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Ivy leaves dry extract EA 575® decreases LPS-induced IL-6 release from murine macrophages

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Received October 28, 2015, accepted November 20, 2015

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Pharmazie 71: 158–161 (2016) *doi: 10.1691/ph.2016.5835*

IL-6 plays a key role in the course of inflammatory processes as well as in the regulation of immune responses by the release of different cytokines. IL-6 is produced e.g. by macrophages recruited to the airways in response to a variety of inflammatory stimuli like allergens and respiratory viruses. Patients with inflammatory airway diseases therefore may benefit from therapies targeting the IL-6 pathway, e.g. reduction of the IL-6 release. Within this context, we tested the influence of the ivy leaves dry extract EA 575® on the LPS-induced release of IL-6 from murine macrophages (J774.2). One point seven μg/ml (5 μM) corticosterone served as positive control and was able to reduce LPS-induced IL-6 release by 46 ± 4 %. EA 575® was tested in concentrations between 40 and 400 μg/ml. EA 575® decreased the LPS-induced IL-6 release in a dose-dependent manner and statistically significant by 25 ± 4 %, 32 ± 4 %, and 40 ± 7 % in concentrations of 80, 160, and 400 μg/ml, respectively. The present data suggest an anti-inflammatory effect of EA 575® used in therapy of chronic- and acute inflammatory airway diseases accompanied with cough.

1. Introduction

Cytokines control the growth and differentiation of cells and are important within cell signaling. Interleukin-6 (IL-6) is one of the cytokines which play an essential role in immunological reactions and inflammatory processes, which results in an elevated IL-6 level in inflammatory diseases. IL-6 is released mainly from monocytes and macrophages through stimulation of Toll-like-receptors (TLR) by the binding of pathogen-associated molecular patterns (PAMPs) like bacterial lipopolysaccharides or through damage-associated molecular patterns (DAMPs) like nuclear or cytosolic proteins released from cells of injured tissues (Cronin et al. 2012; Kawai and Akira 2007; Barton and Medzhitov 2003).

IL-6 receptors (IL-6R) localized in the plasma membrane are expressed in hepatocytes and leukocytes. After binding of IL-6 to the IL-6R, associated glycoprotein 130 (gp130) protein becomes activated by the IL-6/IL-6R complex that subsequently leads to an IL-6 specific cell response. In addition, membrane-bound IL-6Rs are converted by limited proteolysis into a soluble form (sIL-6R) and thus occur in the blood plasma (Scheller et al. 2011). Furthermore, sIL-6Rs are formed by translation of an alternatively spliced mRNA (Iwami et al. 2000). Remarkably, IL-6/sIL-6R complexes can activate different cell types, that do not express the IL-6R e.g. epithelial or smooth muscle cells through the ubiquitous surface protein gp130 (Heinrich et al. 2003). In acute inflammation IL-6 triggers the release of acute-phase proteins from the liver as well as the regulation of the immune response by the release of different cytokines (Gabay 2006). In chronic inflammation IL-6 stimulates the differentiation of B lymphocytes and thereby the production of antibodies as well as T cell activation and subsequently the switch from neutrophil to monocyte recruitment (Gabay 2006).

An impact of IL-6 in acute and chronic airway inflammation e.g. acute and chronic bronchitis, asthma and chronic obstructive pulmonary disease (COPD) is strongly suggested (Bucchioni et al. 2003; Rincon and Irvin 2012; Smiyan et al. 2015; Jahnz-Rozyk et al. 1996).

Ivy leaves dry extracts are used in the treatment of acute and chronic obstructive airway diseases due to its clinically established secretolytic and bronchospasmolytic properties (Mansfeld et al. 1997,1998; Gulyas et al. 1997; Hofmann et al. 2003; Lang et al. 2015).

We were interested in an additional anti-inflammatory effect and therefore investigated the impact of the ivy leaves dry extract EA 575® on the release of IL-6 from lipopolysaccharide-stimulated murine macrophages (J774.2 cells).

