Stress hormones increase cell proliferation and regulates interleukin-6 secretion in human oral squamous cell carcinoma cells

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ABSTRACT

Patients with oral cancer can have high psychological distress levels, but the effects of stress-related hormones on oral cancer cells and possible mechanisms underlying these relationships are unknown. In this study, we have investigated the effects of stress-related hormones on interleukin-6 (IL-6) secretion and proliferation of oral squamous cell carcinoma (OSCC) cells. The effects of norepinephrine (NE), and cortisol were studied in SCC9, SCC15, and SCC25 cells and effects of isoproterenol in SCC9 and SCC25 cells. Real-time PCR studies revealed constitutive β1- and β2-adrenergic receptors (β-ARs) expression in the SCC9, SCC15, and SCC25 cells. The results showed that NE and isoproterenol significantly enhanced IL-6 mRNA expression and protein production in supernatants of SCC9 and SCC25 cells. Physiological stress levels of NE and isoproterenol (10 μM) at 1 h elicited the most robust IL-6 increase. Regarding IL-6 secretion, 10 μM NE induced a 5-fold increase at 1 h, 3.7-fold increase at 6 h, and 3.2-fold at 24 h in SCC9 cells. These effects were blocked by the β-adrenergic antagonist propranolol, supporting a role for β-ARs in IL-6 secretion. The effects of cortisol varied according to the hormone concentration. Pharmacological concentrations of cortisol (1000 nM) inhibited IL-6 production by SCC9 and SCC25 cells. Cortisol dose that simulates stress conditions (10 nM) tended to increase IL-6 expression in SCC9 cells. Hormonal doses that simulate stress conditions (10 μM NE, at 6 h in SCC9 and SCC15 cells and 10 nM cortisol, at 48 h in SCC15 cells) stimulated increased cell proliferation. Treatment of SCC9 cells with IL-6 neutralizing ab (10 μg/mL) partially inhibited NE-induced proliferation. Finally, 20 OSCC biopsies were shown to express β1- and β2-ARs. These findings suggest that stress hormones can affect oral cancer cells behavior.
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1. Introduction

A growing number of studies have shown that hormonal and immune alterations resulting from chronic stress and other behavioral conditions may influence cancer development and progression (Reiche et al., 2004; Thaker et al., 2007; Antoni et al., 2006; Lillberg et al., 2003). Chronic stress is associated with dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, with consequent increase in the production of the hormone cortisol, and elevated levels of norepinephrine (NE) and epinephrine (E), which are catecholamines released from the adrenal medulla and the neurons of the sympathetic nervous system (SNS) (Thaker et al., 2007; Glaser and Kiecolt-Glaser, 2005).

Neurohormonal products derived from chronic stress may reduce the natural killer cell cytotoxicity by inhibiting the response of cells to certain cytokines such as interferon-gamma (IFN-γ) and interleukin-2 (IL-2) (Kiecolt-Glaser and Glaser, 1999; Esterling et al., 1996). Stress hormones also have the ability to act directly on tumor cells and to deregulate the production of cytokines, chemokines, and growth factors that are related to cancer development and progression (Reiche et al., 2004; Antoni et al., 2006; Ardestani et al., 1999). For example, studies on ovarian cancer have shown that catecholamines enhance the expression of substances such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), which are known to influence tumor progression (Lutgendorf et al., 2003; Sood et al., 2006; Yang et al., 2008). Other investigations have demonstrated that the neurohormonal products derived from chronic stress influence skin (Saul et al., 2005), breast (Ben-Eliyahu et al., 1991), lung (Melamed et al., 2005), and colon (Lointier et al., 1992) cancer progression.

Interleukin-6 (IL-6) is a cytokine that plays an important role in angiogenesis and tumoral progression (Heikkilä et al., 2008). The head and neck squamous cell carcinoma (HNSCC) cell line secrete IL-6 (Chakravarti et al., 2006), and a high level of this cytokine has been detected in the saliva and blood of patients with HNSCC

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(Rhodus et al., 2005; Duffy et al., 2008). In-vitro studies have described that IL-6 stimulates cell proliferation (Chakravarti et al., 2006) and bone invasion of OSCC cells (Okamoto et al., 2000). High IL-6 levels in OSCC tissue and plasma have also been associated with recurrence, lymph node involvement, and a poor prognostic survival (Duffy et al., 2008; Nagata et al., 2003). IL-6 has been directly related to chronic stress. Individuals who experience emotional stress display high IL-6 circulating levels (Kiecolt-Glaser et al., 2003). Recently, it has been shown that NE can increase IL-6 expression in malignant melanocytes (Yang et al., 2009) and human ovarian carcinoma cells (Nilsson et al., 2007), thereby contributing to the development of these tumors.

There are few studies investigating the influence of stress hormones on HNSCC. Recently, the presence of β-adrenergic receptors (β-AR) for NE and E has been identified in oral (Shang et al., 2009) and esophagus cancer (Liu et al., 2008) cell lines. These investigations also showed that the proliferation of these cell lines is stimulated by NE and E, respectively. Nevertheless, there is no evidence that IL-6 expression in oral cancer can be influenced by stress hormones. In this study, we have evaluated the effects of stress-related hormones on IL-6 expression and proliferation of OSCC cells, and evidence that OSCC biopsies express β-ARs is provided.

2. Materials and methods

2.1. Cell culture and hormone treatment

The OSCC-derived cell lines SCC9, SCC15, and SCC25 were used in the evaluation of the effects of stress hormones. The cell lines were kindly provided by Dr. Ricardo Della Coletta (School of Dentistry, State University of Campinas, Piracicaba, São Paulo, Brazil). These cells were maintained and propagated in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (DMEM/F12; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, 100 μg/mL streptomycin, and 0.1% gentamicin, at 37 °C, in 5% CO_2 humidified atmosphere. Experiments were carried out with 80% confluent cultures. SCC9, SCC15, and SCC25 cells were seeded in 24-well plates (1.0×10^5 cells per well) and cultured for 24 h in serum-reduced medium (0.1% FBS). The following hormones were tested: NE (Calbiochemical Co, La Jolla, CA), cortisol (Sigma–Aldrich, St. Louis, MO) and isoproterenol (Sigma–Aldrich, St. Louis, MO), a β-adrenergic agonist. The cells SCC9 and SCC25 were then treated with NE or isoproterenol at 0, 0.1, 1, and 10 μM, or cortisol at 0, 1, 10, 100, and 1000 nM. These concentrations were used in the subsequent experiments. The cells SCC15 were treated with NE and cortisol. For blocking experiments, 1 μM propranolol was added to the cell cultures 1 h before addition of 10 μM NE. Cell-free supernatants and cells were collected at 1, 6, and 24 h, and kept at − 80 °C until the assays were performed. The hormone concentrations employed were defined by taking the physiological levels that usually reaching in the tumor microenvironment. NE basal circulating levels range between 10 pM and 1 nM (Sood et al., 2006), and studies have suggested that stress increases these levels to approximately 100 nM, and they may reach 10 μM in the microenvironment of some types of tumors (Antoni et al., 2006; Sood et al., 2006). The concentrations of 10 and 100 nM cortisol reflect similar levels to those found in stress conditions, and higher concentrations (1000 nM) simulate pharmacological doses of glucocorticoids (Miller and O'Callaghan, 2002).

