

# Involvement of the ABC-transporter ABCC1 and the sphingosine 1-phosphate receptor subtype S1P<sub>3</sub> in the cytoprotection of human fibroblasts by the glucocorticoid dexamethasone

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**Abstract** Glucocorticoids (GC) represent the most commonly used drugs for the treatment of acute and chronic inflammatory skin diseases. However, the topical long-term therapy of GC is limited by the occurrence of skin atrophy. Most interestingly, although GC inhibit proliferation of human fibroblasts, they exert a pronounced anti-apoptotic action. In the present study, we further elucidated the molecular mechanism of the GC dexamethasone (Dex) to protect human fibroblasts from programmed cell death. Dex not only significantly alters the expression of the cytosolic isoenzyme sphingosine kinase 1 but also initiated an enhanced intracellular formation of the sphingolipid sphingosine 1-phosphate (S1P). Investigations using S1P<sub>3</sub><sup>(-/-)</sup>-fibroblasts revealed that this S1P-receptor subtype is essential for the Dex-induced cytoprotection. Moreover,

we demonstrate that the ATP-binding cassette (ABC)-transporter ABCC1 is upregulated by Dex and may represent a crucial carrier to transport S1P from the cytosol to the S1P<sub>3</sub>-receptor subtype.

**Keywords** Glucocorticoids · Dexamethasone · Sphingosine kinase · Sphingosine 1-phosphate · ABC-transporter · S1P-receptor subtypes

## Abbreviations

ABC-transporter	ATP-binding cassette transporter
Act	actinomycin D
ASO	antisense-oligonucleotide
BH3	Bcl-2 homology 3 domain
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
cFLIP	FADD-like IL-1 $\beta$ -converting enzyme inhibitory protein
Dex	dexamethasone
FLICE	FADD-like IL-1 $\beta$ -converting enzyme
GC	glucocorticoids
GPCR	G-protein-coupled receptor
GR	glucocorticoid receptor
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IL	interleukin
MKP-1	mapkinase phosphatase 1
NF- $\kappa$ B	nuclear factor k B
ODN	oligonucleotides
PBS	phosphate-buffered saline

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PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3K	phosphoinositol-3-kinase
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SphK	sphingosine kinase
S1P	sphingosine 1-phosphate
TNF $\alpha$	tumour necrosis factor $\alpha$
VEGF	vascular endothelial growth factor

## Introduction

Glucocorticoids (GC) belong to the most commonly used drugs in medicine as they have potent anti-inflammatory and immunosuppressive properties [1].

Topical used GC are able to influence a variety of acute or chronic inflammatory diseases like atopic dermatitis, psoriasis or acne vulgaris. Their long-term use, however, is often associated with severe and partially irreversible adverse effects with atrophy being the most prominent limitation. Histopathologically, GC-induced skin atrophy is characterised by a reduced number of fibroblasts and a rearrangement of the extracellular matrix resulting in a reduced thickness of the skin [2]. Most interestingly, the antiproliferative effect of GC on human fibroblasts is not accompanied by either a cytotoxic action or an increase of programmed cell death [3]. It has been clearly indicated that GC possess cytoprotective properties in human fibroblasts. Thus, the potent synthetic GC dexamethasone (Dex) inhibits tumour necrosis factor (TNF)- $\alpha$ -, UV-, as well as ceramide-induced apoptosis in fibroblasts [3]. On the contrary, an apoptotic action of Dex occurs in thymocytes, lymphocytes and myeloma cells [4].

Several investigations indicate that the anti-apoptotic effect of Dex is mediated by members of the Bcl-2 family, the inhibition of phospholipase A<sub>2</sub>, or the upregulation of cellular FADD-like IL-1 $\beta$ -converting enzyme (FLICE) inhibitory protein (cFLIP) [5, 6]. Moreover, recent studies implicated an involvement of the sphingolipid sphingosine 1-phosphate (S1P) in the anti-apoptotic signalling pathways of Dex [3]. S1P plays a pivotal role in a variety of biological processes like cell growth, differentiation, survival, cell death, migration and adhesion [7]. These important and tightly regulated functions influence several biological processes like angiogenesis, wound healing, neurogenesis and immune cell regulation [8, 9].

The intracellular levels of S1P are regulated by the activation of sphingosine kinases (SphK) whereas specific phosphatases and a pyridoxal-dependent lyase are responsible for the degradation of S1P. Two isoenzymes of SphK, SphK1 and SphK2, with different functions concerning cell

metabolism have been identified yet [10]. Thus, it has been reported that SphK1 activity is affected by various stimuli like platelet-derived growth factor (PDGF), several cytokines or even steroid hormones like 17 $\beta$ -estradiol [8, 11]. More precisely, recent studies revealed that an increased activity of SphK1 is responsible for cytoprotection, whereas the induction of SphK2 triggers apoptosis [10].

S1P acts as second messenger intracellularly as well as a ligand for membrane-bound G-protein-coupled receptors (GPCR) [12]. To date, five members of the S1P receptor family have been identified, namely S1P<sub>1-5</sub>, which are all expressed in human fibroblasts [13]. Up to now, it was unclear how intracellularly generated S1P interacts with membrane-bound receptors, but recently a possible mechanism for the export of S1P has been elucidated in mast cells [14]. In this study, Mitra et al. showed that a distinct member of the ATP-binding cassette (ABC)-transporter family, the ABCC1, is involved in S1P signalling inside and out. Additionally, the ABC-transporter ABCA1 has been characterised to take part in S1P release from astrocytes [15]. Collectively, these data indicate that in a variety of cell types ABC-transporters may be important mediators of S1P-signalling cascades.

In the present study, we investigated the mechanism of anti-apoptotic actions of Dex in human fibroblasts. Furthermore, we assured an involvement of Dex in the export of S1P by the ABC-transporter ABCC1. Moreover, the S1P<sub>3</sub> was elucidated as the crucial receptor subtype to play a decisive role in the anti-apoptotic signalling pathway of Dex.

## Materials and methods

### Materials

S1P was a generous gift from York Pharma (Homberg/Ohm, Germany). Dihydro-S1P was purchased from Biomol (Hamburg, Germany). FuGENE<sup>HD</sup> and LightCycler480 SYBRGreen I MasterMix were from Roche Diagnostics (Mannheim, Germany). SphK1 specific small interfering RNA (siRNA), control-siRNA B, siRNA transfection reagent and siRNA transfection medium were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine serum albumin (BSA), foetal calf serum (FCS) and L-glutamine solution were from Seromed Biochrom (Berlin, Germany). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Gibco (Eggenstein, Germany). Propidium iodide and Annexin V-FITC were acquired from Alexis (Grünberg, Germany). Dex, suramin, actinomycin D (Act), TNF- $\alpha$ , Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, ethylenediaminetetraacetic acid (EDTA), trypsin, Ipegal, sodium desoxycholate, sodium dodecyl sulphate (SDS), phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin, sodium fluoride, sodium orthovanadate, *o*-

phthalaldehyde, ABCC1-siRNA and Nanoparticle siRNA transfection system were purchased from Sigma Aldrich (Deisenhofen, Germany). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Schwalbach, Germany). OptiMEM was from Invitrogen (Karlsruhe, Germany). PCR-primers, Antisense Oligonucleotides (ASO) and control oligonucleotides were synthesised from Tib Molbiol (Berlin, Germany). RNeasy Mini Kit and Quia Shredders were purchased from Quiagen (Hilden, Germany). FermentasAid™ First-strand cDNA synthesis kit was obtained from Fermentas (St. Leon-Roth, Germany). The murine ABCC1-antibody (clone IU2H10), the SphK1-antibody (rabbit, polyclonal), and the  $\beta$ -actin antibody (rabbit, polyclonal) were purchased from Abcam (Cambridge, UK). All other chemicals were purchased from Sigma Aldrich (Deisenhofen, Germany).

#### Isolation of primary human fibroblasts

To isolate human fibroblasts, juvenile foreskin from surgery was incubated at 37°C and 5% CO<sub>2</sub> for 2.5 h in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinisation was terminated by the addition of DMEM containing 10% FCS. Afterwards, cells were washed with phosphate-buffered saline (PBS) and centrifuged at 250×g for 5 min. The pellet was re-suspended in fibroblast growth medium that was prepared from DMEM by the addition of 7.5% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Fibroblasts were pooled from at least three donors and cultured at 37°C and 5% CO<sub>2</sub>. Only cells of the second to fourth passage were used for the experiments.

#### Isolation of S1P<sub>3</sub><sup>(-/-)</sup> and wild-type fibroblasts

All animal experimentation conforms to protocols approved by the institutional animal care committee. Wild-type and S1P<sub>3</sub> knockout mice were generated as recently described [16]. To isolate murine fibroblasts, skin was incubated at 37°C for 2.5 h in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinisation was completed by the addition of DMEM containing 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (murine fibroblasts growth medium). Isolated fibroblasts were washed with PBS and centrifuged at 250×g for 5 min. The pellet was re-suspended in murine fibroblasts growth medium and cultured at 37°C and 5% CO<sub>2</sub>. Cells were genotyped by polymerase chain reaction. The following primers were used: 5'-CACAGCAAGCA GACCTCCAGA-3', 5'-TGGTGTGCGGCTGTCTAGT CAA-3' and 5'-ATCGATACCGTCGATCGACCT-3'.

