NFE2L2/NRF2, OGG1, AND CYTOKINE RESPONSES OF HUMAN GINGIVAL KERATINOCYTES AGAINST OXIDATIVE INSULTS OF VARIOUS ORIGIN

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Abstract

Objective: Bacterial or tobacco-related insults induce oxidative stress in gingival keratinocytes. The aim of this study was to investigate anti-oxidative and cytokine responses of human gingival keratinocytes (HMK cells) against *Porphyromonas gingivalis* lipopolysaccharide (*Pg* LPS), nicotine, and 4-nitroquinoline N-oxide (4-NQO).

Materials and Methods: HMK cells were incubated with *Pg* LPS (1 μl/ml), nicotine (1.54 mM), and 4-NQO (1 μM) for 24 hours. Intracellular and extracellular levels of interleukin (IL)-1β, IL-1 receptor antagonist (IL-1Ra), IL-8, monocyte chemoattractant protein (MCP)-1, and vascular endothelial growth factor (VEGF) were measured with the Luminex® xMAP™ technique, and nuclear factor, erythroid 2 like 2 (NFE2L2/NRF2) and 8-oxoguanine DNA glycosylase (OGG1) with Western blots. Data were statistically analyzed by two-way ANOVA with Bonferroni correction.

Results: All tested oxidative stress inducers increased intracellular OGG1 levels, whereas only nicotine and 4-NQO induced NFE2L2/NRF2 levels. Nicotine, 4-NQO, and their combinational applications with *Pg* LPS induced the secretions of IL-1β and IL-1Ra, while that of IL-8 was inhibited by the presence of *Pg* LPS. MCP-1 secretion was suppressed by nicotine, alone and together with *Pg* LPS, while 4-NQO activated its secretion. Treatment of HMK cells with *Pg* LPS, nicotine, 4-NQO, or their combinations did not affect VEGF levels.

Conclusion: *Pg* LPS, nicotine, and 4-NQO induce oxidative stress and regulate anti-oxidative response and cytokine expressions in human gingival keratinocytes differently. These results may indicate that bacterial and tobacco-related insults regulate distinct pathways.

Introduction

Reactive oxygen species (ROS), which are described as oxygen free radicals, are constantly produced by different types of mammalian cells during their metabolic activity. When at low levels, ROS have beneficial effects and take part in crucial biologic mechanisms, such as cell signaling and regeneration [1]. In contrast, increased accumulation of ROS leads to formation of oxidative stress, which can cause tissue damage, for example, in pathogenesis of peri-implantitis and periodontitis [2]. Bacterial and chemical insults may elevate oxidative stress levels in periodontal tissues, which provoke apoptosis of gingival epithelial cells and fibroblasts via multiple mechanisms such as lipid peroxidation, protein denaturation, oxidation of some important enzymes, stimulation of pro-inflammatory cytokines, and DNA damage [3]. *Porphyromonas gingivalis* lipopolysaccharide (*Pg* LPS) is a well-known ROS inducer and it initiates the secretion of large numbers of inflammatory mediators, including interleukins, chemokines, and adhesion molecules via nuclear factor-kappa B (NF-κB) pathway [4]. Similarly, chemical insults, like nicotine exposure during tobacco use are accepted as an exogenous source of ROS and accelerate the progression of inflammation in periodontal diseases [5]. Another chemical component of tobacco, 4-nitroquinoline N-oxide (4-NQO), can also induce the formation of free radicals in fibroblasts [6]. To our knowledge, however, oxidative stress-inducing effects of this molecule on human gingival keratinocytes have not been studied before.

Human gingival keratinocytes form a barrier to protect underlying tissues from physical, chemical, and microbiological insults by secretion of a wide range of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-1 receptor antagonist (IL-1Ra), and IL-8, as a part of gingival signaling mechanisms [7]. These cytokines are mostly responsible for maintaining the tissue homeostasis, but an unrestrained production of these inflammatory cytokines results in chronic inflammation [8]. In periodontitis patients, elevated ROS levels

trigger the production of pro-inflammatory cytokines, which in turn, increase the neutrophil chemotaxis and neutrophilic ROS production [9].

