immune cells. The thymuses were weighed to eliminate the possibility that eventual differences in the number of lymphocytes in various groups could be attributed to thymus size.

2.2. Animals

Male Sprague–Dawley rats (175–200 g) were purchased from Scanbur BK AB (Sollentuna, Sweden) and housed at the Linköping University animal department. All animals were acclimatised for at least 6 days before being used for experimental procedures. Food and water were supplied *ad libitum*. When the experiment was initiated, most animals weighed between 250 and 270 g. The Swedish National Board for Laboratory Animals' (CFN) guidelines were followed and experimental procedures were approved by the local ethics committee in Linköping.

2.3. Methods

Sephadex beads (G-200 superfine, Pharmacia) were suspended in sterile 0.9% NaCl solution (vehicle) one day before instillation. If uridine was to be co-administered, it was separately diluted at twice the final concentration and mixed with an equal part of SDX of double concentration just prior to instillation.

Rats were weighed and then anaesthetised by inhalation of Isoflurane (Forene, Omnia Mutantur) for a few minutes. While under anaesthesia, the animals were intratracheally instilled with 1 ml/kg of saline, SDX (5 mg/ml), uridine (50 mg/ml, Sigma-Aldrich, Sweden and USA) or a combination of SDX and uridine (50, 25, 12.5, 6.25 or 0.625 mg uridine/ml). Animals were supervised until confirmed fully awake and active. After 24 h the rats were weighed again and then euthanised with approximately 250 mg/kg of sodium pentobarbital.

Blood smears were made using whole blood. The lungs and thymus were excised, rinsed and separately weighed before performing a bronchoalveolar lavage. For the initial experiments, lungs were lavaged with 5 ml of NaCl/Xylocaine (3.33 mg Xylocaine® (AstraZeneca, Sweden)/ml 0.9% NaCl solution) for 60 s; the BALF was then kept on ice until further use. In later experiments, lavages were for practical reasons performed using the same amount of phosphate-buffered saline (PBS: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.136 g, KH_2PO_4 0.2 g, ultra pure water up to 1000 ml, pH 7.4) instead, with comparable results. Lungs were preserved in formalin.

BALF was centrifuged at 400 ×*g* and 4 °C for 5 min before total leukocyte counts and cell smears (Cytospin®) for differential counts were made. Since the lavage cells from rats treated with Sephadex appeared more fragile, the Cytospin method was preferred above the manual smearing technique. BALF supernatants were frozen (−20 °C) until further use.

Blood and lavage cell smears were fixed in methanol for 15–20 min.

Cytospin smears were dyed in May–Grünewald stain (Merck, Sweden) for one minute, rinsed in water and then dyed in Giemsa stain (Merck, Sweden) for about 25 min.

Lungs were sent to the Swedish National Veterinary Institute in Uppsala for analysis. The sections were stained with haematoxylin–eosin, Giemsa or W. Starry, the latter for detection of possible microorganisms.

Lavage-fluid samples were analysed for TNF using a standard ELISA procedure, following the manufacturer's protocol (Quantikine rat TNF immunoassay, R&D Systems, Minneapolis, USA).

2.4. Statistical analysis

Data was analysed with one-way ANOVA and Dunnet's post hoc for significant differences. All significant differences shown in the figures are against the SDX control group.

3. Results

3.1. Macroscopic appearance of lung tissue and oedema

SDX instillation led to pronounced lung oedema and inflammation. Inflamed lungs from the SDX group would consistently appear swollen compared to the saline (vehicle) control group lungs, with a multitude of small inflammatory plaques and frequent spotty bleeding on the surface. When rats had been co-treated with uridine, both the swelling and the size and number of inflammatory plaques were reduced. Lungs from the vehicle control group weighed approximately 1.26 g (SEM±0.02), contrasted by the mean in the SDX group, which was close to 1.78 g (SEM±0.06), see Fig. 1. This demonstrates an influx of fluid corresponding to roughly 40% of the original lung wet weight, caused by the SDX instillation. Only the uridine dose of 50 mg/kg resulted in a statistically significant reduction of SDX-induced oedema ($p=0.001$), although all doses above 12.5 mg/kg appeared to reduce the oedema. A clear dose–response relationship was not observed. While 50 mg/kg reduced the oedema significantly with more than 50%, 25 mg/kg had only a very small impact. 12.5 mg/kg appeared to reduce the oedema by more than 20% but this change was also statistically not significant, probably due to the variation within the SDX-only group. Nor was the apparent increase in lung oedema at 0.625 (data not shown) or 6.25 mg/kg significant. Uridine alone yielded a small increase in lung wet weight (a mean of 1.31 g, SEM±0.03) which was statistically inseparable from the 1.26 g mean lung weight in the NaCl control group.