#### Quantitative real-time polymerase chain reaction

Fibroblasts were first cultured in growth medium and then incubated in DMEM containing 2 mmol/L L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (basal

medium) for 12 h. After stimulation of fibroblasts with Dex for the indicated periods and concentrations, total RNA was collected using the RNeasy Mini Kit. cDNA was generated from total RNA using the FermentasAid™ First-strand cDNA synthesis kit according to the instructions of the manufacturer. Quantitative real-time polymerase chain reaction (PCR) was performed using a LightCycler480 (Roche Diagnostics—Applied Science, Mannheim, Germany) and the LightCycler480 SYBRGreen I MasterMix. CyclophilinA was used as a normalisation control for all experiments. For the measurement of the SphK, the following primers were used: SphK1 AGACCTCCTGACCAACT (forward), ATACTTCTACTCTCTAGGTCC (reverse); SphK2 CCTGGCTGCTAGAGTTG (forward), CCCTCATTGAT CAGGCAC (reverse); cyclophilinA TTTGCTTAATTCTA CACAGTACTTAGAT (forward), CTACCCTCAGGT GGTCTT (reverse). For the measurement of the S1P-receptor subtypes, the following primers were used: S1P<sub>1</sub> 5'-CGTGTT CAGTCTCCTCG-3' (forward), 5'-CTGATGCAG TTCCAGCC-3' (reverse); S1P<sub>2</sub> 5'-GTTAGCCAGGATG GTCTT-3' (forward), 5'-CAACAGA GCGAGACTTCA-3' (reverse); S1P<sub>3</sub> 5'-CGCTTCAGTGTAACAACG-3' (forward), 5'-GAGGGTCACACAGCATT-3' (reverse); S1P<sub>4</sub> 5'-AAGACCAGCCGCGTCTA-3' (forward), 5'-CCAGGCA GAAGAGGATGT-3' (reverse); S1P<sub>5</sub> 5'-GGAATGCAGC CAAAGG-3' (forward), 5'-CCATTATTTTCATCACCGAGTT-3' (reverse); cyclophilinA 5'-TTTGCTTAATTCTACACAG TACTTAGAT-3' (forward), 5'-CTACCCTCAGGTGGTCTT-3' (reverse). For the measurement of the ABC-transporters, the following sequences were used: ABCB1 5'-GGCA AGCTGGAGAGAT-3' (forward), 5'-TAATTACAG CAAGCCTGGAAC-3' (reverse); ABCC1 5'-CTCCT GTGGCTGAATCTG-3' (forward), 5'-CACTTTGATCC CATTGAGAATTT-3' (reverse). Total RNA (10 ng) of at least three different sets of fibroblasts were used to analyse gene expression. Relative mRNA expression was quantified using the comparative threshold cycle method. Data were obtained in at least triplicate and the specific mRNA levels were expressed as the mean±SEM of relative mRNA expression relative to control cells.

#### RNA interference

For silencing SphK1 expression, fibroblasts were transfected for 6 h with SphK1-siRNA or control-siRNA B (final concentration 20 nM) using the siRNA transfection medium and siRNA transfection reagent as described by the manufacturer's protocol. The target sequences for SphK1-siRNA were as follows: 5'-GGGCAAGGCCUUGCAG CUC-3' (sense) and 5'-GAGCUGCAAGGCCUUGCCC-3' (antisense). For silencing ABCC1 expression, fibroblasts were transfected with ABCC1-siRNA or control-siRNA B (final concentration 20 nM) using the Nanoparticle siRNA

Transfection System according to the manufacturer's protocol. After a transfection period of 24 h, cells were incubated with fibroblasts basal medium for 24 h. The target sequences for ABCC1-siRNA were as follows: 5'-GUUCCAAGGUGGAUGCGAA-3' (sense) and 5'-UUCG CAUCCACCUUGGAAC-3' (antisense).

#### Measurement of apoptosis

Fibroblasts ( $1 \times 10^5$  cells per well) were cultured in basal medium and incubated with the indicated agents for 24 h. Then cells were trypsinised and washed twice with binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ). Apoptosis was measured by Annexin V binding using the FACS Calibur (Becton and Dickinson, Heidelberg, Germany) [17]. To discriminate between early apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>) as well as late apoptotic and necrotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>), dye exclusion of the non-vital dye PI was simultaneously measured. Therefore, cells were re-suspended in binding buffer followed by the addition of Annexin V-FITC (final concentration 0.5 µg per ml). The mixture was incubated for 10 min in the dark at room temperature, washed and re-suspended in binding buffer. Then PI was added (1 µg per ml) and samples were analysed by bivariate flow cytometry. The determined apoptotic rates in percent represent Annexin V<sup>+</sup>/PI<sup>-</sup> and Annexin V<sup>+</sup>/PI<sup>+</sup> fibroblasts.

#### Measurement of S1P-levels

S1P levels were determined as described recently [18]. Briefly,  $1 \times 10^6$  fibroblasts were stimulated as described. For the measurement of extracellular S1P, an aliquot of 1 ml was combined with 1 ml methanol containing 2.5 µl concentrated HCl. To determine intracellular S1P, cells were washed with PBS and scraped into 1 ml methanol containing 2.5 µl concentrated HCl. As internal standard dihydro-S1P (50 pmol) was added and lipids were extracted by addition of 1 ml chloroform and 200 µl 4 M NaCl. For alkalisation, 100 µl 3 N NaOH were added. The alkaline aqueous phase was transferred into a siliconised glass tube, and the organic phase was re-extracted with 0.5 ml methanol, 0.5 ml 1 M NaCl and 50 µl 3 N NaOH. The aqueous phases were combined, acidified with 100 µl concentrated HCl and extracted twice with 1.5 ml chloroform. The organic phases were evaporated, and the dried lipids dissolved in 275 µl methanol/0.07 M  $\text{K}_2\text{HPO}_4$  (9:1). A derivatisation mixture of 10 mg *o*-phthalaldehyde, 200 µl ethanol, 10 µl 2-mercaptoethanol and 10 ml 3% boric acid was prepared and adjusted to pH 10.5 with KOH. 25 µl of the derivatisation mixture were added to the resolved lipids for 15 min at room temperature. The derivatives were analysed by a Merck Hitachi LaChrom HPLC system

(Merck Hitachi, Darmstadt, Germany) using a RP 18 Kromasil column (Chromatographie Service, Langerwehe Germany). Separation was done with a gradient of methanol and 0.07 M  $\text{K}_2\text{HPO}_4$ . Resulting profiles were evaluated using the Merck system manager software.

#### Downregulation of S1P-receptors using ASO

ASO were designed to surround the translational initiation site, a place empirically known to be most effective for inhibition of gene expression. Cells ( $1 \times 10^5$  cells per well) were cultured in fibroblast growth medium for 12 h. Control oligonucleotides and ASO were solubilized in OptiMEM and FuGENE<sup>HD</sup> (2 µg of DNA/3 µl) to achieve a final concentration of 500 nmol/L of oligonucleotides. Then, the solution was added to fibroblasts for 72 h. The following specific ASO as well as same-length control oligonucleotides (with the same nucleotides but randomly scrambled sequence) were used: S1P<sub>1</sub> 5'-GACGCTGGTGGGCCCCAT-3' (ASO), 5'-ATGGGGCC CACCAGCGTC-3' (scrambled ODN); S1P<sub>2</sub> 5'-GTTGAG CAGGGAATTCAGGGTGGAGA-3' (ASO), 5'-CATCAC TAGCCACTTGAAGCAGGCCA-3' (scrambled ODN); S1P<sub>3</sub> 5'-CGGGAGGGCAGTTGCCAT-3' (ASO), 5'-ATGG CAACTGCCCTCCCG-3' (scrambled ODN); S1P<sub>4</sub> 5'-GAAGGCCAGCAGGATCATCAGCAC-3' (ASO), 5'-ACCTAGCCAACCCTCCATGAAGGC-3' (scrambled ODN); S1P<sub>5</sub> 5'-CAACATGCCACAAAGGCCAGGAG-3' (ASO), 5'-GCAACAACATAACGGGCCAGCAG-3' (scrambled ODN).

#### Western blot analysis

Fibroblasts were seeded into six-well plates and cultured for 12 h in fibroblast growth medium followed by 12 h of serum-deprivation with basal medium. After stimulation cells were rinsed with ice-cold PBS and harvested in lysis buffer (PBS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , 1% Ipegal, 0.5% sodium desoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM sodium orthovanadate and 50 mM sodium fluoride). Lysates were centrifuged at  $14,000 \times g$  for 30 min. Samples containing 20–40 µg protein were boiled in SDS sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and separated by 10% SDS polyacrylamide gel electrophoresis. Gels were blotted overnight onto PVDF membranes. After blocking with 5% non-fat dry milk for 1 h at 37°C, membranes were incubated with the appropriate primary antibodies at a dilution of 1:1,000 overnight at 4°C, and further incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h. Then, blots were developed according to the manufacturer's protocol. Densitometry measurements were recorded using a Syngene Gene Genius

imaging system (Syngene, Cambridge, UK). Values of protein expression of SphK1 and ABCC1 in response to a treatment with Dex were normalised to  $\beta$ -actin-levels.

