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Glycogen synthase kinase 3 in chronic rhinosinusitis: two faces of a single enzyme in one disease

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ABSTRACT

Background: The origin and pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP) remain unclear. Glycogen synthase kinase 3 (GSK-3) is a unique multitasking kinase involved in the regulation of inflammation and apoptosis and is an important messenger in the downstream signaling of interleukin 6.

Objective: To analyze the possible role of GSK-3 in the pathogenesis of CRSwNP.

Methods: We examined tissue samples of nasal polyps and the inferior turbinate of patients with CRSwNP and the inferior turbinate of individuals without chronic sinusitis (healthy mucosa). Expression levels of GSK-3 and its inactivated form phosphorylated GSK-3 (pGSK-3) were analyzed using DNA microarray, protein array, Western hybridization, and immunohistochemical analysis.

Results: We found increased expression of GSK-3 in both the nasal polyps and the inferior turbinate of patients with CRSwNP compared with those with healthy mucosa ($P < .01$). We did not observe a difference between nasal polyps and the inferior turbinate of patients with CRSwNP, but a highly significant increase in the phosphorylation rate of GSK-3 was detected in the tissue of nasal polyps compared with the turbinates of patients with CRSwNP ($P < .01$).

Conclusion: GSK-3 may play a crucial role in the inflammatory process in CRSwNP. Nasal polyps originate mainly in the mucosa of the middle meatus of the nose and rarely occur in the region of the inferior turbinate. The inhibition of GSK-3 by phosphorylation in nasal polyps, in contrast to the inferior turbinate, is a possible explanation for the different behavior of the mucosa of the middle meatus and the inferior turbinate.

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Introduction

Chronic rhinosinusitis is a common disease worldwide and causes considerable costs in the United States and Western Europe. Chronic rhinosinusitis affects 12.5% of the US population.¹ The overall prevalence of chronic rhinosinusitis was 10.9% in Europe in 2009.² Epidemiologic evidence suggests an association between chronic rhinosinusitis prevalence and air pollution, active cigarette smoking, secondhand smoke exposure, perennial allergic rhinitis, and gastroesophageal reflux.¹ There is a strong association of chronic rhinosinusitis with asthma in all age groups, for both men and women, independent of smoking behavior.³

For simplicity, the disease can be subdivided into 2 types: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps. In fact, other entities within these groups

are suspected.⁴ Although our knowledge of the origin and pathogenesis of the disease continues to increase, many questions are still unresolved. Analog circuits to other inflammatory and proliferative diseases help us to understand the underlying mechanisms.

Glycogen synthase kinase 3 (GSK-3) is a ubiquitously expressed, highly conserved serine/threonine protein kinase found in all eukaryotes. Two isoforms of GSK-3 are known, GSK-3 α and GSK-3 β , and they share 85% overall sequence homology.^{4,5} GSK-3 modulates many fundamental processes, including cell structure, metabolism, gene expression, and in a seemingly paradoxical manner, apoptosis.⁶

GSK-3 is implicated in several diseases, such as mood disorders, neurodegenerative diseases such as Alzheimer disease, cancer, inflammation, cardiovascular disease, and diabetes.⁷ Unlike other kinases, the enzyme is constitutively active and is inactivated in response to cellular signals. GSK-3 has been found to phosphorylate nearly 50 substrates, which generally leads to their inactivation.^{5,7} The best defined regulatory mechanism is the phosphorylation of serine 9 in GSK-3 α or serine 21 in GSK-3 β , which inhibits their activity.^{6,8}

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We analyzed the expression levels and phosphorylation state of GSK-3 α/β in nasal polyps, the inferior turbinates of patients with CRSwNP, and the inferior turbinates of patients with healthy mucosa, suggesting a role of GSK-3 in the pathogenesis of CRSwNP.

Methods

Samples

We examined tissue samples of nasal polyps and the inferior turbinate as an internal control from 11 patients with CRSwNP. Samples were harvested during sinus surgery. Special emphasis was placed on the gentle removal of polyps without bruising or tearing the tissue.