Bacterial and chemical insults can cause protein and DNA destruction via oxidative stress in the host. DNA repair genes, such as 8-oxoguanine DNA glycosylase (OGG1), can inhibit DNA damage resulting from excessive ROS. In addition, nuclear factor, erythroid 2 like 2 (NFE2L2/NRF2), a basic leucine zipper transcription factor, is one of the major regulators of enzymatic antioxidants [10, 11]. It has been demonstrated that NFE2L2/NRF2 can inhibit the 4-NQO-activated oxidative stress [12] and that the activation of NFE2L2/NRF2-dependent anti-oxidative response reduces chemotherapeutic agent-induced ROS levels in an oral-mucositis cell culture model [13]. Down-regulation of NFE2L2/NRF2 in oral PMNs of chronic periodontitis patients has been associated with periodontal tissue destruction due to insufficient anti-oxidative response [14]. In addition, in human macrophages stimulated by *Pg* LPS, NFE2L2/NRF2 induces anti-inflammatory response [15]. NFE2L2/NRF2 also contributes to cellular defense against nicotine-stimulated oxidative stress in human periodontal ligament cells [16].

Oxidative stress is a crucial component in the development of periodontal diseases; however, the regulation of the anti-oxidative NFE2L2/NRF2 pathway in human gingival keratinocytes against bacterial and tobacco-related oxidative stress is still unclear. Moreover, regulations of cytokine and anti-oxidative responses against 4-NQO, alone or together with LPS, have not been studied in human gingival keratinocytes. We hypothesized that bacterial (*Pg* LPS) and tobacco-related (nicotine and 4-NQO) oxidative stress inhibits NFE2L2/NRF2-related anti-oxidative response in gingival keratinocytes and activates the cytokine release leading to inflammation. In this study, the aim was to examine the effects of *Pg* LPS, nicotine, 4-NQO, and their combinations on the production of pro-inflammatory cytokines and anti-oxidative response markers.

Materials and Methods

Cell Culture

Ethics approval was not required for this research as established cell lines were used. The human oral mucosal keratinocyte cell line (HMK), originally obtained from a healthy human gingival biopsy sample [17], was cultured in a medium with keratinocyte-SFM containing human recombinant epidermal growth factor, bovine pituitary extract, and antibiotics (penicillin-streptomycin, 10.000 U/mL, Gibco, Thermo Fisher Sci. Co, Waltham, Massachusetts, USA) at 37°C in 5% CO_2. Culture media were replaced three times per week, and the cells were passaged weekly when reaching the 80-90% confluence. For the stress experiments, HMK cells ($2.5x10^5$/well) were incubated in 12-well plates (Corning Inc., Corning, NY, USA) as described above. After reaching the confluence of 80-90%, the cells were washed with phosphate buffered saline (PBS) three times and fresh media with either *Pg* LPS (1µl/ml, tlrl-pglps, InvivoGen, San Diego, CA, USA), nicotine (1.54 mM, N0267, Sigma Aldrich, Saint Louis, MO, USA), 4-nitroquinoline N-oxide (4-NQO, 1 µM, N8141, Sigma Aldrich), or their combinations (*Pg* LPS+nicotine, *Pg* LPS+4-NQO, nicotine+4-NQO, and *Pg* LPS+nicotine+4-NQO) were added to the wells. The control cells were incubated with fresh cell culture media only. After 24-h of incubation, the medium from each well were collected, and the cells were washed three times with PBS followed by the lysis of the cells with 300 µL of lysis buffer (Tris-HCl pH 7.4). The cells were scraped off and the lysates were collected and sonicated (three cycles of five sec on/off with 50% amplitude and 50% duty cycle). The collected media and lysates were stored in the freezer, at -80 °C for the further tests.

Analysis of Cytokine Levels

Levels of IL-1β, IL-1Ra, IL-8, MCP-1, and VEGF in the cell culture media and in the cell lysates were measured separately with the Luminex® xMAP™ technique (Luminex

Corporation, Austin, TX) using commercially available kits (pro-human cytokine group I assays; Bio-Rad, Santa Rosa, CA). The detection limit of the assay was 0.6 pg/mL for IL-1β, 5.5 pg/mL for IL-1Ra, 1.0 pg/mL for IL-8, 1.1 pg/mL for MCP-1, and 3.1 pg/mL for VEGF. Elute and the media alone were used to determinate the background absorbance. The analyte concentration for which the fluorescence intensity signal was two standard deviations above the background signal was defined as the detection limit. All cytokine detections were carried out in triplicate.