Statistics

Data are expressed as the mean $\pm$ SEM of results from at least three experiments, each run in triplicate. Statistics were performed using Student’s *t* test. \**P*<0.05 and \*\**P*<0.01 indicate a statistically significant difference vs. control experiments.

Results

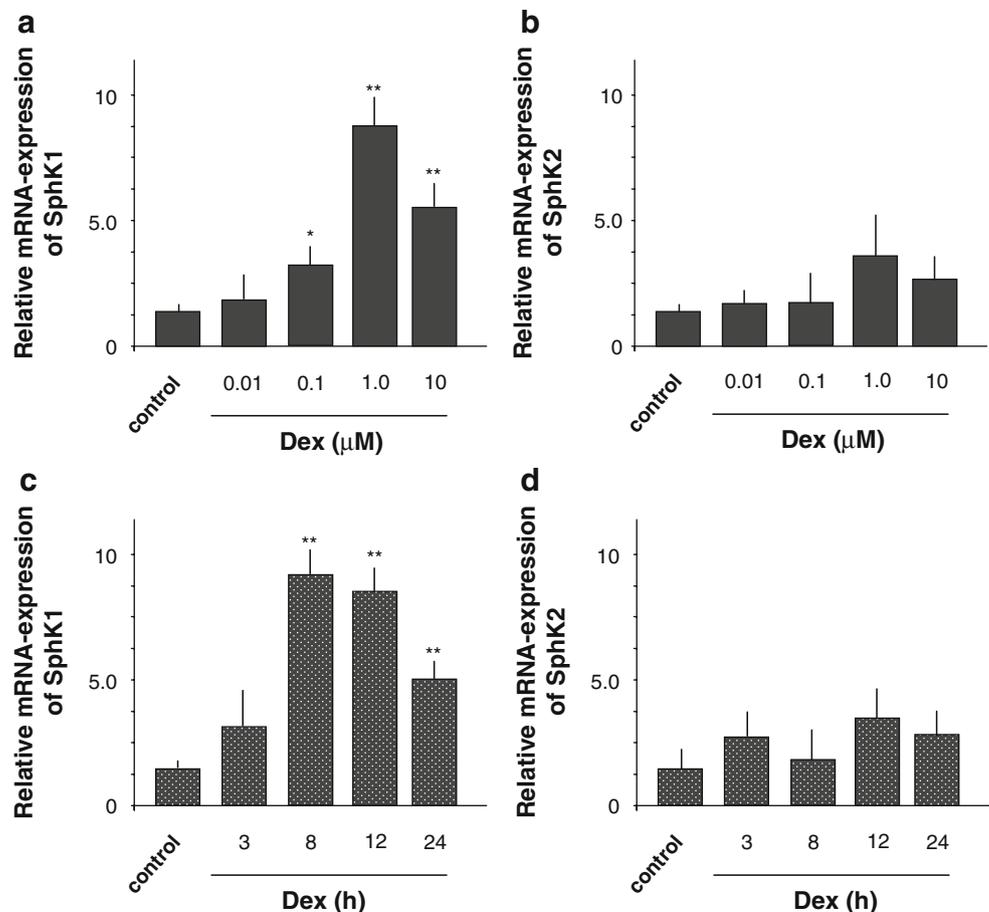
Dex mediates cytoprotection of fibroblasts via activation of SphK1 and formation of S1P

It has been shown that GC possesses anti-apoptotic actions in human fibroblasts via the formation of S1P [3]. But the molecular mechanism is less characterised. Therefore, we proved whether Dex influences the activity of SphK1 and SphK2, the crucial enzymes for the formation of S1P. Indeed, real-time PCR analysis pointed out that Dex

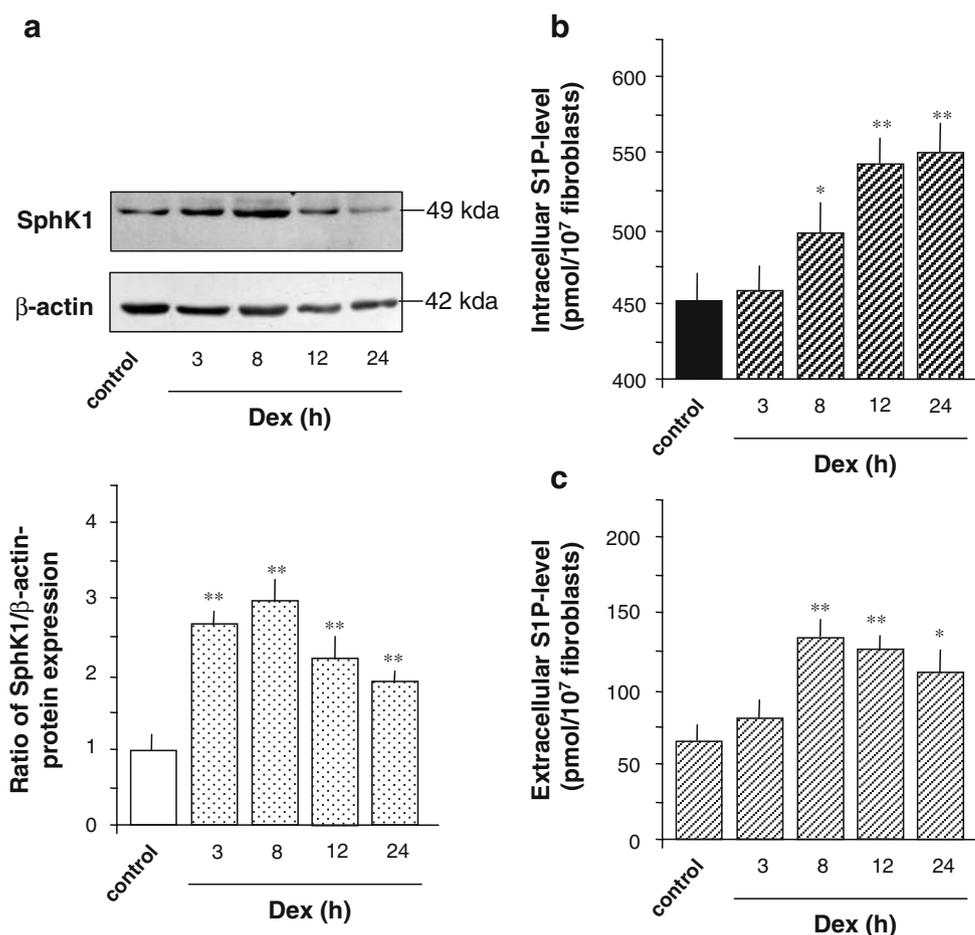
increases mainly mRNA expression of SphK1, whereas no significant enhancement of SphK2-mRNA expression was detected (Fig. 1). More detailed, an enhancement of SphK1 in response to Dex became first visible after 3 h and reached maximal levels after 8 h (Fig. 1c). The most effective dose to augment mRNA levels of SphK1 was at 1  $\mu$ M of Dex and resulted in an eightfold increase of the isoenzyme (Fig. 1a). Next, SphK1 protein expression was analysed by Western blot and densitometric analysis. In analogy to the results of the performed real-time PCR, protein levels of SphK1 were increased in response to Dex (Fig. 2a). Moreover, measurement of S1P-levels in response to Dex confirmed a significant intracellular and extracellular increase of the lipid mediator (Fig. 2b,c).

To prove an involvement of SphK1 in the anti-apoptotic property of Dex, downregulation of SphK1 was performed employing siRNA. Real-time PCR analysis revealed that treatment of fibroblasts with siRNA resulted in a serious reduction of mRNA levels of SphK1. In congruence, downregulation of SphK1 prevented the ability of Dex to enhance SphK1 activity as well as S1P-levels (Fig. 3a,b). Moreover, abrogation of the isoenzyme reduced the aptitude of Dex to protect fibroblasts from apoptosis (Fig. 3c). It should be noted that downregulation of SphK1

**Fig. 1** Dex activates SphK1 in a time- and concentration-dependent manner. Quantitative real-time PCR analysis of SphK1 and SphK2 in response to Dex was performed. Fibroblasts were stimulated with Dex for 8 h with the indicated concentrations of Dex (a,b) or for the indicated time periods with 1  $\mu$ M of Dex (c,d). Real-time PCR starting with 10 ng of total RNA of three different sets of cells was performed with specific primers as indicated and described in the “Materials and methods” section using cyclophilinA as reference gene. Relative mRNA expression was quantified using the comparative threshold cycle method. \**P*<0.05 and \*\**P*<0.001 indicate a statistically significant difference versus vehicle-stimulated cells



