Rheumatol Int (2007) 28:127–136
DOI 10.1007/s00296-007-0411-9

ORIGINAL ARTICLE

EGF activates PI3K-Akt and NF-κB via distinct pathways in salivary epithelial cells in Sjögren's syndrome

Hideki Nakamura · Atsushi Kawakami · Hiroaki Ida · Takehiko Koji · Katsumi Eguchi

Received: 15 January 2007 / Accepted: 23 June 2007 / Published online: 20 July 2007

Abstract Epidermal growth factor (EGF) exerts tropic effects on salivary epithelial cells. We examined EGF-mediated signaling pathways in the salivary epithelial cells of patients with Sjögren's syndrome (SS). We compared the immunohistochemical expression of EGF receptor (EGF-R), phosphatidylinositol 3-kinase (PI3K), Akt and nuclear factor kappa B (NF-κB) in the labial salivary glands of SS patients ($n = 6$) with those of control subjects ($n = 2$). EGF-mediated signaling pathways were further studied in vitro ($n = 3$) using primary salivary epithelial cells; NF-κB p65 nuclear translocation and Akt phosphorylation were examined by immunofluorescence and western blotting, respectively. The phosphorylation of EGF-R and Akt, and the nuclear expression of NF-κB p65, were increased in situ in the salivary epithelial cells of SS patients compared with those of control subjects. Epidermal growth factor induced rapid EGF-R phosphorylation and NF-κB p65 nuclear translocation in primary salivary epithelial cells in vitro. However, EGF also induced late Akt phosphorylation (after 12 h). Chemical inhibition of PI3K-Akt by LY294002/wortmannin did not affect EGF-mediated NF-κB p65 nuclear translocation; and NF-κB inhibition by Bay 11-7082 did not suppress Akt phosphorylation. Our data suggest that EGF stimulates both the PI3K-Akt pathway and NF-κB via distinct mechanisms, promoting tropic effects in SS salivary epithelial cells.

Keywords Akt · Epidermal growth factor · NF-κB · Salivary epithelial cells · Sjögren's syndrome

Abbreviations

APS	Aminopropyltriethoxysilane
DAB	3.3′-diaminobenzidine
DR4	Death receptor 4
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGF-R	EGF receptor
FITC	Fluorescein isothiocyanate
IKK	IkappaB kinase
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PMSF	Polymethylsulfonylfluoride
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SS	Sjögren's syndrome
TBS	Tris-buffered saline
TNF	Tumor necrosis factor
TRITC	Tetramethyl rhodamine isothiocyanate

Introduction

Cell death mechanisms in salivary epithelial cells have been thoroughly examined both in situ and in vitro [1–3], however,

H. Nakamura (✉) · A. Kawakami · H. Ida · K. Eguchi
The First Department of Internal Medicine,
Graduate School of Biomedical Sciences, Nagasaki University,
1-7-1 Sakamoto, Nagasaki City, Nagasaki 852-8501, Japan
e-mail: nhideki@nagasaki-u.ac.jp

T. Koji
Division of Histology and Cell Biology,
Department of Developmental and Reconstructive Medicine,
Graduate School of Biomedical Sciences, Nagasaki University,
1-7-1 Sakamoto, Nagasaki City, Nagasaki, Japan

the tropic pathways acting in salivary epithelial cells remain less understood. Although apoptotic cell death of salivary epithelial cells is increased in the salivary glands of patients with Sjögren's syndrome (SS) [1–5], the defense mechanism of epithelial restoration appears to be activated [6–8]. This latter phenomenon may be up-regulated by the augmented expression of the epidermal growth factor (EGF)/EGF receptor (EGF-R) system, which has been noted in biopsied salivary gland samples from SS patients [9]. Epidermal growth factor binds to and phosphorylates the EGF-R, leading to the activation of intracellular kinase cascades such as the phosphatidylinositol 3-kinase (PI3K)-Akt and IkappaB kinase (IKK)-nuclear factor kappaB (NF-κB) pathways [10–13], both of which are well known anti-apoptogenic survival signals [14, 15]. The NF-κB is a target of Akt-mediated anti-apoptogenic platelet-derived growth factor (PDGF) signaling [16], however, a recent investigation has revealed the cell-type dependent involvement of Akt toward tumor necrosis factor (TNF)-induced NF-κB activation, which is associated at least in part with the degree of IKK expression in each cell type [17].

The present study was designed to determine the distribution of EGF and EGF-R and the mechanisms of the tropic effects of EGF in the salivary epithelial cells of SS patients. Our present immunohistochemical results showed co-localization of phosphorylated EGF-R, phosphorylated Akt and nuclear NF-κB p65 in salivary epithelial cells in situ in patients with SS. Phosphorylation of the EGF-R and Akt, and NF-κB p65 nuclear translocation in the cultured primary salivary epithelial cells in vitro were induced by EGF; however, EGF-mediated activation of Akt and nuclear translocation of NF-κB appear to be regulated by distinct mechanisms.

Materials and methods

Patients

The study subjects were six female patients with primary SS and two female control subjects. All six primary SS patients fulfilled the revised criteria for the diagnosis of SS proposed by the European community [18]. The two control subjects complained of sicca symptoms but did not meet the criteria for SS because they had neither positive anti-SS-A/SS-B antibodies nor mononuclear cell infiltration into minor salivary gland. They also had negative results for sialography. Informed consent was obtained from all participating subjects and the study was conducted in accordance with the human experimental guidelines of our institution.

Biopsy of labial salivary glands

Biopsy of labial salivary glands was conducted in all eight subjects. Under local anesthesia, a biopsy sample of the minor labial salivary glands was obtained from the mucosa of the lower lip at midline. Immediately after biopsy, the tissues were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (pH 7.4), prior to successive immersions in 10, 15 and 20% sucrose. The tissues were then frozen and stored in liquid nitrogen until use.