Analysis of NFE2L2/NRF2 and OGG1 Levels

Protein levels of each sample were determined by the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA). Same amount of protein (23.5 µg) for each sample was mixed with 5 µL of laemmli buffer (4X) and heated at 95°C for 5 min. The samples were loaded on 11% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After electrophoresis, the proteins were transferred to membranes (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories Inc.), which were incubated overnight with primary antibodies against NFE2L2/NRF2 (1:500 dilution, #PA5-14144, Thermo Fisher Scientific, Waltham, MA, USA) and OGG1 (1:500 dilution, #PA1-31402, Thermo Fisher Scientific). As a secondary antibody, goat anti-rabbit IgG (H+L) crossed-absorbed, horseradish peroxidase (HRP) conjugate (1:500 dilution, #G-21234, Thermo Fisher Scientific) was used. For actin determination, a β-actin primary antibody (1:10000 dilution, #MA5-15739 Thermo Fisher) and a goat anti-mouse IgG (H+L) secondary antibody (HRP conjugate, 1:4000 dilution, #62-6520, Thermo Fisher Scientific) were used. The detection of HRP was performed by the Novex® ECL Chemiluminescent Substrate Reagent Kit (0.6 ml/cm^2 area, #WP20005, Thermo Fisher Scientific). For the detection of protein bands on the membranes, the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc.) was used. The bands were

analyzed with the ImageJ software (National Institute of Health, Bethesda, Maryland, USA). Experiments were performed in duplicate in two different time points.

Statistical Analysis

The IBM SPSS V24.0 software (IBM, Armonk, North Castle, New York, USA) was used for statistical analyses. To normalize the cytokine levels to the number of cells, the final cytokine unit was defined as pg of cytokine/μg of total protein. The results are expressed as the values of means and standard deviations. Two-way analysis of variance (ANOVA) with Bonferroni correction was used for the analysis and p values less than 0.05 were accepted as statistically significant.

Results

In the Western blot analyses, OGG1 appeared as two bands, one at 37 kDa and the other around 40 kDa. The 37 kDa polypeptide most likely represented OGG1-1a isoform, while 40 kDa polypeptide represented a processed form of mitochondrial OGG1-2a isoform [18]. A significantly increased OGG1 expression was observed after exposure to *Pg* LPS, nicotine, 4-NQO, and their combinations (Figure 1).

Western blot analyses revealed four NFE2L2/NRF2 bands, two bands at ∼55–65 kDa and two bands at the ∼95–110 kDa. While there is evidence that a biologically relevant molecular weight of NFE2L2/NRF2 is ∼95–110 kDa [19], in the present study, bands at the ∼55–65 kDa were used to run semi-quantitative analyses, as suggested by the manufacturer of the NFE2L2/NRF2 primary antibody. When compared with controls, NFE2L2/NRF2 levels elevated only in the nicotine and 4-NQO groups (Figure 2).

Significantly increased levels of extracellular IL-1β, IL-1Ra, and IL-8 were detected in the nicotine ($p=0.04$ for IL-1 β, $p<0.0.1$ for IL-1Ra, and $p<0.01$ for IL-8) and 4-NQO groups ($p<0.01$ for IL-1 β, $p<0.01$ for IL-1Ra, and $p<0.01$ for IL-8), in comparison to controls.

Combinational applications of *Pg* LPS, nicotine, or 4-NQO elevated extracellular IL-1β ($p=0.031$ for *Pg* LPS+nicotine, $p<0.01$ for *Pg* LPS+4-NQO, $p=0.01$ for both nicotine+4-NQO, and *Pg* LPS+nicotine+4-NQO) and IL-1Ra ($p<0.001$ for all combinations). Extracellular IL-8 level was decreased by *Pg* LPS significantly, while the combinational application of *Pg* LPS with nicotine and 4-NQO elevated its levels ($p<0.01$). Nicotine suppressed ($p=0.02$) and 4-NQO activated ($p<0.01$) extracellular MCP-1. When applied together, 4-NQO and *Pg* LPS ($p<0.01$), and 4-NQO and nicotine ($p=0.04$) increased extracellular MCP-1 levels significantly. Levels of extracellular MCP-1 were decreased in the presence of nicotine, both solely ($p=0.02$) and with *Pg* LPS ($p<0.01$), in comparison to controls (Table 1). Comparisons of solely applications of *Pg* LPS, nicotine, and 4NQO with their combinational applications are given in Table 1.