**Fig. 2** Dex induces SphK1-protein expression and increases intra- and extracellular levels of S1P. For the examination of SphK1-protein expression in response to 1  $\mu$ M Dex, Western blot analysis of fibroblast cell lysates treated with the GC for the indicated time period were performed. For control experiments,  $\beta$ -actin-protein levels were measured (a). Densitometric analysis of Western blots shows the ratio of SphK1/ $\beta$ -actin-protein expression from at least two individual experiments (a). To measure whether activation of SphK1 in response to Dex is associated with an intracellular (b) and extracellular (c) increase of S1P, fibroblasts were stimulated for the indicated time periods with 1  $\mu$ M of Dex. Data are expressed as pmol S1P/ $10^7$  fibroblasts $\pm$ SEM from three experiments. Raw data are presented in the [electronic supplemental material](#). \* $P$ <0.05 and \*\* $P$ <0.001 indicate a statistically significant difference versus vehicle-stimulated cells



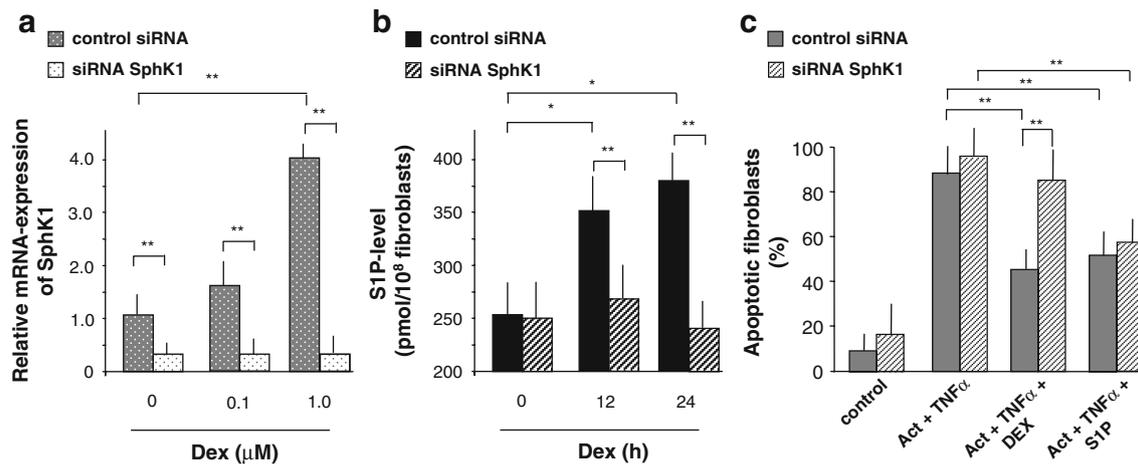
had no influence on the S1P-mediated cytoprotection. These data clearly indicate that activation of SphK1 and subsequent formation of S1P is crucial for the cytoprotective property of Dex.

The ABC-transporter ABCC1 is not only regulated by Dex but also essential for the S1P-export

Although Dex induces an intracellular accumulation of S1P, it is well known that the sphingolipid exerts most of its actions by membrane-bound S1P-receptors. At present, it is not completely understood how intracellular formed S1P is exported out of the cell. Most recently, in mast cells, the involvement of the ABC-transporters ABCC1 and ABCB1 has been discussed to play a decisive role to mediate the export of S1P. Indeed, real-time PCR indicated that mRNA of both ABCC1 and ABCB1 are expressed in human fibroblasts, too (Fig. 4a). Furthermore, it was of interest to investigate whether these ABC-transporters are essential for the anti-apoptotic action of Dex. To this end, the cytoprotective property of Dex was measured in the presence of the ABCB1 inhibitor verapamil and the ABCC1 inhibitor MK571. As shown in Fig. 4b, inhibition of ABCB1 by

verapamil did not abolish the ability of Dex to prevent programmed cell death. Moreover, treatment of fibroblasts with MK571 significantly abrogated the cytoprotective effect of the GC (Fig. 4b). These results suggest a possible involvement of ABCC1 in the anti-apoptotic pathway of Dex. To further substantiate the participation of this transporter, fibroblasts were treated with siRNA against ABCC1. Real-time PCR confirmed a significant reduction of mRNA of ABCC1 (Fig. 4c). In accordance to the findings with MK571, abrogation of ABCC1 by siRNA resulted in a complete reduction of the GC-mediated cytoprotective action (Fig. 4e), whereas S1P-levels in the cells were increased (Fig. 4d). These results clearly show that the ABCC1 is crucial for the export of intracellular formed S1P out of fibroblasts to mediate the anti-apoptotic action of Dex.

As it is well known that GC are able to regulate the expression of ABC-transporters in distinct cell types, it was also of interest to analyse a regulation of the ABCC1 transporter by Dex [19]. Indeed, real-time PCR confirmed that Dex considerably increased mRNA levels of ABCC1 (Fig. 5). There was a transient increase of ABCC1 mRNA with a maximal detectable effect after an 8 h treatment with



**Fig. 3** Dex mediates cytoprotection of fibroblasts via activation of SphK1. For silencing SphK1 expression, fibroblasts were transfected for 6 h with SphK1-siRNA or control-siRNA B (final concentration 20 nM). Then cells were stimulated with the indicated concentrations of Dex for 8 h. Real-time PCR starting with 10 ng of total RNA of three different sets of cells was performed using cyclophilinA as reference gene. Relative mRNA expression was quantified using the comparative threshold cycle method (a). To examine the effect of SphK1-siRNA on intracellular S1P-levels, fibroblasts were transfected for 6 h with SphK1-siRNA or control-siRNA B. Then, cells were

stimulated with 1  $\mu$ M of Dex for the indicated time periods. S1P-levels are expressed as pmol S1P/ $10^8$  transfected fibroblasts  $\pm$  SEM from three experiments (b). To measure the effect of SphK1 on Dex-induced cytoprotection, fibroblasts were transfected for 6 h with SphK1-siRNA or control-siRNA B. Cells were incubated with Dex (1  $\mu$ M, 8 h) or S1P (10  $\mu$ M, 30 min). Then 100 ng/ml Act and 20 ng/ml TNF $\alpha$  were added for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining as described in the “Materials and methods” section (c). \* $P$ <0.05 and \*\* $P$ <0.001 indicate a statistically significant difference versus vehicle-stimulated cells

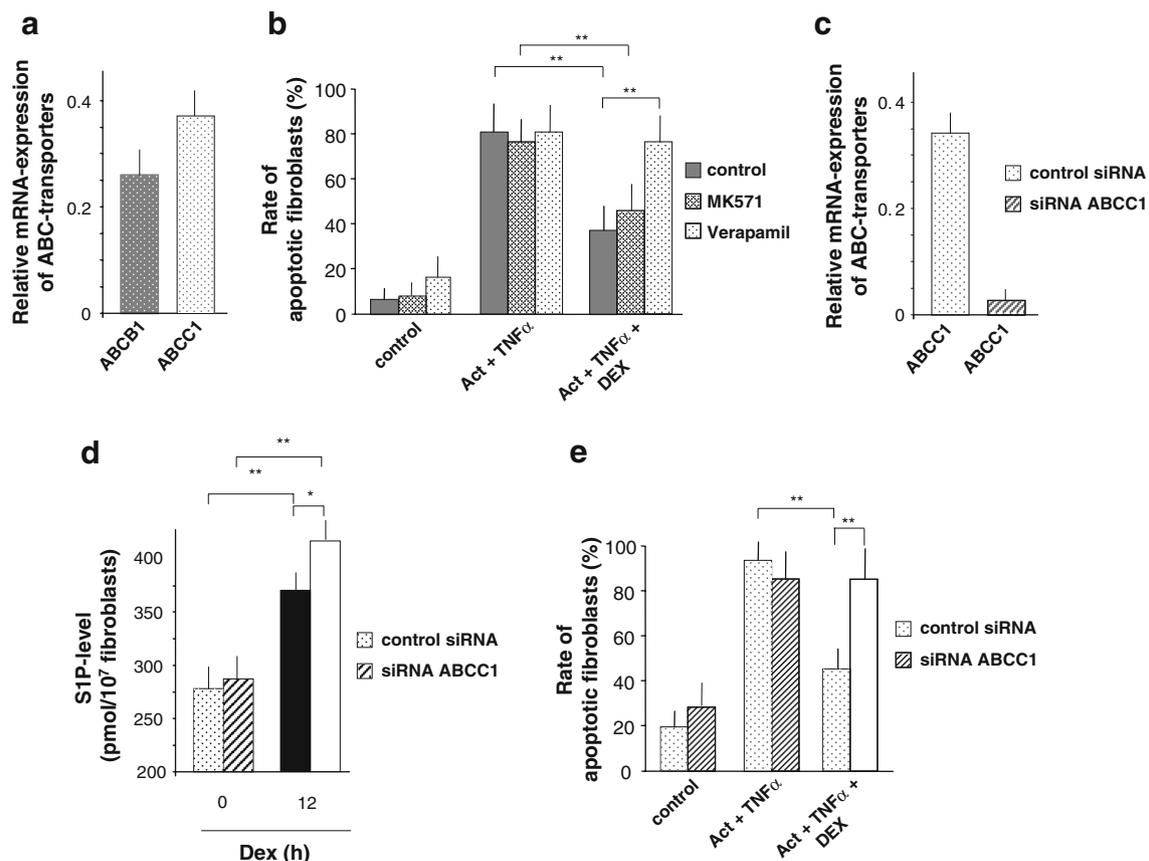
1  $\mu$ M of Dex (Fig. 5a, b). In accordance, protein levels of ABCC1 were enhanced, when cells were treated with Dex (Fig. 5c). Taken together, these results indicate that GC not only induce the formation of intracellular S1P by stimulation of SphK isoenzymes but also promote its export by an increased expression of the ABCC1 in a comparable manner.