2.2. Real-time PCR assessment of IL-6 gene expression

Quantitative real-time reverse transcription-PCR (RT-PCR) was used to assess the IL-6 gene expression in SCC9, SCC15, and SCC25 cells treated with stress hormones. Total RNA from cell lines was isolated with TRIzol, following the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). The RNA concentration was measured by spectrophotometry. First strand cDNAs were synthesized using 1 μg of total RNA and Superscript II RNase H⁻ reverse transcriptase (Invitrogen Life Technologies). IL-6 mRNA levels were measured by means of the SYBR Green system and amplified in the Stepone Real-Time PCR system (Applied Biosystems). The housekeeping gene β-actin was employed as internal positive control. The primers were as follows: β-actin (forward, 5′-TGGATCAGCAAGCAGGAGTATG-3′; reverse, 5′ GCATTTGCGGTG-GACGAT-3′) and IL-6 (forward, 5′-AGGGCTCTTCGGCAAATGTA-3′; reverse, 5′-GAAGGAATGCCCATTAACAACAA-3′). Primers were drawn using the Primer Express software (Applied Biosystems). Reactions were carried out using a volume of 20 μL, and each sample was run in duplicate. The PCR thermal cycle conditions used in the experiments were those recommended by the manufacturer. The IL-6 mRNA expression levels in each sample were normalized to the β-actin mRNA level. The results were analyzed using the comparative threshold cycle (C_T) method. Results were presented on fold increase of the IL-6 mRNA expression in cells treated with hormones as compared to untreated cells.

2.3. Determination of IL-6 protein expression

The total IL-6 protein concentrations in the supernatants of the SCC9 and SCC25 cells treated with stress hormones were determined. Serum-reduced conditioned medium from cultures of oral cancer cells was collected at 1, 6, and 24 h following exposure to NE, isoproterenol, or cortisol. Quantification of serum IL-6 levels was accomplished by the quantitative sandwich enzyme immunoassay technique (ELISA) (R&D Systems, Minneapolis, MN) following the manufacture's protocol. The resulting color was read in a spectrophotometer set to the wavelength of 450 nm.

2.4. β1- and β2-AR expression in OSCC cell lines

To assess whether the SCC9, SCC15, and SCC25 cell lines express mRNA for β1- and β2-AR, real-time PCR assay was performed as described previously. The utilized primers were β1 (forward, 5′-GCGTGTGATGCATCTTTAGATTTT-3′; reverse, 5′- CCTAACCCACC CATCTTCCA-3′) and β2 (forward, 5′-TTGAAGGCCTATGGGAATGG-3′; reverse, 5′-TCCACTCTGCTCCCCTGTGT-3′). Primers were drawn using the Primer Express software. The β-actin gene was used as endogenous control.

2.5. MTT cell proliferation assay

OSCC cells SCC9 and SCC15 were seeded in 96-well plates (1.0×10^3 per well) and grown in 100 μL 10% FBS-supplemented DMEM/F12 medium. After 20% confluence had been reached, cells were cultured for 24 h in serum-reduced medium (0.1% FBS). Cells were treated with NE or cortisol. Blocking experiments were performed with propranolol (1 μM added 1 h before addition of 10 μM NE). The MTT solution was carried out by dissolving 5 mg of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma) in 1 mL of PBS, followed by filtration and sterilization in Millipore filter 0.22 μm. The MTT solution (100 μL) diluted 10× in serum-reduced medium (0.1%) was added to each well after 6, 24, and 48 h of hormone treatment. The cells remained for 4 h at 37 °C and 5% CO_2 humidified atmosphere. The MTT solution was aspirated, and isopropanol (100 μL per well) was added. The plate was then stirred for 30 min at room temperature, to solubilize the blue formazan crystals that stained the mitochondria. Colorimetric quantification was determined by spectrophotometry

set to the wavelength of 570 nm. The experiments were carried out in six replicates and were repeated three times.

2.6. Effects of IL-6 neutralizing ab on NE-induced proliferation

To evaluate whether NE-induced OSCC proliferation is mediated by IL-6, anti-IL-6 ab (R&D Systems, Minneapolis, MN) was employed to neutralize the action of IL-6. Briefly, after SCC9 cells had reached 20% confluence, cells were cultured for 24 h in serum-reduced medium (0.1% FBS). Then, the SCC9 cells were pre-treated with IL-6 neutralizing ab (1 and 10 µg/mL) for 30 min prior to the addition of NE (10 µM). Cells were further incubated for 6 h, and proliferation was evaluated by MTT assay.

2.7. β1- and β2-AR expression in OSCC biopsies, oral leukoplakia biopsies, and normal oral mucosa

To assess whether OSCC cells express β1- and β2-AR, 20 tumor specimens were collected from patients with OSCC who had not received any treatment yet. All the OSCC cases had the diagnosis confirmed histologically. Once removed from the surgery site, the specimens were washed in saline solution, placed in a tube containing TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and immediately stored in liquid nitrogen. For comparative analysis, 17 specimens of oral leukoplakia (considered a precursor lesion of OSCC) and 15 samples of normal oral mucosa were collected and stored following the same protocol. The samples were then thawed and ground in TRIzol with an electric homogenizer.

The total RNA was then extracted, cDNA was synthesized, and real-time PCR assay was performed as previously described.

2.8. Statistical analysis

Data were checked for normality, and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni's multiple-comparison test. P values <0.05 were considered significant.