The weight and appearance of the thymuses did not differ significantly between the control or treatment groups (data not shown).

3.2. Microscopic appearance

Microscopy was performed on lung sections from each treatment group. In lungs from rats given SDX, the beads could be seen interspersed throughout the tissue, though mainly concentrated to alveoli and adjacent tissue. Microgranulomas had formed around the majority of the beads. These were rich in macrophages, monocytes and eosinophils, with the addition of multinucleated giant cells (see Fig. 2).

SDX beads in rats co-treated with uridine were dispersed in much the same way as in the SDX control group, but the microgranulomas surrounding them were, with some exceptions, smaller, and the SDX beads appeared more degraded than in the SDX-only group.

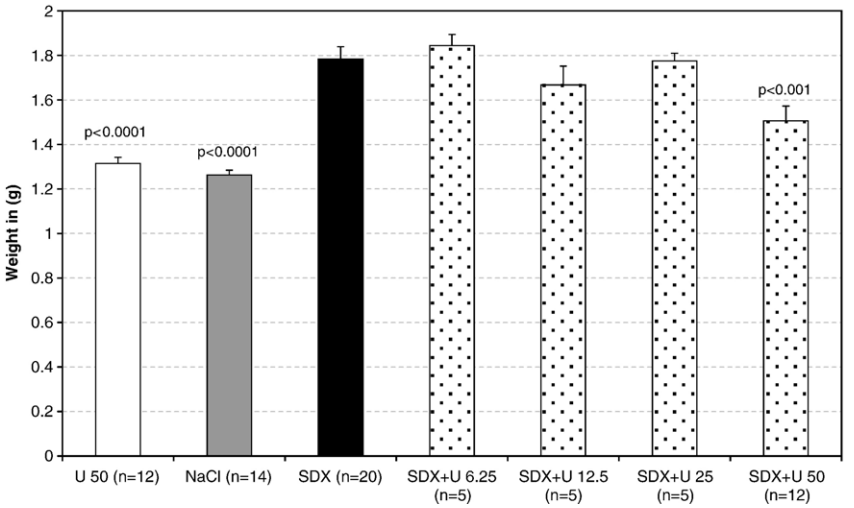


Fig. 1. Lung wet weight as measure of oedema. Lungs were excised 24 h after instillation and cleared of all tissues except lung lobes and trachea before weighing. All comparisons are made against the Sephadex-only group. Values are shown with the standard error of the mean. U = uridine, SDX = Sephadex (5 mg/kg). 50, 25, 12.5 and 6.25 are the administered doses of uridine, in mg/kg.

