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A systematic study on the influence of the main ingredients of an ivy leaves dry extract on the β_2-adrenergic responsiveness of human airway smooth muscle cells

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ABSTRACT

The bronchospasmolytic and secretolytic effects of ivy leaves dry extracts can be explained by an increased β_2-adrenergic responsiveness of the bronchi. Recently, it was shown that α-hederin inhibits the internalization of β_2-adrenergic receptors ($ß_2$AR) under stimulating conditions. α-Hederin pretreated alveolar type II cells and human airway smooth muscle cells revealed an increased $ß_2$AR binding and an elevated intracellular cAMP level, respectively. In order to identify whether additional compounds also mediate an increased β_2-adrenergic responsiveness, we examined the ingredients of an ivy leaves dry extract (EA 575) protocatechuic acid, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, rutin, kaempferol-3-O-rutinoside, 3,4-, 3,5- and 4,5-dicaffeoylquinic acid, hederacoside B, and β-hederin. Within all the tested substances, only β-hederin inhibited the internalization of GFP-tagged $ß_2$AR in stably transfected HEK293 cells. Using fluorescence correlation spectroscopy β-hederin (1 μM, 24 h) pretreated HASM cells showed a statistically significant increase in the $ß_2$AR binding from 33.0 ± 8.9% to 44.1 ± 11.5% which was distributed with 36.0 ± 9.5% for τ_{bound1} and 8.1 ± 2.6% for τ_{bound2}, respectively ($n = 8$, $p < 0.05$). The increased binding was selectively found for the receptor–ligand complex with unrestricted lateral mobility (τ_{bound1} of 0.9 ± 0.1 ms, $D_1 = 9.1 \pm 0.2$ μm^2/s, $n = 8$), whereas the binding of $ß_2$AR with hindered lateral mobility (τ_{bound2} of 64.2 ± 47.6 ms, $D_2 = 0.15 \pm 0.02$ μm^2/s, $n = 8$) was not affected. Compared to control cells, a statistically significant increase of 17.5 ± 6.4% ($n = 4$, $p < 0.05$) and 24.2 ± 5.8% ($n = 4$, $p < 0.001$) in the cAMP formation was found for β-hederin pretreated HASM cells after stimulation with 10 μM of terbutaline and simultaneous stimulation with 10 μM terbutaline and 10 μM forskolin, respectively. Within this systematic study focusing on the influence of the ingredients of an ivy leaves dry extract on HASM cells it was possible to identify β-hederin as further component presumably responsible for the β_2-mimetic effects.

1. Introduction

Preparations of ivy leaves dry extract are used in the treatment of upper respiratory tract conditions characterized by hypersecretion of a viscous mucus and coughing. The extracts are well tolerated by children and adults suffering from acute or chronic obstructive bronchitis and the efficacy of a special extract (EA 575, DER 5-7.5:1, 30% m/m EtOH) has been confirmed by placebo controlled clinical trials and post-marketing surveillance studies [1–5]. Clinical improvements in lung function have been observed, based on spirometric and plethysmographic measurements [1–4]. Ivy extracts show secretolytic and bronchospasmolytic effects, which reduce the airway resistance and clear the respiratory system.

Preclinical studies indicated α-hederin to be the main pharmacologic active compound of ivy leaves dry extracts by increasing the β_2-adrenergic responsiveness in alveolar type II (A549) cells and human airway smooth muscle (HASM) cells [6]. α-Hederin inhibits the internalization of β_2-adrenergic receptors ($ß_2$AR) even under stimulating conditions. In fact, α-hederin pretreated A549 cells showed an increased $ß_2$AR binding [6]. This results in an increased intracellular cAMP level, which subsequently elevates the surfactant production and secretion in alveolar type II cells. Thus, the secretolytic and bronchospasmolytic effect can be explained by an

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indirect β_2-mimetic mode of action. The bronchospasmolytic effect of ivy leaves dry extracts was also demonstrated by an improved isoprenaline mediated relaxation behavior of α-hederin pretreated and methacholine precontracted airway smooth muscles [7].

Mainly, the ingredients of ivy leaves described in literature belong to the natural product classes of saponins, flavonoids and phenolic acids. So far, saponins are considered as the pharmacological relevant compounds influencing the β_2-adrenergic receptor regulation. Besides α-hederin, the saponin hederacoside C is also important. Hederacoside C can be considered as a prodrug due to its conversion to α-hederin, following its resorption into the blood [8]. Whether other ingredients contribute to the indirect β_2-mimetic effect still remains unclear.

In this paper, we investigated the influence of the main saponins, flavonoids and phenolic acids, identified from a therapeutically used ivy leaves dry extract (EA 575, DER 5-7.5:1, 30% m/m EtOH), on β_2-adrenergic receptor-GFP fusion proteins in stably transfected HEK293 cells.

