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The disturbed redox-balance in pulmonary fibrosis is modulated by the plant flavonoid quercetin

C. Veith[a,*], M. Drent[a,b], A. Bast[a], F.J. van Schooten[a,*], A.W. Boots[a]

[a] Department of Pharmacology and Toxicology, NUTRIM School of Nutrition & Translational Research in Metabolism, Faculty of Health, Medicine and Life Sciences, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands

[b] ILD Center of Excellence, St. Antonius Hospital, Koekoekslaan 1, 3435 CM Nieuwegein, The Netherlands

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is characterized by a disturbed pulmonary redox balance associated with inflammation. To restore this balance, antioxidants are often suggested as therapy for IPF but previous clinical trials with these compounds and their precursors have not been successful in the clinic. The exogenous antioxidant quercetin, which has a versatile antioxidant profile and is effective in restoring a disturbed redox balance, might be a better candidate. The aim of this study was to evaluate the protective effect of quercetin on oxidative and inflammatory markers in IPF. Here, we demonstrate that IPF patients have a significantly reduced endogenous antioxidant defense, shown by a reduced total antioxidant capacity and lowered glutathione and uric acid levels compared to healthy controls. This confirms that the redox balance is disturbed in IPF. *Ex vivo* incubation with quercetin in blood of both IPF patients and healthy controls reduces LPS-induced production of the pro-inflammatory cytokines IL-8 and TNFα. This anti-inflammatory effect was more pronounced in the blood of the patients. Our pro-fibrotic *in vitro* model, consisting of bleomycin-triggered BEAS-2B cells, shows that quercetin boosts the antioxidant response, by increasing Nrf2 activity, and decreases pro-inflammatory cytokine production in a concentration-dependent manner. Collectively, our findings implicate that IPF patients may benefit from the use of quercetin to restore the disturbed redox balance and reduce inflammation.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and lethal disease of unknown cause with a median survival of only 3–5 years which is worse than that of several types of cancer (*e.g.*, breast, bladder, and colorectal) (Woodcock and Maher, 2014; Raghu et al., 2011a; Vancheri et al., 2010). It is the most common and severe form of idiopathic interstitial lung diseases that affects mainly 60- to 70-year old ever smokers (Raghu et al., 2011a). This disease is characterized by an excessive and uncontrolled reparative process following chronic alveolar epithelial microinjuries, leading to excessive scarring of the lungs which impairs the ability of the lungs to transport oxygen (Zoz et al., 2011; King et al., 2011). While IPF is by definition "idiopathic" (*i.e.*, of unknown cause), the list of potential fibrogenic triggers that have been associated with IPF includes, among others, cigarette smoking, exposure to man-made fibers and chronic infection (King et al., 2011). Key clinical feature is a cruelly impaired lung function, ultimately leading to death from respiratory failure (King et al., 2011). In the US only, IPF affects between 150,000–200,000 people, and as many as 40,000 people die from this disease each year (Raghu et al., 2014). Similar incidence, prevalence and mortality rates have been reported in Europe with for example a yearly incidence of approximately 1000–1500 new cases in the Netherlands (Meltzer and Noble, 2008; Navaratnam et al., 2011).

IPF is characterized by a disturbed pulmonary redox-balance due to a chronic overload of ROS in comparison to protective antioxidants (Kuwano et al., 2003; Rahman et al., 2012). The increased ROS production is suggested to play a critical role in epithelial activation and injury of the alveolar cells including damage to DNA, lipids and proteins which ultimately causes severe tissue damage thereby contributing to the progression of the disease (Lenz et al., 1996; Hecker et al., 2012; Ushio-Fukai and Nakamura, 2008).

Moreover, increased ROS levels are critical in driving the progression of IPF mainly *via* triggering the activation and release of active TGF-β1, that augments the existing inflammation and lung scarring (Arts et al., 2004). Furthermore, ROS mediate TGF-β1-induced fibrogenesis including activation and proliferation of fibroblasts, differentiation into myofibroblasts causing secretion of ECM molecules and excessive collagen deposition, activation of pro-inflammatory cells and induction of EMT (Cheresh et al., 2013).

* *Corresponding authors at: Department of Pharmacology and Toxicology, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands.
E-mail addresses: c.veith@maastrichtuniversity.nl (C. Veith), f.vanschooten@maastrichtuniversity.nl (F.J. van Schooten).

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Under normal circumstances, the endogenous antioxidant defense system provides sufficient protection against ROS (Bast et al., 1991) but in patients burdened with IPF this system may be overloaded. Part of the pulmonary antioxidant defense system is regulated by the redox sensitive transcription factor nuclear factor eryrhroid 2-related factor 2 (Nrf2) (Cheresh et al., 2013). In the presence of oxidative stress, Nrf2 translocates into the nucleus where it will bind to the antioxidant response elements (ARE) that will induce the transcription of antioxidant genes (Cho et al., 2006). Nrf2 is upregulated in IPF to restore the pulmonary redox status but not to a sufficient enough level, leaving IPF patients exposed to elevated ROS levels (Singh et al., 2010a). Consequently, antioxidant therapy to strengthen such a reduced antioxidant defense might be efficacious in IPF treatment. Since ROS are capable of initiating and mediating inflammation, antioxidant therapy might also mitigate the elevated pro-inflammatory response.