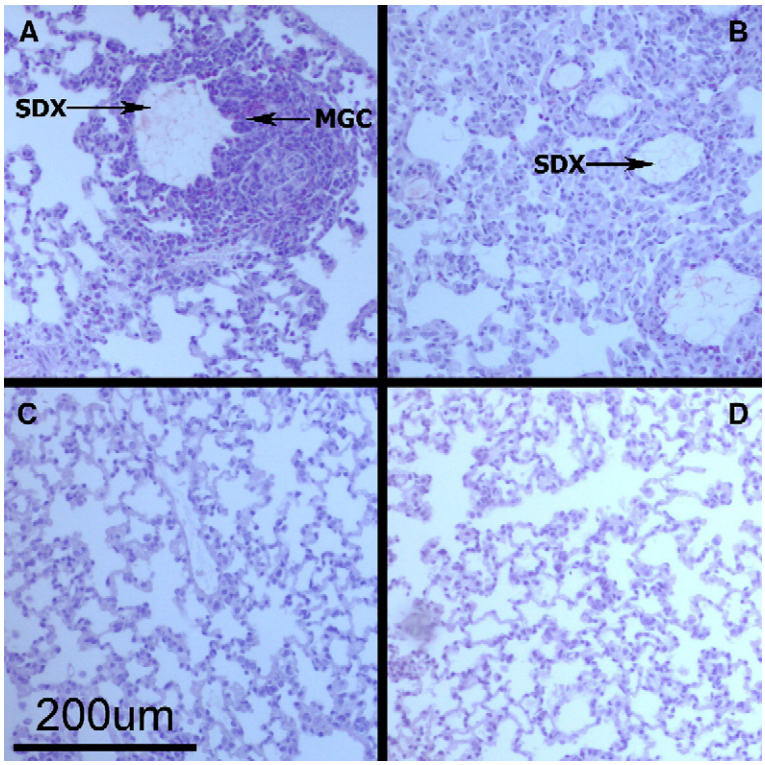


Fig. 2. Histological sections of lungs from Sprague–Dawley rats intratracheally instilled with A Sephadex (SDX, 5 mg/kg), B SDX (5 mg/kg) and uridine (25 mg/kg), C NaCl (vehicle control) and D uridine control (50 mg/kg). Sections A and B show pulmonary granulomas associated with partially degraded Sephadex beads, indicated with arrows marked SDX. In addition to macrophages, monocytes, eosinophils and lymphocytes, the Sephadex-induced lesions often displayed multinucleated giant cells (macrophage polykaryons) with peripheral nuclei, indicated with the arrow marked MGC. The selected sections are haematoxylin–eosin stained.

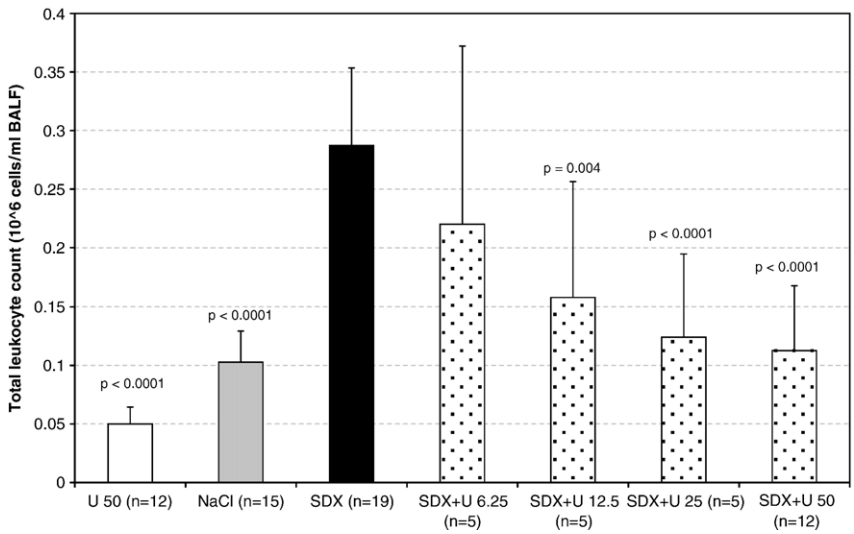


Fig. 3. Total leukocyte count in bronchoalveolar lavage fluid. The lavage was performed in excised lungs with 5 ml of phosphate-buffered saline or a mixture of saline and Xylocaine. For differential counts, see Fig. 4. Values are shown with the standard error of the mean and all comparisons are against the Sephadex group. U = uridine, SDX = Sephadex (5 mg/kg). 50, 25, 12.5 and 6.25 are the administered doses of uridine, in mg/kg.