2. Materials and methods

2.1. Chemicals

Ivy leaves dry extract EA 575 was provided by Engelhard Arzneimittel GmbH Co.KG (Niederdorfelden, Germany).

3,4-, 3,5- and 4,5-dicaffeoylquinic acid, protocatechuic acid, chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid were provided from Phytolab (Vestenbergsgreuth, Germany). Rutin, kaempferol-3-O-rutinoside, and α-hederin were obtained from HWI (Rülzheim, Germany). Hederacoside B was delivered by ChromaDex (Irvine, CA, USA). β-Hederin was obtained by alkaline hydrolysis of hederacoside B and subsequent isolation from the reaction mixture by HPLC. Terbutaline hemisulfate was purchased from Sigma (Taufkirchen, Germany). Synthesis, identity, and binding behavior of Alexa532-NA at β_2-adrenergic receptors were reported by our group [9].

2.2. Cell culture

Human airway smooth muscle (HASM) cells obtained from I. Hall (Institute of Pharmaceutical Science and Experimental Therapeutics, University of Nottingham, Nottingham, UK) were cultivated in DMEM-F12 medium supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. For fluorescence correlation spectroscopy experiments, cells were seeded at a density of $1.7-3.4 \times 10^4$ cells/cm^2 on heat-sterilized glass coverslips (#1, L18 mm, Marienfeld, Lauda-Königshofen, Germany) and cultured in a 12-well plate (Nunc, Langenselbold, Germany) at 37 °C and 5% CO_2. For experimental procedure cells of a confluency of 80–90% were used. For the cAMP assay, 1×10^3 cells per well were seeded in a 96-well plate (Nunc) and cultivated at 37 °C and 5% CO_2 until reaching a confluency of 90%.

HEK293 cells overexpressing β_2-adrenergic receptor-GFP fusion proteins produced by our group [6] were cultivated and plated onto heat-sterilized glass coverslips as described above for HASM cells. After reaching 70–80% confluency, cells were used for live cell imaging.

2.3. Live cell imaging

Pictures of live HEK293 cells stably expressing β_2AR-GFP were taken with an Axiovert® 200M (Zeiss, Jena) fluorescence microscope equipped with argon laser, beam splitter HFT 488/543, oil immersion objective (Plan Apochromat 63×/1.4), bandpass filter BP 505-530, and pinhole 96 mm was used. Pictures were recorded with a AxioCam® (Zeiss, Jena, resolution of 512 × 512 pixels) camera. The camera was controlled by the Axiovision® (Zeiss, Jena, release 4.7) software. Pictures shown are representatives of three independently performed experiments. Prior to measurements cells pretreated for 24 h with 1 μM of test compound or 0.1% methanol as vehicle (control cells) were washed three times with Locke's solution (154.0 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$ dihydrate, 1.0 mM $MgCl_2$ hexahydrate, 3.6 mM $NaHCO_3$, 5.0 mM HEPES, and 2.0 mM D-(+)-glucose monohydrate, pH 7.3). Coverslips were mounted on a coverslip carrier above the microscope objective. Incubation volume was 300 μL. For internalization experiments, cells were incubated for 30 min with 1 μM terbutaline hemisulfate. All measurements were performed at 20 °C.

2.4. Fluorescence correlation spectroscopy (FCS)

Measurements were performed with a ConfoCor 1 instrument (Zeiss, Jena, Germany) equipped with an argon laser (LGK 7812 ML 2, Lasos, Jena, Germany). For excitation, the 514 nm line of the laser was used and focused through a water immersion objective (C-Apochromat, 63 × /1.2) into the sample (excitation filter 515 FS 10-25, Andover, Salem, MA, laser power $p514 = 2.4$ kW/cm^2). The emitted fluorescence was separated from the excitation light with a dichroic filter (FT540, Andover) and from the background noise with a bandpass filter (EF530-600, Andover). After passing a variable pinhole (40 μm), the intensity fluctuations were detected by an avalanche single-photon counting module (SPCM-AG Series, PerkinElmer Optoelectronics, Fremont, ON) and correlated over time using a digital hardware correlator (ALV-5000, ALV, Langen, Germany) to generate autocorrelation curves. For FCS measurements, the laser focus was positioned on the upper plasma membrane of the cell by motoraided scanning of the cell in the z-direction. Using the MATLAB Software (version R2009a, The MathWorks Inc., Natick, MA) autocorrelation curves were evaluated with the autocorrelation function.

