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NF-κB and Nrf2 interfere in the inhibition of LPS-stimulated cytokine release by the resin monomer HEMA

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H. Schweikl, Wolfgang Buchalla
Department of Conservative Dentistry and Periodontology, University Hospital Regensburg, Germany

INTRODUCTION

Dental adhesives or dental composites release monomers such as 2-hydroxyethyl methacrylate (HEMA) before polymerization when applied in cavities created as a consequence of the removal of carious dentine from deep caries lesions. These monomers interfere with effects induced by bacterial stressors such as lipopolysaccharide (LPS) set free from cariogenic microorganisms [1]. Consequently, immunocompetent dental pulp cells will activate defense mechanisms through the secretion of pro- or ant-inflammatory cytokines such as TNF-α, IL-6, or IL-10 after the activation of the redox-sensitive transcription factor NFκ-B. Monomers induce oxidative stress based on the formation of reactive oxygen (ROS) or nitrogen species (RNS) thereby activating the expression of enzymatic antioxidants under the control of the transcription factor Nrf2 [2].

AIM

The role of NFκ-B and Nrf2 in the interference of HEMA with the LPS-activated release of cytokines was analyzed in the present investigation.

METHOD

RAW264.7 mouse macrophages as a model of the cellular innate immune system were exposed to HEMA (0-8 mM) for 1h or 24h , both in the presence or absence of LPS (0.1 µg/ml), or 25 µM tBHQ .

The formation of ROS and RNS was determined by flow cytometry (FACS) after staining of cells with specific fluorescent dyes.

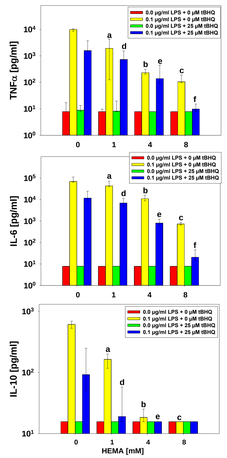
Release of TNF-α, IL-6, or IL-10 was detected using standard ELISA kits.

Protein fractions isolated from cell nuclei and the cytosol of exposed cells were analyzed for the expression of Nrf2, NF-κB, and enzymes regulating oxidative stress by routine Western blotting.

Viable cells and cells in the various phases of cell death were determined using an annexin V-FITC apoptosis detection kit and flow cytometry.

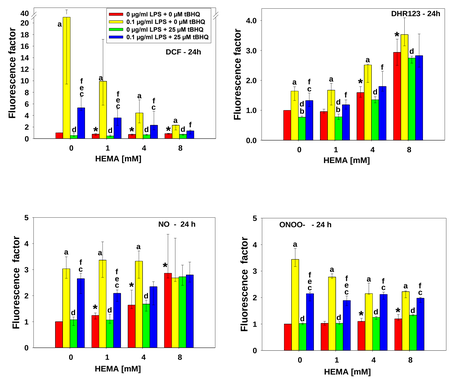
RESULTS

Secretion of Cytokines



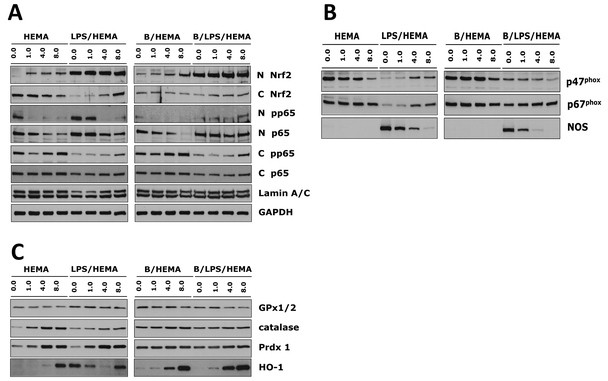
Secretion of cytokines in HEMA-exposed RAW264.7 macrophages. Cells were stimulated with LPS (0.1 µg/ml) and exposed to HEMA (0-1-4-8 mM) for 24h. Cell cultures were preincubated with tBHQ (25 µM, tert-butylhydroquinone tBHQ) for 18h, and then stimulated with LPS (0.1 µg/ml) and HEMA (0-1-4-8 mM) for 24h. Bars show median values (plus 25%/75% percentiles) calculated from quadruplicate samples obtained in four independent experiments (n=16)

