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ORIGINAL ARTICLE

The MEK1/2–ERK1/2 Pathway is Activated in Chronic Rhinosinusitis with Nasal Polyps

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Abstract Chronic rhinosinusitis with nasal polyps (CRSwNP) is a common disease that has a considerable impact on the quality of life. Alterations in signalling pathways may contribute to the ongoing inflammation and proliferation in CRSwNP. The MEK1/2–ERK1/2 pathway transmits signals from many extracellular molecules to regulate cellular processes. We examined tissue samples from nasal polyps and the inferior turbinate of patients with CRSwNP and the inferior turbinate from subjects with healthy mucosa. The expressions of MEK1/2, ERK1/2, and their active phosphorylated forms pMEK1/2 and pERK1/2 were analysed using DNA microarray, quantitative real-time PCR, protein array, Western hybridisation, and immunohistochemistry. We detected increased MEK1/2 protein expression in nasal polyps compared to the inferior turbinates of patients with CRSwNP or healthy mucosa. We also found a higher amount of MEK1/2 in the inferior turbinates of patients with CRSwNP compared to those with healthy mucosa. Most importantly, we observed a significant increase in the phosphorylation of MEK1/2 and ERK1/2 in nasal polyps compared to both types of controls. We observed activation of the MEK1/2–ERK1/2 pathway in nasal polyps. Interestingly, we did not see the same activation pattern in different tiers of the MEK1/2–ERK1/2 signalling cascade. One explanation for this result is that the components enhance the complex MEK–ERK cascade in a distinct manner, enabling a wide variety of functions. The MEK1/2–ERK1/2 pathway appears to play a pivotal role in the pathogenesis of CRSwNP.

Keywords Chronic rhinosinusitis · ERK1/2 · MEK1/2 · Nasal polyp · Phosphokinase

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Introduction

Chronic rhinosinusitis (CRS) affects more than 10 % of the European population (Bachert and Zhang 2012). CRS has a large social impact and an economic burden, accounting for billions of dollars in health care and loss of working days worldwide each year (Sun et al. 2012).

Chronic rhinosinusitis with nasal polyps is a distinct pathologic sub-type of CRS with an estimated prevalence of 2–5 % (Fokkens et al. 2012). CRSwNP is a multifactorial disease, with numerous host and environmental factors, including reactive airway disease; allergic rhinitis; viral, bacterial and/or fungal colonisation/infection; osteitis; superantigens; and biofilms having been implicated (Fokkens et al. 2012; Sun et al. 2012). Taken together, CRSwNP is a recurrent, benign, extensively proliferative disease; the pathogenesis of CRSwNP is not fully understood.

One of the major mechanisms for controlling cellular functions in response to disturbances, including hormones and growth factors, is protein phosphorylation (Edelman et al. 1987). The mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases,

which are among the most ancient signal transduction pathways; they have been widely implicated throughout evolution in many physiological processes (Cargnello and Roux 2011; Widmann et al. 1999). MAPKs are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence. Generally, the phosphorylation of both threonine and tyrosine is required for MAPK activation (Rossomando et al. 1989).

All eukaryotic cells possess multiple MAPK pathways, which together regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation (Widmann et al. 1999). By far, the most extensively studied groups of mammalian MAPKs are the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases, and p38 MAPK isoforms (Cargnello and Roux 2011). MAPK signalling cascades lead to activation of the MAPK-activated protein kinases (Cargnello and Roux 2011).

The ERK cascade was the first elucidated MAPK cascade (Seger and Krebs 1995) and has been extensively analysed over the years (Rubinfeld and Seger 2005). The ERK cascade is composed of up to six tiers of sequentially activated protein kinases, which allow for the amplification and regulation of the transmitted signals. The main phosphorylation cascade includes rapidly accelerated fibrosarcoma (Raf) kinases, MAPK/ERK kinases (MEK), ERKs, and ribosomal s6 kinases (RSK) (Yoon and Seger 2006). The most important regulatory step is the activation of ERKs by MEKs (Rubinfeld and Seger 2005).

The main members of the MEK family are MEK1 (45 kDa) and MEK2 (46 kDa) (Seger and Krebs 1995). The mechanism of MEK1 activation involves protein phosphorylation of serine 218 (S218) and S222 within its activation loop. The phosphorylation of both S218 and S222 is important for full MEK1 activity (Cowley et al. 1994; Zheng and Guan 1994). The phosphorylation of each of these residues alone is sufficient to cause partial activation, although S222 most likely plays a major role in this activation (Seger et al. 1994). MEK2 is activated by phosphorylation on S222 and S226. MEKs provide specificity and an amplification step in the cascade, which makes them a central regulatory component in this signalling pathway. In addition to the activation loop, an important regulatory domain is located in the NH2-terminal region of MEKs (Crews et al. 1992; Seger et al. 1992). This domain contains a nuclear export signal (Fukuda et al. 1996; Jaaro et al. 1997) and an ERK-binding region (Tanoue et al. 2000).

ERK1 (44 kDa) and ERK2 (42 kDa) were first described by Boulton et al. (1991a, 1991b). ERK1 and ERK2 have a sequence identity of 83 %, with most of the differences falling outside the kinase core (Lu and Xu 2006). The MEK-catalysed phosphorylation of the two residues on ERKs seems to be a sequential reaction in which tyrosine phosphorylation (Y204 in ERK1; Y187 in ERK2) precedes threonine phosphorylation (T202 in ERK1; T185 in ERK2) (Pulido et al. 1998). The phosphorylation of a single residue on ERK fails to activate the enzyme; thus, activation requires the phosphorylation of both activation segment residues (Roskoski 2012).

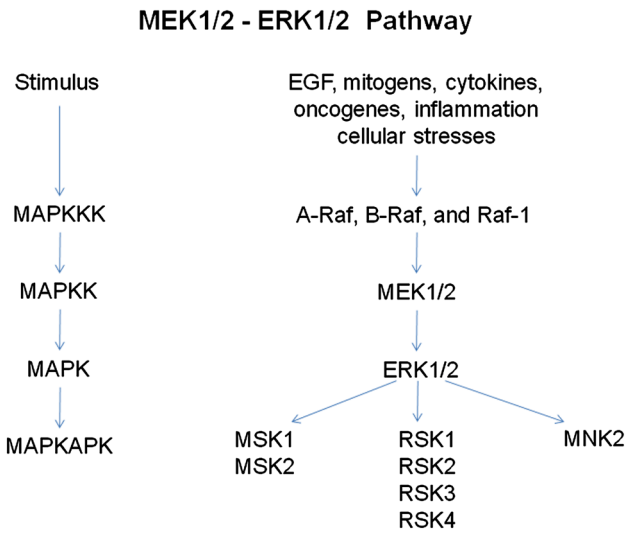

Fig. 1 Scheme of the MEK1/2–ERK1/2 pathway. The pathway is a four-component kinase module consisting of the MAPK ERK1/2, the upstream MAPKK MEK1/2, and the MAPKKK A-Raf, B-Raf or Raf-1, which receive signals from cell surface cytokine receptors or from other stimulating factors to trigger the downstream pathway. ERK1/2 is activated by a distinct kinase cascade in which the MAPKKK A-Raf, B-Raf, or Raf-1 phosphorylates and activates the downstream dual-specificity MAPKK MEK1/2, which in turn stimulates MAPK ERK1/2 activity through dual phosphorylation of threonine and tyrosine residues. Upon activation, ERK1/2 itself can phosphorylate the serine and threonine residues of the MAPK-activated protein kinases (MAPKAPK) MSK1/2, RSK1/2/3/4 or MNK2. *EGF* epidermal growth factor

The activation of ERK1/2, induced by different initiating signals, results in the phosphorylation of different substrates; thus far, more than 150 substrates have been identified. These substrates can be categorised as transcription factors, protein kinases, protein phosphatases, cytoskeletal proteins, scaffolding proteins, receptors, signalling molecules, apoptosis-related proteins, and other types of proteins (Fig. 1).