3. Results

3.1. Adrenergic stimulation upregulates IL-6 gene expression in OSCC cells

In all the evaluated times (1, 6, and 24 h), treatment of SCC9, SCC15, and SCC25 cells with physiological stress levels of NE (10 µM) elevated IL-6 mRNA expression. Maximum IL-6 expression peaked 1 h after stimulation with 10 µM NE, leading to an increase of 501.5 ± 34.8%, 317.1 ± 32.65%, and 237.7 ± 37.6% in IL-6 mRNA expression in SCC9 ($p < 0.001$), SCC15 ($p < 0.05$), and SCC25 cells ($p < 0.05$), respectively (Fig. 1A–C). A smaller but significant enhancement in IL-6 mRNA levels in the SCC9 and SCC25 cell lines was also observed after 6 h of stimulation with NE, which did not continue after 24 h (Fig. 1A and B). The synthetic β-adrenergic receptor agonist isoproterenol also induced a significant rise in IL-6 mRNA expression in SCC9 and SCC25 cells (SCC15 cells were not tested for isoproterenol). Specifically, after 1 h of treatment of SCC9 cells with 1 and 10 µM isoproterenol, IL-6 RNAm levels
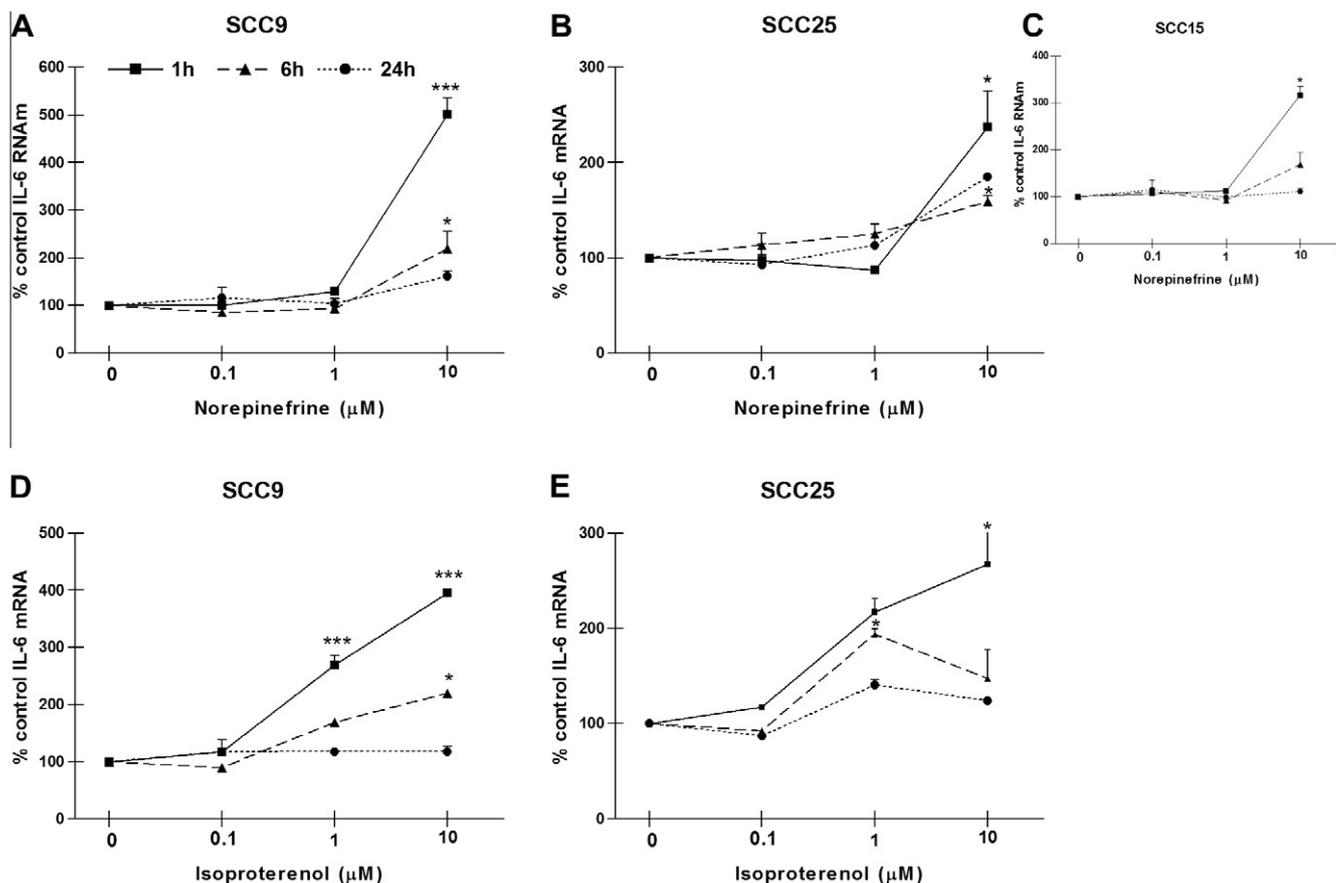
Fig. 1. Human OSCC cell lines were stimulated with norepinephrine (A, SCC9; B, SCC25; and C, SCC15) and isoproterenol (0.1, 1, or 10 µM) (D, SCC9 and E, SCC25). Cells were collected 1 h (square), 6 h (triangle), or 24 h (oval) after stimulus. Total RNA was isolated, and real-time PCR was used to quantify IL-6 RNA. Values are presented in points as percentage of IL-6 mRNA expression of stimulated cells relative to control (non-stimulated cells – 0 µM or 0 nM), which was set as 100%. Bars, SE. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$.

increased $269.7 \pm 16.4\%$ ($p < 0.001$) and $395.6 \pm 4.4\%$ ($p < 0.001$), respectively (Fig. 1D). As in the case of SCC9 cells, after 1 h, 10 μM isoproterenol induced a significant increase in IL-6 mRNA production by SCC25 cells ($267.2 \pm 43.5\%$; $p < 0.05$). However, after longer periods, higher IL-6 mRNA levels were observed with 1 μM isoproterenol, where only the increase after 6 h was significant ($194.1 \pm 5.8\%$; $p < 0.05$) (Fig. 1E).
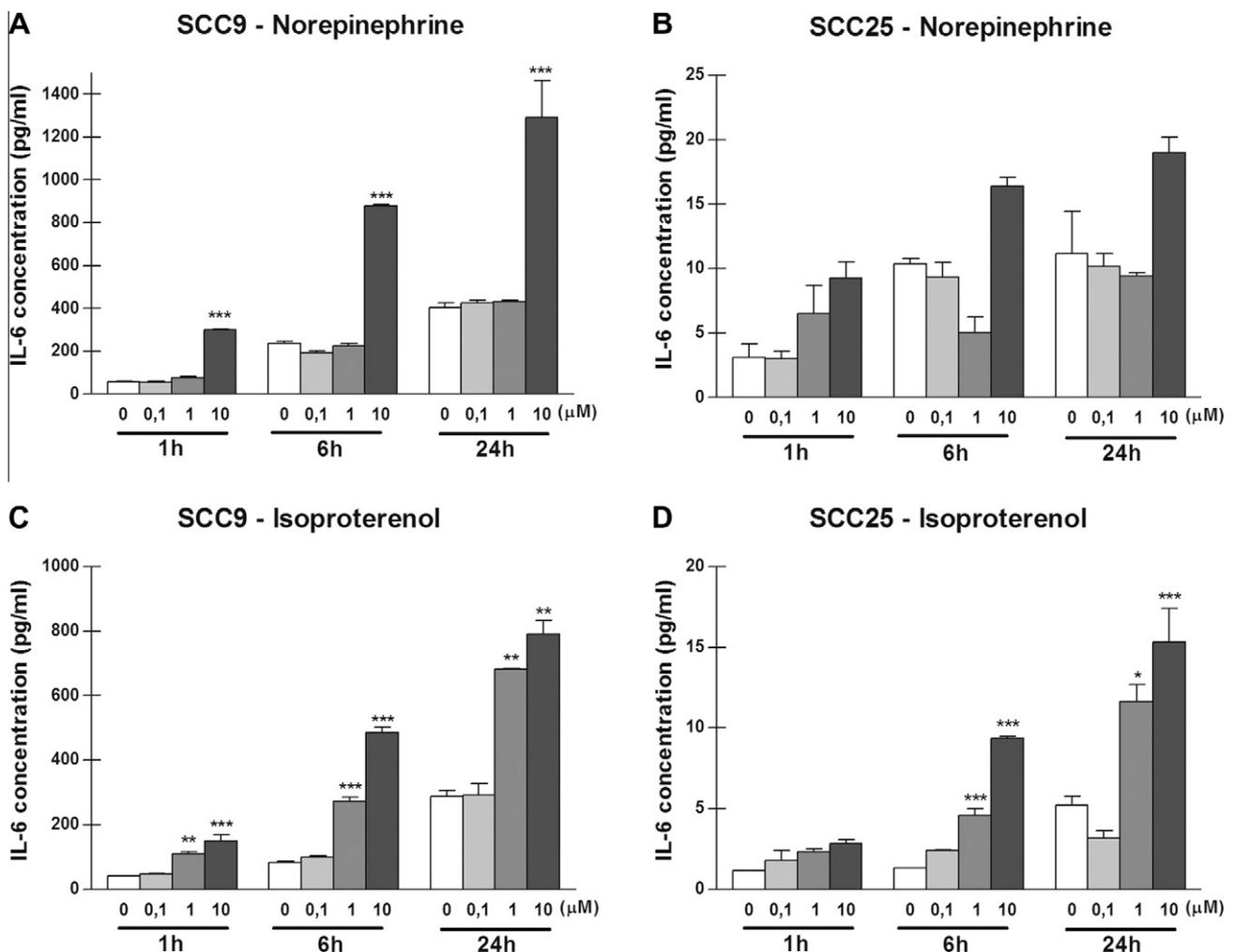
3.2. Adrenergic stimulation increases IL-6 protein levels in culture supernatants