Monoclonal and polyclonal antibodies, recombinant protein and chemical inhibitors

The proteins expressed in the labial salivary glands were determined using several types of antibodies (Table 1). Polyclonal anti-NF-κB p65 antibody and anti-Akt antibody (reacted with whole Akt) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and polyclonal anti-phosphorylated Akt antibody (Ser 473) and anti-EGF-R antibody (reacted with whole EGF-R) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyclonal anti-phosphorylated EGF-R antibody (Tyr 1173) was purchased from BioSource International, Inc. (Camarillo, CA, USA), and anti-β-actin and recombinant human EGF were purchased from Sigma (St Louis, MO, USA). Donkey anti-mouse IgG and rabbit IgG (H + L chain) polyclonal antibody, conjugated with peroxidase (for western blotting), were purchased from MBL (Nagoya, Japan). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (H + L) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit IgG (H + L), used for immunofluorescence, were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA). The PI3K inhibitors LY294002 and wortmannin, and the IKK inhibitor Bay11-7082 were purchased from Calbiochem (La Jolla, CA, USA). Normal rabbit serum was purchased by Dakocytomation (Glostrup, Denmark).

Culture of primary salivary epithelial cells

Salivary epithelial cells from the labial salivary gland were cultured immediately after biopsy following the method reported previously by Ogawa et al. [19] and Ping et al. [20] Briefly, tissues were rinsed with cold sterile PBS containing 100 units/ml of penicillin and 100 µg/ml of streptomycin prior to mincing into fragments. One piece of the tissue was placed in a 6-well plate coated with type I collagen (Sigma). The culture medium was a defined keratinocyte-SFM (Invitrogen Life Technologies, Carlsbad, CA, USA) with the attached supplement, 0.4 µg/ml of hydrocortisone (Sigma) and 25 µg/ml of bovine pituitary extract (Kurabo, Osaka, Japan). Outgrowth of the cells was observed within 1 week, and when the cells reached confluence, they were subcultured into a 100 mm^2 plate coated with type I collagen. For immunofluorescence, the primary salivary epithelial cells were cultured onto 12 mm^2 cover

Table 1 Antibodies used in the present experiments

Antibodies	Immunogen	Poly/mono	Working dilution	Application	Source
Anti-p65	N terminus of human p65	Poly	1:100	IHC	Rabbit
Anti-p65	N terminus of human p65	Mono	1:100	IF	Mouse
Same as above			1:500	WB	
Anti-pAktSer^{473} (IHC-specific)	Ser^{473} of mouse Akt	Poly	1:100	IHC, IF	Rabbit
Anti-pAktSer^{473}	Ser^{473} of mouse Akt	Poly	1:500	WB	Rabbit
Anti-Akt	human Akt1	Poly	1:100	IHC	Rabbit
Same as above			1:1,000	WB	
Anti-pEGFRTyr^{1173}	Tyrosine^{1173} of human EGFR	Poly	1:50–100	IF	Rabbit
Anti-EGFR	Human EGFR	Poly	1:50–100	IF	Rabbit

Poly polyclonal antibody, *Mono* monoclonal antibody, *IHC* immunohistochemistry, *IF* immunofluorescence, *WB* western blot

slips precoated with type I collagen, Cellmatrix (Nitta Gelatin, Inc., Osaka, Japan).

Immunohistochemical examination of labial salivary glands

Tissue sections (7 μm thick) from labial salivary glands were cut and mounted onto glass slides precoated with aminopropyltriethoxysilane (APS). The sections were then stained using the labeled streptavidin-biotin method (Histofine Staining Kit, Nichirei Co., Tokyo, Japan) as described previously [21]. Briefly, endogenous peroxidase was inactivated in a 3% H_2O_2 solution. These sections were then incubated with 10% goat or rabbit serum, followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with biotinylated anti-rabbit or mouse IgG for 12 min prior to washing and incubation with peroxidase-conjugated streptavidin. The color was developed by soaking the sections in 3.3′-diaminobenzidine (DAB) and H_2O_2 for 10 min followed by counterstaining soaking the sections in 10% methyl green solution. Negative control sections were treated with normal mouse IgG or normal rabbit serum. To enable precise comparisons of the same cells stained with two different antibodies, two consecutive "mirror" sections were stained for anti-NF-κB p65 antibody and anti-phosphorylated Akt antibody, as described previously [22].

Pixel intensity measurement

To quantify and visualize stronger intensity of DAB development of ducts in the LSG, the tissue samples were scanned by Axioscope microscopy (Carl Zeiss, Jena, Germany) using Axioscan CCD camera (Carl Zeiss, Jena, Germany). Then, photographed images were analyzed by DAB Analysis System (Carl Zeiss, Jena, Germany). The stronger intensity of staining compared to other lesions was demonstrated as red signal automatically determined by the DAB Analysis System. To obtain accurate intensity of nucleus and cytoplasm of ducts in LSG, red signal of lesions except ducts was erased before intensity measurement. Pixel intensity in the three different fields are examined.

Immunofluorescence study

Primary salivary epithelial cells cultured onto 12 mm^2 cover slips were incubated for the indicated time in the presence or absence of EGF. After cultivation, the cells were briefly rinsed in PBS and then incubated for 10 min in PBS containing 4% PFA. After incubation, the cells were immediately immersed in methanol at −20°C for 10 min, rinsed in PBS, and incubated in blocking buffer (5% normal horse serum in PBS) for 1 h. The cells were incubated in the diluted antibody for 1 h, washed several times in PBS, and further incubated for 1 h in diluted isotype-specific secondary antibodies (1/100 for FITC-labeled and TRITC-labeled secondary antibodies) in blocking solution, supplemented with Hoechst dye 33,258 at 50 ng/ml. The cells were washed three times in PBS, mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and scanned and photographed by confocal microscopy (LSM5, PASCAL; Carl Zeiss, Jena, Germany) equipped with epifluorescence optics and appropriate filters for the detection of FITC, TRITC or Hoechst dye. Control experiments were performed to ensure the isotype specificity of each secondary antibody used. Specimens were scanned into Paint shop Pro8 software (Jasc Software, Inc., MN, USA) and compiled.