The pathogenesis of CRS and the underlying mechanisms of cell signalling are poorly understood, and alterations in the signalling pathways may contribute to ongoing inflammation and proliferation in CRSwNP patients. Our focus was to study whether the MEK1/2–ERK1/2-signalling cascade is involved in the pathogenesis of nasal polyps.

Materials and Methods

Patients

Endoscopic sinus surgery was performed on 22 patients with CRSwNP. The same batch of samples was analysed by multiple methods. We removed the polyps gently without bruising or tearing them. The duration of sinus complaints was more than 3 months, with a mean duration of 1.5 years (±0.7 years), and all of the patients had failed conservative therapy. The Lund–Mackay scoring system (0–24) was used to grade the radiographic extent of sinus disease (Lund and Mackay 1993). All of the patients suffered from bilateral polyposis, with a mean score of 16.2 (±5.1). The Sino-Nasal Outcome Test-20 German Adapted Version (SNOT-20-GAV) was used to evaluate the patients' quality of life (Baumann et al. 2008; Piccirillo et al. 2002), and the mean score was 34.5 (±11.5). The patients were skin tested for pollens, dust mites, pet dander, and moulds using standardised extracts (Allergopharma Joachim Ganzer KG, Reinbek, Germany) in a time frame of 4 weeks prior to surgery. Eosinophilic CRSwNP was determined by histopathological examination, and patients with neutrophilic polyps or mucoviscidosis were not included in the immunological study. No patient had undergone treatment with systemic or topical corticosteroids within 4 weeks before surgery.

As an internal control, we used samples from the inferior turbinates of the same patients. In addition, we examined ten inferior turbinate samples from patients without a history of sinusitis or allergy who underwent septal surgery or a septorhinoplasty as an external control.

The study was approved by the Human Ethics Committee of the University of Luebeck (AZ 10-201) and was conducted in accordance with the ethical principles for medical research formulated in the WMA Declaration of Helsinki. All of the patients provided signed informed consent.

DNA Microarray

Fresh tissue samples of nasal polyps and the inferior turbinates from eight patients with CRSwNP were immediately snap frozen in liquid nitrogen, stored at −80 °C, and shipped on dry ice to Miltenyi Biotec (Bergisch Gladbach, Germany) for microarray analysis. RNA was isolated and quality checked using the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA, USA), and the RNA integrity number was calculated. Briefly, 1.65 µg Cy3-labelled fragmented cRNA in hybridisation buffer was hybridised overnight (17 h, 65 °C) to Agilent Whole Human Genome Oligo Microarrays 4 × 44 K V1 using Agilent's recommended hybridisation chamber and oven. Gene expression was calculated using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware, Seattle, WA, USA).

Quantitative Real-Time PCR

RNA was isolated from 12 pairs of nasal polyps and the inferior turbinate of the same patient with CRSwNP. Quantitative real-time PCR (qRT-PCR) was performed using the following TaqMan® Gene Expression Assays: MEK1 (Assay ID Hs00983247_g1), MEK2 (Hs00360961_m1), ERK1 (Hs00385075_m1), ERK2 (Hs01046830_m1), ERK2 transcript variant 1 (Hs01052196_m1) and ß-actin (Hs99999903_m1; all Applied Biosystems, Foster City, CA, USA). The transcriptional activity of the genes studied was analysed using a LightCycler 1.5 (Roche, Basel, Switzerland). One microgram of each RNA sample was synthesised to cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), according to the manufacturer's instructions. The qRT-PCR reaction mixture consisted of 10 µl TaqMan® Gene Expression Master Mix (Applied Biosystems), 1 µl TaqMan® Gene Expression Assay, and 3 ng of cDNA. The thermal cycling conditions for qRT-PCR were as follows: 50 °C for 2 min for UDG incubation and 95 °C for 10 min for AmpliTaq Gold, UP enzyme activation, 50 cycles at 95 °C for 15 s and 60 °C for 1 min. ß-Actin was used as an internal standard. The qRT-PCR data were analysed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Protein Array

The Human Phosphokinase-Kinase Array Kit (Proteome Profiler™ Human Phospho-Kinase Array Kit, R&D Systems Inc., Minneapolis, USA) allows for the simultaneous detection of the relative levels of phosphorylation of 46 kinase phosphorylaton sites. The protein array contains MAPKs, MAPK kinases, signal transducer and activator of transcription proteins, other transcription factors, and signal transduction adaptor proteins. Capture and control antibodies were spotted in duplicate on nitrocellulose membranes. Tissue homogenates of the polyps from five patients with chronic polypoid sinusitis using the inferior turbinate as an internal control (250 µg of total proteins per array) were applied to the phosphoprotein array according to the manufacturer's instructions. The tissue extracts were diluted and incubated overnight with the array. The array was washed to remove unbound proteins followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-horseradish peroxidise (HRP) and chemiluminescent detection reagents were applied, and a signal was produced at each capture spot corresponding with the amount of bound phosphorylated protein.

The images were acquired using Fusion FX 7®, and the pixel density was analysed using Bio-1D software® (both Vilber Lourmat, Marne-la-Vallée, Cedex, France).

Western Blot Analysis

The tissue lysates from ten nasal polyps and ten inferior turbinates from patients with CRSwNP and ten inferior turbinates from patients with healthy mucosa were denatured at 95 °C for 5 min in 1× SDS sample buffer (Carl Roth GmbH, Karlsruhe, Germany). The protein concentrations were determined using Bradford's assay, and 30 µg of total protein was resolved by Tris–glycine gel electrophoresis (Mini-Protean TGX®, Any kD, BioRad, Hercules, CA, USA) and then transferred to nitrocellulose membranes. The membranes were initially blocked with Tris-buffered saline (TBS) containing 0.1 % TWEEN 20 (TBS-T) and 3 % non-fatty dry milk for MEK1/2 and ERK1/2, and TBS-T and 3 % bovine serum albumin (BSA; BioRad) for pMEK1/2 and pERK1/2 for 1 h at room temperature (RT).

The membranes were then probed overnight at 4 °C with the following primary antibodies: (1) anti-MEK1/2 (R&D Systems Inc., catalogue no. MAB2678) in TBS-T and 2 % non-fatty dry milk using a dilution of 1:2,000, (2) anti-phospho-MEK1/2 (S218/S222; S222/S226) (R&D Systems Inc., catalogue no. AF2506) using a dilution of 1:1,000, (3) anti-ERK (Novus, Littleton, Co, USA, catalogue no NBP-30128) using a dilution of 1:5,000, and (4) anti-phospho-ERK1/2 (T185/Y187; T202/Y204) (Invitrogen Corp, Camarillo, CA, USA, catalogue no 44-680G) using a dilution of 1:1000 in TBS-T and 3 % BSA.