$$G(\tau) = 1 + \frac{\sum_{j=1}^{M} Q_j^2 N_j}{\left(\sum_{j=1}^{M} Q_j N_j\right)^2} \times \frac{1}{1 + \tau / \tau_{D_j}} \times \sqrt{\frac{1}{1 + \mathrm{SP}^2 \times \tau / \tau_{D_j}}} \times \left(1 + \frac{t}{1-t} e^{-\frac{\tau}{\tau_{\mathrm{trip}}}}\right)$$

with

$$Q_j = \sigma_j \eta_j g_j$$

and

$$\tau_{D_j} = \frac{\omega_0^2}{4 D_j}$$

where N_j is the average number of molecules of species j in the volume element, τ_{D_j} is the diffusion time constant of species j, τ is the correlation time, ω_0 is the radius of the observation volume in the focal plane, z_0 is the radius of the observation volume in the z-direction, D_j is the translational diffusion coefficient of species j, t is the triplet state, τ_{trip} is the triplet time, Q_j is the quantum yield factor, σ_j is the absorption coefficient, η_j is the fluorescence quantum yield, and g_j is the fluorescence detection efficiency of species j.

HASM cells pretreated for 24 h with 0.01 μM, 0.1 μM, and 1.0 μM β-hederin, respectively, and subsequently washed three times with Locke's solution were used for receptor binding studies. FCS

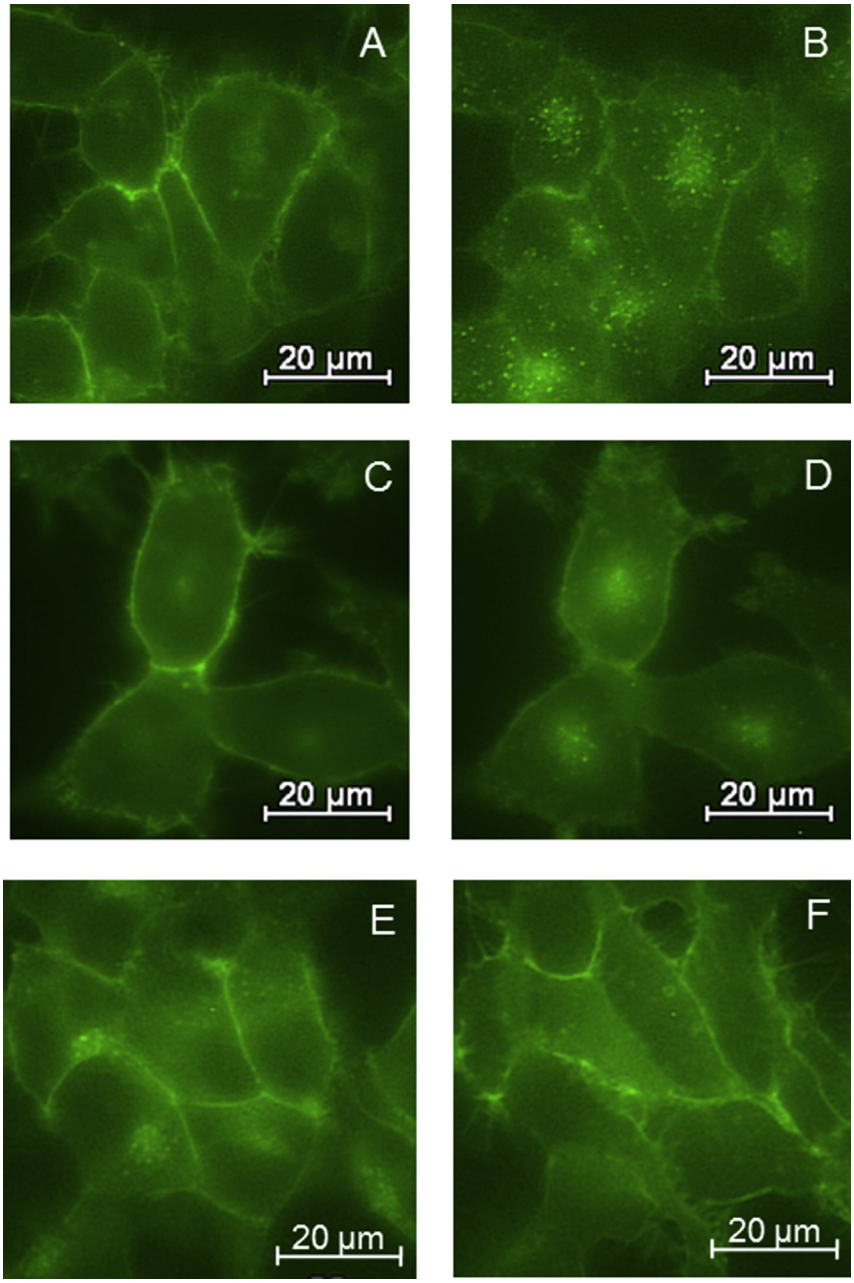

Fig. 1. Live cell images of HEK293 cells overexpressing β_2AR-GFP. Unstimulated control cells without pretreatment (A) and pretreated for 24 h with 1 µM α-hederin (C) and β-hederin (E). Following stimulation with 1 µM terbutaline for 30 min (B, D, F).

measurements were performed 15 min after adding 5 nM of Alexa532-NA ($n = 8$).