Despite the fact that numerous large clinical trials have been conducted over the last few years since Raghu et al. published the IPF guidelines for diagnosis and management, there is still no effective cure for IPF that increases survival (Raghu et al., 2011b). Consequently, current standard treatment still consists of anti-inflammatory and immunosuppressive agents, aiming at reducing symptoms and the underlying inflammation (Raghu et al., 2011a). Moreover, current IPF treatments such as pirfenidone and *N*-acetyl-L-cysteine (NAC) aiming at decreasing oxidative stress in the lungs do show a clinical benefit but fail to be completely successful. The latest update of these guidelines therefore acknowledges the need for further research regarding the pathology of the disease as well as the efficacy and long-term safety of IPF medications to improve the outcome of the disease and the patient's quality of life (Raghu et al., 2015).

Alternatively, other strong antioxidants with a more versatile character, including those present in our daily diet, have already been suggested as good candidates for antioxidant therapy in interstitial lung diseases (ILD) including IPF (Bast et al., 2010). The dietary antioxidant quercetin (3,3′,4′,5,7-pentahydroxyflavone) for instance is a prototypical polyphenolic plant flavonoid which is mainly present in vegetables and fruit as well as in tea and red wine (D'Andrea, 2015). It is suggested that quercetin is the most potent scavenger of ROS, including superoxide, within the flavanoid family (D'Andrea, 2015). By activating the Nrf2 pathway, it also indirectly exerts its function through induction of Nrf2-regulated genes to further increase specific protection against oxidants (Numazawa and Yoshida, 2004; Surh et al., 2008). However, quercetin is not only known for its strong anti-oxidative but also for its anti-inflammatory capacities both *in vitro* (Boots et al., 2008; Gauliard et al., 2008) and in various pulmonary models (Impellizzeri et al., 2015; Wang et al., 2014; Takashima et al., 2014). Additionally, studies indicate that it is also effective in restoring a disturbed redox balance and reducing inflammation *in vivo* in other interstitial lung diseases (ILD) such as sarcoidosis (Boots et al., 2009; Boots et al., 2011).

The aim of the present study is to evaluate the antioxidant and inflammatory status in IPF as well as the possible beneficial effects of the dietary antioxidant quercetin hereon. To this end, the protective effects of quercetin on the antioxidant defense and inflammatory markers have been determined both *in vitro* in pulmonary epithelial cells and *ex vivo* in LPS-stimulated blood of IPF patients. We hypothesize that the antioxidant and inflammatory status in IPF are compromised and that quercetin can offer protection against oxidant-induced lung damage by restoring the disturbed redox balance and decreasing levels of inflammatory markers.

2. Materials & methods

2.1. Clinical study

11 non-smoking patients with IPF, diagnosed according to the guidelines (Raghu et al., 2011a), were enrolled in this study *via* recruitment in our outpatient clinic (Table 1). The control group, matched on age and gender, consisted of 9 non-smoking healthy controls. Based on food questionnaires, all patients and controls had comparable dietary habits with an average daily quercetin intake of 15 mg. The Medical Ethical Committee of the Maastricht University Medical Center had approved the protocol and the study was registered at http://www.clinicaltrials.gov (NCT-00512967). All participants have given their written informed consent.

Table 1
Patient characteristics: non-smoking IPF patients and healthy matched controls. Age is expressed in years, length in cm, weight in kg, time since diagnosis in years and DLCO (diffuse capacity of the lung for carbon monoxide), FEV_1 (forced expiratory volume in 1 s) and FVC (forced vital capacity) in percentage of the predicted value based on age and gender. Data are expressed as mean ± SEM.

	IPF patients	Matched controls
Number (m/f)	11 (8/3)	9 (5/4)
Age (years)	39–74 (63 ± 3)	45–60 (57 ± 3)
Length (cm)	158–174 (167 ± 2)	160–179 (170 ± 3)
Body mass index (kg/m^2)	18.5–31 (27 ± 1)	18.5–30 (26 ± 1)
Time since diagnosis (years)	0–4 (2 ± 0,4)	–
Biopsy taken	7/11	–
DLCO (percentage predicted)	19–66 (42 ± 5)	–
FEV1 (percentage)	44–86 (72 ± 4)	–
FVC (percentage)	36–140 (78 ± 8)	–
Medication	NAC (1500 mg daily): 4 × Prednisone (30 mg daily): 1 × NAC (1500 mg daily) + Prednisone (25–30 mg daily):5 ×	–

2.2. Lung function measurement

Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were measured with a pneumotachograph (Masterlab, Jaeger, Würzburg, Germany). The diffusing capacity of the lung for carbon monoxide (DLCO) was measured using the single-breath method (Masterlab) (Quanjer et al., 1993).

2.3. Cell culture and treatments

The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM/F12 supplemented with cholera toxin (10 ng/mL), epidermal growth factor (10 ng/mL), insulin (5 μg/mL), transferin (5 μg/mL), dexamethasone (0.1 μM), bovine pituitary extract (15 μg/mL) and bovine serum albumin (0.5 mg/mL). Cells were pretreated for 30 min with 10, 25 or 50 μM quercetin (Sigma, St. Lois, MO) or 0.1% ethanol (vehicle control) and subsequently exposed to different concentrations (0.1, 0.3, 1, 3, 10, 30 μg/mL) bleomycin for various time periods (2 h, 4 h, 24 h or 48 h).

2.4. Preparation of blood samples

Blood was collected in EDTA-containing vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) and kept on ice prior to processing.

2.5. Trolox antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC value) is a measurement for the total antioxidant status, assessing the capacity of a compound to scavenge ABTS radicals. This assay has been performed as described earlier (Fischer et al., 2005) with some minor modifications. In short, blood was centrifuged (3000 rpm, 5′ at 4 °C) and plasma was afterwards deproteinized, using 10% TCA (1:1) followed by