2. Investigations and results

The murine macrophage cell line J774.2 was used to determine the influence of ivy leaves dry extract EA 575® on the lipopolysaccharide (LPS)-induced IL-6 release. Compared to non-treated control cells (basal) stimulation with 10 ng/ml LPS for 12 h showed a pronounced increase in IL-6 release which could be significantly reduced by 46 ± 4 % by co-incubation with 1.7 μg/ml (5 μM) corticosterone (positive control, Fig. 1). Co-incubation of LPS with EA 575® showed a dose-dependent decrease in IL-6 release from J774.2 cells compared to pure LPS stimulation. Whereas 40 μg/ml EA 575® did not show any influence on the LPS-induced IL-6 release, EA 575® concentrations of 80, 160, and 400 μg/ml decreased the IL-6 levels by

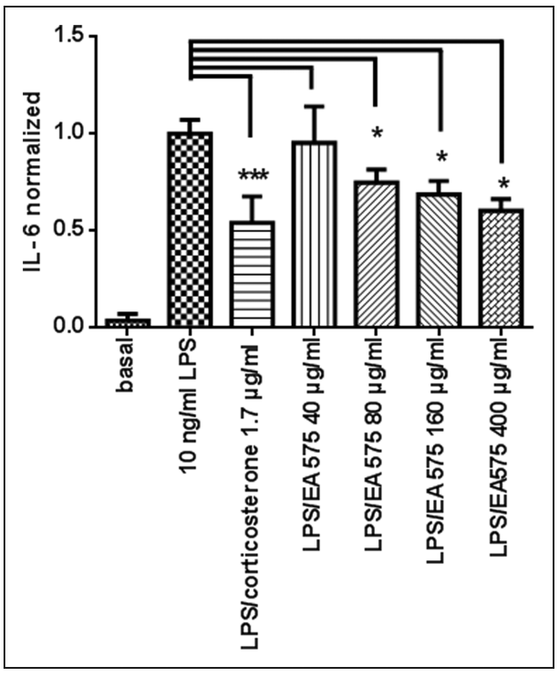

Fig. 1: Influence of corticosterone and EA 575® on the LPS-induced IL-6 release from J.774.2 cells. Results represent the mean ± S.D. ($N \geq 4$, $^{*}p < 0.05$, $^{***}p < 0.001$).

25 ± 4 %, 32 ± 4 %, and 40 ± 7 %, respectively (Fig. 1). The decrease was statistically significant for extract concentrations of 80 µg/ml or higher.

3. Discussion

The relevance of IL-6 is well-known in different inflammatory diseases like rheumatoid arthritis (Bauer and Hermann 1991) and is also accounted in the therapy with humanized antibodies like tocilizumab (Nishimoto 2006). Furthermore, IL-6 is involved in inflammatory airway diseases. In induced sputum of patients with COPD elevated IL-6 levels can be observed as well as an anti-proportional relation between IL-6 serum levels and the forced expiratory volume in 1 second (FEV_1) in patients with asthma. It is supposed that the effect of IL-6 in asthma is mediated through elevated release of IL-4 from $CD4^+$ cells and the inhibition of type 1 T helper cell (Th1) differentiation (Rincon and Irvin 2012). It could also be shown that an IL-6 antibody significantly decreases the neutrophilic and eosinophilic airway inflammation in mice. Thus, patients with inflammatory airway diseases may benefit from therapies targeting the IL-6 pathway, e.g. reduction of IL-6 release (Chu et al. 2015).

In this paper we investigated the influence of the ivy leaves dry extract EA 575® on the LPS-induced release of IL-6 from murine macrophages (J774.2 cells). Cellular IL-6 release was significantly reduced by co-incubation with EA 575® concentrations $\geq$ 80 µg/ml (Fig. 1). Remarkably, an EA 575® concentration of 400 µg/ml reduced the IL-6 release by 40 ± 7 % and thus showed a comparable effectiveness to the positive control (5 µm corticosterone) *in vitro*.