Association kinetic of Alexa532-NA in concentrations of 5 nM and 20 nM at β_2-adrenergic receptors were measured time-dependently for 25 min. Using the Prism® (version 5.04, GraphPad Software Inc., La Jolla, CA) software and the evaluation tool "nonlinear regression, association kinetics – two and more concentration" maximum number of binding sites (B_{max}) of HASM cells pretreated with 1 µM β-hederin and control cells were determined.

2.5. cAMP assay

The cAMP assay was performed using the HitHunter cAMP assay kit for adherent cells (Amersham Bioscience, Freiburg, Germany) following the manufacturer's instructions. HASM cells were incubated for 24 h with 1 µM β-hederin. 0.1% methanol was added as a vehicle to control cells. Before the cAMP concentration was examined, cells were further pretreated with 10 µM terbutaline, 10 µM forskolin or simultaneously with 10 µM terbutaline and 10 µM forskolin for 10 min at 37 °C ($n = 4$). The fluorescence was measured using a GENios microplate reader (Tecan, Crailsheim, Germany) applying an excitation wavelength of 530 nm and an emission wavelength of 610 nm. Data processing was conducted with Magellan 3.0 software (Tecan).

2.6. Statistical data evaluation

Statistical data evaluation was performed with one factorial analysis of variance (ANOVA), after their normal distribution was ensured by means of a D'Agostino & Pearson normality test. A

Dunnett's post hoc test was subsequently performed. The results were considered to be significant for p values of <0.05.

3. Results

3.1. HPLC-MS analysis of an ivy leaves dry extract

By means of high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis 17 ingredients were identified from an ivy leaves dry extract (EA 575, DER 5-7.5:1, 30% m/m EtOH), leading to the detection of protocatechuic acid (1), neochlorogenic acid (2), chlorogenic acid (3), rutin (4), kaempferol-3-O-rutinoside (5), 3,4-, 3,5- and 4,5-dicaffeoylquinic acid (6,7,8) as well as hederacoside B, C, D and F (9,10,11,12), hederaginin-3-O-glucoside (13), α-, β- and δ-hederin (14,15,16). In addition, cryptochlorogenic acid (17) was identified in ivy leaves dry extracts for the first time. Assignment of the ingredients in the HPLC chromatogram was performed through comparison of UV and MS data and retention times of corresponding reference substances (Table 1, Supplemental material). The molecular structures of the compounds tested are given in Figs. 1–2 of the Supplemental material.

3.2. Internalization of β_2-adrenergic receptors

The main ingredients 1–9, 14, 15, 17 were investigated on their influence on the internalization of β_2-adrenergic receptor-GFP fusion proteins (β_2AR-GFP) in stably transfected HEK293 cells using fluorescence microscopy. Stimulation with 1 μM terbutaline (β_2-agonist, positive control) for 30 min led to a pronounced endocytotic β_2-adrenergic receptor (ß$_2$AR) internalization, which was recognized by large intracellular vesicles positive for the receptor-ligand complexes (Fig. 1B). Preincubation with 1 μM α-hederin (negative control) for 24 h clearly led to an inhibition of ß$_2$AR internalization under stimulating conditions. A similar result was found for a 24 h pretreatment with 1 μM β-hederin. Compared to the positive control, similar vesicles were not observed in α-hederin and β-hederin pretreated cells, respectively (Fig. 1B, D, F).

Live cell recordings of α-hederin and β-hederin pretreated cells, respectively, clearly demonstrate the inhibition of ß$_2$AR internalization for the duration of 30 min after addition of 1 μM terbutaline, compared to control cells (video 1–3, supplemental material).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.pupt.2014.09.002.

In contrast, all other tested compounds (1–9, 17) showed no effect under identical experimental conditions (1 μM test compound for 24 h, then 1 μM terbutaline for 30 min). The corresponding fluorescence microscope images clearly demonstrated internalization of ß$_2$AR after stimulation that did not differ qualitatively from control cells (Fig. 3, Supplemental material). For this reason, these compounds (1–9, 17) were not considered any further for the following investigations.