All patients had a history of sinusitis of more than 3 months with a mean (SD) duration of 1.5 (0.6) years and did not respond to conservative therapy. The Lund-Mackay scoring system (score of 0–24) was used to grade the radiographic severity of sinus disease.⁹ All patients had a diffuse polyposis; the mean (SD) score of the patients was 16.2 (5.2). Subjective symptoms were evaluated by the Sino-Nasal Outcome Test-20 German Adapted Version.^{10,11} The mean (SD) score was determined to be 34.2 (12.0).

Patients were skin tested for pollens, dust mites, pets, and molds using standardized extracts (Allergopharma Joachim Ganzer KG, Reinbek, Germany) within a time frame of 4 weeks before surgery. Eosinophilic CRSwNP was determined by histopathologic examination. No patient with neutrophilic polyps or a mucoviscidosis was included in the study. No patient received systemic or topical corticosteroids within 4 weeks before surgery.

In addition, we examined 8 samples of the inferior turbinate of patients with no 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), conducted in accordance with the ethical principles for medical research formulated in the World Medical Association Declaration of Helsinki, and all patients gave signed informed consent.

Microarray Analysis

Fresh tissue samples of nasal polyps and the inferior turbinate of 8 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 via an Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, California), and the RIN value was calculated. Agilent whole human genome microarrays (4 × 44,000) were performed following the manufacturer's protocols, and the gene expression was calculated using the Rosetta Resolver gene expression data analysis system (Rosetta Bio-software; Seattle, Washington).

Protein Array

The human phosphokinase-kinase array kit (Proteome Profiler Human Phospho-Kinase Array Kit; R&D Systems Inc, Minneapolis, Minnesota) allows the simultaneous detection of the relative levels of phosphorylation of 46 kinase phosphorylation sites. Capture and control antibodies are spotted in duplicate on nitrocellulose membranes.

Tissue homogenates from samples of the polyps from 5 patients with chronic polypoid sinusitis and the inferior turbinate as an internal control (250 μ g of total proteins per array) were applied to the phosphoprotein array following 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 peroxidase and chemiluminescent detection

reagents were applied, and a signal was produced at each capture spot corresponding to the amount of phosphorylated protein bound.

The images were acquired by Fusion FX 7. Pixel density was analyzed using Bio-1D software (both Vilber Lourmat, Marne-la-Vallée, Cedex, France).

Western Hybridization

Tissue lysates of samples of 11 nasal polyps, 11 inferior turbinates of patients with CRSwNP, and 8 inferior turbinates of patients with healthy mucosa were denatured by boiling for 5 minutes in 1 × sodium dodecyl sulfate sample buffer (Carl Roth GmbH, Karlsruhe, Germany). The protein concentrations were determined using a Bradford assay, and 30 μ g of total protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Mini-Protein TGX; Any kD; BioRad, Hercules, California) and then transferred to nitrocellulose membranes. The real representation of all protein bands was controlled with Ponceau staining.

The membranes were initially blocked with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% bovine serum albumin (BSA) (BioRad) for 1 hour at room temperature (RT) and were then probed overnight at 4°C using a dilution of 1:1,000 with the following primary antibodies in TBS-T and 5% BSA: anti-GSK-3 α/β (Abcam, Cambridge, United Kingdom; catalog No. ab62368) and anti-phospho-GSK-3 α/β (Ser-9/Ser-21; Cell Signaling Technology Inc, Danvers, Massachusetts; catalog No. 9331). After washing with TBS-T, the membranes were incubated with a 1:3,333 dilution of goat anti-rabbit specific secondary antibody (BioRad) in TBS-T and 5% BSA for 1 hour at RT. Protein bands were visualized using an AP conjugate substrate kit (BioRad).

Nitrocellulose membranes were reprobed overnight at 4°C with a dilution of 1:1,000 of a primary antibody of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology Inc; catalog No. 14C10) to determine the loading in each lane. GAPDH is a well-established housekeeping gene in our research group and has already been used in numerous research projects concerning the characterization of nasal polyps. The membranes were washed and then incubated with a 1:1,000 dilution of species-specific horseradish peroxidase–linked secondary antibody in TBS-T and 5% BSA for 3 hours at RT. Membranes were washed and protein bands visualized as described. Pixel densities were quantified and documented with Fusion FX7 using Bio-1D software.