The mechanism by which EA 575® decreases the release of IL-6 from macrophages is not yet investigated and should be subject of future research. However, Schröffelbauer et al. (2010) showed that glycyrrhizin, a saponine from licorise (*Glycyrrhiza glabra L.*), inhibits TLR 3, TLR 4, and TLR 9 mediated IL-6 release from RAW 264.7 macrophages. IL-6 release not mediated through TLR receptors was not influenced by glycyrrhizin and therefore they supposed exclusively receptor-mediated effects. In addition they showed that glycyrrhizin inhibits the activation of the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) by inhibition of the phosphorylation of the inhibitor of κB (IκB). NF-κB is a key regulator in the signaling pathway of TLR3, TLR4, and TLR9 and induces among others the expression of IL-6. Additionally, LPS-induced activation of TLR4 leads to an internalization of TLR4/LPS-complexes in form of early endosomes. Endosomal TLR4/LPS is required for the association of the adaptor proteins TRIF-related adaptor protein (TRAM) and TIR-domain-containing adapter-inducing interferon-β (TRIF) to generate a TLR4/LPS/TRAM/TRIF-complex which finally activates IRF3 (McGettrick and O'Neill 2010). IRF3 as a transcription factor increases among others the expression of IL-6. Schröfelbauer et al. (2010) showed that glycyrrhizin inhibits the internalization of TLR4. This leads to an impaired signal transduction through the adaptor proteins TRAM and TRIF (Kagan et al. 2008). Interesting to note, that α-hederin from ivy leaves dry extracts is structurally related to glycyrrhizin and therefore we suppose that EA 575® may act in an analogous manner. Recently, the influence of α-hederin on lung inflammation and blood cytokines in ovalbumin sensitized guinea pigs was investigated (Keyhanmanesh et al. 2015). In comparison to sensitized animals, pathological changes like vascular and airway membrane hyperplasia, mucosal plugs, respiratory epithelial denudation, cellular infiltration, and atelectasis were significantly less pronounced in the α-hederin pretreated group. Additionally, α-hederin decreased the blood IL-4 level and increased the serum interferon-γ (IFN-γ) concentration compared to sensitized animals, indicating inhibitory effects on Th2 helper cells and stimulatory effects on Th1 helper cells. The authors suggest that α-hederin may be of therapeutic benefit in inflammatory diseases associated with decreased balance of Th1/Th2. Finally, α-hederin pretreatment reduced the ovalbumin-induced increase in IL-17 levels (Keyhanmanesh et al. 2015). Saponins are among the main pharmacological relevant compounds in ivy leaves dry extracts. Saponin enriched extracts showed anti-inflammatory effects in carrageenan- and cotton-pellet-induced acute and chronic inflammation models in rats (Süleyman et al. 2003). Escin, a mixture of saponins isolated from the seeds of the horse chestnut, shows anti-edematous and anti-inflammatory effects and is therefore used in venous diseases (Sirtori 2001). Recently it was shown, that escin exerts synergistic anti-inflammatory effects with low doses of glucocorticoids in the inhibition of carrageenan-induced paw edema in rats and in decreasing nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and IL-1β levels in LPS-stimulated murine macrophages (RAW264.7 cells) (Xin et al. 2011).

Stimulation of TLR4 by LPS results in the activation of NF-κB as a transcription factor, which promotes the expression of IL-6. β_2-adrenergic receptor (β_2AR) signaling negatively regulates the NF-κB activation through binding of β-arrestin 2 to inhibitor of κB (IκBα)/NF-κB complexes in RAW264.7 cells and thus down regulation of β_2AR in response to LPS increases NF-κB activation (Fig. 2) (Kizaki et al. 2009).

α-Hederin inhibits β_2AR internalization under stimulating conditions and therefore increases β_2AR signaling, which leads to an increased β_2AR binding and subsequently increased formation of the second messenger cyclic adenosine monophosphate (cAMP) in alveolar type 2 cells (A549) and human airway smooth muscle (HASM) cells (Sieben et al. 2009). We assume that α-hederin in turn leads to an increased formation of β-arrestin 2 and thus to a negative regulation of NF-κB activation. This could possibly explain an EA 575® mediated anti-inflammatory effect by the decrease in IL-6 lev-