IL-6 protein levels were measured in supernatants of the SCC9 and SCC25 cells. Production of IL-6 protein by SCC9 cells at the three tested times was enhanced compared to the production by SCC25 cells. For example, the mean basal levels of IL-6 production by SCC9 and SCC25 cells at 1 h with no stimulation were 58.63 ± 3.42 pg/mL and 3.11 ± 1.06 pg/mL, respectively. The basal level of IL-6 production by SCC9 and SCC25 cells with no stimulation were detectable at 1 h and increased over the time period examined (Figs. 2 and 3). For both cell lines, physiological stress levels of NE (10 μM) elicited the most robust IL-6 increase. Maximum elevations in IL-6 occurred at 1 h of incubation. As depicted in Fig. 2A, stimulation of SCC9 cells with 10 μM NE for 1 h produced 301.3 ± 3.45 pg/mL of IL-6 protein, resulting in an approximately 5-fold increase ($p < 0.001$) compared to the control. After 6 h, 10 μM NE induced a 3.7-fold increase, whereas after 24 h a

3.2-fold enhancement in IL-6 production ($p < 0.001$) was detected. As for SCC25 cells, treatment with 1 μM NE for 1 h produced a 2.1-fold increase in IL-6 production, and 10 μM NE induced an elevation of approximately 3-fold (Fig. 2B). For both SCC9 and SCC25 cells, a maximum IL-6 rise was observed after 6 h in the presence of 10 μM isoproterenol. The mean basal level of IL-6 secretion by SCC9 cells after 6 h was 83.18 ± 3.23 pg/mL. The IL-6 levels increased to 272.3 ± 12.42 pg/mL after treatment with 1 μM isoproterenol ($p < 0.001$), and to 487.1 ± 15.27 pg/mL after treatment with 10 μM isoproterenol ($p < 0.001$) (Fig. 2C). The patterns of the IL-6 increase in SCC25 cells after isoproterenol stimulation were similar to those found in SCC9 cells, except for the stimulus with 0.1 μM isoproterenol after 24 h, which reduced IL-6 levels (but this result was not significant) (Fig. 2D).

3.3. The effects of cortisol on IL-6 expression in OSCC cells

The pattern of IL-6 mRNA expression after treatment with cortisol was distinct from that found for NE and isoproterenol. The effects of cortisol varied according to the hormone concentration. In SCC9 cells, in general, higher concentrations of cortisol (100 and 1000 nM) determined lower IL-6 mRNA and protein production. For 1000 nM cortisol, a dose that is approximately equivalent to pharmacological levels of glucocorticoid, there was a significant decrease in IL-6 mRNA expression at all the tested periods. A larger suppression in IL-6 mRNA expression and IL-6

Fig. 2. Adrenergic stimulation increases IL-6 secretion by OSCC cells. Human OSCC cell lines (SCC9 and SCC25) were stimulated with norepinephrine (A and B) and isoproterenol (C and D) (0.1, 1, or 10 μM). Supernatants were collected 1 h, 6 h, or 24 h after the stimulus, and IL-6 was measured by ELISA. Data represent the mean (±SEM) of triplicate determinations of the IL-6 concentration. $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$.
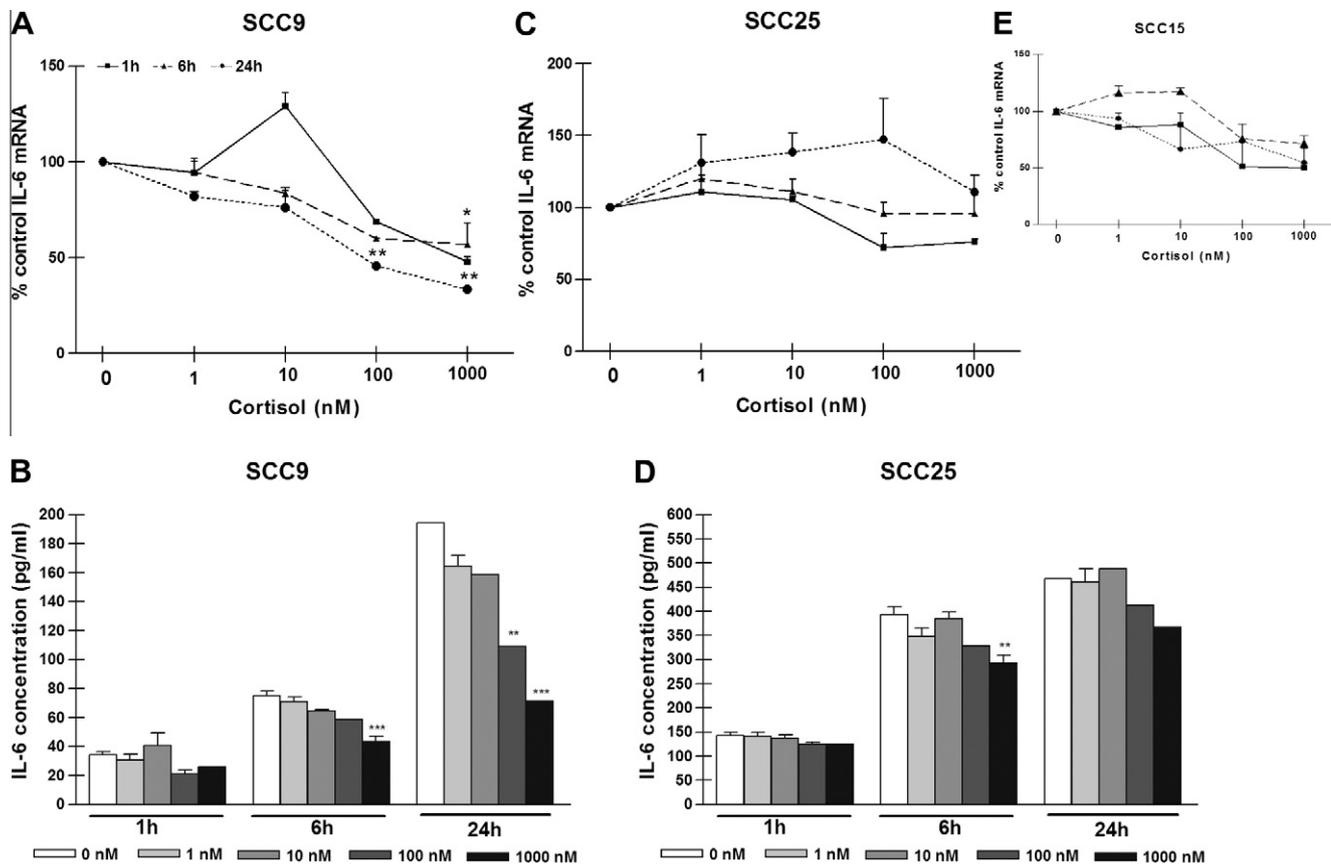
Fig. 3. Cortisol influences IL-6 expression and secretion by tumor cells. SCC9, SCC25, and SCC15 cells were stimulated with cortisol (1, 10, 100, or 1000 nM). Cells were collected 1 h (square), 6 h (triangle), or 24 h (oval) after stimulus and real-time PCR was used to quantify IL-6 RNA (A, C, and E). Supernatants were collected 1, 6, or 24 h after the stimulus, and IL-6 was measured by ELISA (B and D). *P ⩽ 0.05; **P ⩽ 0.01; ***P ⩽ 0.001.