When used alone or in combination, none of the tested compounds induced any effect on intracellular cytokine levels of HMK cells (Table 2). Combinational applications of 4-NQO with *Pg* LPS or with nicotine suppressed the intracellular IL-8 and MCP-1 levels in comparison to 4-NQO alone (Table 2).

Discussion

To our knowledge, the effect of bacteria- and tobacco-related oxidative stress on NFE2L2/NRF2 and OGG1 expressions has not been studied in human gingival keratinocytes before. Our study provides experimental evidence how nicotine and 4-NQO induce NFE2L2/NRF2 and OGG1 expressions in HMK cells, while *Pg* LPS stimulates OGG1 expression. Also for the first time, we demonstrated that 4-NQO stimulates MCP-1 expression in human gingival keratinocytes, whereas nicotine suppresses its expression.

LPS of *P. gingivalis* is able to activate the inflammatory response, which leads to the accumulation of ROS that induces the oxidative stress in different types of oral cells [20].

Two main components of cigarette smoke, nicotine and 4-NQO, take part in lipid peroxidation and formation of free radicals [21]. In the present study, the exposure to *Pg* LPS, nicotine, 4-NQO, and their combinations induced oxidative stress in the HMK cell line, as demonstrated by elevated OGG1 levels. Overexpression of OGG1 causes DNA demethylation in response to oxidative stress [22], and DNA demethylation and a hypoxic microenvironment are known to elevate NFE2L2/NRF2 expression [23, 24]. These observations may explain the underlying mechanism of nicotine and 4-NQO-induced NFE2L2/NRF2 expression shown in our study. Instead, it is unclear why *Pg* LPS, an activator of OGG1 expression, did not stimulate NFE2L2/NRF2 expression. Although NFE2L2/NRF2 is the major mediator of anti-oxidative response, there are proteins like forkhead box-O (FOXO), which is involved in the transcription of antioxidants [25]. The activation of the FOXO transcription by *P. gingivalis* has been demonstrated in gingival epithelial cells [26], indicating that antioxidant response against *Pg* LPS can be regulated by pathways other than NFE2L2/NRF2. Further studies should be performed to elucidate the role of other anti-oxidative mechanisms in bacteria-induced oxidative stress.

In the present study, both nicotine and 4-NQO exposure lead to an increase of IL-1Ra, IL-1β, and IL-8 extracellularly, whereas nicotine suppressed and 4-NQO elevated extracellular MCP-1. No additional effect of *Pg* LPS was observed when it was used together with nicotine or 4-NQO, indicating that the observed effect was dependent on nicotine or 4-NQO. There is evidence that the nicotine stimulation leads to an increased the expression of IL-1α, IL-6, and IL-8 in keratinocyte and fibroblast cultures [27]. Although increased levels of IL-8 and MCP-1 have been demonstrated after incubation of gingival keratinocytes and fibroblasts with *P. gingivalis* [28, 29], no alteration was seen in MCP-1 levels after exposure of HMK cells to *Pg* LPS in our study. The divergence between the studies may be due to differences in components of *P. gingivalis* (sonic extract of *P. gingivalis* vs LPS of *P.*

gingivalis) used to stimulate the cell lines. In a study by Johnson et al. [30], no change in the IL-8 expression of gingival epithelial cells was found after nicotine treatment. This is most likely related to different nicotine molarities used: i.e., 0.1 µM and 1 mM the study of Johnson et al. and 1.5 mM in our study. In addition, our results showed that nicotine and *Pg* LPS stimulated IL-8 secretion from the HMK cells at similar levels. It has been demonstrated that both *Pg* LPS and nicotine stimulate IL-8 secretion but using different pathways; *Pg* LPS activates TLR-2, while nicotine activates the ERK1/2 pathway [28, 31]. An interesting finding was that the combinational use of nicotine and *Pg* LPS did not induce any change in IL-8 expression, but this remains unexplained in the limits of this study.