The next day, the membranes were blocked again and then incubated with a 1:3,000 dilution of goat anti-mouse specific secondary antibody (BioRad) for MEK and a 1:3,333 dilution of goat anti-rabbit specific secondary antibody (BioRad) in TBS-T and 3 % BSA for pMEK, ERK, and pERK for 1 h at RT. The protein bands were visualised using an AP conjugate substrate kit (BioRad).

The pixel density of the Western blots was quantified and documented using Fusion FX7® and Bio-1D software®.

Nitrocellulose membranes for the Western blots to detect ERK1/2 were re-probed with a primary antibody for β-tubulin (Abcam, Cambridge, UK, catalogue no. 8226), and membranes for the Western blots to detect MEK1/2 were re-probed with a primary antibody for GAPDH (Cell Signaling Technology Inc., Danvers, MA, USA, catalogue no. 14C10), both with a dilution of 1:1,000 overnight at 4 °C to determine the loading in each lane. The membranes were washed and then incubated with a 1:3,333 dilution of secondary antibody in TBS-T and 3 % BSA for 1 h at RT. The membranes were washed, and the proteins bands were visualised as described above.

Immunostaining Analysis

Fresh frozen tissue sections of six nasal polyps and six inferior turbinates from patients with CRSwNP and six inferior turbinates from patients with healthy mucosa were air dried and fixed in ice-cold (−20 °C) 100 % methanol for 10 min. After a drying time of 10 min, the sections were washed three times with room temperature TBS (RT-TBS). Then, the tissue sections were incubated with 3 % H_2O_2 in RT-TBS for 15 min to inhibit the endogenous peroxidase.

After being washed with RT-TBS, the samples were permeabilised with 0.3 % Triton X-TBS for 15 min and then washed with RT-TBS. The sections were incubated with (1) a 1:50 dilution of anti-MEK1/2 (R&D Systems Inc., catalogue no. MAB2678), (2) a 1:100 dilution of anti-phospho-MEK1/2 (S218/S222; S222/S226) (R&D Systems Inc., catalogue no. AF2506), (3) a 1:200 dilution of anti-ERK (Novus, catalogue no NBP-30128), and (4) a 1:50 dilution of anti-pospho-ERK1/2 (T185/Y187; T202/Y204) (Invitrogen Corp, catalogue no 44-680G) overnight at 4 °C.

The next day, the slides were washed with RT-TBS and then incubated for 20 min at RT with a polylink secondary antibody. After washing with RT-TBS, the samples were incubated with an HRP-conjugated avidin–biotin-complex (both DCS Detection line), followed by incubation with AEC (DCS Chromoline, all Innovative Diagnostik-Systeme, Hamburg, Germany). The reaction was stopped using RT-TBS.

Finally, nuclear staining was performed using Mayer's haematoxylin. The sections were embedded in Dako Faramount aqueous medium (Dako North America Inc., Carpinteria, CA, USA). The immunostained sections were viewed and photomicrographed using an Axiovert 200 inverse microscope (Carl Zeiss, Oberkochen, Germany).

Statistics

The data were normally distributed. All of the cases were included in the analyses. The data are presented as the mean and standard deviation (SD). The microarray data were analysed using the Rosetta Resolver® gene expression data analysis system. The "Agilent/Intensity—Pairwise Ratio Builder" pipeline from the Rosetta software was used for data normalisation and to build ratio experiments. Within this pipeline, the "Build Pairwise Ratios" step applies the "Agilent Single Colour Ratio Error Model" to the profiles and builds the ratios. The applied normalisation was based on the Rosetta Affymetrix error model (Weng et al. 2006). The indicated p value was calculated from X Dev, which considers both the log(ratio) and log(error). The data were analysed using one-way ANOVA and sign test using Stat View 5.0 software (SAS Institute, Cary, NC,

USA). Differences between the groups were considered to be significant when $p < 0.05$.

Results

Gene Expression of ERK2 Transcription Variant 2 in the Polyps

We used microarray ($n = 8$) and qRT-PCR analysis ($n = 12$) of the nasal polyps and the inferior turbinates from patients with CRSwNP to identify a possible modification in the mRNA levels.

We detected equal amounts of MEK1 mRNA (NM_002755) on average and in each pair of samples of nasal polyps and inferior turbinates from the same patient by microarray; the mean fold change was 1.08 (SD 0.10). The microarray (NM_030662) revealed equal amounts of MEK2 mRNA on average with a mean fold change of 1.20 (SD 0.55). Only one pair had a significantly higher mRNA level in the polyp.

The microarray (NM_002746) did not show a significant difference in the amount of ERK1 mRNA on average, with a mean fold change of 1.20 (SD 0.59). However, we found a mixed picture with significant fold changes and a higher mRNA level in the polyp from three pairs and a significant fold change with a lower mRNA level in the polyps from the other three pairs.

The microarray (NM_002745) showed no quantitative difference on average in the amount of ERK2 mRNA transcript variant 1 between the samples of nasal polyps and the inferior turbinate from the same patient; the mean fold change was 1.00 (SD 0.08). We did not find a difference in any single pair.

Otherwise, ERK2 transcript variant 2 (NM_138957) had a significant mean fold change of 1.34 (SD 0.22; $p < 0.05$) by microarray analysis, indicating a higher amount of ERK2 mRNA transcript variant 2 in the polyps compared to the inferior turbinate of patients with CRSwNP. In seven of the eight pairs, we identified a significant fold change, with a higher mRNA level in the polyp and an equal amount in one case (Fig. 2).