Western blot analysis

After cultivation, the cells was collected and lysed by the addition of lysis buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate SDS, 1 mM sodium orthovanadate, and 1 mM polymethylsulfonylfluoride

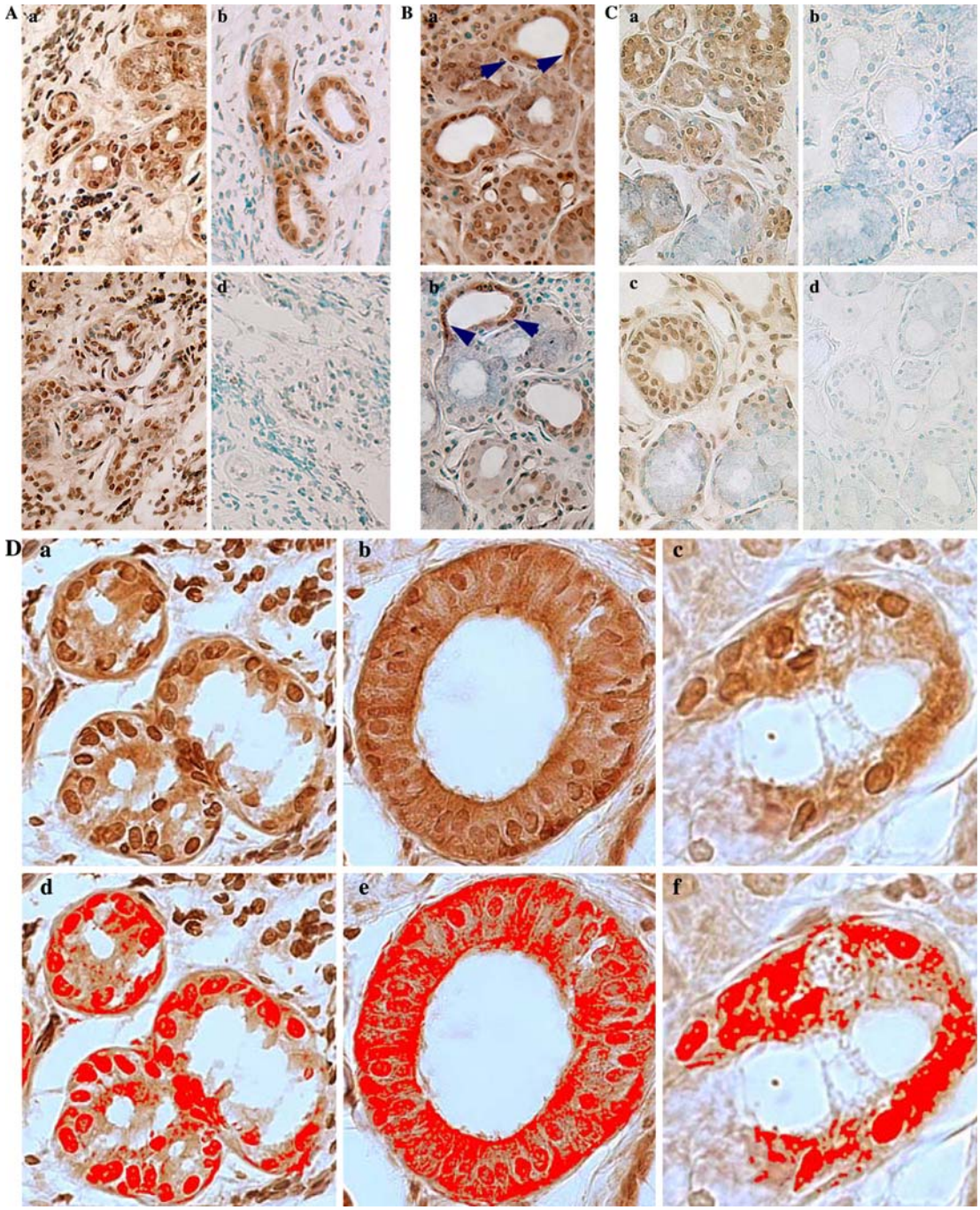

Fig. 1 Expression of NF-κB p65 and phosphorylated Akt in the labial salivary glands of patients with SS. **A** Expression of nuclear NF-κB p65 (**a**), phosphorylated Akt (**b**), whole Akt (**c**) and negative control (normal rabbit serum) (**d**) by serial sections. In situ salivary epithelial cells in SS patients clearly expressed both nuclear NF-κB p65 and cytoplasmic phosphorylated Akt in the ducts. With regard to the cytoplasmic expression of Akt, clearer expression was seen in the case of phosphorylated Akt than with whole Akt. **B** Co-localization of nuclear NF-κB p65, (**a**) and cytoplasmic phosphorylated Akt of the ducts (**b**) determined by mirror sections. *Arrowheads* indicate the same portion. **C** Neither nuclear p65 (**a**) nor phosphorylated Akt (**b**) was observed in control subjects. (**c**) and (**d**) represent whole Akt in nucleus of the ducts and negative controls (normal rabbit serum), respectively. Methyl green was used for counterstaining of nuclei. The data shown are the results from one representative case of the six SS patients and the two control subjects. (Original magnification $\times$200). **D** Results of pixel intensity measurement study for the staining by DAB without counter staining by methyl green of ducts of both a SS patient and a control subject. *Upper panels* show results of p65 staining of a SS patient (**a**) and control subject (**b**) and phosphorylated Akt of a SS patient (**c**). The stronger intensity of p65 was demonstrated as red signal of the ducts of both a SS patient (**d**) and a control subject (**e**). Furthermore, the pixel intensity of phosphorylated Akt of a SS patient was demonstrated (**f**). Red signal of other regions except the colored ducts was erased before visualizing intensity of focused ducts (Original magnification $\times$400)