According to the present results, exposure to *Pg* LPS, nicotine or 4-NQO of HMK cells does not have any stimulatory or inhibitory effect on VEGF levels. To our knowledge, this kind of study protocol using HMK cells is not available for comparison. In a recent study where gingival fibroblasts were used, the incubation with *Pg* LPS elevated IL-8 and MCP-1 secretions but not that of VEGF [29].

One limitation of the present study was its short-term culture protocol 24 hours, which was selected on the basis of our initial tests, where we observed that keratinocytes reach to full confluency after 24 hours and then they start to change their morphology and lose their viability. On the other hand, the combined application of 4-NQO together with *Pg* LPS and nicotine was a novel to approach. Together with nicotine, 4-NQO is one of the potent mutagens in tobacco and it induces carcinogenesis via oxidative DNA damage [32]. Despite that, most studies use nicotine as an inducer, ignoring oxidative stress caused by 4-NQO. One interesting outcome of the present study was that nicotine decreased MCP-1 levels with 50%, whereas 4-NQO elevated them with 100%. It is possible that these two tobacco-related oxidative stress inducers use different receptors or pathways in regulating cytokine expression.

In conclusion, nicotine and 4-NQO, two common components of tobacco, stimulate both NFE2L2/NRF2 and OGG1 expression in human gingival keratinocytes. *Pg* LPS, on the other hand, induces oxidative stress (OGG1) but not NFE2L2/NRF2 expression. In addition, regulation of cytokine differs between bacteria- (*Pg* LPS) and tobacco-related oxidative stress inducers.

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Table 1: Comparison of the extracellular cytokine levels between the control and *Porphyromonas gingivalis* lipopolysaccharide (LPS), nicotine (NIC), and 4-Nitroquinoline N-oxide (4-NQO) treated groups. Data are given as mean (standard deviation). * indicates a statistically significant difference with the control, α indicates a statistically significant difference with the LPS, § indicates a statistically significant difference with the nicotine, and † indicates a statistically significant difference with the 4-NQO.

		Control	LPS	NIC	4-NQO	LPS+NIC	LPS+4-NQO	NIC+4-NQO	LPS+NIC+4-NQO
Extracellular (pg/ µg protein) mean (std)	IL-1β	0.130 (0.009)	0.151 (0.01)	0.196* (0.01)	0.280* (0.01)	0.185* (0.01)	0.260* (0.01)	0.205* † (0.01)	0.209* † (0.01)
	IL-1Ra	7.733 (0.73)	8.130 (0.27)	15.238* (0.05)	16.543* (1.06)	15.252* α (0.18)	12.764* α † (0.26)	13.505* † (0.30)	13.347* α † (0.57)
	IL-8	26.307 (0.83)	18.548* (0.27)	33.633* (0.31)	34.763* (0.88)	27.283 α § † (0.58)	24.541 α (1.06)	34.177* § (1.78)	28.340 α § † (1.081)
	MCP-1	7.184 (0.65)	7.529 (0.69)	3.363* (0.28)	17.732* (0.61)	2.718* α § (0.11)	12.789* † (0.07)	10.693* § † (0.29)	7.977§ † (1.58)
	VEGF	141.959 (11.73)	143.998 (2.97)	130.632 (16.61)	147.744 (9.43)	122.789 (8.19)	155.412 (11.44)	129.042 (7.09)	129.036 (13.49)

Table 2: Comparison of the intracellular cytokine levels between the control and *Porphyromonas gingivalis* lipopolysaccharide (LPS), nicotine (NIC), and 4-Nitroquinoline N-oxide (4-NQO) treated groups. Data are given as mean (standard deviation) and asterisk (*) indicates a statistically significant difference with the 4NQO.

		Control	LPS	NIC	4-NQO	LPS+NIC	LPS+4-NQO	NIC+4-NQO	LPS+NIC+4-NQO
Intracellular (pg/ µg protein) mean (std)	**IL-1β**	9.134 (1.78)	9.290 (1.55)	8.113 (0.46)	7.962 (0.70)	7.989 0.17	6.249 (0.28)	7.253 (1.18)	6.154 (1.19)
	IL-1Ra	133.450 (17.84)	149.785 (12.94)	155.241 (11.90)	150.552 (28.28)	127.285 (4.73)	117.606 (12.15)	108.809 (22.82)	137.401 (7.26)
	IL-8	0.525 (0.10)	0.429 (0.03)	0.570 (0.03)	0.944 (0.26)	0.395 (0.04)	0.401* (0.02)	0.497* (0.07)	0.734 (0.12)
	MCP-1	0.600 (0.03)	0.720 (0.06)	0.447 (0.12)	0.957 (0.09)	0.340 0.01	0.446* (0.01)	0.443* (0.17)	0.489* (0.19)
	VEGF	5.595 (0.29)	5.697 (0.60)	6.845 (0.68)	6.383 (0.51)	4.360 (0.19)	5.415 (0.10)	4.994 (0.70)	6.399 (0.11)