The S1P<sub>3</sub> receptor subtype is increased by Dex and part of its cytoprotective property

Next, it was of interest to characterise the specific S1P receptor subtype involved in the anti-apoptotic signalling pathway of the GC. Although it is well established that all five S1P receptor subtypes are present in human fibroblasts, a regulation of these GPCRs by GC has not been reported in human fibroblast or other cell types, yet. In analogy to previous results [13], in human fibroblasts a dominant expression of the S1P<sub>3</sub> was observed (Fig. 6a). Moreover, when cells were stimulated with Dex a drastic enhancement of the S1P<sub>3</sub> receptor subtype occurred (Fig. 6a). In fact, real-time PCR showed a transient augmentation of the S1P<sub>3</sub> mRNA in response to Dex with a maximum at 8 h (Fig. 6c). Thereby, the most effective dose to mediate S1P<sub>3</sub> upregulation was detected with 1  $\mu$ M of the GC (Fig. 6b). It should be mentioned that there exist no appropriate antibodies for the analysis of S1P<sub>3</sub> protein levels up to now.

Employing antisense technique we further sought to elucidate which receptor subtype is responsible for the anti-apoptotic action of Dex-induced S1P formation. Real-time PCR analysis confirmed that treatment of fibroblasts with S1P<sub>1</sub>-, S1P<sub>2</sub>-, S1P<sub>3</sub>-, S1P<sub>4</sub>- and S1P<sub>5</sub>- ASO resulted in a significant abrogation of mRNA levels (data not shown). As depicted in Fig. 7, downregulation of the receptor subtypes S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>4</sub>, and S1P<sub>5</sub> did not reduce the inhibition of programmed cell death induced by Dex. It is of interest that only abrogation of the S1P<sub>3</sub> influenced the ability of Dex to inhibit apoptosis of fibroblasts, suggesting that this receptor subtype is crucial for cytoprotection (Fig. 8a). Furthermore, suramin, which has also been identified to block the S1P<sub>3</sub> receptor subtype, almost completely abrogated the anti-apoptotic effect of Dex (Fig. 8b). Finally, primary fibroblasts were isolated from wild-type or S1P<sub>3</sub> knockout mice. In agreement with human fibroblasts, Dex possessed a distinct cytoprotective property in fibroblasts isolated from wild-type mice (Fig. 8c). On the contrary, in S1P<sub>3</sub>-knockout fibroblasts, Dex was no longer able to protect cells from Act/TNF $\alpha$ -induced apoptosis confirming the involvement of S1P<sub>3</sub> in the anti-apoptotic signalling cascade of Dex (Fig. 8d).

These results clearly indicate that Dex exerts its anti-apoptotic effect by modulation of inside-out signalling of S1P. In the cytosol, SphK1 is upregulated by Dex leading to an increased intracellular amount of S1P. At the cell surface, the S1P<sub>3</sub> receptor subtype is essential for the anti-apoptotic action of Dex. The crucial connection between intracellular formed



**Fig. 4** ABCC1 is critical for the Dex-induced cytoprotection in human fibroblasts. Quantitative real-time PCR analysis of ABCB1 and ABCC1 starting with 10 ng of total RNA of three different sets of cells was performed using cyclophilinA as reference gene (a). To measure the effect of ABCB1 and ABCC1 inhibitors on Dex-induced cytoprotection, fibroblasts were preincubated with the ABCB1 inhibitor verapamil (10  $\mu$ M) and the ABCC1 inhibitor MK571 (100  $\mu$ M) for 30 min. Then, cells were treated with Dex (1  $\mu$ M, 8 h) and apoptosis was induced by the addition of 100 ng/ml Act and 20 ng/ml TNF $\alpha$  for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining (b). For silencing ABCC1 expression, fibroblasts were transfected with ABCC1-siRNA or control-siRNA B (final concentration 20 nM) for

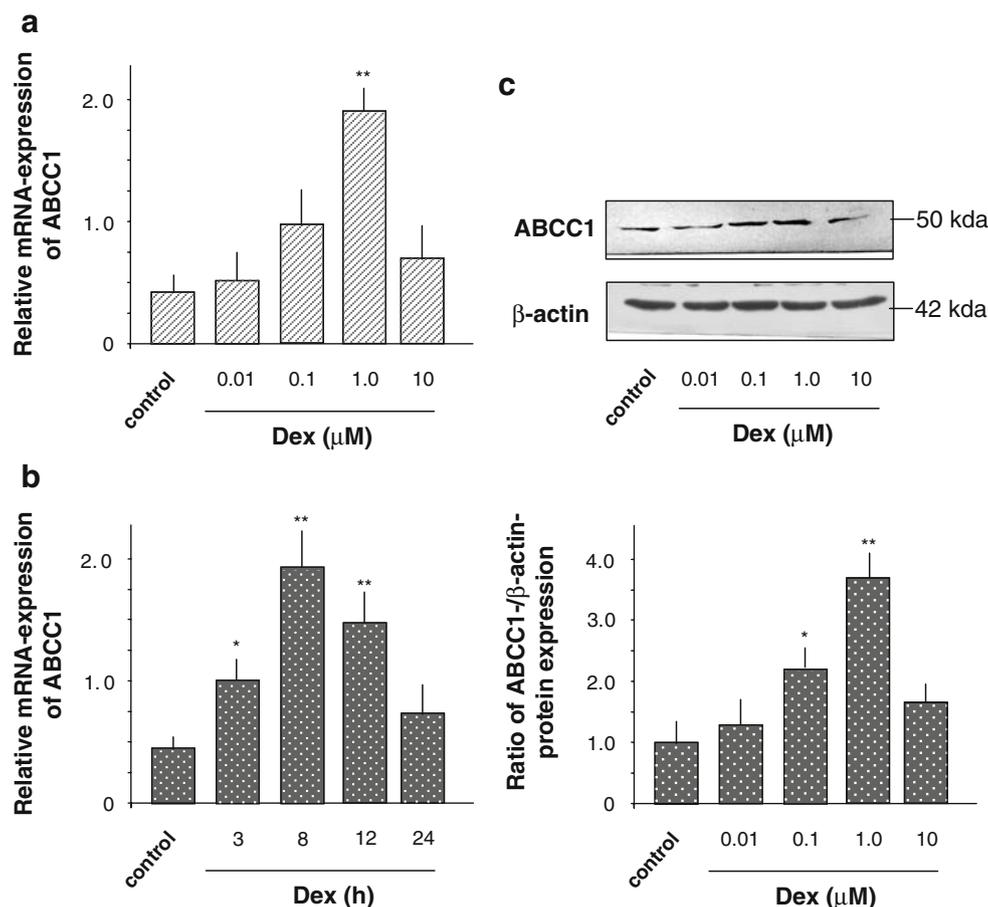
24 h. Real-time PCR starting with 10 ng of total RNA of three different sets of cells was performed using cyclophilinA as reference gene. Relative mRNA expression was quantified using the comparative threshold cycle method (c). To examine the effect of ABCC1 on Dex-induced cytoprotection, fibroblasts were transfected for 24 h with ABCC1-siRNA or control-siRNA B. Measurement of S1P-levels confirmed an enhancement of the lipid mediator in the cells (d). Then, cells were incubated with Dex (1  $\mu$ M, 8 h) followed by the addition of 100 ng/ml Act and 20 ng/ml TNF $\alpha$  for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining as described in the “Materials and methods” section (e). \* $P$ <0.05 and \*\* $P$ <0.001 indicate a statistically significant difference versus vehicle-stimulated cells

S1P and its extracellular action is the ABCC1-transporter which exports S1P to the cell surface receptor. Thus, it is not surprising that Dex not only enhances SphK-activity but also S1P<sub>3</sub>. Furthermore, Dex upregulates the mRNA as well as the protein level of the ABCC1 transporter and the S1P<sub>3</sub>-receptor subtype punctuating the importance of these two proteins for the anti-apoptotic signalling pathway of Dex (Fig. 9).