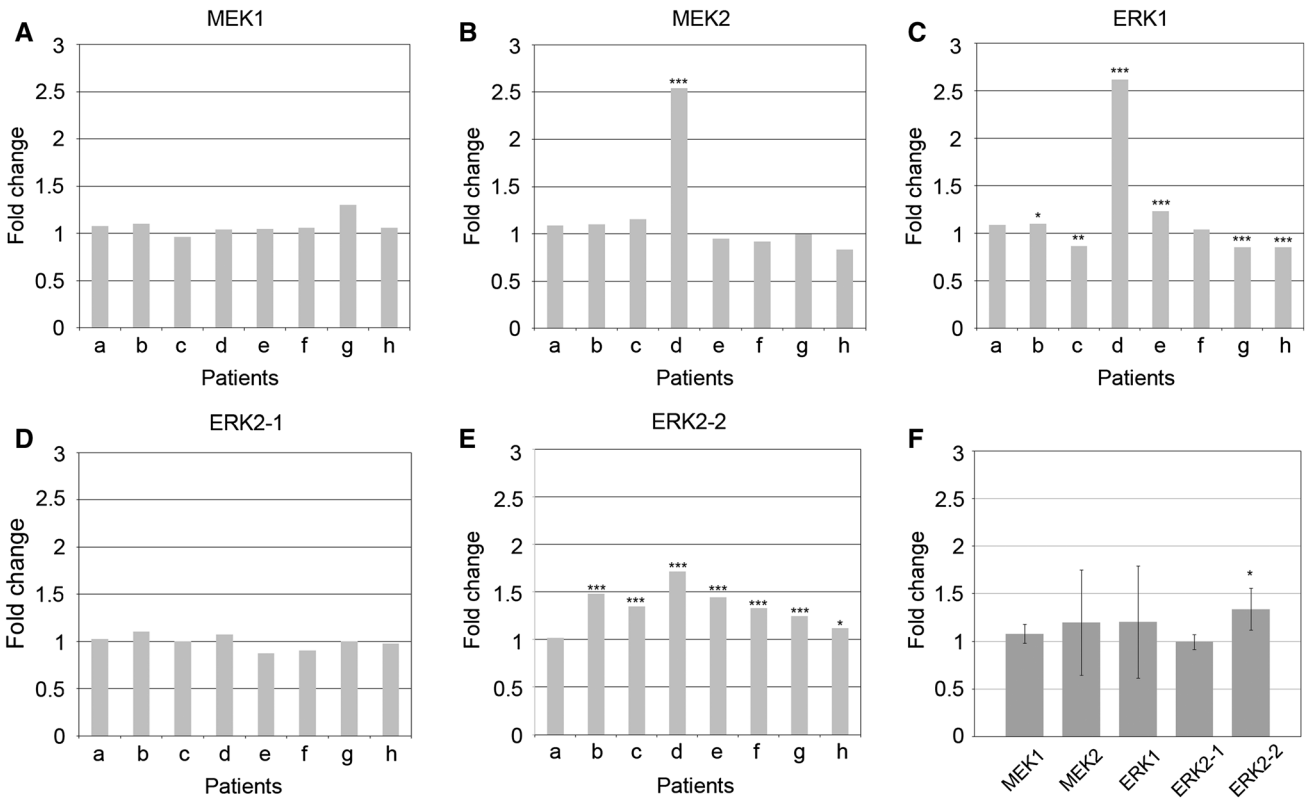

Fig. 2 DNA microarray results. Fold change in mRNA levels of the MEK1 (**a**), MEK2 (**b**), ERK1 (**c**), ERK2 transcript variant 1 (**d**), and ERK2 transcript variant 2 (**e**) between polyps and inferior turbinates for each sample pair from the same patient. The subject number is indicated (*a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*). We detected a significant fold change with a higher amount of ERK2 transcript variant 2 mRNA in the polyps compared to the inferior turbinate from the same patient with CRSwNP in seven out of eight samples. **f** Mean fold changes in mRNA levels (MEK1, MEK2, ERK1, ERK2 transcript variant 1 and 2) between polyps and inferior turbinates from the same patient. The *bars* indicate the standard deviation. *$0.01 \leq p < 0.05$, **$0.001 \leq p < 0.01$, ***$p < 0.001$

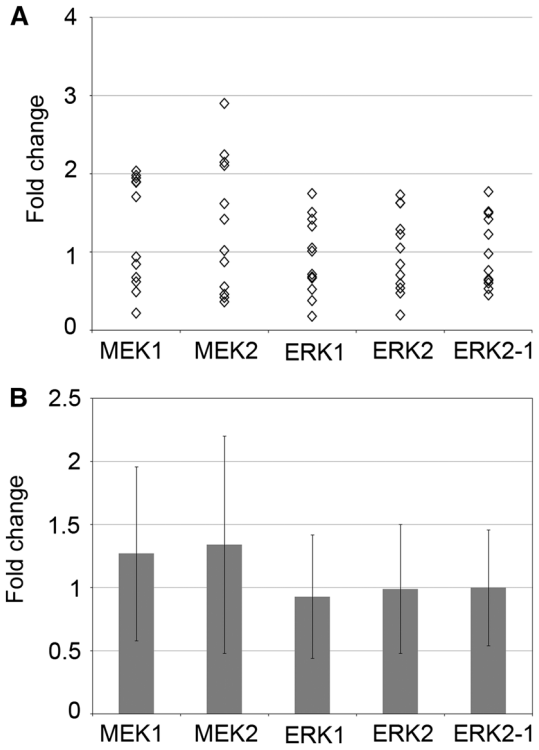

Fig. 3 qRT-PCR results. **a** Fold change in mRNA levels of the MEK1, MEK2, ERK1, ERK2, and ERK2 transcript variant 1 between polyps and inferior turbinates for each sample pair from the same patient ($n = 12$). **b** Mean fold changes in mRNA levels (MEK1, MEK2, ERK1, ERK2, and ERK2 transcript variant 1) between polyps and inferior turbinates from the same patient. The *bars* indicate the standard deviation

These microarray results were validated by qRT-PCR. We detected equal mean mRNA levels of MEK1, MEK2, ERK1, ERK2 total amount of transcript variant 1 and 2 and ERK2 transcript variant 1 in the polyps and the inferior turbinates from the same patient (Fig. 3; Table S1). We could detect only the total amount of ERK2 mRNA and ERK2 transcript variant 1 mRNA by qRT-PCR. Therefore, we were not able to confirm the microarray observation of an increase of the mRNA level of ERK2 transcript variant 2 in the polyps compared to the inferior turbinate of patients with CRSwNP by qRT-PCR.

The results of the DNA microarray for the kinases upstream from MEK1/2 and ERK1/2′s downstream targets are listed in Table S2. Additionally, the microarray (NM_003942) showed significant fold changes in the mitogen- and stress-activated protein kinase 2 (MSK2); there were higher mRNA levels in the polyps of five sample pairs and lower mRNA levels in the polyps of one case sample. The mean fold change was 1.60 (SD 0.95). The microarray (NM_002953) revealed a significant mean fold change of 1.62 (SD 0.40; $p < 0.05$), indicating a higher amount of RSK1 RNA in the polyps compared to the inferior turbinate of patients with CRSwNP on average. In seven of the eight pairs, we identified a significant fold change, with higher mRNA levels in the polyps and lower mRNA levels in the polyp in one case.

We observed upregulation at the transcription level of the direct downstream target paired box protein 6 (Pax6)/(NM_001604) and of the protein budding uninhibited by benzimidazoles 1 (BUB-1)/(NM_004336), which is located two steps downstream.

We detected downregulation of nuclear receptor 77 (Nur77)/(NM_002135) and microphthalmia-associated transcription factor (MITF)/(NM_198159) at the transcription level, both of which are located two tiers downstream from ERK1/2.