3.3. β_2-adrenergic receptor binding studies

Receptor binding studies were performed using fluorescence correlation spectroscopy (FCS) to investigate the influence of β-hederin on the binding behavior of ß$_2$AR in human airway smooth muscle (HASM) cells. 5 nM of Alexa532-NA (arterenol, labeled with Alexa-532) was used as fluorescent ß$_2$AR ligand. After an incubation time of 15 min, the laser focus was positioned on the upper membrane surface and fluctuations in fluorescence intensity were measured for 60 s. Evaluation of the autocorrelation curve revealed a diffusion time constant τ_{free} of 0.056 ± 0.001 ms ($n = 8$) for freely diffusing Alexa532-NA and two diffusion time constants for receptor-ligand complexes with unrestricted and hindered lateral mobility of τ_{bound1} of 1.1 ± 0.4 ms ($n = 8$) and τ_{bound2} of 64.2 ± 47.6 ms ($n = 8$), respectively (Fig. 2). In these experiments diffusion time constants were distributed with 67.0 ± 8.9% for τ_{free}, 24.0 ± 7.1% for τ_{bound1}, and 9.0 ± 2.3% for τ_{bound2}. The total binding was 33.0 ± 8.9% (Fig. 3). Diffusion coefficients of $D_1 = 9.1 \pm 0.2$ μm^2/s and $D_2 = 0.15 \pm 0.02$ μm^2/s were calculated for the different receptor-ligand complexes from τ_{bound1} and τ_{bound2} values.

After pretreatment of HASM cells with 1 μM β-hederin for 24 h the ß$_2$AR binding displayed a statistically significant increase to 44.1 ± 11.5% which was distributed with 36.0 ± 9.5% for τ_{bound1} and 8.1 ± 2.6% for τ_{bound2}, respectively ($n = 8$, $p < 0.05$) (Fig. 3). Thus, the increased binding was selectively found for the receptor–ligand complex with unrestricted lateral mobility. In order to clarify whether the increase in ligand binding is due to an inhibition of receptor internalization or possibly to an increased receptor expression, the receptor density was determined by measuring the association kinetic of Alexa532-NA at concentrations of 5 nM and 20 nM over 25 min. Data evaluation revealed maximum number of binding sites (B_{max}) values of 27.6 ± 1.8 nM ($n = 10$) and 31.6 ± 2.3 nM ($n = 3$) for β-hederin pretreated cells and control cells, respectively, which were not statistically different. While the diffusion time constant with τ_{bound1} of 0.9 ± 0.1 ms was not affected by the β-hederin pretreatment, hindered diffusion with τ_{bound2} of 206.8 ± 218.7 ms was further restricted, compared to control cells. Whereas a pretreatment with 0.01 μM of β-hederin did not show any effect, the receptor binding was slightly increased with 0.1 μM of β-hederin, which was not statistically significant (Fig. 3).

3.4. cAMP levels in human airway smooth muscle cells

The increased ß$_2$AR binding was further confirmed by determination of intracellular cAMP levels of human airway smooth muscle (HASM) cells under stimulating conditions (10 μM forskolin and 10 μM terbutaline) after pretreatment with 1 μM β-hederin for 24 h. Compared to control cells, a statistically significant increase of 17.5 ± 6.4% ($n = 4$, $p < 0.05$) in the cAMP formation was found for β-hederin pretreated HASM cell after stimulation with 10 μM terbutaline for 10 min. cAMP formation was further enhanced by simultaneous stimulation with 10 μM terbutaline and 10 μM forskolin to a value of 24.2 ± 5.8% ($n = 4$, $p < 0.001$). The sole stimulation with 10 μM forskolin of α-hederin pretreated cells also tended to a higher cAMP level, which was not statistically significant compared to the corresponding control (Fig. 4).

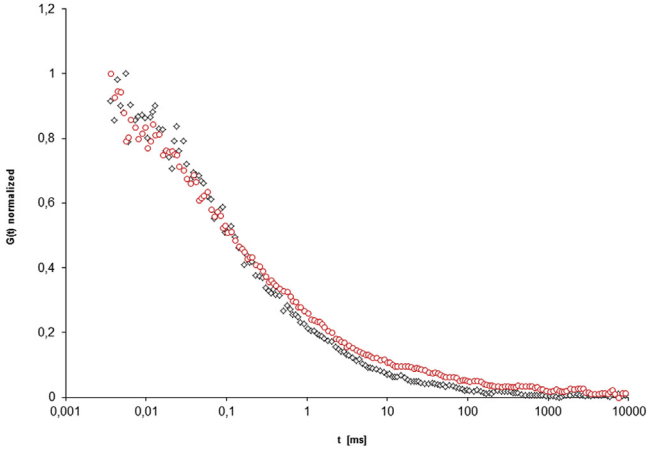

Fig. 2. Autocorrelation curves for the Alexa532-NA binding to untreated (◊, black) and α-hederin pretreated (○, red) HASM cells. Right-shift of the autocorrelation curve indicates an increased Alexa532-NA binding to ß$_2$AR of β-hederin (1 μM, 24 h) pretreated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