## Discussion

GC has been identified to mediate strikingly different biological responses regarding programmed cell death or

survival depending on the cell type [20]. In a variety of hematopoietic cells such as monocytes, T-lymphocytes and lymphoma cells, GC are able to trigger apoptosis [21, 22]. Thus, GC, in combination with further chemotherapeutics belongs to standard therapy regimes for the treatment of various leukaemias. Controversially, recent studies indicate that GC may also protect some endothelial and epithelial cells from programmed cell death [5]. Although it is well established that GC inhibit cell proliferation of human fibroblasts, they maintain survival of the epidermal cells by promoting anti-apoptotic processes [3]. But the molecular mechanism by which GC mediate apoptotic and especially anti-apoptotic actions are not well defined. Several models have been suggested in order to elucidate signalling path-



**Fig. 5** Dex increases ABCC1 in a time- and concentration-dependent manner. Quantitative real-time PCR analysis of ABCC1 in response to Dex was performed. Fibroblasts were stimulated with Dex for 8 h with the indicated concentrations of Dex (a) or for the indicated time periods with 1 μM of Dex (b). Real-time PCR starting with 10 ng of total RNA of three different sets of cells was performed using cyclophilinA as reference gene. Relative mRNA expression was quantified using the comparative threshold cycle method (a, b). For

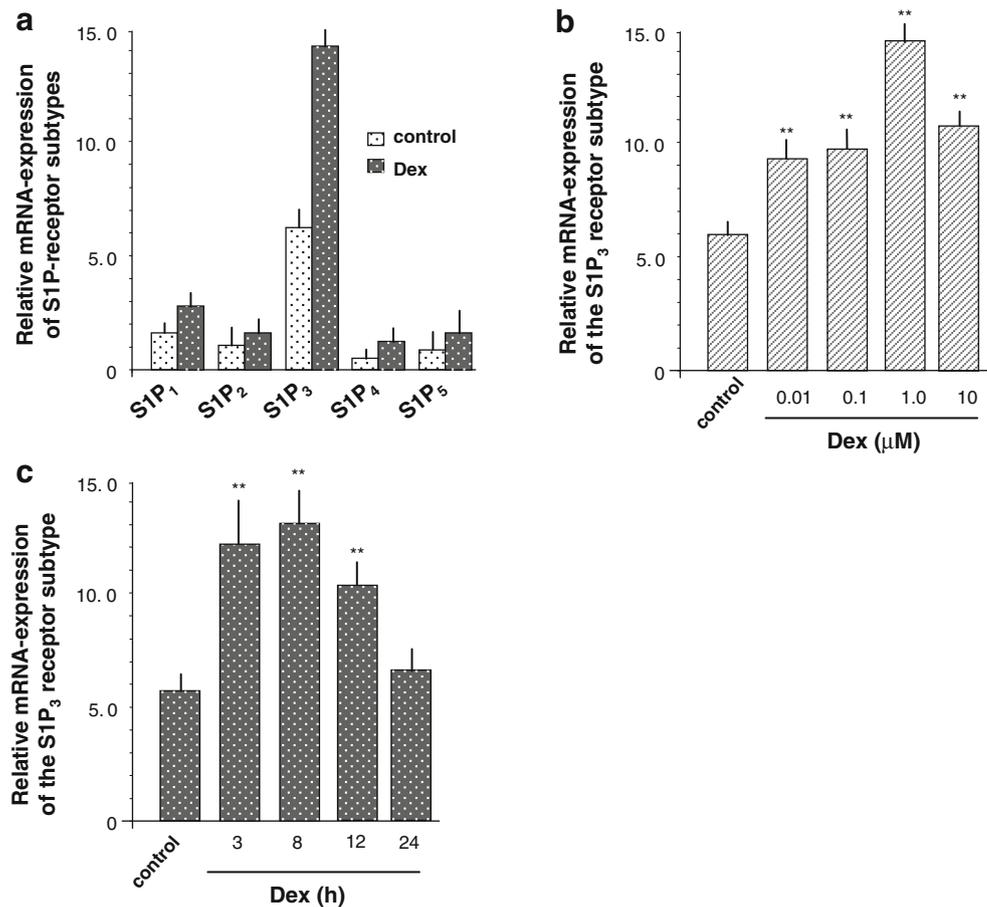
the examination of ABCC1-protein expression in response to 1 μM Dex, Western blot analysis of fibroblast cell lysates treated with the indicated GC concentrations for 8 h were performed. For control experiments β-actin-protein levels were measured. Densitometric analysis of Western blots shows the ratio of ABCC1/β-actin-protein expression from at least two individual experiments (c). Raw data are presented in the [electronic supplemental material](#). \* $P < 0.05$  and \*\* $P < 0.001$  indicate a statistically significant difference versus vehicle-stimulated cells

ways how GC can alter apoptosis and survival [23]. A variety of studies provide evidence that resistance to cell death of hematopoietic cells in response to GC is a result of defects in glucocorticoid receptor (GR), in GR-binding partners or GR target genes leading to a dysregulation of the rheostat of Bcl-2 proteins [23]. Thus, the BH3-only (Bcl-2 homology 3 domain) protein family members Bad, Bid, Bim and Puma serve as transmitters of apoptotic stimuli by activation of the multidomain family members Bak and Bax [5, 24, 25]. These proteins are critical for the formation of pores in the outer mitochondrial membrane, which is accompanied by the release of mitochondrial proteins such as cytochrome *c* [25]. Finally, by the formation of an apoptosome complex, cytochrome *c* provokes the activation of caspase 3. This process is tightly regulated by the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL, which

interact with pro-apoptotic Bcl2-proteins leading to their inactivation [26]. In contrast to the pro-apoptotic actions, an anti-apoptotic effect of GC has been elucidated depending on cell type and dosage. It is of interest that several studies indicate that a functional GR is required for a GC-induced anti-apoptotic signalling [5, 20].

Recent investigations have figured out that GC are able to activate the phosphoinositol-3-kinase (PI3K)/Akt kinase signalling pathway, which is known to be an essential mediator of cellular survival [27]. Moreover, NF-κB has been identified to participate as intracellular regulator of programmed cell death [3]. In hepatoma cells, it has been shown that GC mediate anti-apoptotic effects by an enhanced nuclear translocation of NF-κB [28]. It has been suggested that MKP-1 is not only involved in the cytoprotective action of NF-κB but is also increased in response to GC [5]. On the contrary, in human

**Fig. 6** Dex activates the S1P<sub>3</sub> receptor subtype in a time- and concentration-dependent manner. Quantitative real-time PCR starting with 10 ng of total RNA of three different sets of cells was performed using cyclophilinA as reference gene. Fibroblasts were stimulated with 1 μM Dex for 8 h (a) or with the indicated concentrations of Dex (b) or for the indicated time periods with 1 μM of Dex (c). Relative mRNA expression was quantified using the comparative threshold cycle method. Human fibroblasts were stimulated with Dex (1 μM, 8 h) followed by an immunofluorescence analysis of S1P<sub>3</sub> (d). \**P*<0.05 and \*\**P*<0.001 indicate a statistically significant difference versus vehicle-stimulated cells



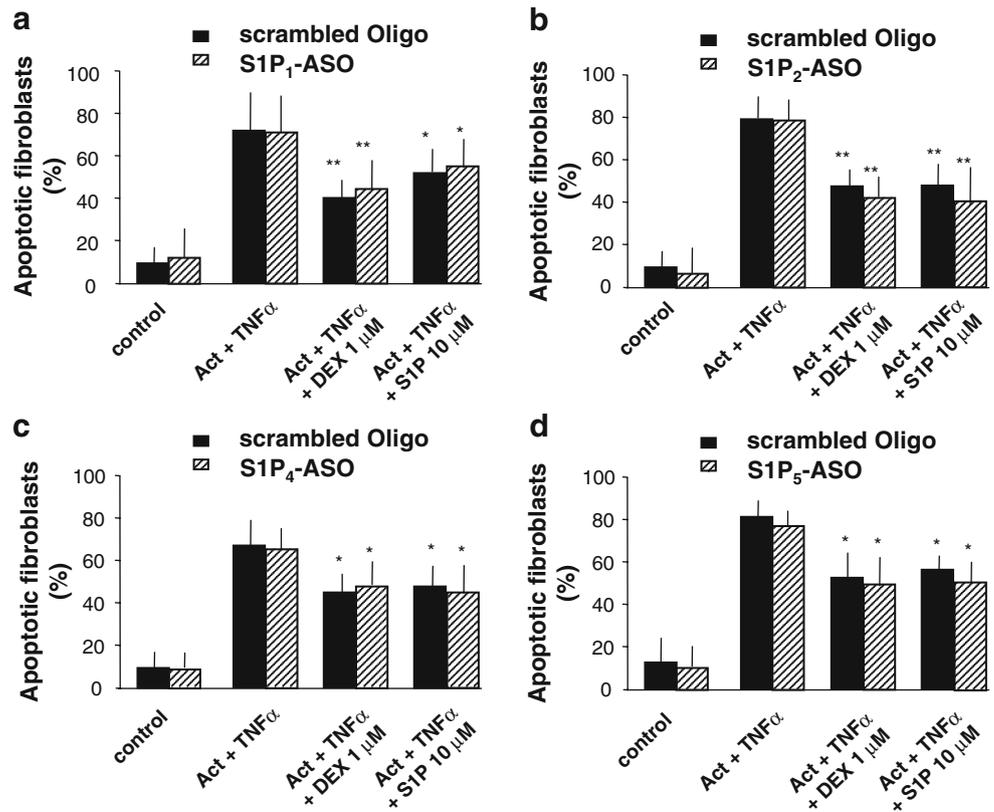
fibroblasts GC inhibit NF-κB activation suggesting that this signalling pathway is not involved in GC-mediated protection of the dermal cells. In this cell type, the sphingolipid S1P has been identified as a potent molecule to inhibit apoptosis [5].