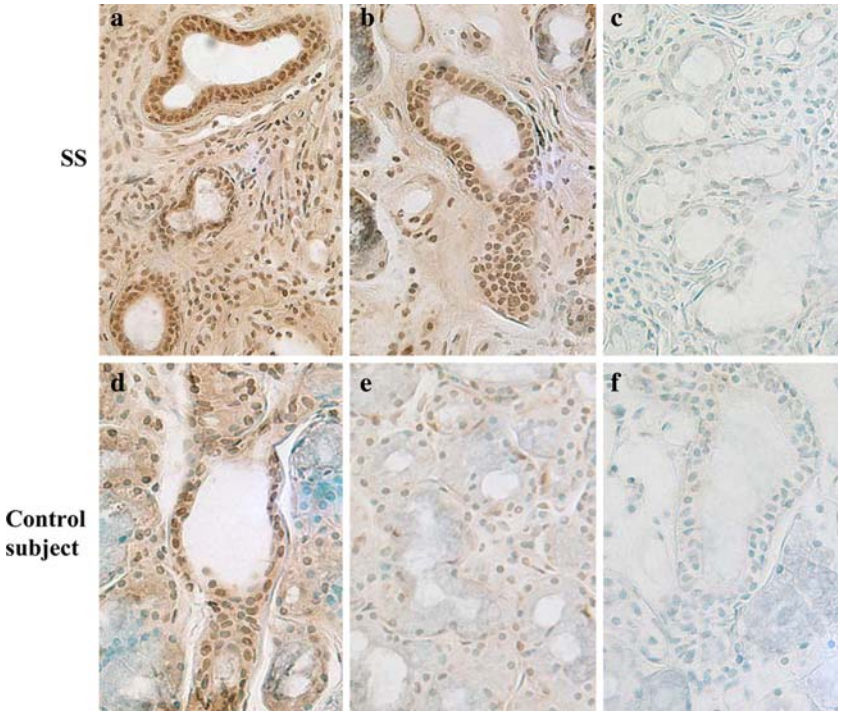

Fig. 2 Expression of phosphorylated EGF-R in the labial salivary glands of SS patients. Whole EGF-R was expressed in both SS patients (**a**) and control subjects (**d**), however, phosphorylated EGF-R Y^{1173} was clearly detected only in the salivary epithelial cells of SS patients (**b**) but not in control subjects (**e**). (**c**) and (**f**) show the negative controls (normal rabbit serum)of SS and control subjects, respectively. The data shown are the results from one representative case of the six SS patients and the two control subjects. (Original magnification ×200)

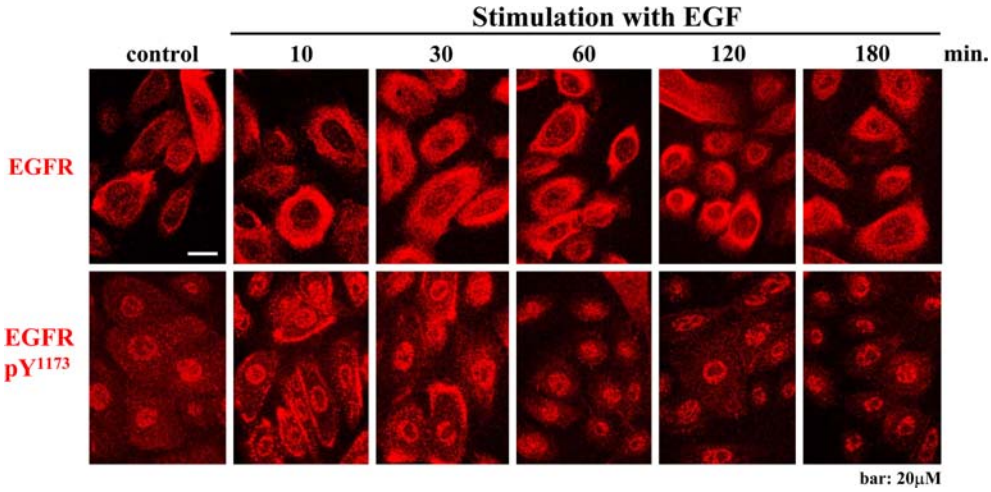

Fig. 3 EGF induces rapid phosphorylation of EGF-R in cultured primary salivary epithelial cells. The expression of EGF-R on salivary epithelial cells was examined by probing anti-EGF-R (*upper panel*) and anti-phosphorylated EGF-R (*lower panel*) with or without stimulation of EGF for up to 180 min (3 h). Confocal microscopy demonstrated rapid phosphorylation of EGF-R by EGF within 30 min. The results shown here are from representative experiments on individual cells from our three SS patients. (*bar* 20 μM)

(PMSF)) for 25 min on ice. Insoluble material was removed by centrifugation (14,000 rpm, 15 min, 4°C). Supernatants were collected and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Melville, NY, USA). In the cases in which nuclear expression of NF-κB was observed, a nuclear extraction kit (Panomics, Inc., Redwood City, CA, USA) was used. Identical amounts of protein (10 μg) were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Pagel (Bio-Rad, Tokyo, Japan). The proteins were transferred to a polyvinylidene fluoride (PVDF) filter, which was blocked for 2 h using 5% non-fat dried milk in Tris-buffered saline (TBS, 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at 4°C overnight in a 1:500–1:1,000 dilution of each antibody. The filter was washed with TBS and incubated with a 1:1,000 dilution of donkey anti-mouse IgG or rabbit IgG, coupled with horseradish peroxidase. An enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) was used for detection.