Table 1 Western blotting results

	Polyp	Turbinate (CRSwNP)	Turbinate (healthy mucosa)
MEK1/2	5.34 (1.89)	2.13 (0.57)	1 (0.23)
pMEK1/2 (S218/S222; S222/S226)	2.18 (0.43)	0.94 (0.27)	1 (0.16)
ERK1/2	1.13 (0.22)	0.87 (0.27)	1 (0.11)
pERK1/2 (T185/Y187; T202/Y204)	2.41 (0.63)	1.18 (0.48)	1 (0.35)

Amount of MEK1/2, pMEK1/2, ERK1/2, and pERK1/2 in polyps, turbinates of patients with CRSwNP, and healthy mucosa; mean (SD)

MEK1/2 Protein Expression is Higher in the Polyps Compared to the Inferior Turbinates of Patients with CRSwNP and much Higher Compared to Healthy Mucosa

Western blot analysis was performed to study the protein expression levels of MEK1/2 and ERK1/2. We detected significantly higher expression of MEK1/2 in the nasal polyps ($n = 10$) compared to the inferior turbinates from patients with CRSwNP ($n = 10$, 2.51-fold, $p < 0.01$) and patients with healthy mucosa ($n = 10$, 5.34-fold, $p < 0.01$). The difference between the amount of MEK1/2 in the inferior turbinates of patients with CRSwNP compared with those with healthy mucosa was also significant (2.12-fold, $p < 0.05$). Western blot analysis showed no difference in the expression of unphosphorylated ERK1/2 between the polyps and turbinates of patients with CRSwNP and those with healthy mucosa (Table 1; Fig. 4).

MEK1/2 and ERK1/2 are Activated in Nasal Polyps

MEK1/2 and ERK1/2 are activated by phosphorylation at defined sites. Hence, the phosphorylation level was

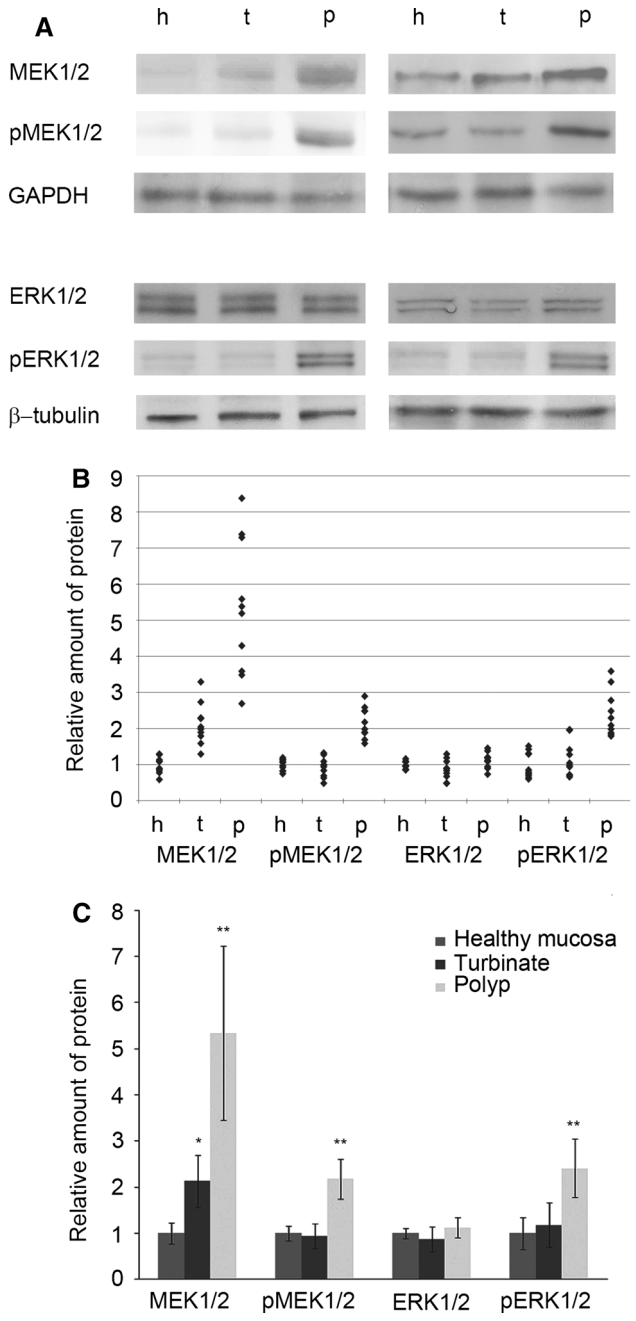

Fig. 4 Western hybridisation results. **a** Representative samples of MEK1/2, pMEK1/2, ERK1/2, and pERK1/2 in polyps (p), turbinates (t), and healthy mucosa (h). Reprobing with an antibody against GAPDH or ß-tubulin demonstrated equal protein loading in *each lane*. **b** The levels of MEK1/2, pMEK1/2, ERK1/2 and pERK1/2 from samples of polyps (p), turbinates of patients with CRSwNP (t), and healthy mucosa (h). **c** The mean levels of MEK1/2, pMEK1/2, ERK1/2, and pERK1/2 in polyps and inferior turbinates of patients with CRSwNP compared to healthy mucosa. We observed a higher amount of MEK1/2 in polyps compared to both the turbinates of patients with CRSwNP and healthy mucosa, and a higher amount of MEK1/2 was observed in the turbinates of patients with CRSwNP compared to healthy mucosa. We detected a higher amount of pMEK1/2 and pERK1/2 in the polyps compared to both control types. The *bars* indicate the standard deviation. *$0.01 \leq p < 0.05$, **$p < 0.01$

analysed using a phosphokinase protein array and Western hybridisation experiments. The quantitative analysis of the protein array showed significantly increased levels of pMEK1/2 and pERK1/2 in the polyps from four of the five tested pairs of polyp and turbinate samples from patients with CRSwNP. The mean concentration of pMEK1/2 and pERK1/2 in the polyps was 1.79-fold and 2.19-fold higher, respectively, compared to the inferior turbinates of patients with CRSwNP ($n = 5$, $p < 0.05$; Fig. 5). The Western blot analysis confirmed the results of the protein array. The amounts of pMEK1/2 and pERK1/2 were approximately twofold in the polyps ($n = 10$) compared to the inferior turbinates of patients with CRSwNP ($n = 10$) and those with healthy mucosa ($n = 10$, $p < 0.01$). No difference between the inferior turbinates from patients with CRSwNP and those with healthy mucosa was observed (Table 1; Fig. 4).

The protein array results for the kinases downstream from ERK1/2 are listed in Table S3. Additionally, we observed marginal evidence of higher phosphorylated mitogen- and stress-activated protein kinase 1/2 (pMSK1/2) levels in the polyps compared to the inferior turbinates of the same patient with CRSwNP in the protein array ($p = 0.79$).

Spatial Distribution of MEK1/2, pMEK1/2, ERK1/2, and pERK1/2

We observed nearly the same spatial distribution of MEK in the polyps and turbinates of patients with CRSwNP and those with healthy mucosa. In all types of tissue, MEK was localised, particularly to the cytosol of the cells lining the basal membrane. We did not observe notable staining of the stroma cells. Additionally, the staining pattern of pMEK was very similar to that of MEK in the cytosol of a few epithelial cells and not noteworthy in the submucosal space. ERK was found in only some of the cells from the turbinates of patients with healthy mucosa and those with CRSwNP, while we detected clear ERK staining in the cytosol of nearly all of the cells in the basal epithelial layer of the polyps. The cells in the connective tissue were rarely stained in all three types of tissue. We detected pERK staining in all of the layers of the epithelium in the three tissue types, but more cells were stained in the epithelium of the polyps compared to the turbinates of patients with CRSwNP and many more were stained compared to patients with healthy mucosa. In the stroma of the polyps, pERK was stained in nearly all of the connective tissue cells and infiltrating cells of the immune system. The rate of stained cells in the stroma of the turbinates of patients with CRSwNP was more than double that of patients with healthy mucosa (Fig. 6).