For the intracellular generation of S1P from the precursor molecule sphingosine, two mammalian isoforms of SphK, SphK1 and SphK2, are essential [8]. Although these two enzymes possess five conserved domains and produce the same product, namely, S1P, they exert controversial effects concerning cell fate [10]. In the present study, we were able to show that GC induce a significant increase of mRNA and protein level of SphK1, whereas SphK2 is not affected. It is also well established that there exists a rapid activation of SphK1 by a wide range of growth factors including PDGF, vascular endothelial growth factor (VEGF) as well as epidermal growth factor (EGF). Nevertheless, recent work provides evidence that also steroid hormones like 17β-estradiol or calcitriol provoke an enhanced expression of this isoenzyme [8, 11, 29]. Moreover, silencing of SphK1 by siRNA completely prevented the anti-apoptotic action mediated by Dex indicating that SphK1 is crucial for the cytoprotective effect of the steroid hormone Dex. This is consistent with

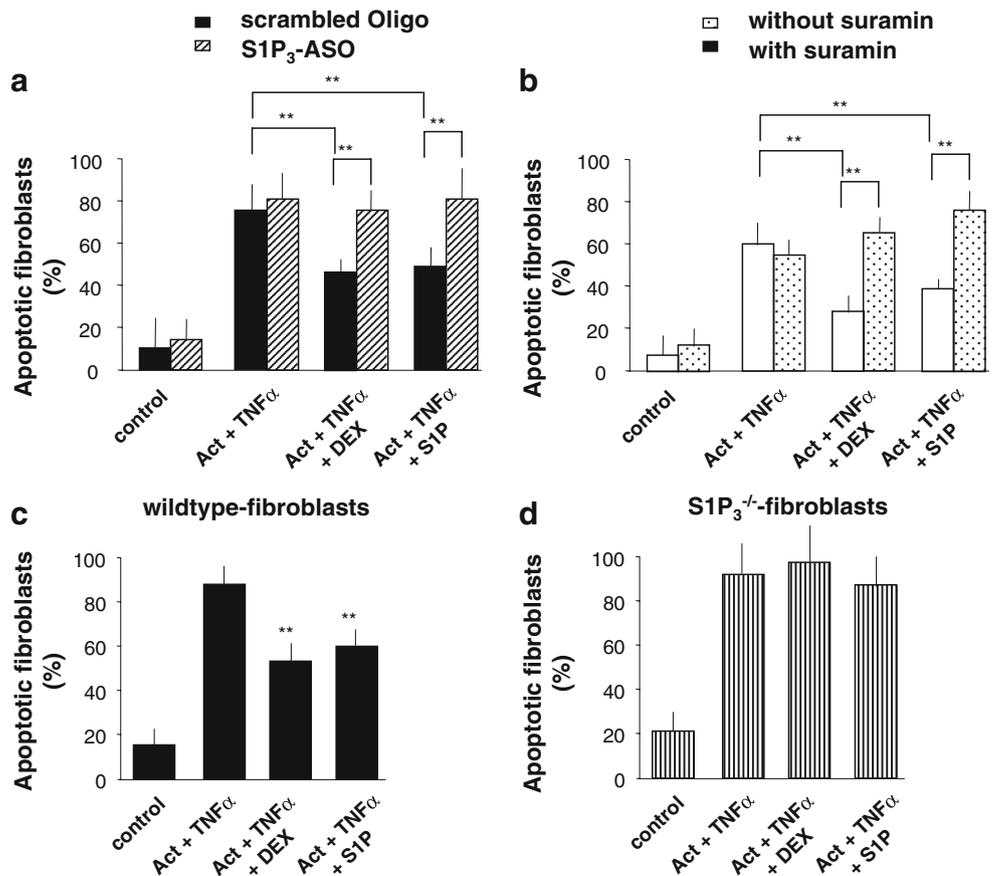
studies showing that SphK1 is the prominent isoenzyme to protect cells from programmed cell death [10, 30]. Thus, overexpression or agonist stimulation of SphK1 promote not only cell survival but also makes cells resistant against radiation and chemotherapy [31]. Although Dex enhances the expression of SphK1 it cannot be excluded that the GC induces a rapid activation of SphK1. Most interestingly, in contrast to anti-apoptotic SphK1, upregulation of SphK2 has been identified to contribute to cytotoxic actions [5, 25, 32]. Recent studies revealed that SphK2 contains a peptide sequence reminiscent of the BH3 domain of pro-apoptotic Bcl-2 family members. In analogy with BH3-domain-only proteins, it has been figured out that SphK2 interacts and neutralises prosurvival proteins like Bcl-2 and Bcl-xL [25].

A tremendous number of investigations clearly show that overexpression or activation of SphK1 due to various stimuli causes a translocation of the isoenzyme to the plasma membrane where its substrate sphingosine resides resulting in the formation of cytosolic S1P [8]. Our investigations encourage that also GC attribute to the formation of S1P in fibroblasts by an increase of SphK1 expression. Indeed, we were able to point out that intracellular S1P levels in fibroblasts are augmented in

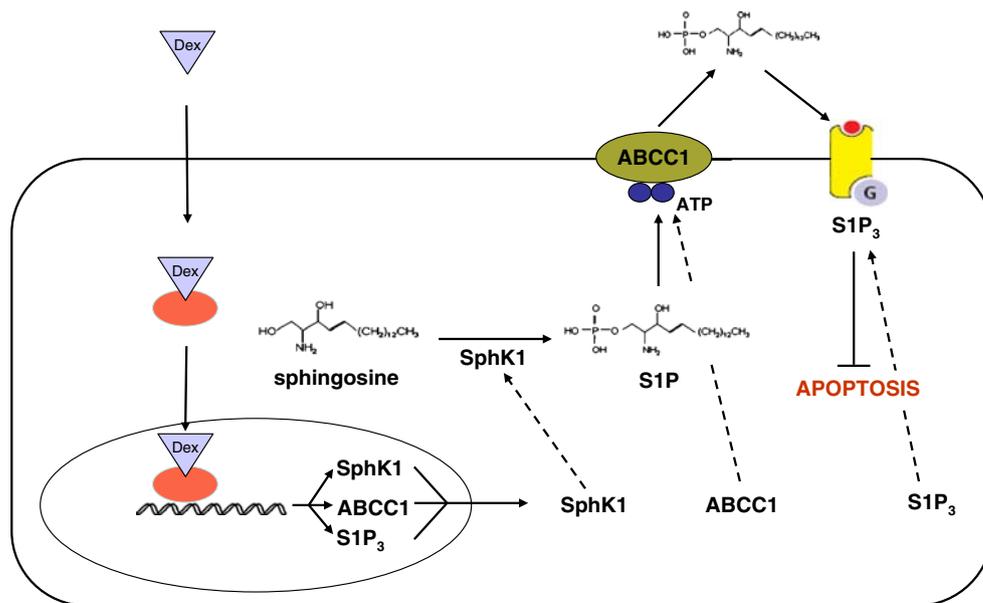
**Fig. 7** Involvement of S1P-receptor subtypes in Dex-mediated cytoprotection of human fibroblasts. Human fibroblasts were pretreated with control oligonucleotides (500 nM) or S1P<sub>1</sub><sup>-</sup>, S1P<sub>2</sub><sup>-</sup>, S1P<sub>4</sub><sup>-</sup>, S1P<sub>5</sub><sup>-</sup>-ASO (each 500 nM) for 72 h. Then, cells were incubated with Dex (1 μM, 8 h) or S1P (10 μM, 30 min) followed by the addition of 100 ng/ml Act and 20 ng/ml TNFα for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining as described in the “Materials and methods” section (a–d). \**P*<0.05 and \*\**P*<0.001 indicate a statistically significant difference versus vehicle-stimulated cells



**Fig. 8** Involvement of S1P<sub>3</sub> in Dex-mediated cytoprotection of fibroblasts. Human fibroblasts were pretreated with control oligonucleotides (500 nM) or S1P<sub>3</sub>-ASO (500 nM) for 72 h (a) or with suramin (10 μM) for 30 min (b). Then, cells were incubated with Dex (1 μM, 8 h) or S1P (10 μM, 30 min) followed by the addition of 100 ng/ml Act and 20 ng/ml TNFα for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining. Wild-type (c) and S1P<sub>3</sub><sup>-/-</sup>-fibroblasts (d) were stimulated with Dex (1 μM, 8 h) or S1P (10 μM, 30 min) followed by the addition of 100 ng/ml Act and 20 ng/ml TNFα for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining (c,d). \**P*<0.05 and \*\**P*<0.001 indicate a statistically significant difference versus vehicle-stimulated cells