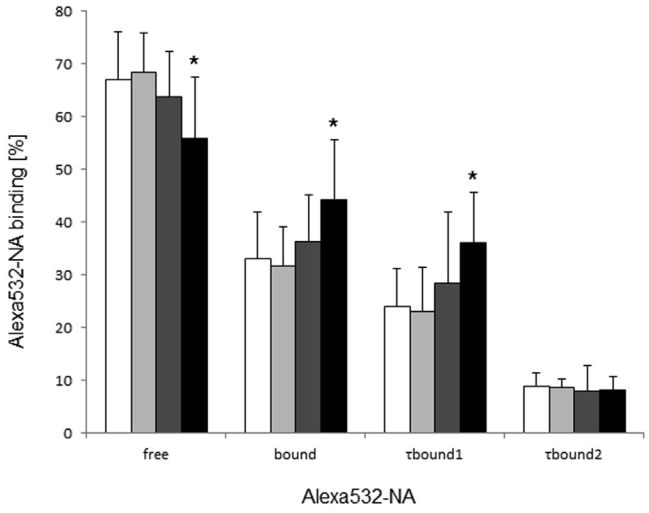

Fig. 3. Alexa532-NA binding to ß$_2$AR on HASM cells. Occurrence of free and bound Alexa532-NA and lateral mobility of β_2AR-ligand complexes with τ_{bound1} and τ_{bound2} of control cells (white) and cells pretreated with 0.01 μM (light gray), 0.1 μM (dark gray) and 1 μM (black) β-hederin for 24 h (b) ($n = 8$, $p < 0.05$).

4. Discussion

The β_2-adrenergic receptor (ß$_2$AR) is mainly responsible for the relaxation of smooth muscle by adrenaline stimulation. Thus, activation of ß$_2$AR e.g. leads to a relaxation of the bronchial tubes. Alveolar type II cells of the alveoli synthesize and secrete surfactant, a surface-active lipoprotein complex, that increases pulmonary compliance and prevents collapse of the lung at the end of expiration. The surfactant production is mediated among others by the activation of ß$_2$AR [10].

Ivy leaves dried extracts are used for the treatment of acute and chronic obstructive airway diseases. The secretolytic and bronchospasmolytic effects have been explained by an indirect β_2-mimetic mode of action, mediated by α-hederin through an inhibition of internalization of ß$_2$AR [6,7]. The thus elevated β_2-adrenergic responsiveness was confirmed by an increased ß$_2$AR binding and elevated cAMP levels of α-hederin pretreated alveolar type II (A549) cells and human airway smooth muscle (HASM) cells, respectively [6]. This increased cAMP signaling induces alveolar type II cells to enhance the formation and secretion of surfactant, which reduces the viscosity of viscous mucus and therefore results in the secretolytic effect of ivy preparations proven in clinical studies [1-4]. Bronchial muscle cells respond to the increased cAMP level with the PKA-induced phosphorylation of the myosin light-chain kinase (MLCK) and therefore to a diminished activation potential of the MLCK by the calmodulin/Ca^{2+} complex [11]. Furthermore, the release of Ca^{2+} from intracellular stores and the influx through calcium channels is blocked [12]. Via the inhibition of the MLCK and the reduced intracellular calcium concentration of bronchial muscle cells it is possible to explain the bronchospasmolytic activity of ivy preparations seen within patients [4]. Wolf et al. could demonstrate an explicit α-hederin dose-dependent improvement of the isoprenaline-induced muscle relaxation on pre-contracted bovine tracheal muscle stripes by isometric tension measurements [7]. Additionally, in vitro antispasmodic activity of saponins, flavonoids and dicaffeoylquinic acids was investigated using isolated guinea-pig ileum with acetylcholine as spasmogen. Obviously, saponins contribute most to the antispasmodic activity [13].

