Effect of 2-Hydroxyethyl Methacrylate on Human Pulp Cell Survival Pathways ERK and AKT

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Abstract

Previous investigations have revealed that dental monomers could affect intracellular pathways leading to cell survival or cell death. Mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) might mediate cell responses as well as cell survival and apoptosis. The purpose of this study was to evaluate the effects of 2-hydroxyethyl methacrylate (HEMA) on the ERK1/2 and AKT pathways in human primary pulp fibroblasts (HPCs). HPCs were treated with various concentrations of HEMA, after which viability and reactive oxygen species levels were determined by flow cytometry with Annexin V-PI staining and 2,7-dichlorofluorescine diacetate, respectively. Whole-cell extracts were immunoblotted with anti-P-Akt or anti–P-ERK1/2. Cell viability decreased in a dose-dependent manner after HEMA exposure, showing a significant decrease with 10 mmol/L HEMA ($p < .05$). HEMA treatment resulted in a 4-fold increase in reactive oxygen species formation ($p < .05$). A short HEMA exposure (30–90 minutes) increased ERK1/2 phosphorylation, whereas a decrease in the AKT phosphorylation was observed. Selective inhibitors of the ERK (PD98059) and AKT (LY294002) pathways amplified HPC cell damage after HEMA exposure. Our findings demonstrated that HEMA exposure modulates the ERK and AKT pathways in different manners, and that in turn, they function in parallel to mediate pro-survival signaling in pulp cells subjected to HEMA cytotoxicity. *(J Endod 2008;34:684–688)*

Key Words

Dental pulp cells, HEMA, PI3K/AKT, survival pathways

It is well-established that dental monomers like tetraethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) can be released from dental resin materials (1–4). HEMA is commonly a major component of dental bonding resins and adhesive systems. It has been shown that HEMA diffuses rapidly across the dentin toward the pulp, and this might cause pulp irritation (5). In clinical trials, toxic concentrations depend on the procedures used as well as the remaining dentin thickness (6).

Many studies have examined the cytotoxicity of this monomer in different cell cultures by using a wide range of assay techniques and have reported a variable level of cytotoxicity (4, 7). Recent studies focused on the possible mechanisms and cell responses induced by dental monomers (7). It has been demonstrated that HEMA induces an increase in reactive oxygen species (ROS) levels, which in turn could affect cell damage, proliferation, and cell survival. HEMA-induced cell death and apoptosis might be related to an increase in ROS in primary fibroblasts (8, 9) and in an immortalized cell line (10, 11). Moreover, ROS induced by HEMA might modulate the cell cycle in primary pulp cells and gingival epithelial cells (12). Leachables from resin-based materials might also modulate intracellular signal pathways by modulating intracellular ROS levels (7). We have provided evidence that ROS production caused by HEMA activates the protective transcription factor nuclear factor kappa B (NF-κB) in primary fibroblasts (8). Furthermore, apoptosis induced by HEMA and TEGDMA seems to be mediated by ROS and by a differential activation of the mitogen-activated protein kinases (MAPKs) p38, JNK, and ERK in rat submandibular salivary gland acinar cells (10). Recently it has been shown that TEGDMA might affect the PI3K/AKT survival pathway but not the ERK1/2 pathway in human primary pulp cells (HPCs) (13). In general, the ERK pathway is associated with proliferation and survival (14, 15), but it has also been reported that induction of apoptosis might be mediated via ERK (16, 17). Another major pathway that is central to the mediation of cellular responses, including cell survival, proliferation, apoptosis, and metabolism, is the PI3-kinase/AKT pathway (18).

Although previous reports have studied the potential molecular mechanisms of HEMA cell damage (7), so far the effects of HEMA on the ERK and PI3K/AKT pathway in HPCs remain to be analyzed. Therefore, we tested the hypothesis that HEMA might influence these major intrinsic cellular survival pathways in HPCs.