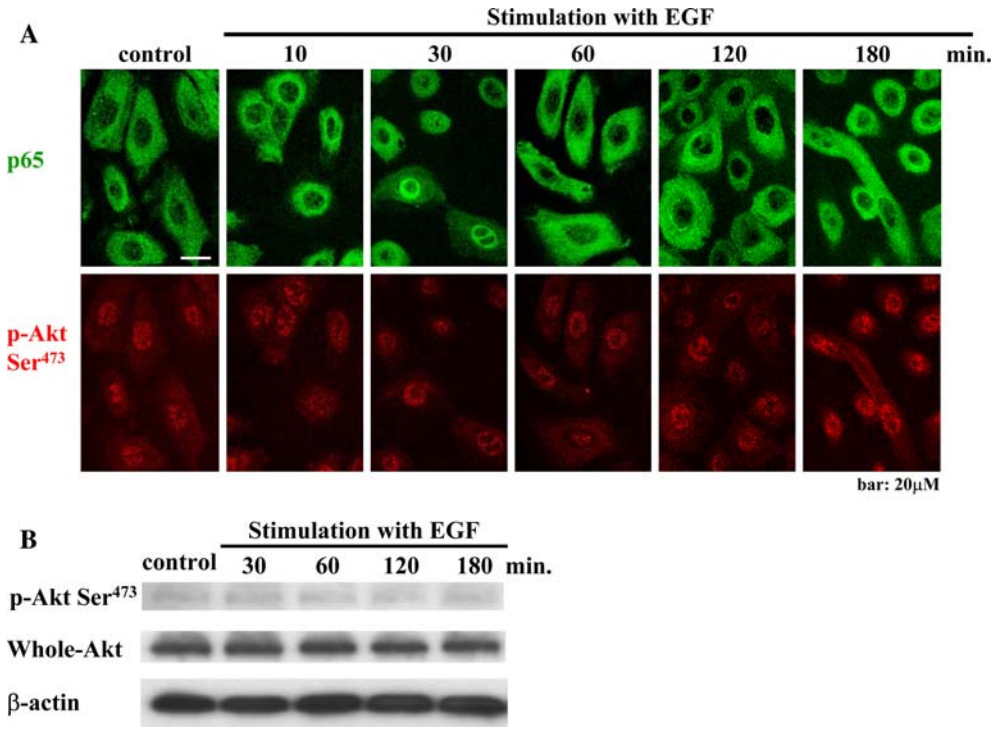

Fig. 4 EGF induces rapid nuclear translocation of NF-κB p65 in salivary epithelial cells but not Akt phosphorylation within 3 h. **a** Immunofluorescence study of NF-κB p65 nuclear translocation. Confocal microscopy clearly indicated that EGF induced NF-κB p65 nuclear translocation within 30 min (*upper panels*), while the phosphorylation of Akt could not be detected a test periods of up to 3 h (*lower panels*). The epithelial cells were double labeled using anti- p65 antibody with FITC-conjugated secondary antibody (*upper panels*) and anti-phosphorylated Akt antibody with TRITC-conjugated secondary antibody (*lower panels*). (*bar* 20 μM). **b** Phosphorylation of Akt by western blotting was not detected within 3 h of EGF stimulation. As a control β-actin was used. The results shown here are from representative experiments on individual cells from our three SS patients

Results

Immunohistochemical detection of NF-κB, Akt and EGF-R in the labial salivary glands of SS patients

Figure 1 shows representative results of the immunohistochemical detection of NF-κB and Akt in the labial salivary glands of patients with SS, compared with control subjects. As shown in Fig. 1A, nuclear NF-κB p65 was expressed in the ducts of the salivary epithelial cells of SS patients, as were Akt and phosphorylated Akt. Whole Akt was predominantly observed in nuclei. Although phosphorylated Akt was also found in nuclei, it was more clearly detected in cytoplasm than in nuclei as compared with whole Akt. The mirror section technique identified co-localization of the two molecules in the same ducts although intracellular distribution of these molecules was different (Fig. 1B). In contrast to the data of SS patients, neither nuclear NF-κB p65 nor phosphorylated Akt was detected in the salivary epithelial cells of control subjects, although cytoplasmic NF-κB p65 and whole Akt were observed (Fig. 1C). Using pixel intensity measurement system, strong nuclear distribution of p65 in SS was found (Fig. 1D-d), although p65 of the control subject was observed both cytoplasm and nucleus (Fig. 1D-e). In Fig. 1D-e, intensity of some nucleuses was much weaker than cytoplasmic signal intensity or traced in a control subject, which was obviously different from nuclear distribution of the intensity in a SS patient. With regard to phosphorylated Akt in LSG of a SS patient, both nuclear and cytoplasmic staining pattern was observed (Fig. 1D-f). We also examined EGF-R expression in situ in the salivary glands, finding that whole EGF-R was expressed in the salivary epithelial cells of both SS patients and control subjects, while phosphorylated EGF-R was seen only in the cells of SS patients (Fig. 2).

EGF induces rapid NF-κB p65 nuclear translocation in primary salivary epithelial cells independent of Akt activation

We initially studied the phosphorylation of EGF-R in primary salivary epithelial cells in vitro, demonstrating that EGF rapidly phosphorylates EGF-R within 30 min, as observed by confocal microscopy (Fig. 3). Furthermore, EGF also induced nuclear translocation of NF-κB p65 in the cells within 30 min (Fig. 4a), but did not induce the phosphorylation of Akt at time periods up to 3 h (Fig. 4a,

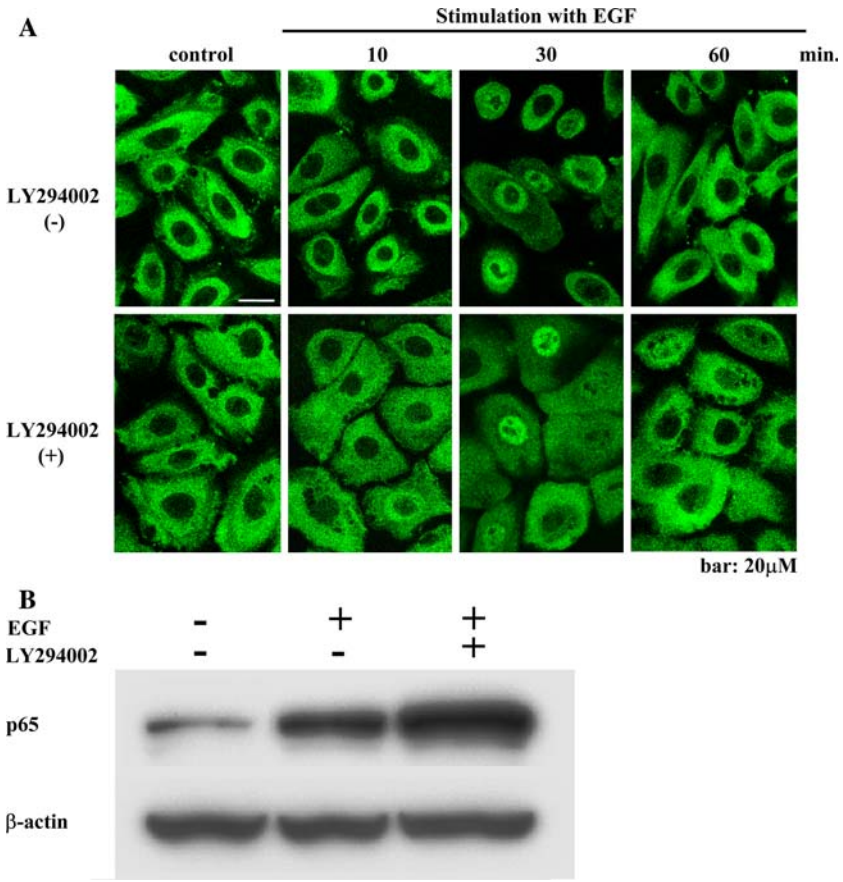

Fig. 5 EGF-mediated NF-κB p65 nuclear translocation is not modulated by the PI3K inhibitor LY294002 in salivary epithelial cells. **a** Primary salivary epithelial cells were preincubated with 50 μM of LY294002 for 1 h prior to stimulation with EGF (50 ng/ml). Pretreatment with LY294002 did not inhibit EGF-mediated NF-κB p65 nuclear translocation, as observed by confocal microscopy. The results shown here are from representative experiments on individual cells from our three SS patients. (*bar* 20 μM). **b** Nuclear NF-κB p65 expression by western blotting. Stimulation with EGF clearly increased the amount of nuclear NF-κB p65, and pretreatment with LY294002 did not inhibit EGF-mediated NF-κB p65 nuclear expression. As a control β-actin was used

by immunofluorescence; Fig. 4b, by western blotting). Although constant level of faint nuclear staining of phosphorylated Akt continued during 180 min stimulation with EGF, that was much weaker than the staining at longer stimulation and low level of Akt expression was confirmed by western blotting.