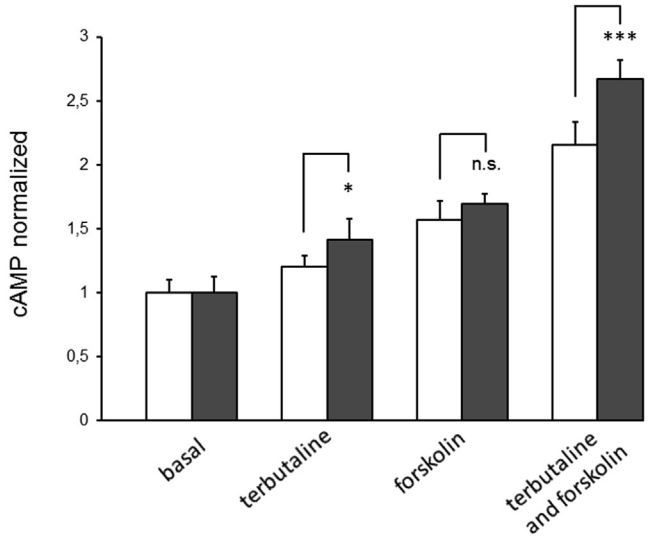

Fig. 4. Accumulation of cAMP in HASM cells comparing control cell levels (basal) with cells stimulated with 10 μM terbutaline, 10 μM forskolin or simultaneously with 10 μM terbutaline and 10 μM forskolin for 10 min before (white) and after pretreatment with 1 μM β-hederin for 24 h (gray) ($n = 4$, $p^* < 0.05$, $p^{***} < 0.001$, n.s. = not significant).

The ivy leaves dried extract (EA 575, DER 5-7.5:1, 30% m/m EtOH) mainly consists of saponins, flavonoids and phenolic acids. The main constituents of each natural product class were investigated on their influence on the internalization of ß$_2$AR under stimulating conditions. Stably transfected HEK293 cells overexpressing the ß$_2$AR as a GFP fusion protein were used for these experiments. β-Hederin and hederacoside B were tested as representatives of the saponins.

β-Hederin inhibited internalization of ß$_2$AR under stimulating conditions (Fig. 1F). This effect has already been described for the structurally related α-hederin which served in the present study as a negative control (Fig. 1D). In contrast, hederacoside B showed no effect and thus behaved similarly to hederacoside C (Fig. 1, Supplemental material) [6,8].

The flavonoids rutin and kaempferol-3-O-rutinoside did not influence the internalization of ß$_2$AR (Fig. 1, Supplemental material). On the other hand, flavonoids may have the ability to affect the regulation of β-adrenergic receptors. It has recently been described that hyperoside mediates an internalization of β_1-adrenergic receptors (ß$_1$AR) under non-stimulating conditions, whereas the ß$_2$AR remains in the plasma membrane [14,15].

Finally, the phenolic acids protocatechuic acid, neochlorogenic acid, chlorogenic acid, 3,4-, 3,5- and 4,5-dicaffeoylquinic acid, and cryptochlorogenic acid were investigated. Remarkably, cryptochlorogenic acid was identified as an ingredient of an ivy leaves dry extract (EA 575, DER 5-7.5:1, 30% m/m EtOH) for the first time. Cells pre-treated with these substances showed a clear internalization of ß$_2$AR after subsequent stimulation with terbutaline (Fig. 1, Supplemental material). Obviously, the tested phenolic acids were not able to inhibit this regulatory process.

For further investigations β-hederin was only used because all other test substances had no effect on the internalization of ß$_2$AR. Receptor binding studies were conducted using fluorescence correlation spectroscopy (FCS) to confirm the increased β_2-adrenergic responsiveness, expected for β-hederin pretreated HASM cells. Evaluation of the autocorrelation curve indicated a statistically significant increase in the total Alexa532-NA binding from 33.0 ± 8.9% to 44.1 ± 11.5% which was selectively found for receptor-ligand complexes with unrestricted lateral mobility (τ_{bound1}) (Fig. 3). This effect was dose-dependent for β-hederin. Evaluation of the association kinetics of Alexa532-NA revealed comparable β_2AR densities with B_{max} values of 27.6 ± 1.8 nM and

31.6 ± 2.3 nM for β-hederin pretreated cells and control cells, respectively. Since β-hederin does not affect the β_2AR expression in HASM cells, inhibition of receptor internalization appears to be responsible for the increased ligand binding. Comparable results were found for α-hederin with alveolar type II (A549) cells [6]. Sieben et al. have been able to demonstrate that under stimulating conditions the number of ß$_2$AR increases with hindered lateral mobility [16]. This finding indicates a possible internalization, since it can be assumed that ß$_2$AR are nearly immobile within coated pits. A redistribution of β_2AR-Alexa532 complexes to slower diffusion time constants was observed in A549 cells after terbutaline stimulation, which correlated temporally with the internalization prozess [9]. The increase of receptor-ligand complexes with unrestricted lateral mobility in β-hederin pretreated HASM cells indicates a decreased ß$_2$AR internalization. This should lead to an increased β_2-adrenergic responsiveness and consequently to increased cAMP formation. In fact, in β-hederin pretreated HASM cells the cAMP level was statistically significant enhanced by 17.5 ± 6.4% and 24.2 ± 5.8% under terbutaline and terbutaline/forskolin stimulating conditions, respectively, compared to control cells (Fig. 4). Comparable results were recently found for α-hederin pretreated HASM cells [6].