**Fig. 9** Schematic overview of the hypothetical mechanism how Dex mediates cytoprotection in human fibroblasts



response to Dex. In this context, it has been suggested that cytosolic S1P may act either intracellularly or mediates its actions by activation of GPCR [12]. Taking a closer look regarding the anti-apoptotic signalling mediated by S1P, several studies suggest that extracellular receptors are involved in the S1P-mediated cytoprotection [33]. Nevertheless, it remains unclear how cytosolic-generated S1P is exported to the outer cell membrane of fibroblasts in order to take part in the interplay of Dex-mediated cytoprotection. The present study provides evidence that the ABC-transporter, ABCC1, is involved in the anti-apoptotic signalling cascade resulting in the export of intracellular S1P to the extracellular membrane. This so-called S1P signalling ‘inside and out’ has already been described in a variety of cell types and displays an important factor of the enigmatic signalling pathways of S1P [8]. Recent studies provide evidence that in human platelets, the release of S1P from the cytosol works in a carrier-dependent manner [34], moreover an involvement of the ABC-transporter ABCA1 in the export of S1P to the extracellular space has been discovered in astrocytes [15]. Additionally, another member of the ABC-transporter family, the ABCC1, has been identified as the crucial carrier for the S1P-export in mast cells. In these cells, ABCC1 accounts for the abundant constitutive stimulated secretion of S1P by antigens [14]. It remains unknown, however, how the preference of a specific ABC-transporter for S1P release is regulated by a certain cell type. In this context, the regulation of cell type expression of ABC-transporters may play a critical role [15]. Indeed, the present study corroborates not only an involvement of ABCC1 in the anti-apoptotic signalling

cascade of Dex in fibroblasts but also characterises a tight regulation of this carrier in response to the GC. Thus, Dex promotes an upregulation of ABCC1 mRNA and protein levels in order to transport S1P to the outer cell membrane of fibroblasts. Consistent with these results, pharmacological inhibition of ABCC1 or the use of siRNA captured S1P in the cell reflected by increased intracellular levels of S1P. Despite enhanced cytosolic S1P levels, the anti-apoptotic effect of Dex vanished. Moreover, it should be mentioned that several other investigations indicate an alteration of ABC-transporter expression by GC in a variety of cell types [19]. More precisely, an increase of ABCB1 mRNA has been observed after stimulation with Dex not only in HepG2 but also in CACO-2 cells [35].

The critical S1P-export suggests that S1P acts as ligand for its extracellular located S1P receptors. Indeed, down-regulation of S1P-receptors by specific ODN indicated that Dex mediates its anti-apoptotic effect via the S1P<sub>3</sub>-receptor subtype. Moreover, we were able to confirm our results analysing the cytoprotective effect of Dex on S1P<sub>3</sub>-knockout fibroblasts. Taken together, in the present study, we provide evidence that GC mediate their cytoprotective action via promotion of the inside-out signalling of S1P. In detail, Dex potently upregulates the cytosolic enzyme SphK1 leading to an increased intracellular amount of S1P. At the cell surface of fibroblasts, the S1P<sub>3</sub> was identified as the essential receptor subtype for the anti-apoptotic action of Dex. The decisive connection between intracellularly formed S1P and its extracellular action is the ABCC1-transporter which exports S1P to the cell surface receptor.

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**Conflict of interest statement** The authors declare that they have no competing financial interests.

## References

- Rhen T, Cidlowski JA (2005) Antiinflammatory action of glucocorticoids new mechanisms for old drugs. *N Engl J Med* 353:1711–1723
- Schoepe S, Schacke H, May E et al (2006) Glucocorticoid therapy-induced skin atrophy. *Exp Dermatol* 15:406–420
- Hammer S, Sauer B, Spika I et al (2004) Glucocorticoids mediate differential anti-apoptotic effects in human fibroblasts and keratinocytes via sphingosine-1-phosphate formation. *J Cell Biochem* 91:840–851
- Sharma S, Lichtenstein A (2008) Dexamethasone-induced apoptotic mechanisms in myeloma cells investigated by analysis of mutant glucocorticoid receptors. *Blood* 112:1338–1345
- Herr I, Gassler N, Friess H et al (2007) Regulation of differential pro- and anti-apoptotic signaling by glucocorticoids. *Apoptosis* 12:271–291
- Oh HY, Namkoong S, Lee SJ et al (2006) Dexamethasone protects primary cultured hepatocytes from death receptor-mediated apoptosis by upregulation of cFLIP. *Cell Death Differ* 13:512–523
- Spiegel S, Milstien S (2002) Sphingosine 1-phosphate, a key cell signaling molecule. *J Biol Chem* 277:25851–25854
- Takabe K, Paugh SW, Milstien S et al (2008) "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. *Pharmacol Rev* 60:181–195
- Watterson KR, Lanning DA, Diegelmann RF et al (2007) Regulation of fibroblast functions by lysophospholipid mediators: potential roles in wound healing. *Wound Repair Regen* 15:607–616
- Hofmann LP, Ren S, Schwalm S et al (2008) Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. *Biol Chem* 389:1399–1407
- Sukocheva OA, Wang L, Albanese N et al (2003) Sphingosine kinase transmits estrogen signaling in human breast cancer cells. *Mol Endocrinol* 17:2002–2012
- Goetzl EJ, Wang W, McGiffert C et al (2007) Sphingosine 1-phosphate as an intracellular messenger and extracellular mediator in immunity. *Acta Paediatr Suppl* 96:49–52
- Keller CD, Rivera Gil P, Tolle M et al (2007) Immunomodulator FTY720 induces myofibroblast differentiation via the lysophospholipid receptor S1P3 and Smad3 signaling. *Am J Pathol* 170:281–292
- Mitra P, Oskeritzian CA, Payne SG et al (2006) Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc Natl Acad Sci USA* 103:16394–16399
- Sato K, Malchinkhuu E, Horiuchi Y et al (2007) Critical role of ABCA1 transporter in sphingosine 1-phosphate release from astrocytes. *J Neurochem* 103:2610–2619
- Ishii I, Friedman B, Ye X et al (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP (B3)/EDG-3. *J Biol Chem* 276:33697–33704
- Manggau M, Kim DS, Ruwisch L et al (2001) 1 $\alpha$ , 25-Dihydroxyvitamin D3 protects human keratinocytes from apoptosis by the formation of sphingosine 1-phosphate. *J Invest Dermatol* 117:1241–1249
- Ruwisch L, Schafer-Korting M, Kleuser B (2001) An improved high-performance liquid chromatographic method for the determination of sphingosine-1-phosphate in complex biological materials. *Naunyn Schmiedeberg's Arch Pharmacol* 363:358–363
- Ayaori M, Sawada S, Yonemura A et al (2006) Glucocorticoid receptor regulates ATP-binding cassette transporter-A1 expression and apolipoprotein-mediated cholesterol efflux from macrophages. *Arterioscler Thromb Vasc Biol* 26:163–168
- Amsterdam A, Tajima K, Sasson R (2002) Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. *Biochem Pharmacol* 64:843–850
- Schmidt M, Pauels HG, Luger N et al (1999) Glucocorticoids induce apoptosis in human monocytes: potential role of IL-1 beta. *J Immunol* 163:3484–3490
- Davis MC, McColl KS, Zhong F et al (2008) Dexamethasone-induced inositol 1, 4, 5-trisphosphate receptor elevation in murine lymphoma cells is not required for dexamethasone-mediated calcium elevation and apoptosis. *J Biol Chem* 283:10357–10365
- Tissing WJ, Meijerink JP, den Boer ML et al (2003) Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. *Leukemia* 17:17–25
- Chittenden T (2002) BH3 domains: intracellular death-ligands critical for initiating apoptosis. *Cancer Cell* 2:165–166
- Liu H, Toman RE, Goparaju SK et al (2003) Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J Biol Chem* 278:40330–40336
- Letai A, Bassik MC, Walensky LD et al (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2:183–192
- Saffar AS, Dragon S, Ezzati P et al (2008) Phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase regulate induction of Mcl-1 and survival in glucocorticoid-treated human neutrophils. *J Allergy Clin Immunol* 121:492–498
- Evans-Storms RB, Cidlowski JA (2000) Delineation of an antiapoptotic action of glucocorticoids in hepatoma cells: the role of nuclear factor-kappaB. *Endocrinol* 141:1854–1862
- Sauer B, Gonska H, Manggau M et al (2005) Sphingosine 1-phosphate is involved in cytoprotective actions of calcitriol in human fibroblasts and enhances the intracellular Bcl-2/Bax rheostat. *Pharmazie* 60:298–304
- Shida D, Takabe K, Kapitonov D et al (2008) Targeting SphK1 as a new strategy against cancer. *Curr Drug Targets* 9:662–673
- Taha TA, Argraves KM, Obeid LM (2004) Sphingosine-1-phosphate receptors: receptor specificity versus functional redundancy. *Biochim Biophys Acta* 1682:48–55
- Sankala HM, Hait NC, Paugh SW et al (2007) Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. *Cancer Res* 67:10466–10474
- Hait NC, Oskeritzian CA, Paugh SW et al (2006) Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. *Biochim Biophys Acta* 1758:2016–2026
- Kobayashi N, Nishi T, Hirata T et al (2006) Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner. *J Lipid Res* 47:614–621
- Martin P, Riley R, Back DJ et al (2008) Comparison of the induction profile for drug disposition proteins by typical nuclear receptor activators in human hepatic and intestinal cells. *Br J Pharmacol* 153:805–819