protein levels was observed after treatment with 1000 nM cortisol at 24 h. This treatment reduced IL-6 mRNA expression by $298 \pm 1.9\%$ compared to the control ($p < 0.01$) (Fig. 3A) and induced a 2.7-fold decrease in IL-6 protein levels ($p < 0.001$) (Fig. 3B). In contrast, after 1 h, 10 nM cortisol (simulating physiological stress levels) promoted increase IL-6 mRNA expression (129% compared to control) (Fig. 3A) and protein levels (Fig. 3B) in SCC9 cells, but these changes did not reach significance. These cortisol effects were blocked by glucocorticoid inhibitor Mefipristone (data not shown). SCC25 cells did not exhibit a significant response to cortisol treatment. Specifically, SCC25 cells treated with 1000 nM cortisol at 6 h produced 292.2 ± 17.40 pg/mL of IL-6, resulting in a 1.25-fold decrease compared to the control ($p < 0.05$) (Fig. 3D). In these same cells, lower IL-6 mRNA levels were detected at 1 h with 100 nM cortisol ($131.1 \pm 0.03\%$ compared to the control) and 1000 nM cortisol ($152.1 \pm 2.7\%$), while an increase in IL-6 mRNA levels took place at 24 h using 10 nM cortisol ($138 \pm 12.96\%$) and 100 nM cortisol ($147 \pm 28.75\%$), but these results were not significant (Fig. 3C).

Similar results were found in SCC15 cells, in which lower cortisol concentrations (1 and 10 nM) did not determine large variations in IL-6 mRNA levels, whereas high concentrations simulating pharmacological concentrations (e.g., 1000 nM) decreased IL-6 expression (but these results were not significant) (Fig. 3E).
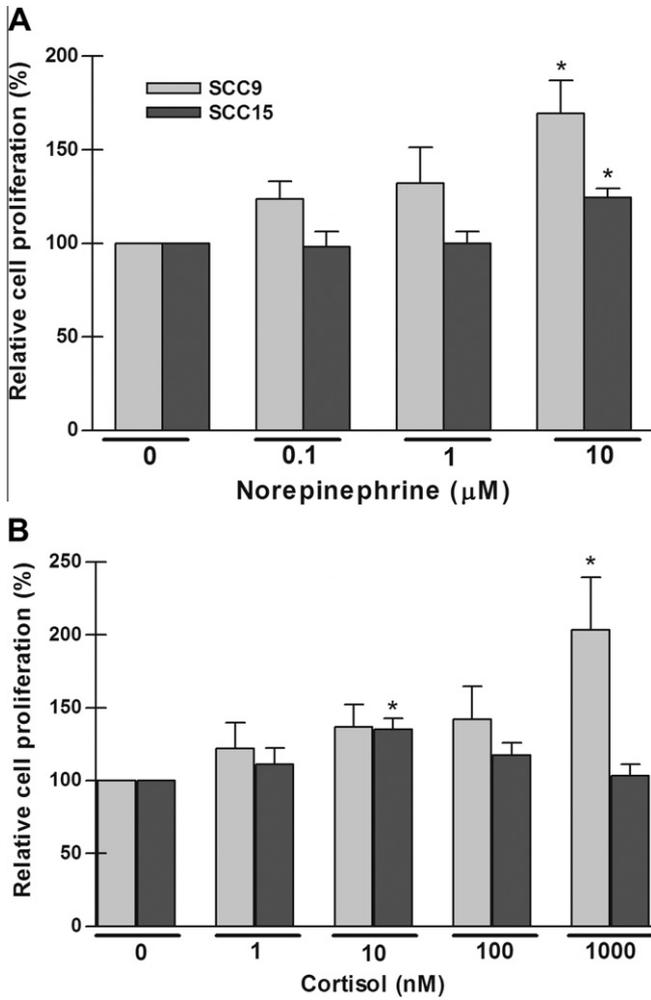
3.4. Stress hormones increase OSCC cell proliferation

To examine the effects of stress hormones on OSCC cell proliferation, SCC9 and SCC15 cells were treated with different doses of NE and cortisol, and cell proliferation was assayed by MTT at 6, 24, and 48 h. The SCC25 cell line was not assayed by MTT because it did not respond well (absence of cell growth) to culture in serum-reduced medium (0.1% FBS). Stimulation of SCC9 and SCC15 cells with physiological NE stress levels (10 μM) induced an enhancement of $170 \pm 17.7\%$ ($p < 0.05$) and $124 \pm 13.7\%$ ($p < 0.05$) in cell proliferation at 6 h compared with non-treated cells, respectively (Fig. 4A). These NE-induced effects of SCC9 and SCC15 cells were not significant at subsequent times (24 and 48 h) (data not shown). In SCC9 cells, treatment with pharmacological levels of cortisol (1000 nM) produced at later time point (48 h) a rise of approximately $200 \pm 36.1\%$ in cell proliferation ($p < 0.05$) (Fig. 4B). Cortisol doses that simulate stress conditions (10 nM) induced at 48 h an increase in cell proliferation in SCC9 (non-significant) (Fig. 4B) and in SCC15 cells ($135 \pm 17.5\%$; $p < 0.05$) (Fig. 4B). There was no significant increase in the cell proliferation index after 6 and 24 h of stimulus with cortisol (data not shown).

3.5. Regulation of IL-6 expression and OSCC cell proliferation by stress hormones requires β-ARs activation

Real-time PCR assays confirmed that SCC9, SCC15, and SCC25 cells express mRNA for β1- and β2-AR (Fig. 5A). To determine whether the increase in IL-6 expression was mediated through β-adrenergic receptors, the cell lines were pre-treated with a non-specific β antagonist (propranolol), at the time point of maximum mRNA IL-6 expression (10 μM NE at 1 h). Propranolol pretreatment inhibited NE-induced IL-6 mRNA expression in the three cell lines investigated (Fig. 5B). Next, whether the increase in cell proliferation induced by NE was also mediated by β-ARs was assessed. SCC9 cells were treated with propranolol before stimulation with 10 μM

Fig. 4. Stress hormones increase OSCC cell proliferation. The human OSCC cell lines SCC9 and SCC15 were stimulated with 0.1, 1, and 10 μM norepinephrine or with 1, 10, 100, and 1000 nM cortisol, and MTT cell proliferation assays were determined at 6, 24, and 48 h. The non-stimulated cells (0 μM or 0 nM) were set as 100%, and the treated cells were determined relatively to non-stimulated cells. Significant results were found with NE at 6 h (A) and cortisol at 48 h (B). Bars represent the mean ± SEM of six replicates, and the assays were performed in triplicate. $^{*}P \leqslant 0.05$.