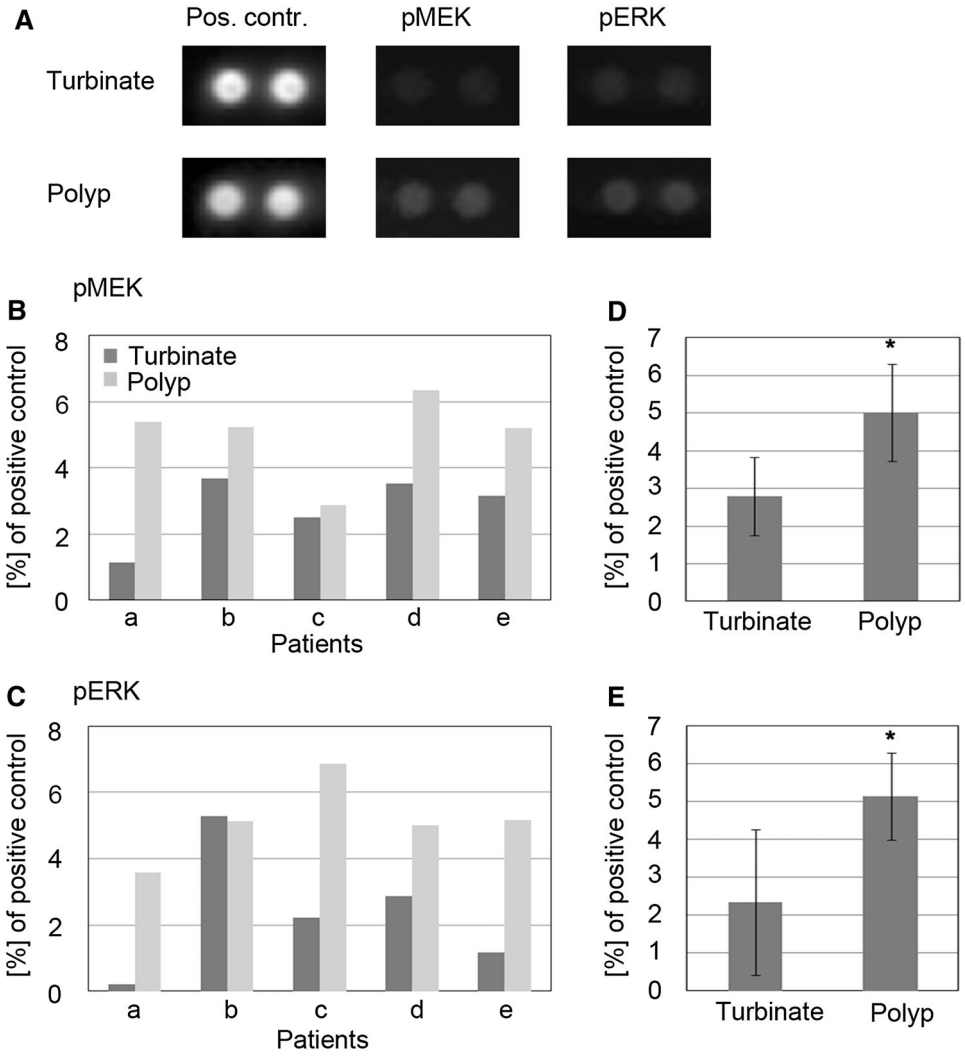

Fig. 5 Protein array results. **a** Representative protein array of a polyp and an inferior turbinate from the same patient. **b**, **c** Amount of pMEK1/2 and pERK1/2 in polyps and inferior turbinates from the same patient. The subject number is indicated (*a*, *b*, *c*, *d*, *e*). We observed a higher amount of pMEK1/2 in the polyps of all the sample pairs and a higher amount of pERK1/2 in the polyps in four out of five sample pairs. **d**, **e** Mean levels of pMEK1/2 and pERK1/2 in polyps and turbinates from the same patient with CRSwNP. We detected significantly higher mean levels of both pMEK1/2 and pERK1/2 in the polyps. The *bars* indicate the standard deviation. $*p < 0.05$

Discussion

Knowledge About CRSwNP is Increasing, but the Intracellular Mechanisms are Poorly Understood

Chronic rhinosinusitis is a clinically heterogeneous group of sinus diseases (Van Zele et al. 2006) that can be classified as CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP; Fokkens et al. 2012). CRSwNP is often associated with bronchial asthma (Sejima et al. 2012).

CRS is associated with a general inflammatory reaction, and numerous inflammatory mediators are activated in this mucosal infection (Tóth et al. 2011). CRS positively correlates with increased interleukin 4 (IL-4), IL-5, IL-10 and IL-13, which play an important role in the resolution of infections. Additionally, an increase in IL-12, which is involved in pathogen-related immune activation by antigen-presenting cells, has been observed (Riechelmann et al. 2005). IL-19 is overexpressed in the epithelium of patients with CRSwNP (Pace et al. 2012).

In the study of CRSwNP, there have been numerous new findings related to biofilms (Hirotsu et al. 2011; Tóth et al. 2011), the role of *Staphylococcus aureus* (Sachse et al. 2010), inflammatory cells (Sejima et al. 2012; Van Zele et al. 2006), Th1/Th2 polarisation (Riechelmann et al. 2005; Sejima et al. 2012; Tóth et al. 2011; Van Zele et al. 2006), remodelling processes linked to fibrosis or oedema formation (Garavello et al. 2005), and disturbances in innate or adaptive immunity (Pacova et al. 2009; Ratjen and Döring 2003; Schubert 2004).

Although knowledge of this multifactorial disease has increased, the involvement of the respiratory epithelium in the mechanisms of CRS is poorly understood. Thus, the lack of an understanding of the molecular mechanisms by which a cell deciphers extracellular signals remains a fundamental problem (Rosen 1987). However, the link