Figures

Figure.1

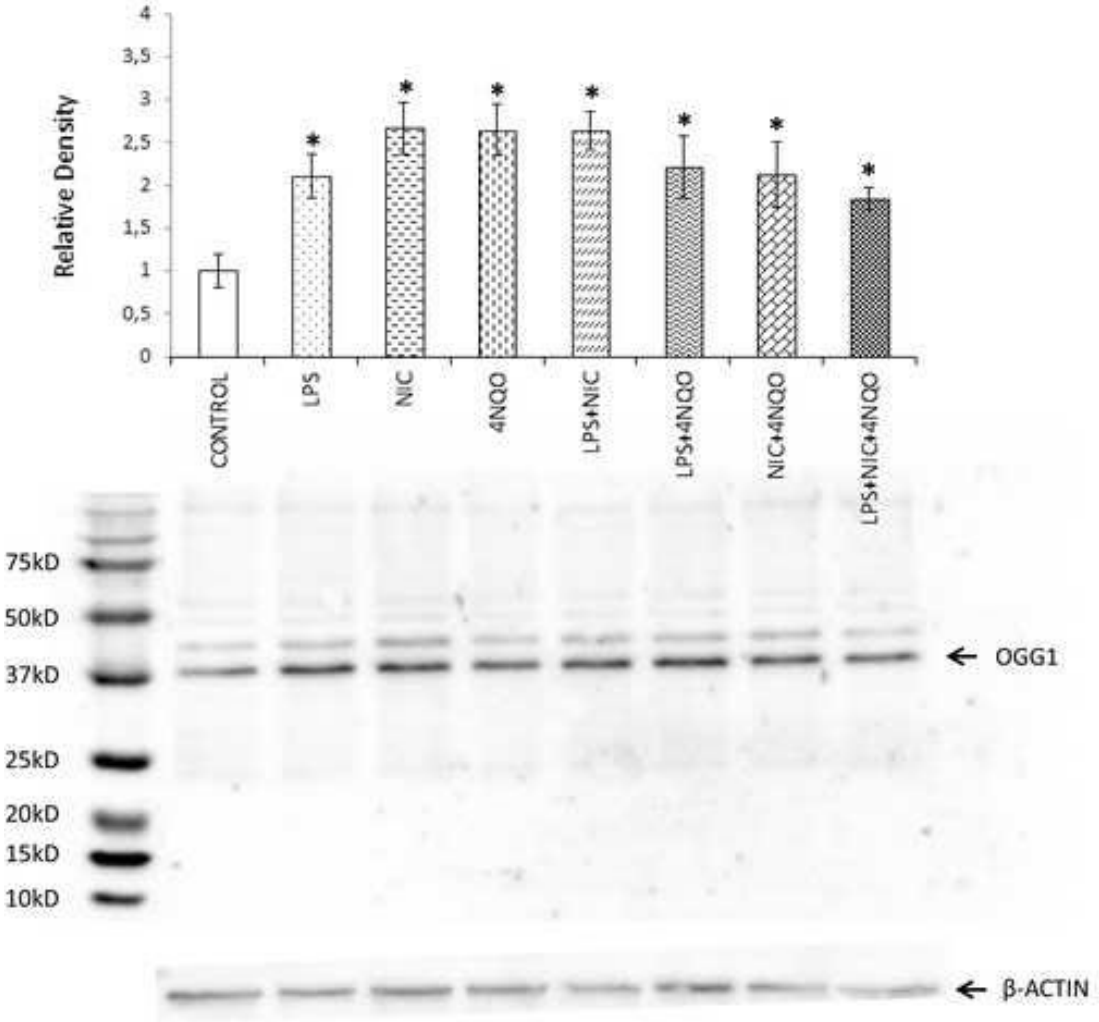

Figure.2

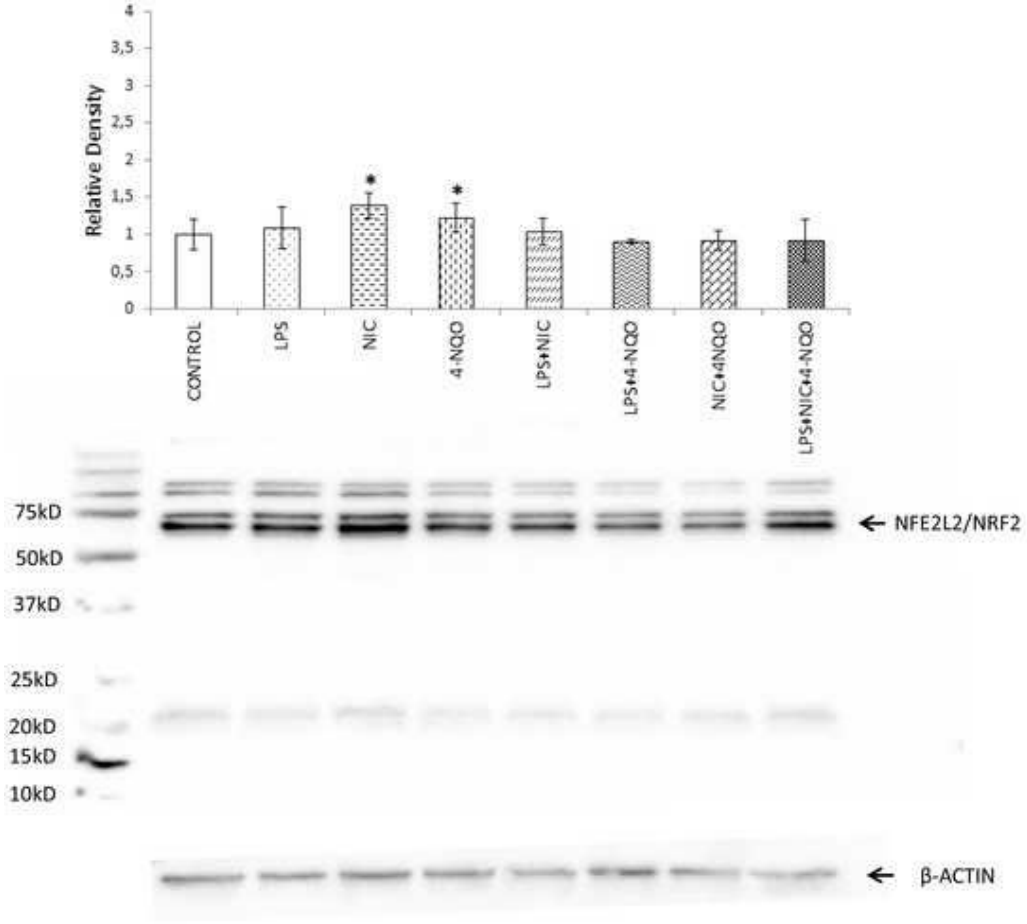
Relative Density
4
3,5
3
2,5
2
1,5
1
0,5
0
CONTROL
LPS
NIC
4-NQO
LPS+NIC
LPS+4-NQO
NIC+4NQO
LPS+NIC+4-NQO
75kD
50kD
37kD
25kD
20kD
15kD
10kD
NFE2L2/NRF2
β-ACTIN

Figure legends

Figure 1: Stimulation of the HMK cells with *Porphyromonas gingivalis* lipopolysaccharide (LPS), nicotine (NIC), 4-Nitroquinoline N-oxide (4-NQO), and their combinations increased OGG1 expression of HMK cells. β-Actin was assed as loading control. Asterisk (*) indicates a statistically significant difference with control.

Figure 2: The NFE2L2/NRF2 protein expression in untreated and, *Porphyromonas gingivalis* lipopolysaccharide (LPS), nicotine (NIC), and 4-Nitroquinoline N-oxide (4-NQO) treated HMK cells. NFE2L2/NRF2 expression was significantly higher in NIC and 4-NQO groups in comparison with control. β-Actin was assed as loading control. Asterisk (*) indicates a statistically significant difference with control.

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