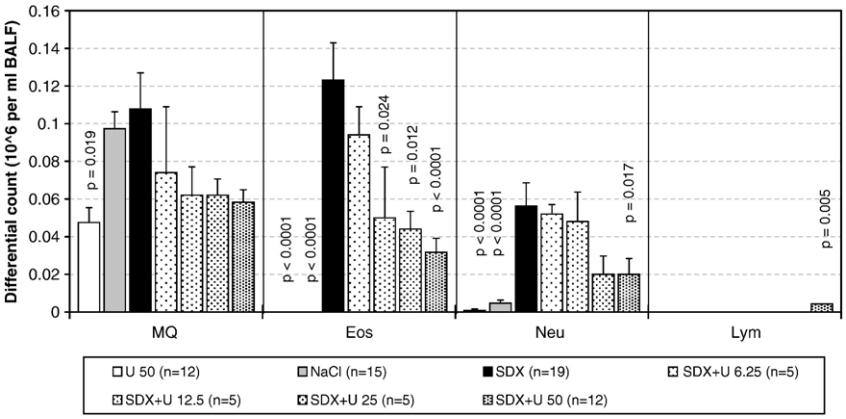


Fig. 4. Differential cell count in bronchoalveolar lavage fluid. The counts were made on May–Grünewald-stained Cytospin smears. Mean values are shown with the standard error of the mean. MQ = macrophages, Eos = eosinophils, Neu = neutrophils, Lym = lymphocytes. All *p* values are for comparisons with the Sephadex control group. U = uridine, SDX = Sephadex (5 mg/kg). 50, 25, 12.5 and 6.25 are the administered doses of uridine, in mg/kg.

In a few of the lung sections from the vehicle control group local, minor increases in inflammatory cell numbers were observed, but the cells appeared more concentrated around the trachea and were probably attracted by minor damages caused by the installation procedure. Bacteria were not detected in any section.

3.3. Total leukocyte and differential counts

SDX instillation led to a massive increase in total leukocyte counts in the BALF (Fig. 3), the numbers almost tripling compared to the NaCl group (0.29×10^6 vs. 0.10×10^6 cells/ml BALF). Macrophages, neutrophils and eosinophils had all increased (Fig. 4), most noticeably eosinophils to 0.12×10^6 cells/ml BALF (SEM ± 0.02) and neutrophils to 0.06×10^6 cells/ml BALF (SEM ± 0.01). Both cell types were nearly absent in BALF from the NaCl control group. BALF from the uridine-only group (50 mg/kg), on the contrary, almost exclusively comprised macrophages and contained only about half the number of cells of that seen in the NaCl controls (0.05×10^6 vs. 0.10×10^6 cells/ml). Co-administered at the time of SDX provocation, uridine treatment led to decreases of mainly macrophages and eosinophils. These decreases were more pronounced at higher uridine concentrations and

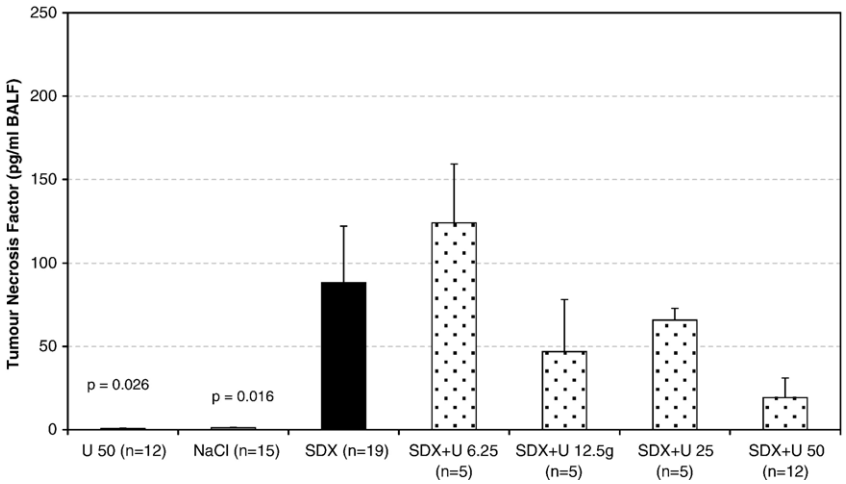


Fig. 5. Tumour necrosis factor levels in bronchoalveolar lavage fluid. A commercial ELISA kit (Quantikine, R&D Systems) was used for detection of the cytokine. Values are shown as means plus the standard error of the mean. All *p* values are for comparisons with the Sephadex group. U = uridine, SDX = Sephadex (5 mg/kg). 50, 25, 12.5 and 6.25 are the administered doses of uridine, in mg/kg. Samples from each animal have been analysed in triplicates and *n* indicates the number of animals in each group.

significant at all concentrations at and above 12.5 mg/kg. Notably, macrophage numbers appeared to be affected already at lower concentrations, while the effects on eosinophils and neutrophils occurred at higher concentrations. However insignificantly, neutrophils seemed to increase at a very low (0.625 mg/kg, results not shown) concentration of uridine, while higher concentrations seemed to suppress the infiltration. Lymphocytes, which were not observed in lavage from any other groups, increased at the highest concentration of uridine (50 mg/kg).