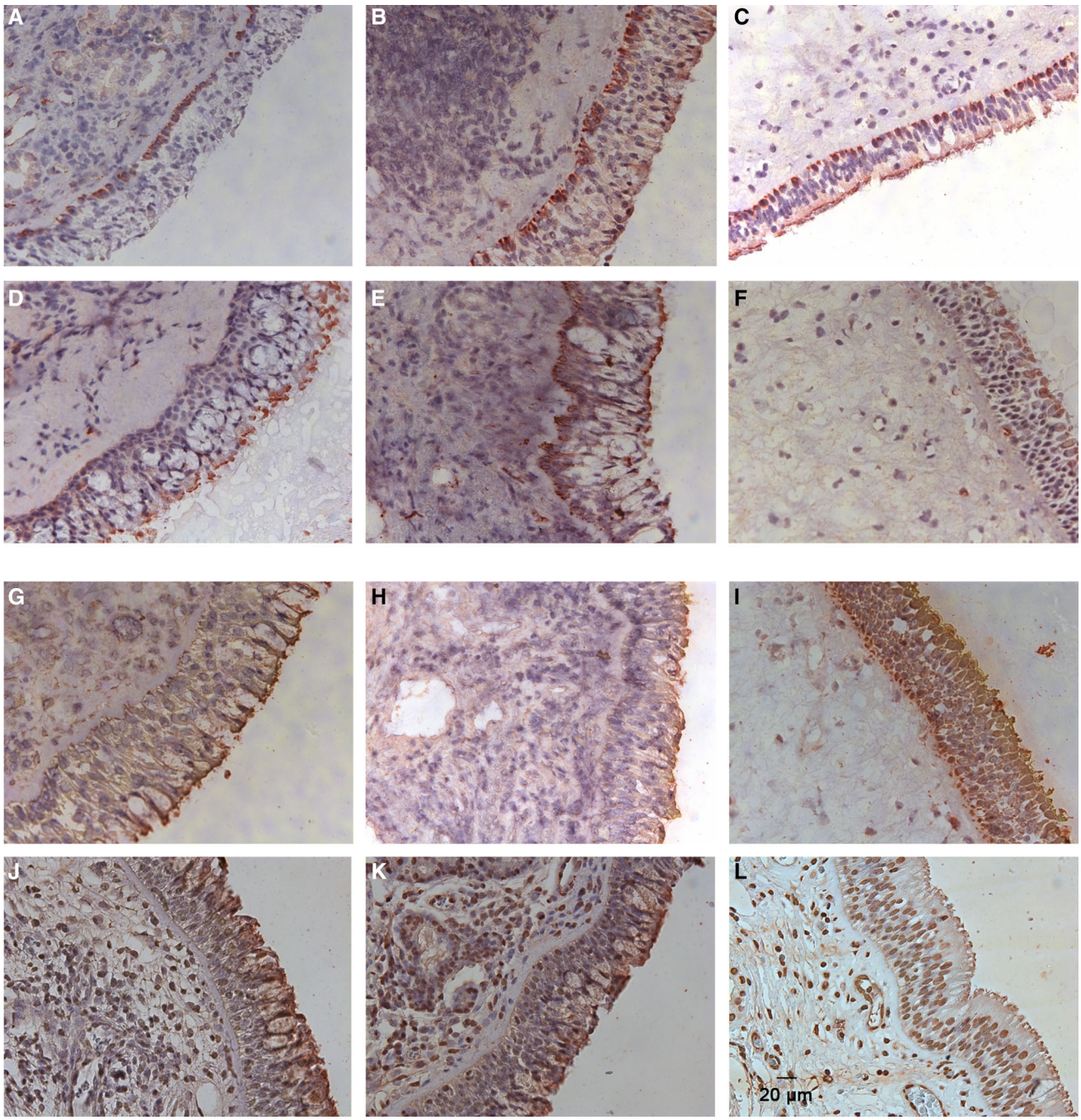

Fig. 6 Representative examples of immunostaining with a haematoxylin counterstain. **a**–**c** MEK1/2, **d**–**f** pMEK1/2, **g**–**i** ERK1/2, and **j**–**l** pERK1/2 (**a**, **d**, **g**, **j** healthy mucosa; **b**, **e**, **h**, **k** turbinate/CRSwNP; **c**, **f**, **i**, **l** polyp). Both MEK and pMEK were localised particularly in the cytosol of the epithelial cells facing the basal membrane, and only a few cells from the stroma were stained. ERK1/2 showed cytosol staining in a few cells of the basal layer of the epithelium in the turbinates of patients with CRSwNP and with healthy mucosa, but almost every cell of the basal layer of the epithelium from the polyps exhibited staining. pERK was found in cells of all layers of the epithelium in the three different types of tissue; however, pERK was found in more cells in the polyps than the turbinates from patients with CRSwNP and in much greater quantities compared to patients with healthy mucosa. All of the stroma cells (fibroblasts and invading cells from the immune system) were stained in the polyps, and more cells in the turbinates were stained in patients with CRSwNP than in those with healthy mucosa

between altered signalling pathways and the local inflammatory response and proliferation remains poorly defined in CRSwNP. The MEK/ERK cascade is a central pathway in many cellular processes; the MAPKs regulate diverse cellular programs by relaying extracellular signals to intracellular responses (Cargnello and Roux 2011).

Distinct Activation Patterns in the Different Tiers of MEK–ERK Signalling

We observed a higher amount of ERK2 transcript variant 2 mRNA in the nasal polyps by microarray in a small number of samples. We found increased MEK1/2 protein expression in the nasal polyps compared to the inferior turbinates from patients with CRSwNP and those with healthy mucosa, and a higher level of MEK1/2 protein expression in the inferior turbinates of patients with CRSwNP compared to those with healthy mucosa by Western hybridisation. This difference was not identified by the immunostaining experiments. This might be due to a different sensitivity of the antibody used for the various applications. We detected a significant increase in the MEK1/2 and ERK1/2 phosphorylation rates in the nasal polyps compared to the turbinates of patients with CRSwNP or healthy mucosa.

There are different ways to generate this higher amount of phosphorylated MEK1/2 and ERK1/2. The first possibility is a higher expression at the transcription level, with a higher quantity of mRNA leading to higher protein expression and therefore a higher amount of substrate for activated upstream signalling proteins in the cascade. We observed a higher gene expression at the transcription level for one transcript variant (transcript variant 2) of ERK2 by microarray, but this result could not be confirmed by qRT-PCR. ERK2 transcript variant 1 and ERK2 transcript variant 2 encode for the same protein, and the total amount of ERK2 transcript variant 1 and ERK2 transcript variant 2 did not differ between polyps and turbinates. In line with this observation, we detected an equal amount of ERK1/2 protein in all three types of tissue. The second feasible mechanism is higher protein expression resulting from a faster protein biosynthesis process regardless of the mRNA amount. This mechanism could explain the case of MEK1/2. A more intense phosphorylation of the existing protein by upstream signalling molecules is the third potential process. Thus, it is possible that the higher amount of pERK1/2 is due to a higher quantity of pMEK1/2.

In general, we observed activation of the MEK1/2–ERK1/2 pathway in the nasal polyps, but our results showed no consistent increase across all stages of the cascade. We found distinct activation patterns in different tiers of the pathway, which was mediated by sequential phosphorylation and activation of protein kinases.

However, we observed an increased amount of pERK1/2 in nasal polyps compared with the inferior turbinate of patients with CRSwNP and healthy mucosa, suggesting that the preliminary end point of the MEK1/2–ERK1/2 cascade is altered before dissemination of signalling. This higher amount of pERK1/2 may be attributed to a higher quantity of pMEK1/2, and this higher quantity of pMEK1/2 again is explained by a higher total MEK1/2 protein amount. We conclude that in CRSwNP, the regulation of MEK and ERK is disrupted at several points, but the key event is possibly the higher protein expression of MEK1/2.