Fig. 5. (A) β1-AR and β2-AR RNA expression in the SCC9, SCC15, and SCC25 cell lines. (B) IL-6 RNA expression is regulated through the β-AR. Human OSCC lines (SCC9, SCC15, and SCC25) were stimulated with NE (10 μM) in the presence or absence of the β-blocker propranolol (PROP) (1 μM). Propranolol was added 1 h before the NE stimulus. Cells were collected 1 h after the NE stimulus, total RNA was isolated, and real-time PCR was used to quantify IL-6 RNA. Data are presented as the mean fold increase in IL-6 RNA expression relative to non-stimulated cells. Bars represent means ± SEM. $^{*}P \leqslant 0.05$; $^{***}P \leqslant 0.001$. (C) Cell proliferation is β-AR-dependent and anti-IL-6 ab partially inhibited the increase NE-induced cell proliferation. For the inhibition experiments, propranolol (Prop) at 1 μM was added 1 h before stimulus with 10 μM NE in SCC9 cells and IL-6 neutralizing ab (1 and 10 μg/mL) for 30 min prior to the stimulus. MTT cell proliferation assay were determined at 6 h. Bars represent means ± SEM. $^{*}P \leqslant 0.05$: 10 μM NE group compared to control group (NE 0); $^{**}P \leqslant 0.05$: 10 μM NE + 1 μM Prop group compared to 10 μM NE group; $^{\#}P \leqslant 0.05$: 10 μM NE + anti-IL-6 (10 μg/mL) group compared to 10 μM NE group.
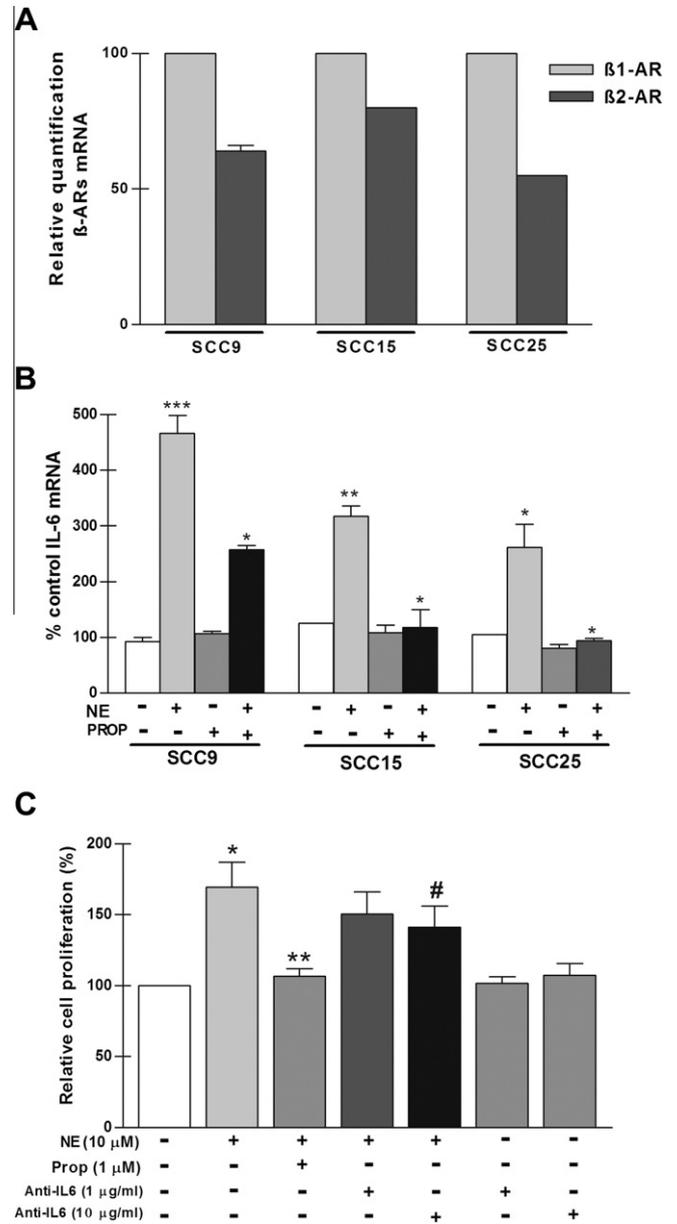
NE at 6 h, and cell proliferation was assayed by MTT. Inhibition of β-ARs produced significant decrease in NE-induced cell proliferation, showing that this event is β-AR-dependent (Fig. 5C). This decreasing in NE-induced cell proliferation after β-ARs inhibition also was found in the SCC15 cells (results not shown).

3.6. Effects of IL-6 neutralizing ab on NE-induced proliferation

Since NE may stimulate IL-6 production by OSCC, whether NE-induced OSCC proliferation is mediated by IL-6 was subsequently tested. To this end, anti-IL-6 ab was used to neutralize the action of IL-6 in SCC9 cells. As illustrated in Fig. 5C, treatment of SCC9 cells with 10 μg/mL of anti-IL-6 induced significant inhibition of NE-induced proliferation ($p < 0.05$). Anti-IL-6 in lower concentration (1 μg/mL) was not able to inhibit NE-induced proliferation (Fig. 5C). Recombinant IL-6 increased SCC9 cell proliferation (data not shown).

3.7. OSCC biopsies express β1- and β2-ARs

To determine the clinical relevance of our results, expression of β1- and β2-ARs mRNAs were examined in 20 tumor specimens of

OSCC and compared with the expression in 17 specimens of oral leukoplakia and 15 specimens of normal oral mucosa. Clinical characteristics of patients from whom samples were obtained are summarized in Table 1. β1- and β2-AR mRNAs were expressed in all 20 cases of OSCC. Of the 17 cases of leukoplakia, five were negative for β1-AR and one was negative for β2-AR. Of the 15 specimens of normal mucosa, three did not express β1-AR and one was negative for

Table 1
Clinical characteristics of patients from whom samples were collected for evaluation of mRNA expression for β1- and β2-AR by real-time PCR.