Generation of ROS or RNS



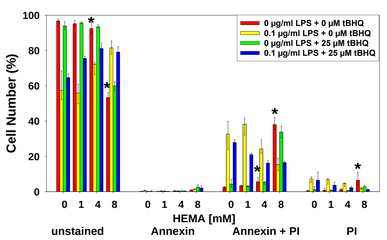
Generation of reactive oxygen species (ROS), nitric oxide (NO) and peroxynitrite (ONOO⁻) in HEMA-exposed RAW264.7 mouse macrophages. Cell cultures were preincubated with tBHQ (25 µM, tert-butylhydroquinone tBHQ) for 18h, and then stimulated with LPS (0.1 µg/ml) and HEMA (0-1-4-8 mM) for 1h (A,C,E) or 24h (B,D,F). ROS production was analyzed by flow cytometry after staining of cells with 2',7'-dichlorodihydrofluorescin diacetate (DCF fluorescence) (A,B) or dihydrorhodamin123 and RNS production was *was determined using Cell Meter™ fluorimetric assay kits* (AAT Bioquest)
Bars show median values (plus 25%/75% percentiles) calculated from individual histograms obtained in independent experiments (n=6-7). Significant differences between median values calculated from untreated cultures (0 mM HEMA) and cultures exposed to 1, 4, or 8 mM HEMA are shown by asterisks (*)

Protein Expression



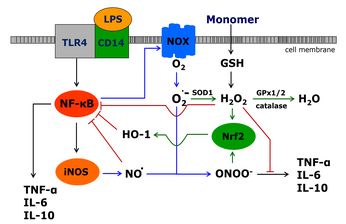
Expression of Nrf2, NF-κB, and related proteins in HEMA- and LPS-exposed cell cultures. RAW264.7 mouse macrophages were incubated with HEMA (0-1-4-8 mM), or 0.1 µg/ml LPS in the presence or absence of tBHQ (B) for 24h. The expression of proteins was then detected by immunoblotting.
Panel A: Expression of Nrf2 and the phosphorylated (pp65) subunit p65 of NF-κB in cell nuclei (N) and cytosol (C). The expression of lamin A/C and GAPDH was used as a marker for cell nuclei or the cytosol, respectively.
Panel B) Expression of regulatory subunits p47 ^{phox} and p67^{phox} of Nox2 and nitric oxide synthase (NOS) in the cytosol.
Panel C) Expression of glutathione peroxidase (GPx1/2), catalase, peroxiredoxin 1 (Prdx1), and heme oxygenase1 (HO-1) in the cytosol.

Apoptosis and Cell Survival



HEMA-induced apoptosis in RAW264.7 mouse macrophages. Cell cultures were treated with HEMA (0-1-4-8 mM), lipopolysaccharide (LPS; 0.1 µg/ml) or tert-butylhydroquinone (tBHQ, 25 µM) for 24h, and subsequently analyzed by flow cytometry after the staining of cells with annexin V-FITC (Annexin) or propidium iodide (PI). Bars show percentages of viable cells (unstained) or cells in the various phases of cell death (apoptosis, late apoptosis, or necrosis) as median values (plus 25% / 75% percentiles) summarized from duplicate samples per experimental condition in seven independent experiments. Proportions of cell populations detected as viable (unstained) cells or as cells in apoptosis (annexin), late apoptosis (annexin +PI), or necrosis (PI) (apoptosis, late apoptosis, or necrosis). Significant differences between median values calculated from untreated cultures (0 mM HEMA) and cultures exposed to 1, 4, or 8 mM HEMA are shown by asterisks (*).

CONCLUSIONS



The present findings identify downregulation of NF-kB activity as the cause of the HEMA-induced inhibition of LPS-stimulated cytokine secretion as summarized in a hypothetical model . Binding of LPS to TLR4 and CD14 induces the activation of NF-κB. Then, NF-κB induces the formation of NO· and superoxide anions through iNOS and Nox2. Levels of the non-enzymatic antioxidant glutathione are reduced after the covalent binding of the resin monomer. Consequently, the formation of H_2O_2 through SOD1 activity exceeds the capacity of GPx1/2 and catalase to reduce H_2O_2. Next, LPS-induced NF-κB signaling is inhibited through multiple mechanisms. At the non-transcriptional level, the redox-sensitive nuclear translocation of NF-κB is primarily prevented due to the physiologically relevant LPS-stimulated formation of NO· and by the HEMA-induced formation of H_2O_2. Monomer-induced production of H_2O_2 may also negatively interfere with the formation of ONOO- needed for the immediate release of cytokines from secretory granules. In addition, nitrosative and oxidative stress produced by the formation of ONOO- and H_2O_2 stimulate the transcription factor Nrf2, leading to the differential activation of HO-1 followed by the inhibition of NF-kB.

REFERENCES

[1] Schweikl H et al. Cell responses to cariogenic microorganisms and dental resin materials-Crosstalk at the dentin-pulp interface? *Dent Mater 2017;33:514-524.*

[2] Gallorini M et al. Activation of the Nrf2-regulated antioxidant cell response inhibits HEMA-induced oxidative stress and supports cell viability. *Biomaterials 2015;56:114-28.*

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CONTACT INFORMATION

Prof. Dr. Helmut Schweikl, University Hospital Regensburg, D-93042 Regensburg, Germany; e-mail: Helmut.Schweikl@ukr.de

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