Immunostaining Analysis

Fresh frozen tissue sections were fixed in 100% methanol for 10 minutes. After a drying time of 10 minutes, the sections were washed with TBS at RT (RT-TBS). Thereafter, the tissue sections were incubated with 3% hydrogen peroxide in RT-TBS for 15 minutes to inhibit the endogenous peroxidase.

After washing with RT-TBS, the samples were permeabilized with 0.3% Triton X-TBS for 15 minutes, followed by washing with RT-TBS. The sections were incubated with a 1:100 dilution of anti-phospho-GSK-3 α/β (Cell Signaling Technology Inc; catalog Nos. 5676 and 9331) overnight at 4°C.

The next day, the slides were washed with RT-TBS and then incubated for 20 minutes at RT with a polylink secondary antibody. After washing with RT-TBS, the samples were incubated with a horseradish peroxidase–conjugated avidin-biotin complex (both DCS Detection Line; Innovative Diagnostik-Systeme, Hamburg, Germany), followed by incubation with 3-Amino-9-Ethylcarbazole (AEC) (DCS Chromoline; Innovative Diagnostik-Systeme).

After washing with RT-TBS the sections were incubated with AEC (DCS Chromoline; Innovative Diagnostik-Systeme, Hamburg, Germany). The reaction was stopped with RT-TBS.

Finally, nuclear staining was performed with Mayer hematoxylin. Sections were embedded in Dako Faramount Aqueous Medium (Dako

North America Inc, Carpinteria, California). The immunostained sections were viewed, and photomicrographs were obtained on an Axiovert 200 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Data were analyzed with 1- and 2-way analysis of variance, followed by the Fisher exact test, using Stat View 5.0 software (SAS Institute Inc., Cary, North Carolina). Differences between groups were considered significant at $P < .01$.

Results

Equal GSK-3 Messenger RNA Levels in Polyps and Turbinates of Patients With CRSwNP

The evaluation of the microarray (NM_019884) revealed no quantitative difference in the amount of GSK-3 α messenger RNA (mRNA) between the samples of nasal polyps and the inferior turbinate of the same patient on average ($n = 8$). The mean (SD) fold change was 0.99 (0.21). We found a significant fold change with a higher mRNA level in the polyp in only one pair of samples.

The evaluation of the microarray (NM_002093) also revealed equal amounts of GSK-3 β mRNA on average, with a mean (SD) fold change of 0.97 (0.15). We observed a significantly higher mRNA level in the polyp in 2 pairs and a significantly lower mRNA level in the polyp in one pair (Fig 1).

Increased Expression of GSK-3 in Nasal Polyps and Inferior Turbinates of Patients With CRSwNP

We detected significantly higher expression levels of GSK-3 α in both the tissue of the nasal polyps ($n = 11$, 3.35-fold, $P < .01$) and the inferior turbinates of patients with CRSwNP ($n = 11$, 2.72-fold, $P < .01$) compared with healthy mucosa ($n = 8$).

In addition, the expression level of GSK-3 β was significantly elevated both in the tissue of the nasal polyps ($n = 11$, 1.8-fold, $P < .01$) and the inferior turbinates of patients with CRSwNP ($n = 11$, 2.29-fold, $P < .01$) compared with healthy mucosa ($n = 8$; Fig 2).

Increased Phosphorylation of GSK-3 in Nasal Polyps

Western blot analysis revealed a 3.27-fold increase in phosphorylated pGSK-3 α in the tissue of the nasal polyps ($n = 11$) compared with the inferior turbinates of patients with CRSwNP ($n = 11$, $P < .01$) and a 4.33-fold increase compared with healthy mucosa ($n = 8$, $P < .01$). We observed no significant difference between the turbinates of patients with CRSwNP and those with healthy mucosa.

We observed a similar pattern for pGSK-3 β by Western blotting. The amount of phosphorylated pGSK-3 β was 4.45-fold higher in the tissue of the nasal polyps ($n = 11$) compared with the inferior turbinates of patients with CRSwNP ($n = 11$, $P < .01$) and 2.87-fold higher compared with healthy mucosa ($n = 8$, $P < .01$) (Fig 2).