Clinical Characteristics	Normal mucosa ($n = 15$)	Leukoplakia ($n = 17$)	OSCC ($n = 20$)
Mean age (years)	62.2 (±8.36)	56.5 (±8.31)	60.25 (±11.22)
Range	40–79	42–82	43–80
Gender: n (%)			
Male: n (%)	7 (47)	8 (47)	15 (75)
Female: n (%)	8 (53)	9 (53)	5 (15)
Sample site: n (%)			
Floor of mouth	2 (13.3)	2 (11.8)	4 (20)
Gum	1 (6.7)	1 (5.9)	0 (0)
Tongue	2 (13.3)	3 (17.6)	4 (20)
Buccal mucosa	3 (20)	4 (23.5)	2 (10)
Hard palate	1 (6.7)	3 (17.6)	2 (10)
Alveolar ridge	4 (26.7)	2 (11.8)	4 (20)
Retromolar area	2 (13.3)	2 (11.8)	4 (20)
Tobacco use: n (%)			
No	2 (13)	1 (5.9)	3 (15)
Yes	13 (87)	16 (94.1)	17 (85)
Alcohol consumption: n (%)			
No	13 (86.7)	11 (64.7)	5 (8.8)
Yes	2 (13.3)	6 (35.3)	15 (35.4)
Clinical stage: n (%)			
I	–	–	2 (10)
II			4 (20)
III			4 (20)
IV			10 (50)

β2-AR. Quantitatively, the mean expression of the β1-AR mRNA levels in OSCC specimens was 2.7-fold higher compared to normal mucosa ($p < 0.05$), while in specimens of leukoplakia the expression was 1.6-fold higher ($p > 0.05$) (Fig. 6A). In contrast, β2-AR mRNA mean expression was lower in leukoplakia compared to normal mucosa and OSCC, but these results were not significant (Fig. 6A). The β-AR expression for each studied case can be better seen in Fig. 6B and C.

4. Discussion

This study provides strong evidence that OSCC cells are influenced by neurohormonal mediators. The results demonstrated that stress-related mediators (NE and isoproterenol) can enhance the production of the pro-angiogenic cytokine IL-6 in human OSCC cell lines. IL-6, originally identified as a B-cell growth factor, is produced by many cell types, including T-cells, macrophages, and stromal cells. As seen in this study, OSCC cells are also capable of producing IL-6, and basal levels are already detectable at 1 h. Secreted cytokine products, including IL-6, are available to interact with cellular receptors; thus, they are able to exert paracrine or autocrine effects. The concentrations of IL-6 secreted by OSCC cells in this study, even by non-stimulated cells, are clearly within the range expected to have biological activity. This activity is related to autocrine growth stimulation of cancer cells (Hodge et al., 2005) and could explain the non-stimulated increase in IL-6 observed over the time period. Previous studies on melanoma (Yang et al., 2009) and ovarian cancer cells (Nilsson et al., 2007) have shown that IL-6 expression is upregulated via adrenergic stimulation. Enhanced IL-6 production after NE treatment has also been reported in myocytes (Briest et al., 2003) and human pancreatic duct epithelial cells (Chan et al., 2008).

The NE and isoproterenol concentrations that determined maximum increase in IL-6 expression were within the levels that would be produced from stress-related catecholamine secretion (10 μM). Maximum elevations in IL-6 occurred at an early time

(1 h), giving evidence of fast metabolism of adrenergic mediators by OSCC cells. Nilsson et al. (2007) found that maximum increases in IL-6 expression in ovarian carcinoma cells occurred only after 6 h of incubation with NE. Nilsson's results after 3 h of treatment of these same cells with NE showed just a minimum rise in IL-6 production. These data indicate that distinct tumors may have variable sensitivity to catecholamines. The responses to NE were mediated by β-adrenergic receptors, whereas the β1- and β2-ARs antagonist propranolol inhibited the NE-dependent upregulation of IL-6 expression and protein release. This inhibition reached control levels in SCC15 and SCC25 cells and was partial in SCC9 cells, indicating that other receptors can be involved in the SCC9 cell activation during the NE-induced IL-6 production.

To our knowledge, this is the first study showing that IL-6 expression and production in OSCC cells can be upregulated by NE. The activation of the IL-6 complex is related to growth stimulation of OSCC cells (Chakravarti et al., 2006). Moreover, high IL-6 production in tumor cells and plasma of patients with OSCC has been associated with recurrence, regional metastasis, and poor survival (Duffy et al., 2008; Nagata et al., 2003). As a result, upregulated IL-6 production in response to NE found in this study can be a way for stress-related OSCC progression. It has also been found that NE treatment increase the expression of other substances that contribute to angiogenesis (such as VEGF) in nasopharyngeal carcinoma tumor cells, an EBV-associated malignant tumor (Yang et al., 2006), and multiple myeloma-derived cells (Yang et al., 2008).

Similarly to what happens in terms of IL-6 expression, treatment with NE at physiological stress levels (10 μM) induced SCC9 and SCC15 cell proliferation. Furthermore, IL-6 neutralizing ab partially inhibited the NE-induced proliferation in SCC9 cells, indicating a possible pathway among NE/IL-6/cell growth in OSCC cells. The NE-induced SCC9 and SCC15 cell proliferation was mediated by β-adrenergic receptors and was significant at 6 h, compared to 24 and 48 h. The effects of β-adrenergic agonists on malignant cell proliferation has been reported for several tumors, including lung (Schuller et al., 1999), pancreas (Askari et al., 2005), breast (Cakir et al., 2002), and gastric (Shin et al., 2007) cancers, all of which are adenocarcinomas. Studies investigating the influence of catecholamines on human HNSCC cell proliferation, as in our case, are still scarce. Liu et al. (2008) have demonstrated that epinephrine stimulates esophageal squamous cell carcinoma cell proliferation. This effect occurred via β-AR-dependent transactivation of the extracellular signal-regulated kinase/cyclooxygenase-2 pathway. Recently, Shang et al. (2009) have reported that the OSCC cell line TCa8113 expresses β2-AR and presents NE-induced proliferation, an effect that was also inhibited by propranolol. However, the authors presented no data concerning the expression of the β1-receptor subtype. Here, constitutive expression of both β1- and β2-ARs in the three studied OSCC cell lines has been demonstrated. Collectively, the results obtained by us and by Shang et al. (2009) provide evidence that catecholamines such as NE may play an important role in the progression of oral cancer.