3.4. TNF

Untreated or uridine-treated rats which had not been provoked with SDX had low or negligible amounts of TNF in their BALF (Fig. 5), while BALF from rats given SDX only, in general had markedly increased TNF levels but ranged from 4 to 805 pg/ml BALF (mean 88 ± 34 (SEM) pg/ml). Concentrations of <5 pg/ml were probably due to the instillation procedure only, and not SDX-related. Uridine treatment at and above 12.5 mg/kg appeared to lower the TNF levels, but statistical significance could not be obtained due to the large variation in the SDX control group.

4. Discussion

Our previous results [3] with uridine in an *in vitro* adhesion assay showed that exogenous uridine affected TNF-induced static adhesion between granulocytes and cultured human umbilical cord endothelial cells (HUVEC). The *in vitro* model was designed primarily to mimic the up-regulation of adhesion molecules on the endothelium and subsequent granulocyte adhesion — both pivotal steps in leukocyte migration. Since uridine has been reported to have pleiotropic biological effects, we wished to evaluate and complement our previous results with *in vivo* data. The SDX model is well established and has several advantages with respect to ethically suitable handling of animals and simple drug administration. More importantly, as mentioned in the introduction, the model also displays several features of chronic (and in particular asthma-like) inflammation, for instance the formation of multinucleated giant cells, which is known to be a hallmark of chronic granulomatous reactions [5,6].

Our ambition with this study was to investigate whether uridine actually holds any directly measurable anti-inflammatory properties. In the current setting, characteristic hallmarks of an ongoing SDX-induced inflammation were markedly reduced by treatment with exogenous uridine.

We observed a considerable decrease in lung weight demonstrating that uridine treatment reduces oedema. Uridine treatment also led to a pronounced decrease in total leukocyte influx. The significant decrease in the influx of neutrophils and eosinophils is especially interesting given the established role of these cells as key ingredients in many inflammatory processes. Though not systematically quantified, the microscopic appearance of lung sections from inflamed animals also indicated that the size of microgranulomas was reduced when uridine was administered. Animals in the uridine control group appeared to have fewer macrophages in their airways (as indicated by BALF samples) compared to the vehicle control group, but it is difficult to conclude whether this reflects an actual reduction or simply an inhibition of an inflammatory response caused by the instillation procedure.

In healthy humans, uridine is under tight homeostatic control. Orally administered uridine is almost completely cleared in a single pass through the liver — only 7–8% of an administered dose reaches the plasma, according to Al Safarjalani [7]. In rats, the plasma clearance of uridine is reportedly near to total. Due to this low bioavailability and to minimize possible side effects of high oral doses, we chose an administration regimen where uridine could be directly administered to the location of interest, namely to the lungs by intratracheal instillation.

Uridine is a pyrimidine nucleoside, consisting of the nucleobase uracil linked to a ribose by a 1β-glycosidic bond. Like all nucleosides, uridine is a hydrophilic molecule, and as such does not easily diffuse across the cell membrane. To overcome this, most mammalian cells express at least one type of nucleoside transporter. In Sprague–Dawley rat lungs, the concentrative nucleoside transporter (CNT) 3 and the equilibrative nucleoside transporter (ENT) 1 dominate, although CNT2 and ENT2 are expressed in low amounts [8]. CNT2 prefer purines, while the other transporters accept both purine and pyrimidine nucleosides.

Once inside the cell, uridine is converted into uridine-5′-monophosphate (UMP) by uridine kinases. This phosphorylation is most likely the rate-limiting step [9]. Thereafter, uridine is phosphorylated into its corresponding di- and tri-phosphates UDP and UTP. Studies on primary cell cultures and different cell lines of human and animal origin, isolated leukocytes and lymphocytes have revealed the expression of functional purinergic nucleotide (P2) receptors of various subtypes, several of which are preferentially activated by UTP and UDP [10,11]. Uridine can therefore directly affect both the epithelium and the inflammatory cells in the form of UTP or UDP.