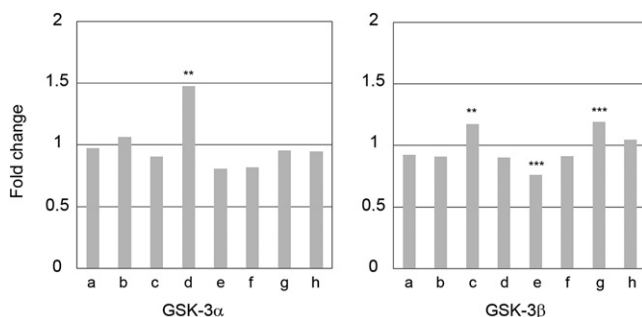


Figure 1. Results of the DNA microarray for each tested pair of polyps and turbinates of patients with chronic rhinosinusitis with nasal polyps ($n = 8$). ** $.001 \leq P < .01$; *** $P < .001$. GSK-3 indicates glycogen synthase kinase 3.

The quantitative analysis of the protein array revealed a significantly increased level of pGSK-3 α/β in the tissue of the polyp in each tested pair of samples of polyp and turbinate of patients with CRSwNP ($P < .01$). The mean concentration of pGSK-3 α/β in the tissue of polyps was 3.73-fold higher compared with the tissue of the inferior turbinate of patients with CRSwNP ($n = 5$, $P < .01$) (Fig 3).

Spatial Distribution of pGSK-3

Figure 4 displays representative examples illustrating the distribution of pGSK-3 α/β in tissue samples of nasal polyps, the inferior turbinate of a patient with CRSwNP, and healthy mucosa. Hematoxylin-eosin staining of the nasal turbinate of patients with both healthy mucosa and CRSwNP revealed the typical multirow ciliated epithelium, which included goblet cells. Variable degrees of hyperplasia and focal squamous metaplasia were found in the epithelium of the nasal polyps.

Homogeneous staining of pGSK-3 α/β was observed by immunohistochemical analysis in the cytoplasm of the epithelial cells of both the nasal turbinate of patients with CRSwNP and the healthy mucosa (Fig 4, A and B). In the polyp tissue, a uniform strong expression of pGSK-3 α/β was revealed in the cytoplasm of almost all cells of the epithelial layer (Fig 4C). In the tissue of nasal polyps with squamous cell metaplasia, we observed a strong expression of pGSK-3 α/β in the outer two-thirds of the lining cells. Interestingly, the cytoplasm of the innermost epithelial cells facing the basal membrane revealed weak staining (Fig 4D). In nasal polyps and in the stroma of the nasal turbinates of patients with both CRSwNP and with healthy mucosa, the cytoplasm of the cells of the connective tissue and of inflammatory cells showed weak staining (Fig 4, A, B, C, and D).

The data also revealed that our immunohistochemical results correlated with pGSK-3 band intensities in Western blot and protein array investigations.

Discussion

The formation of nasal polyps is connected with a chronic inflammatory process and with the activation of different cytokines.¹² A distinct set of inflammatory and remodeling factors has been found to be elevated in chronic rhinosinusitis.¹³

Innate and adaptive immune responses are regulated by GSK-3. GSK-3 controls the activity of more than 20 transcription factors, including several that are important for immune function.¹⁴ GSK-3 is a crucial regulator of the balance between proinflammatory and anti-inflammatory cytokine production.¹⁵ GSK-3 dampens the production of anti-inflammatory interleukin (IL) 10 by suppressing the transcriptional activity of CREB and AP-1, both of which contribute to IL-10 expression.¹⁶ These discoveries led to the rapid application of GSK-3 inhibitors, such as lithium, to animal models of sepsis, arthritis, colitis, multiple sclerosis, and others, demonstrating their potential use as therapeutic interventions.¹⁴

In our study, we demonstrate that GSK-3 may be highly expressed in both the nasal polyps and the inferior turbinates of patients with CRSwNP. This finding suggests that the nasal mucosa of the inferior turbinate is also involved in the inflammatory process in CRSwNP. Therefore, we suggest that one can term both nasal polyps and the inferior turbinate of patients with CRSwNP as *inflammatory nasal mucosa*.