To examine the role of Akt in EGF-mediated NF-κB p65 nuclear translocation, we used chemical inhibitors of PI3K, LY294002 and wortmannin. Neither LY294002 (Fig. 5a) nor wortmannin (data not shown) affected EGF-mediated NF-κB p65 nuclear translocation. In addition to our immunofluorescence study, similar results were obtained by the use of nuclear extracts of salivary epithelial cells in which EGF-mediated NF-κB nuclear expression was not suppressed in the presence of LY294002 (Fig. 5b).

EGF induces late phosphorylation of Akt in primary salivary epithelial cells independent of IKK-NF-κB activation

Although EGF did not induce Akt phosphorylation in primary salivary epithelial cells within 3 h (Figs. 4a, b), it was found to strongly phosphorylate Akt mainly in cytoplasm 12–24 h of stimulation with EGF (Fig. 6a), with a distribution similar to that observed by immunohistochemical study (Fig. 1a). The EGF-mediated PI3K activation appeared to be essential to the process since the use of the PI3K chemical inhibitors LY294002 and wortmannin, significantly suppressed EGF-induced Akt phosphorylation (Fig. 6b). The use of the IKK inhibitor Bay 11-7082 did not modulate the phosphorylation of Akt, suggesting that EGF-mediated PI3K-Akt activation is achieved distinctively through the IKK-NF-κB pathway (Fig. 6b). The results of western blotting (Fig. 6a, b) were confirmed by the results of immunofluorescence studies (Fig. 6c, d). In Fig. 6d, the staining of phosphorylation of Akt was double labeled with Hoechst 33258 to examine whether the major part of staining is nucleus or cytoplasm. When the salivary epithelial cells were treated with EGF, most of activated Akt was found in cytoplasm although strong signal was also detected in the nucleus. Although activated Akt signal was found when the cells were pretreated with Bay 11-7082, the signal was detected in the nucleus, which was different from the distribution with stimulation of EGF only.

Discussion

In agreement with other investigators, we found that the expression of death receptors such as Fas, death receptor 4