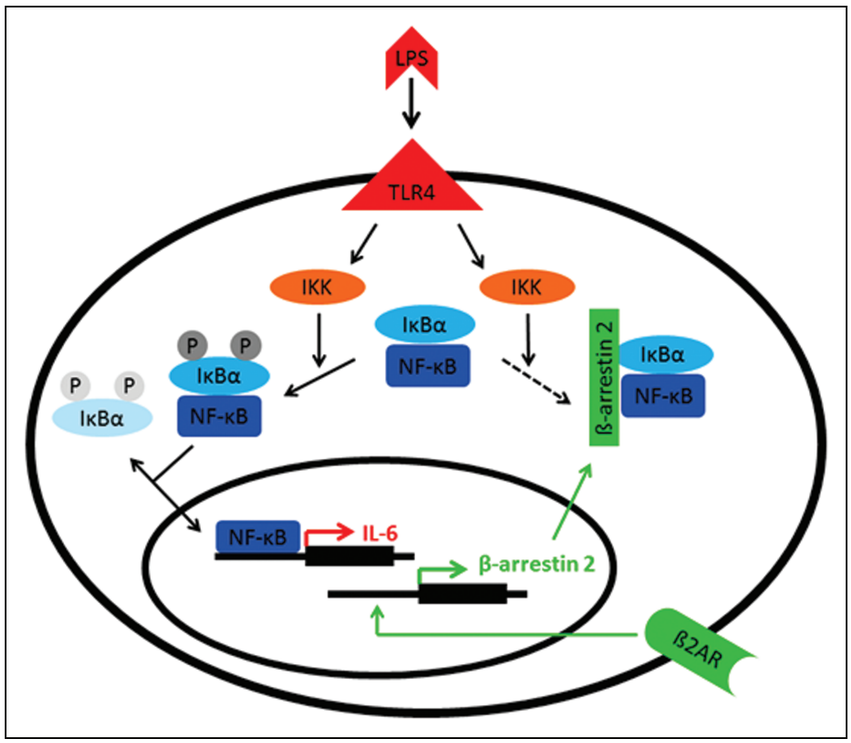

Fig. 2: NF-κB-mediated IL-6 expression is negatively regulated by β-arrestin 2, Stimulation of TLR4 by LPS activates IκB kinase (IKK), Twofold phosphorylation of IκBα by IKK leads to a cleavage of the IκBα/NF-κB-complex, NF-κB is translocated in the cell nucleus and induces expression of IL-6. Activation of the β_2-adrenergic signaling pathway induces the expression of β-arrestin 2 which forms a complex with IκBα/NF-kB in the cytosol. β-arrestin 2 stabilizes IκBα/NF-κB and prevents phosphorylation of IκBα by IKK (Kizaki et al. 2009, modified).

els of LPS-stimulated murine macrophages. Investigations using β-arrestin 2 assays have to be performed in addition to confirm this hypothetical mode of action and to substantiate a possible influence of EA 575® on the cross-talk between TLR4 and β_2AR signaling.

4. Experimental

4.1. Chemicals

Ivy leaves dry extract EA 575® (DER 5-7.5:1, 30 % m/m ethanol) was provided by Engelhard Arzneimittel (Niederdorfelden, Germany). All other reagents were obtained from Sigma Aldrich (Taufkirchen, Germany) if not stated otherwise.

4.2. Cell culture

The mouse macrophage cell line J774.2 was obtained from Sigma Aldrich (Cat. Nr.: 85011428). Cells were seeded in DMEM media (Cat. Nr.: 31053, Life Technologies, Carlsbad, CA) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamin, and 10 % fetal calf serum. Cells were used for experiments after reaching a confluence of 70-80 %.

4.3. Determination of IL-6 in J774.2 cells

For interleukin 6 (IL-6) measurements J774.2 cells were seeded in 12-well plates at a density of 6 x 10^5 cells per well and were allowed to grow for 24 h. Increased release of IL-6 into cell supernatant was induced by 10 ng/ml LPS (Cat.Nr.: L2630, Sigma Aldrich, Taufkirchen, Germany) for 12 h. One point seven µg/ml (5 µM) corticosterone together with 10 ng/ml LPS for 12 h was used as positive control. Ivy leaves dry extract EA 575® was tested in concentrations of 40, 80, 160, and 400 µg/ml incubated for 12 h simultaneous with LPS. Cell supernatants were used for IL-6 determination with a Novex® mouse IL-6 ELISA kit (Cat. Nr.: KMC0061, Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Absorbance was measured on a Tecan® Genius Reader (Tecan, Maennedorf, Switzerland) at 450 nm. The results are shown as relative absorbance at 450 nm, normalized to the positive control.

4.4. Statistical data evaluation

Statistical data evaluation was performed with Kruskal-Wallis test followed by Dunn's multiple comparison test. Results were considered to be significant for p-values of < 0.05.

Acknowledgements: This work was supported by a research grant of Engelhard Arzneimittel GmbH & Co.KG, Niederdorfelden, Germany

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