The P2 receptors are divided into P2X and P2Y subfamilies, the P2Y receptors preferentially G_q-protein

coupled [12]. Uracil nucleotides binding to P2Y receptors lead to activation of various signalling pathways, including activation of phospholipase C, activation of protein kinase C and mitogen-activated protein kinases [2,4,12]. These signalling pathways are known to be of great importance in many of the cellular responses that are activated in inflammation. Studies like those performed by Lazarowski et al. [13] have shown that at least $P2Y_2$ and $P2Y_6$ receptors are present on human airway epithelia and, interestingly in the context of our study, Chootip et al. [14] have demonstrated the presence of multiple UTP- and UDP-responsive P2Y receptors on Sprague–Dawley rat pulmonary arteries. It has also been speculated that patients with congenital defects in the synthesis of pyrimidine nucleotides or who supercatabolise pyrimidines are more susceptible to recurrent respiratory infections and impaired cell-mediated immunity [15,16].

Somewhat contradictory to our results, the effects of P2Y receptor activation by uracil nucleotides on neutrophils and eosinophils are usually described as pro-inflammatory, in the sense that oxygen radical production, adhesion, degranulation and release of e.g. eosinophilic cationic protein (ECP) are stimulated [10,11,17–19]. Recent evidence show that UTP is involved in the stimulation of inflammatory cell migration [20,21] and haematopoietic stem cells [1], sometimes with greater efficacy than ATP. However, the interpretation of the *in vivo* effects of P2Y receptor signalling is often confused by the fact that several other nucleotide receptors with sometimes opposing effects may be expressed on the cells, thus exerting a modulating role. Often, the nucleotide signal contributes to a response synergistically or requires another priming stimulus which ultimately shapes and directs the final response. This should be considered in relation to the reports where uridine has been shown to have a protective role, likely through its nucleotide derivatives (e.g. [7,22–25]).

Several research groups have observed a substantial increase in TNF in response to SDX [26–30]. This was also seen in our previous study where a uridine analogue, 4-thiouridine, was found to reduce the TNF release with nearly 80% (see Ref. [3]), but this time we found a large variation in the TNF levels within the SDX group. None of the samples showed such high levels as those seen in the previous study. TNF levels vary considerably between studies made by others, probably reflecting different SDX administration routes, rat strains and detection techniques, but the differences between our previous study and the current one cannot be explained by these factors. Instead, it is probably due to differences in experimental procedures (particularly in the collection of lavage fluid) or environmental factors. The strong effect on TNF by 4-thiouridine was not paralleled in the current study, although there appears to be a tendency for TNF to decrease with increasing uridine doses.

Within the time-frame (24 h) and by the administration route of our experiment, uridine alone does not appear to have any harmful systemic or local effects. This is in congruence with case reports of patients with congenital disorders of pyrimidine synthesis [16,31], or supercatabolism of pyrimidines [32]. These disorders have been treated successfully with high oral doses (up to 3 g/kg body weight [15]) of uridine, with very few severe side effects. Side effects such as fever and diarrhoea have been seen in subjects with normal pyrimidine synthesis and catabolism after ingestion of excessive oral doses or prolonged intravenous infusion. These side effects are not believed to be caused by uridine itself, but by the accumulation of its catabolites [7]. Aerosolized uridine has been well tolerated by cystic fibrosis patients, where UTP and UDP binding to $P2Y_2$ and $P2Y_6$ receptors have been shown to induce Cl^- secretion.

Uridine has been demonstrated to have protective, supporting and even directly anti-inflammatory functions in cells and tissues under different kinds of cellular stress or pathological conditions. Although the current study supports an anti-inflammatory role for uridine, the exact mechanisms by which uridine reduces or alters the inflammatory response are unclear. The mechanisms could include an indirect effect as a building block of RNA, an indirect effect through its role in transport of chemical energy or a specific direct role mediated by binding of uridine derivatives to the G_q-protein coupled P2Y receptors leading to activation of for example kinases in inflammatory cells. The available literature gives reason to suspect that both direct and indirect mechanisms could act in concert. In conclusion, the present study demonstrates an anti-inflammatory effect of uridine in an animal model of lung inflammation. The mechanism(s) of this interesting biological effect demands further study.

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