GSK-3 has the perplexing capacity to either increase or decrease the apoptotic threshold. These apparently paradoxical effects are because GSK-3 is differentially regulating the 2 major apoptotic signaling pathways.

GSK-3 favors cell death caused by the mitochondrial intrinsic apoptotic pathway activated by cell damage. The facilitation of signals that cause disruption of mitochondria and the regulation of transcription factors that control the expression of anti-apoptotic or proapoptotic proteins are promoted by GSK-3.

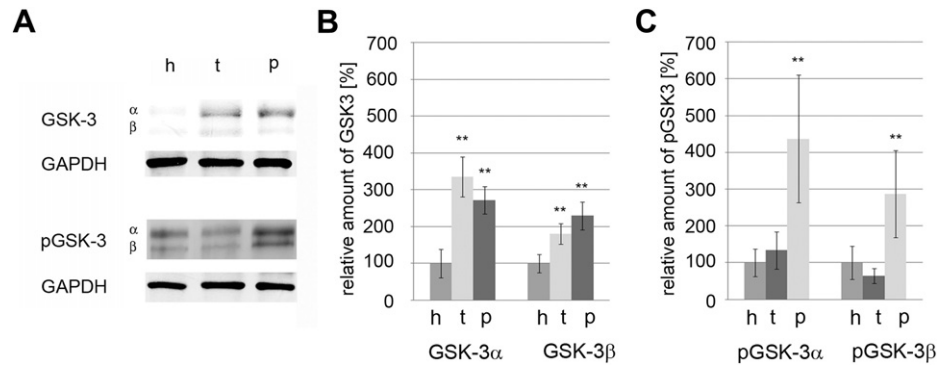


Figure 2. Results of Western blotting. A, Representative example. Mean levels of glycogen synthase kinase 3 (GSK-3) (B) and phosphorylated glycogen synthase kinase 3 (pGSK-3) (C) in nasal polyps (p), the inferior turbinates of patients with chronic rhinosinusitis with nasal polyps (t), and healthy mucosa (h). The error bars indicate the SDs. $^{**}.001 \leq P < .01$. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.

The extrinsic apoptotic pathway entails extracellular ligands stimulating cell-surface death receptors that initiate apoptosis by activating caspase 8. This early step in extrinsic apoptotic signaling is inhibited by GSK-3.⁶

Our results reveal a significantly higher phosphorylation rate of GSK-3α/β in nasal polyps compared with the inferior turbinate of patients with CRSwNP. Moreover, we observed a uniform

expression of pGSK-3α/β in all cells of the epithelial layer in polyps with hyperplasia of the epithelium. Because intrinsic apoptosis signaling is inhibited by phosphorylation of GSK-3, our findings indicate that the inhibition of apoptosis could contribute to the formation of nasal polyps. Nasal polyps rarely occur in the region of the inferior turbinate; most of the nasal polyps originate from the mucosa surrounding the middle meatus.¹⁷ Our findings provide