We also observed marginal evidence of higher pMSK1/2 levels with the protein array screening method, but this change was not significant and this protein was therefore not studied further. Additionally, the DNA microarray data suggest upregulation of MSK2, RSK1, a kinase (BUB-1), and a transcription factor (Pax6) downstream from ERK1/2, and downregulation of another transcription factor (MITF) and an orphan nuclear receptor (Nur77) downstream from ERK1/2 at the transcription level. However, taken together, these findings do not provide clear evidence of the activation of MSK1/2, RSK1/2/3/4, or the mitogen-activated protein kinase-interacting kinase 2 (MNK2), which are considered the main direct downstream targets of ERK1/2. The functional consequence of MAP kinase signalling activation is determined not only by phosphorylation processes, but also by different combinations of the three tiers of kinases, the regulation and involvement of distinct scaffolding proteins, and a selective interaction between MAP kinase and its binding proteins, such as MAP kinase substrates, MAP2Ks, phosphatases, and scaffolding proteins (Lu and Xu 2006). In this way, the ERK signals can be disseminated through the multiplicity of the cascade's approximately 160 substrates. In addition, cross talk and feedback loops exist between the different MAPK pathways, and these pathways also have relationships with other pathways. ERK1/2 activation regulates proliferation, differentiation, survival, migration, angiogenesis, and even chromatin remodelling through the phosphorylation of both cytoplasmic and nuclear targets, including phosphatases, transcriptional factors, and cytoskeletal proteins (Dhillon et al. 2007). These processes are not fully understood, and the involvement of processes regulated by the ERK cascade is often opposed. Therefore, future research is needed to study the downstream targets and downstream events of the MEK1/2–ERK1/2 pathway and to investigate cross talk with other pathways to elucidate the relevance of MEK1/2 and ERK1/2 activation in CRSwNP.

Apparently, in CRSwNP, MEK1/2, and ERK1/2 regulation first occurs at the phosphorylation level. However, the expression levels of the unphosphorylated protein are also controlled, leading to inactive signalling proteins and at least one subunit of the mRNA. In the special case of this benign, highly proliferative disease, the different components seem to enhance the complex MEK1/2–ERK1/2 cascade in a distinct manner, enabling a wide variety of functions.

pERK has an Additional Distribution Pattern Compared to the Other Kinases

MEK, pMEK, and ERK are localised primarily to the cells facing the basal membrane and scarcely in the upper layers of the epithelium and stroma. Interestingly, pERK was found in the nuclei of all of the cell layers in the epithelium of the polyps and was very strongly stained in the cells from the stroma of the turbinates of patients with CRSwNP. We conclude that ERK is activated in the epithelium of nasal polyps, indicating a role in the accelerated cell cycle. The ERK pathway could play a pivotal role in regulating the inflammatory process in CRSwNP in polyps and turbinates.

First Description of a Possibly Specific Role for ERK2 Transcript Form 2

ERK1 and ERK2 have a very similar sequence and are often thought of interchangeably (Yoon and Seger 2006). Other studies, however, have presented compelling evidence that the ERK kinases are not functionally redundant and might have very different roles (Lloyd 2006; Vantaggiato et al. 2006).

There are two transcription variants of ERK2 mRNA and both encode the same protein. ERK2 transcript variant 1 represents the longer transcript (5,916 bp) compared to ERK2 transcript variant 2 (1,499 bp). We observed a higher quantity of ERK2 transcript variant 2 mRNA in nasal polyps compared to the inferior turbinate in patients with CRSwNP by microarray. There is a lack of data concerning the role of this transcript variant. Further studies on the role of different transcript variants of ERK 2, not only in rhinology but also in cell biology in general, are required.

Effect of ERK1/2 on Apoptosis

The activation of ERK1/2 generally promotes cell survival, but under certain conditions ERK1/2 can have pro-apoptotic functions (Lu and Xu 2006). The MEK1/2–ERK1/2 pathway is dysregulated in approximately one-third of all human cancers. In addition to this pathway, ERK1/2 has been shown to be activated by a variety of pathways depending on the individual ligand, cell surface receptor, and cell type (Dhillon et al. 2007). Therefore, the pathway has attracted considerable attention as a target for anticancer therapy (Roskoski 2012).

The activation of ERK1/2 has been shown to inhibit apoptosis in response to a wide range of stimuli, such as tumour necrosis factor (TNF), Fas ligand, TNF-related apoptosis-inducing ligand (Tran et al. 2001), radiation (Kitagawa et al. 2002), osmotic stress (Foncea et al. 2000; Lu et al. 2002; Nagata and Todokoro 1999), hypoxia (Buckley et al. 1999), growth factor withdrawal (Erhardt et al. 1999), nitric oxide (Kim et al. 2002), hydrogen peroxide (Wang et al. 1998), matrix detachment (Le Gall et al. 2000), and chemotherapeutic agents (Anderson and Tolkovsky 1999). In response to most of these stimuli, ERK1/2 undergoes either transient or prolonged activation, resulting in an anti-apoptotic effect (Lu and Xu 2006).

We observed activation of the MEK1/2–ERK1/2 pathway, which can explain the proliferation of the epithelium of the polyps as a result of the anti-apoptotic effects of ERK1/2.

Effect of ERK1/2 on Inflammation

The ERK1/2 MAP kinase pathway is typically activated, as the name suggests, by mitogenic stimuli, such as epidermal growth factor or platelet-derived growth factor. ERK signalling is also involved in pro- and anti-inflammatory responses. The ERK1/2 pathway is a dominant, highly responsive pathway in traumatic skeletal muscle injury-induced inflammation. In skeletal muscle injury, a bimodal regulation of ERK1/2 has been observed in acute inflammation, which was responsible for the coupling and timing of pro- and anti-inflammatory processes (Szelenyi and Urso 2012).

ERK1/2 activation plays a central role in chronic obstructive pulmonary disease (COPD). Rapid and lasting cigarette smoke-induced activation of ERK1/2 has been demonstrated in cultured lung cells. Elevated pulmonary ERK1/2 phosphorylation has been detected in mice (Mercer et al. 2004) and rats (Chang et al. 2001) exposed to cigarette smoke. Most importantly, the relevance of these events to COPD was established by the discovery of significantly elevated ERK1/2 activity in airway and alveolar epithelial cells from patients with emphysema compared to nonemphysematous controls (Mercer et al. 2004). Thus, MAP kinase inhibitors are beginning to emerge as a promising therapeutic strategy for COPD (Mercer and D'Armiento 2006).

Two studies have investigated the involvement of the MEK1/2–ERK1/2 pathway in CRSwNP. Lipopolysaccharide stimulation induces increased ERK1/2 phosphorylation in nasal polyp fibroblasts (NPFs), but this phosphorylation could be inhibited by treatment of NPFs with clarithromycin (Furuya et al. 2010). The expression of the C–C-chemokine ligand 2 gene in fibroblasts from human nasal polyps is stimulated by TNF-α through the MAPK pathways (Lin et al. 2007).

We showed activation of the MEK1/2–ERK1/2 pathway, which indicates the involvement of this signalling cascade in the inflammatory process in CRSwNP.

In our study, we found increased expression of the signalling protein MEK1/2 and activation of MEK1/2 and ERK1/2, which are the two essential tiers of the signalling cascade. Furthermore, we observed an isolated higher expression of ERK2 mRNA transcript variant 2 by a single method in a small number of samples, which has not been described for any tissue before. Our results provide evidence for a crucial role of the MEK1/2–ERK1/2 pathway in the pathogenesis of CRSwNP.

This signalling cascade alone cannot mediate a disease with such complex pathology. Further research should be conducted to improve our understanding of the activation of signalling pathways and how these pathways direct cellular responses in the mucosa of the noses of CRSwNP patients. Deciphering the mechanisms of intracellular signalling in CRSwNP continues to be an important and challenging task.

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