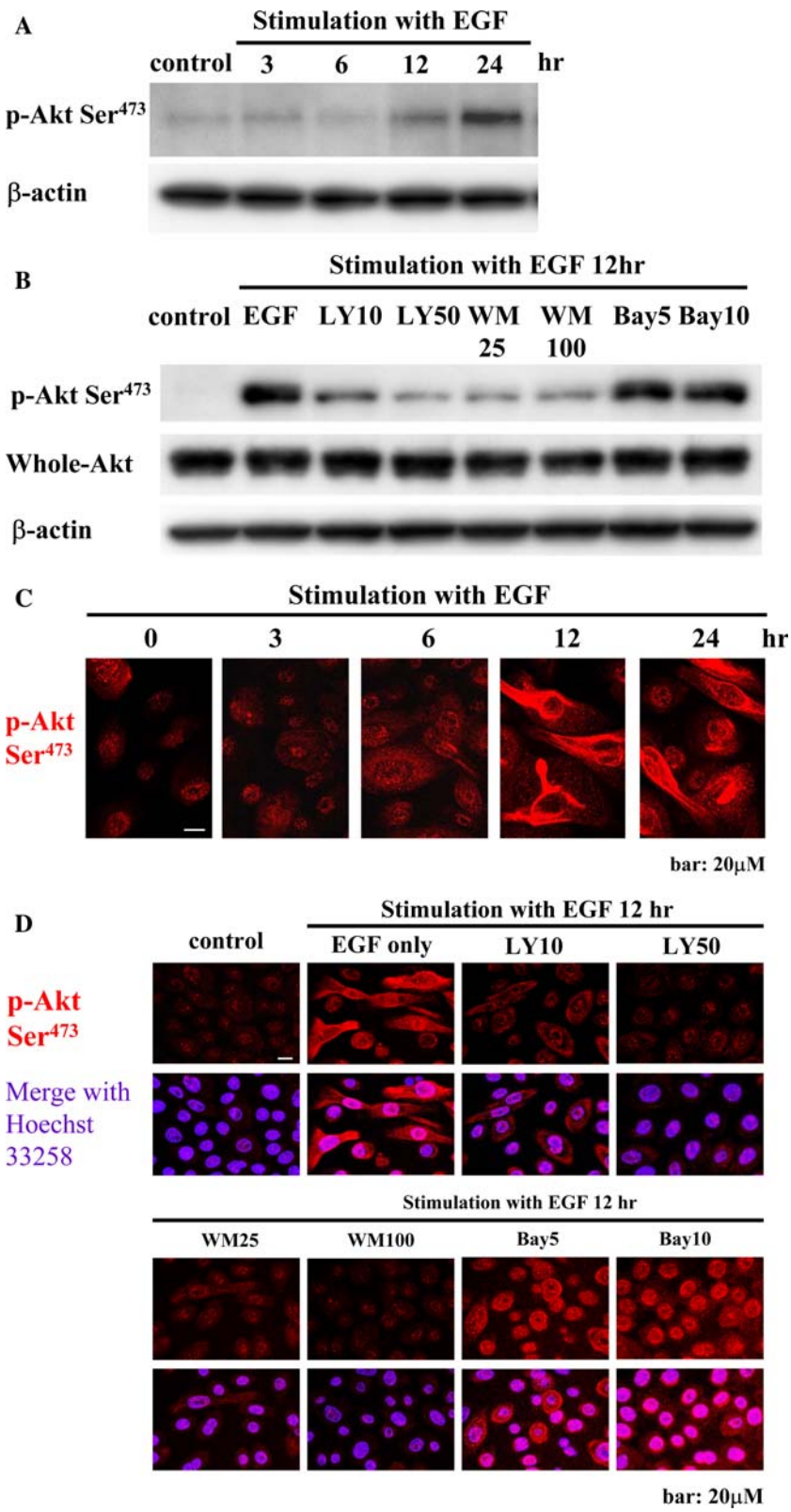

Fig. 6 Late EGF-induced phosphorylation of Akt via PI3K independent of IKK-NF-κB by western blot analysis and confocal microscopy. **a** EGF induced Akt phosphorylation at 12–24 h. **b** Pretreatment with PI3K inhibitors (LY294002 at 10 and 50 μM, and wortmannin at 25 and 100 nM) was found to inhibit the phosphorylation of Akt, while pretreatment with an IKK inhibitor (Bay 11-7082 at 5 and10 μM) did not affect Akt phosphorylation. Salivary epithelial cells were stimulated with EGF for 12 h. As a control β-actin was used. **c** EGF phosphorylates Akt at 12–24 h based on immunofluorescence data, similar to the data depicted in Fig. 6a. **d** Confirmation of the results shown in Fig. 6b by immunofluorescence study. To clarify distribution of phosphorylated Akt at each panel, the nucleus was stained with 50 ng/ml of Hoechst 33258 (blue staining) and presented as merged view with phosphorylated Akt, showing the nuclear distribution of phosphorylates Akt as purple signal. Results are representative experiments of individual cells from three SS patients. (*bar* 20 μM)

(DR4), DR5 and double-stranded DNA breaks in situ in patients with SS [2, 3, 23]. In addition, our present data also show the expression of phosphorylated Akt and nuclear NF-κB p65 in the same cells by immunohistochemistry. In the pathogenesis of sialadenitis in SS, apoptotic cell death induced by Fas/Fas ligand, granzymes or perforin in salivary

epithelial cells is thought to be associated with salivary tissue destruction [1, 2]. Hence, mechanisms that counteract cell death also appear to be activated; the appearance of lymphoepithelial islands may reflect the proliferative response of ductal epithelial cells to inflammatory stimuli in SS patients; Yamamura et al. [8] describe their study, in which they found that lymphoepithelial islands were surrounded by inflammatory lymphocytes.

Epidermal growth factor and EGF-R are considered to be tropic factors for salivary epithelial cells in the salivary glands of SS patients based on evidence presented by Gorgoulis et al. [9] that EGF and EGF-R are densely expressed in the epithelial duct cells of lymphoepithelial or destructive lesions in secondary SS patients. Since the expression of phosphorylated EGF-R as well as co-localization of phosphorylated Akt and nuclear NF-κB p65 were found in situ in the salivary epithelial cells of SS patients in our immunohistochemical study, we focused on the regulatory mechanisms of EGF as an inducer for the activation of PI3K-Akt and IKK-NF-κB in vitro. Ahn et al. [24] recently reported that nerve growth factor (NGF)-treated PC-12 cells are resistant to apoptosis induced by activated cell-free apoptosome through PI3K signaling, suggesting that the PI3K-Akt pathway is essential in promoting cell survival. Their explanation implied that growth factors possess the potential to activate the PI3K-Akt pathway and subsequent anti-apoptotic effect.

In the present study, EGF phosphorylated EGF-R on primary salivary epithelial cells, and NF-κB p65 nuclear translocation and phosphorylation of Akt in the cells were clearly induced by EGF. As concerns sustained EGF-induced phosphorylation of EGF-R for 10–30 min, Keates et al. [25] recently demonstrated that EGF as well as Helicobacter pylori augmented sustaining expression of EGF-R messenger RNA in the gastric epithelial cells up to 6 h, indicating that in their case activation of MAP kinase pathway might be involved. Although expression of MAP kinases was not detected in our previous study [26], relevant mechanism is supposed to be related to activation of EGF-R in the salivary epithelial cells. The anti-apoptogenic function of PI3K-Akt and IKK-NF-κB are convincing [14, 15]. However, the dependency of each pathway on the other for stimulation may be cell-type dependent or stimulus-dependent such as the cases of Akt located up-stream of NF-κB [27–29] and of NF-κB located up-stream of Akt [30], with each activated in a distinct fashion, as previously reported by Wang et al. [31]. In the present study, we found that, in primary salivary epithelial cells, NF-κB was rapidly activated, while Akt was phosphorylated later during EGF stimulation. Regarding morphological change of the epithelial cells stimulated with EGF 12 h, Ackland et al. [32] previously reported that EGF stimulation for 6 days induced ''ruffling'' of PMC42-LA cells with increased fibronectin deposition, implying that these changes might be related to up-regulation of cell adhesion system. The chemical inhibition by kinase inhibitor strongly suggests that each pathway (PI3K-Akt and IKK-NF-κB) appears to be distinctively activated by EGF. Furthermore, EGF stimulation significantly inhibited pro-apoptogenic stimuli toward primary salivary epithelial cells in vitro [33]. Thus, we believe that EGF-mediated activation of both PI3K-Akt and IKK-NF-κB is functionally anti-apoptogenic. Further studies are necessary to determine the molecular role of each pathway in protecting against salivary epithelial cell death.

Research studies investigating anti-apoptogenic factors in SS are scarce. In the present study, our results suggest EGF/EGF-R involvement in anti-apoptogenic signals via PI3K-Akt and IKK-NF-κB. These results may provide new insight into the mechanism of salivary epithelial cell repair in patients with SS.

Acknowledgments The authors would like to thank Dr. Noriyoshi Ogawa from the Third Department of Internal Medicine, Hamamatsu Medical University, for advice regarding the primary cell culture of epithelial cells obtained from minor salivary gland biopsy. We would also like to thank Dr. Yoshitaka Hishikawa from the Division of Histology and Cell Biology, Department of Developmental and Reconstructive Medicine, Graduate School of Biomedical Sciences, Nagasaki University, for his kind advice and technical instruction of pixel intensity measurement.