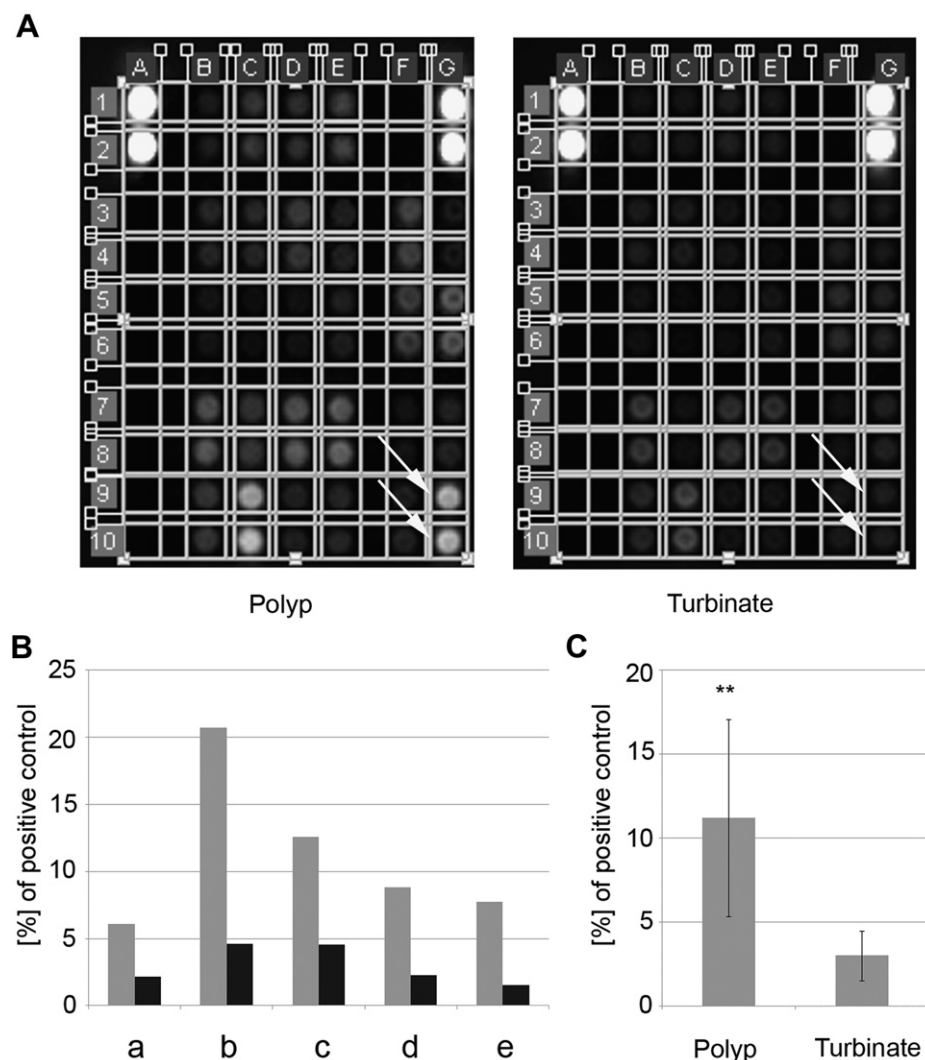


Figure 3. Results of the protein array. A, Representative example. B, Results of each tested pair of polyps (white) and turbinates of patients with chronic rhinosinusitis with nasal polyps (black). C, Mean levels of phosphorylated glycogen synthase kinase 3α/β. The error bars indicate the SDs. $^{**}.001 \leq P < .01$.