Effects of cortisol on IL-6 expression differ according to the hormone dose. At different times, cortisol at a concentration compatible with physiological stress levels in humans (10 nM) enhanced IL-6 expression in SCC9, SCC15, and SCC25 cells, but these results were not significant. In contrast, cortisol concentrations closer to pharmacological levels (1000 nM) promoted reduction in IL-6 expression at all analyzed time points in SCC9 and SCC15 cells. These data suggest the possibility of cortisol have a dual role on IL-6 expression in OSCC cell, in which doses that simulate physiological stress levels (e.g., 10 nM) could have a proinflammatory effect, while pharmacological doses inhibit the proinflammatory cytokine IL-6. Inhibitory effects of glucocorticoids on the
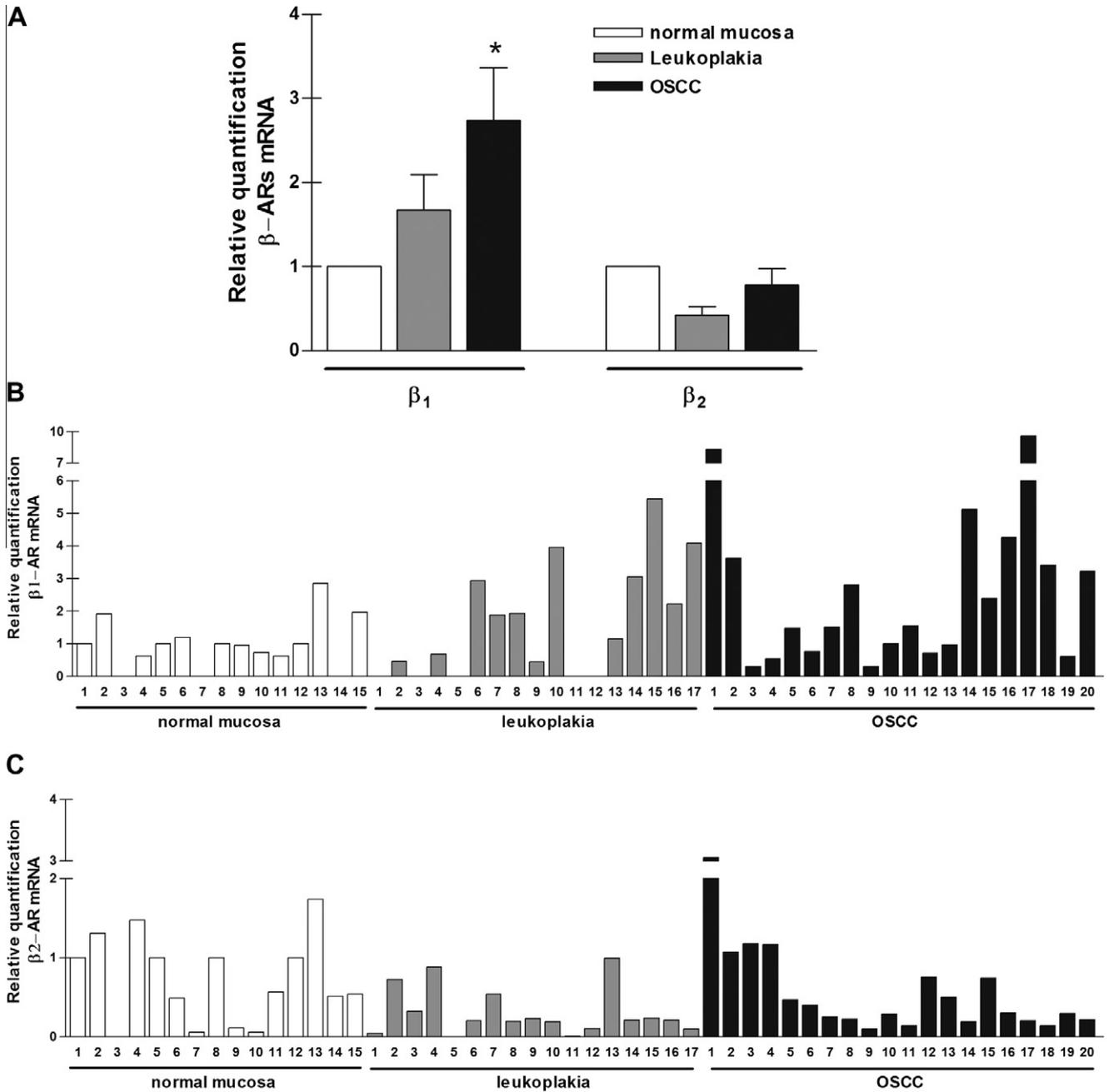
Fig. 6. (A) β1-AR and β2-AR mRNA expression levels in specimens of human OSCC tumors (n = 20), human leukoplakia (n = 17), and normal human mucosa (n = 15). Each bar represents the mean of the relative quantification (RQ) of the β1-AR and β2-AR mRNA expression concerning the normal mucosa. *P < 0.05, OSCC group compared with healthy controls (normal mucosa) for β1-AR. (B and C) Individual representation of each patient according to their β1-AR (C) or β2-AR (B) mRNA expression.

expression of cytokines such as IL-6 and IL-8 have been reported previously (Hasan et al., 2003; Yano et al., 2006). Nevertheless, in these studies the cortisol was generally tested at pharmacological concentrations (1000 nM or more). Lutgendorf et al. (2003) also found different effects of cortisol on VEGF in ovarian carcinoma cells, depending on the hormone dose. In line with our results on IL-6, pharmacological doses of cortisol inhibited VEGF secretion, while cortisol simulating physiological stress levels (10 nM) induced significant increase in VEGF. Although some types of non-steroidal anti-inflammatory drugs (NSAIDs) cause antiproliferative effects and induce apoptosis in HNSCC cell lines (Thurnher et al., 2001; Pelzmann et al., 2004), it seems that the effects of glucocorticoids on the growth of these cells are not as clear. For example, previous experiments with a high dose of hydrocortisone

(3000 nM) did not reveal relevant effects on the HNSCC cell proliferation rate (Thurnher et al., 2001). Our findings showed that 10 nM cortisol increased the number of SCC15 cells after 48 h of treatment, while doses of 1000 nM increase the number of SCC9 cells after the same time period. As observed in the case of cytokines expression regulation, this result may suggest that the cortisol effect on the cell cycle proteins may be dependent on the hormone levels. Further studies are necessary to evaluate which underlying mechanisms are activated in OSCC cells after variations of the systemic and tissue levels of cortisol in response to chronic and acute stress conditions.

In addition to confirming that OSCC cell lines express β1- and β2-AR, we have also demonstrated that these receptors are expressed in specimens of OSCC, oral leukoplakia, and normal oral

mucosa. The β-adrenergic receptors are members of the large family of G protein-coupled receptors (GPCR), and their activation involves protein-tyrosine-kinase-activated pathways, as well as cyclic-adenosine-monophosphate (cAMC)-linked pathways. It has been shown that several types of cancer express β-AR, which may affect proliferation and migration as well as induce metastasis (Askari et al., 2005; Cakir et al., 2002; Shin et al., 2007). β1-AR expression in OSCC and oral leukoplakia specimens has not yet been reported. Quantitatively, the mean β1-AR expression level in OSCC was approximately 3- and 2-fold those encountered in the normal mucosa and leukoplakia, respectively. These findings suggest that the changes in epithelial and mesenchymal cells during oral carcinogenesis can be accompanied by modifications in β1-AR expression. Moreover, β1-adrenergic receptor agonists, such as NE, could determine more pronounced effects in neoplastic tissues compared to normal tissues. β2-AR expression in OSCC biopsies has been previously analyzed by Shang et al. (2009). Immunohistochemistry analysis showed that 67.7% of OSCC cases were positive for β2-AR protein expression, while only 20% of adjacent normal mucosa specimens were positive for β2-AR staining (Shang et al., 2009). However, β1-AR expression was not evaluated. In our cases, only one specimen of normal mucosa was negative for β2-AR, and there was no expressive difference in its expression when tumor and normal mucosa specimens were compared. This distinct result in terms of β2-AR expression obtained by us and Shang et al. may be due to the use of different methods. In real-time PCR assay other cells of the tumor microenvironment that also express β-ARs in addition to epithelial cells are also included in the analysis.

Previous studies have shown that patients with oral cancer can have high psychological distress levels (Kugaya et al., 2000; Chen et al., 2009). The effects of stress-related hormones on oral cancer cells are still poorly understood. Although this study has limitations because it is composed mainly of in-vitro assays, the results reveal that stress-related mediators, mainly NE at concentration compatible with physiological stress levels in humans, can upregulate IL-6 expression and induce OSCC cell proliferation. These findings provide one of the first evidences that stress hormones may act directly on OSCC cells and possibly affect tumor progression.

Conflict of interest statement

No potential conflicts of interest are disclosed.

Acknowledgments

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