References

1. Fox RI, Stern M, Michelson P (2000) Update in Sjögren's syndrome. Curr Opin Rheumtol 12:391–398
2. Kong L, Ogawa N, Nakabayashi T, Liu GT, D'Souza E, McGuff HS et al (1997) Fas and Fas ligand expression in the salivary glands of patients with primary Sjögren's syndrome. Arthritis Rheum 40:87–97
3. Nakamura H, Koji T, Tominaga M, Kawakami A, Migita K, Kawabe Y et al (1998) Apoptosis in labial salivary glands from Sjögren's syndrome (SS) patients: comparison with human T lymphotropic virus-I (HTLV-I)-seronegative and -seropositive SS patients. Clin Exp Immunol 114:106–112
4. Mullighan CG, Heatley S, Lester S, Rischmueller M, Gordon TP, Bardy PG (2004) Fas gene promoter polymorphisms in primary Sjögren's syndrome. Ann Rheum Dis 63:98–101
5. Jonsson R, Gordon TP, Konttinen YT (2003) Recent advances in understanding molecular mechanisms in the pathogenesis and antibody profile of Sjögren's syndrome. Curr Rheumatol Rep 5:311–316
6. Ohlsson M, Szodoray P, Loro LL, Johannessen AC, Jonsson R (2002) CD40, CD154, Bax and Bcl-2 expression in Sjögren's syndrome salivary glands: a putative anti-apoptotic role during its effector phases. Scand J Immunol 56:561–571
7. Polihronis M, Tapinos NI, Theocharis SE, Economou A, Kittas C, Moutsopoulos HM (1998) Modes of epithelial cell death and repair in Sjögren's syndrome (SS). Clin Exp Immunol 114:485–490
8. Yamamura Y, Onodera K, Ichinohasama R, Ooya K (2000) A histopathological study of lymphoepithelial island formation in labial salivary glands in patients with primary Sjögren's syndrome. J Oral Pathol Med 29:110–117

9. Gorgoulis V, Giatromanolaki A, Iliopoulos A, Kanavaros P, Aninos D, Ioakeimidis D et al (1993) EGF and EGF-R immunoexpression in Sjögren's syndrome secondary to rheumatoid arthritis. Correlation with EBV expression? Clin Exp Rheumatol 11:623–627
10. Anest V, Cogswell PC, Baldwin AS Jr (2004) IkappaB kinase alpha and p65/RelA contribute to optimal epidermal growth factor-induced c-*fos* gene expression independent of IkappaBalpha degradation. J Biol Chem 279:31183–31189
11. Kitagawa K, Hamada Y, Kato Y, Nakai K, Nishizawa M, Ito S et al (2004) Epidermal growth factor and interleukin-1beta synergistically stimulate the production of nitric oxide in rat intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 287:G1188–G1193
12. Dhar A, Young MR, Colburn NH (2002) The role of AP-1, NF-kappaB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. Mol Cell Biochem 234–235:185–193
13. Navolanic PM, Steelman LS, McCubrey JA (2003) EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review). Int J Oncol 22:237–252
14. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C (2003) PI3K/Akt and apoptosis: size matters. Oncogene 22:8983–8998
15. Viatour P, Merville MP, Bours V, Chariot A (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci 30:43–52
16. Romashkova JA, Makarov SS (1999) NF-kappaB is a target of AKT in anti-apoptotic PDGF signaling. Nature 401:86–90
17. Gustin JA, Ozes ON, Akca H, Pincheira R, Mayo LD, Li Q et al (2004) Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. J Biol Chem 279:1615–1620
18. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE et al (2002) Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American–European consensus group. Ann Rheum Dis 61:554–558
19. Ogawa N, Ping L, Zhenjun L, Takada Y, Sugai S (2002) Involvement of the interferon-gamma-induced T cell-attracting chemokines, interferon-gamma-inducible 10 kd protein (CXCL10) and monokine induced by interferon-gamma (CXCL9), in the salivary gland lesions of patients with Sjögren's syndrome. Arthritis Rheum 46:2730–2741
20. Ping L, Ogawa N, Sugai S (2005) Novel role of CD40 in fas-dependent apoptosis of cultured salivary epithelial cells from patients with Sjögren's syndrome. Arthritis Rheum 52:573–581
21. Nakamura H, Eguchi K, Nakamura T, Mizokami A, Shirabe S, Kawakami A et al (1997) High prevalence of Sjögren's syndrome in patients with HTLV-I associated myelopathy. Ann Rheum Dis 56:167–172
22. Nakamura H, Kawakami A, Tominaga M, Migita K, Kawabe Y, Nakamura T et al (1999) Expression of CD40/CD40 ligand and Bcl-2 family proteins in labial salivary glands of patients with Sjögren's syndrome. Lab Invest 79:261–269
23. Matsumura R, Kagami M, Tomioka H, Tanabe E, Sugiyama T, Sueishi M et al (1996) Expression of ductal Fas antigen in sialoadenitis of Sjögren's syndrome. Clin Exp Rheumatol 14:309–311
24. Ahn JY, Rong R, Liu X, Ye K (2004) PIKE/nuclear PI 3-kinase signaling mediates the antiapoptotic actions of NGF in the nucleus. EMBO J 23:3995–4006
25. Keates S, Keates AC, Katchar K, Peek RM Jr, Kelly CP (2007) Helicobacter pylori Induces up-regulation of the epidermal growth factor receptor in AGS gastric epithelial cells. J Infect Dis. 196:95–103
26. Nakamura H, Kawakami A, Yamasaki S, Kawabe Y, Nakamura T, Eguchi K (1999) Expression of mitogen activated protein kinases in labial salivary glands of patients with Sjögren's syndrome. Ann Rheum Dis. 58:382–385
27. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB (1999) NF-kappaB activation by tumor necrosis factor requires the Akt serine-threonine kinase. Nature 401:82–85
28. Dhawan P, Singh AB, Ellis DL, Richmond A (2002) Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression. Cancer Res 62:7335–7442
29. Kao SJ, Lei HC, Kuo CT, Chang MS, Chen BC, Chang YC et al (2005) Lipoteichoic acid induces nuclear factor-kappaB activation and nitric oxide synthase expression via phosphatidylinositol 3-kinase, Akt, and p38 MAPK in RAW 264.7 macrophages. Immunology 115:366–374
30. Meng F, Liu L, Chin PC, D'Mello SR (2002) Akt is a downstream target of NF-kappa B. J Biol Chem 277:29674–29680
31. Wang HQ, Quan T, He T, Franke TF, Voorhees JJ, Fisher GJ (2003) Epidermal growth factor receptor-dependent, NF-kappaB-independent activation of the phosphatidylinositol 3-kinase/Akt pathway inhibits ultraviolet irradiation-induced caspases-3, -8, and -9 in human keratinocytes. J Biol Chem 278:45737–45745
32. Ackland ML, Newgreen DF, Fridman M, Waltham MC, Arvanitis A, Minichiello J et al (2003) Epidermal growth factor-induced epithelio-mesenchymal transition in human breast carcinoma cells. Lab Invest 83:435–448
33. Nakamura H, Kawakami A, Ida H, Koji T, Eguchi K. Epidermal growth factor inhibits Fas-mediated apoptosis in salivary epithelial cells of patients with Sjögren's syndrome accepted in Clin Exp Rheumatol