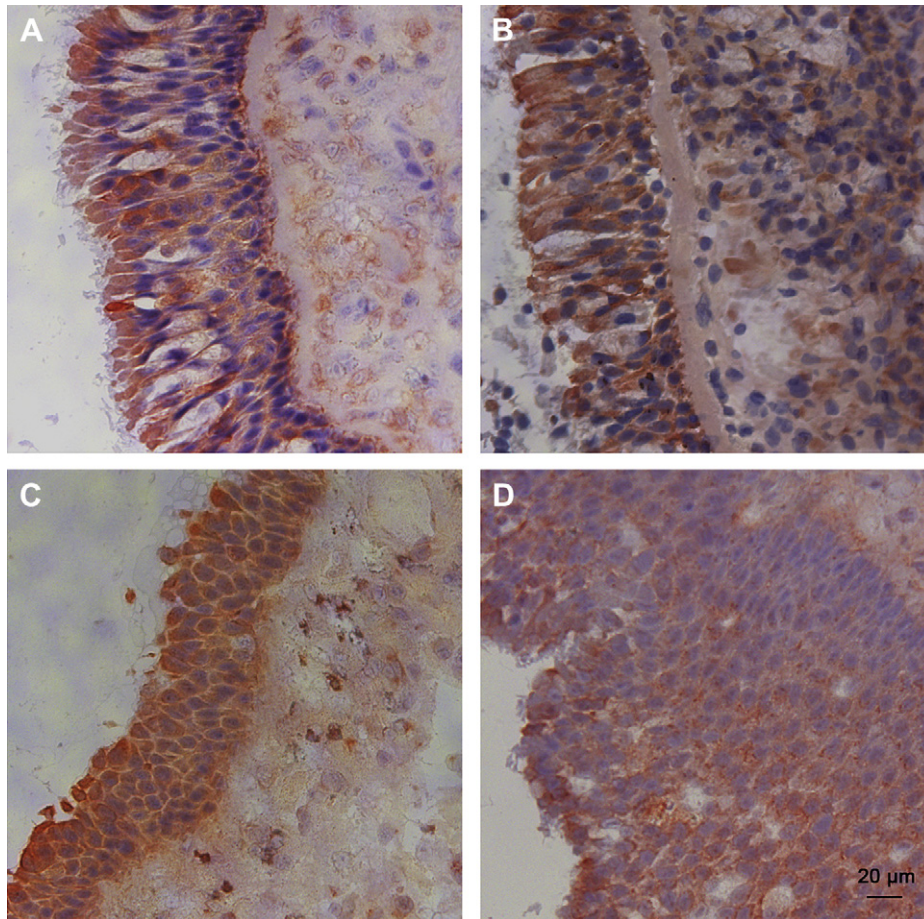


Figure 4. Representative examples of immunostaining of phosphorylated glycogen synthase kinase 3. A, Healthy mucosa. B, Turbinate of a patient with chronic rhinosinusitis with nasal polyps. C, Nasal polyp with hyperplasia. D, Nasal polyp with focal squamous metaplasia.

a possible explanation for the different behavior of the mucosa in the middle meatus and of the inferior turbinate.

We could only detect pGSK-3 α/β in the outer two-thirds of the epithelial layer of polyps with squamous cell metaplasia; the innermost lining cells facing the basal membrane had weak staining. Nasal polyposis, even in the presence of metaplasia, is in fact always a benign disease. A possible conclusion is that in this type of tissue, the antiapoptotic effect of pGSK-3 turns into a proapoptotic effect. As a consequence, proliferation of nasal polyps is a self-limiting process. The phosphorylation of GSK-3, in particular in the outer cell layers of the epithelium of nasal polyps with squamous cell metaplasia, may decrease the extrinsic apoptotic threshold, and this decrease may protect the cells from malignant transformation.

The inflammatory response in chronic rhinosinusitis is associated with elevated levels of proinflammatory cytokines. Various studies have reported increased amounts of IL-6 in patients with CRSwNP.¹⁸ It has been demonstrated that small compounds of the bacterial cell wall, such as lipoteichoic acid and peptidoglycan derived from *Staphylococcus aureus*, could induce IL-6 synthesis.^{15,18}

After treatment with cortisone, the level of IL-6 was significantly decreased when compared with the pretreatment level. The beneficial effect of oral corticosteroids may be due to the significantly reduced levels of IL-6.¹⁹ In myeloma cells, GSK-3 as a downstream element of Akt/GSK-3 signaling is phosphorylated (inactivated) by IL-6.²⁰ We detected a higher phosphorylation rate of GSK-3 in nasal polyps. We conclude that we can provide new evidence for the role of IL-6 by demonstrating its downstream signaling.

GSK-3 is a fascinating enzyme with a diverse number of actions in intracellular signaling systems. GSK-3 is located downstream of

several major signaling pathways, including the phosphatidylinositol 3-kinase pathway, the Wnt pathway, Hedgehog signaling, and Notch.⁵

Numerous stimuli lead to inactivation of GSK-3.^{21,22} Interactions of GSK-3 with nuclear factor- κ B,^{14,23,24} mTOR,²⁴ CREB-binding protein,²⁵ β -catenin,^{24,26} transforming growth factor β ,²⁶ and p38²⁷ are known.

We observed activation of GSK-3 in inflammatory nasal mucosa in general and inactivation of GSK-3 in nasal polyps. The number of cross-connections and relationships with other signaling pathways is immense. Systematic studies on the cross-ties are required to grasp the significance of the changes found in this single enzyme on the overall system of cell signaling in CRSwNP.

Conclusion

We demonstrate that GSK-3 is highly expressed in inflammatory nasal mucosa in both polyps and the inferior turbinate. The phosphorylation rate of GSK-3 in nasal polyps is significantly higher compared with the inferior turbinate of patients with CRSwNP. GSK-3 is a key signaling molecule in the regulation of inflammation and apoptosis and is an important messenger in the downstream signaling of IL-6. The results of our study provide evidence that GSK-3 plays a pivotal role in the pathogenesis of CRSwNP.

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