Although the β_2-mimetic effect of the ivy leaves dry extrakt (EA 575, DER 5-7.5:1, 30% m/m EtOH) according to the data available so far is presumably mediated mainly by the existing saponins, the flavonoids and phenolic acid may also contribute to the clinical efficacy. As an example, rutin inhibits the NO-synthesis in lipopolysaccharide (LPS) stimulated RAW 264.7 cells via the NF-$_\kappa$B-dependent iNOS gene transcription [17]. Additionally, an anti-inflammatory effect could be shown within an animal model. Here, rutin inhibited the carrageenan-induced (i) edema formation of rat paws and (ii) arthritis in rats within the acute as well as in the chronic phase, respectively [18,19]. Antioxidative effects of rutin have been found within a variety of analysis including the lipid peroxidation assay [20].

An anti-inflammatory and antioxidative effect has also been described for caffeoylquinic acid derivatives. With the ovalbumin-induced allergic asthma mouse model, the inhibitory effect of chlorogenic acid on the synthesis of IgE and TH2-cell specific cytokines (e.g. IL-4, IL-5) could be shown [21]. Upon oral application, chlorogenic acid diminished the carrageenan-induced edema formation of rat paws, in a dose-dependent manner. The authors exclude an inhibition of the prostaglandin E2 (PGE_2) synthesis as mode of action and actually suggest the inhibition of the TNF-α or IL-6 synthesis [22]. Within the mouse model it could be shown that chlorogenic acid application protect from LPS-induced acute lung injury [23]. Chlorogenic acid inhibits the LPS-induced upregulation of the cyclooxygenase 2 (COX2) expression in RAW 264.7 cells, mediated through the inhibited NF-$_\kappa$B-dependent gene activation, which as a result reduces the PGE_2 synthesis and explains the anti-inflammatory effect [24]. In bovine liver microsomes chlorogenic acid reduces the iron-induced lipid peroxidation in a dose dependent manner. The production of hydroxyl radicals is hereby clearly prevented by the formation of chlorogenic acid–iron complexes [25]. An antioxidative effect of chlorogenic acid was also found in the intestinal ischemia-reperfusion model in rats [26]. The antioxidative activity of neochlorogenic acid, cryptochlorogenic acid and chlorogenic acid could be determined by means of electron spin resonance (ESR) spectroscopy within a xanthine oxidase (XOD) assay. All three compounds were able to scavenge superoxide anion radicals. In addition, a preventive effect toward the oxidation of linoleic acid methyl ester could also be identified [27]. 3,4-dicaffeoylquinic acid inhibits the phorbol 12-myristate 13-acetate (PMA) induced COX-2 expression and subsequently the formation of PEG_2 in RAW 264.7 cells. As a consequence, the catalytic activity of JNK/p38 MAP kinases is blocked and the activation of C/EBPβ and AP-1 is inhibited [28]. 3,5- and 3,4-dicaffeoylquinic acid showed within the DPPH assay, which utilizes the redox reaction of stable *2,2*-diphenyl-1-picrylhydrazyl radicals, antioxidative properties, that are more pronounced in comparison to ascorbic acid. This antioxidative properties are assumed to result from the *ortho*-dihydroxy-phenolic moiety as well as from the −CH═CH−COOR group. 3,5- and 4,5-dicaffeoylquinic acid inhibit the LPS-induced NO-Synthesis in RAW 264.7 cells and also the LPS-induced expression of the NO-synthase (iNOS) and the cyclooxygenase-2 (COX-2) as well as their respective mRNA synthesis in human keratinocytes (HaCaT) [29]. Anti-inflammatory effects of an ivy leaves dried extract (EA 575, DER 5-7.5:1, 30% m/m EtOH), as testified by a reduced leucocyte count and mucoprotein content within the blood sera of children suffering from chronic bronchitis, following inhalative treatment, can be probably assigned to the presence of flavonoids and phenolic acids [30]. Preclinical studies suggest that also α-hederin may have an antioxidative effect, found in different assays like DPPH, free radical scavenging, hydrogen peroxide scavenging. As an example, α-hederin showed an inhibition on lipid peroxidation of linoleic acid emulsion [31].

5. Conclusions

Herbal drugs represent an indispensable option for the treatment of a variety of diseases. The ivy leaves dry extract (EA 575) is used for the treatment of acute and chronic obstructive airway diseases. The main ingredients belong to the group of saponins, flavonoids and phenolic acids and their influence on the β_2-adrenergic receptor of human airway smooth muscle cells was investigated by fluorescence correlation spectroscopy measurements, internalization studies and cAMP assays. Within this present work it was possible to identify two saponins β-hederin and hederacoside B as further components of an ivy leaves dry extract presumably responsible for the β_2-mimetic effects.

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Appendix A. Supplemental material

Supplemental material related to this article can be found at http://dx.doi.org/10.1016/j.pupt.2014.09.002.

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