Materials and Methods

Reagents

HEMA was purchased from Sigma Chemical Co (St Louis, MO). 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR); Annexin V

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and Propidium Iodide (PI) Kit from MBL Medical & Biological Laboratories Co, Ltd. (Nagoya, Japan). Medium and reagents were from Gibco, Life Technologies (Grand Island, NY). 4-Morpholinyl-8-phenyl-4H-1-benzopyran-4-one (LY294002; CAS-No. 154447-36-6) and 2-amino-3-methoxyflavone (PD98059; CAS-No. 167869-21-8) were obtained from Calbiochem (Bad Soden, Germany).

Cell Culture

Human pulp fibroblasts were obtained from human third molars that were freshly extracted from 4 healthy young patients, with proper informed consent. The protocol was reviewed and approved by the Institutional Review Board (University of Naples "Federico II"). Pulp tissue was minced into small tissue pieces and cultured in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. In all experiments, cells were pooled and used between passage 2 and passage 6.

Viability and Cell Death Detection

HPCs (1×10^5) were plated in 35-mm culture dishes and incubated at 37°C for 24 hours. Cells were then exposed to HEMA (0–14 mmol/L) in the presence or absence of 50 μmol/L LY294002 and 40 μmol/L PD98059 for 24 hours at 37°C. Flow cytometry was used to detect viability and cell death. After treatment, floating and adherent cells were collected and harvested by centrifugation and then washed once with phosphate-buffered saline. Next, the cells were suspended in 500-μL binding buffer. Untreated and treated cells were stained with Annexin V–fluorescein isothiocyanate and PI and incubated at room temperature for 15 minutes before being analyzed by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA). Viable cells (no staining), apoptotic cells (Annexin V+), and necrotic cells (both PI+/Annexin V+ or PI+ alone) were detected and quantified as a percentage of the entire population (19). The sum of apoptotic and necrotic cells was considered to be the cell death population (20). Data were analyzed by the WinMDI 2.8 program (The Scripps Research Institute, San Diego, CA). Data from at least 4 independent experiments, performed in duplicate, were pooled to determine the mean viable cell population.

Measurement of ROS Levels

Production of ROS in HPC cells was quantified by using the cell permeant fluorescence probe DCFH-DA. One $\times 10^5$ cells were plated in 35-mm culture dishes and incubated at 37°C for 24 hours. Then HPCs were incubated with HEMA for 30, 60, and 120 minutes in the presence or absence of 50 μmol/L LY294002 and 40 μmol/L PD98059. At the end of each exposure time, cells were stained with 10 μmol/L DCFH-DA for 30 minutes at 37°C, detached with trypsin/ethylenediaminetetraacetic acid (EDTA), washed, resuspended in phosphate-buffered saline, and then immediately analyzed by flow cytometry. We used a FACScan flow cytometer to measure ROS generation on the basis of the fluorescence intensity (FL-1, 530 nm) of 20,000 cells. Mean fluorescence intensity was obtained by histogram statistics with WinMDI 2.8. Each independent experiment was performed at least 4 times in duplicate.

Western Blotting

Human pulp cells were grown in 100-mm culture dishes until 70%–80% confluent and exposed to HEMA for different time periods (0–120 minutes). Western blotting analysis was performed as previously described (13). Briefly, antibodies to the endogenous proteins were the following: P-Akt ser473 (mouse monoclonal; UBI), Akt (rabbit polyclonal; Cell Signaling Technology, Danvers, MA), P-Erk 1/2 (mouse monoclonal; Santa Cruz Biotechnology Inc, Santa Cruz, CA), and Erk 2 (rabbit polyclonal; Santa Cruz Biotechnology Inc). Total extracts were prepared in 50 mmol/L Tris-HCl (pH 7.4), 1% Nonidet NP-40, 100 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 0.1 mmol/L NaVO3, 1 mmol/L β-glycerolphosphate, 2.5 mmol/L sodium pyrophosphate, and a protease inhibitor cocktail for 30 minutes. Whole-cell extracts were separated on 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose filter. Immunoblots were performed according to the manufacturer's recommendations. Each independent experiment was performed at least 4 times.

Statistical Analysis

Values were expressed as the mean ± standard deviation, and the data were analyzed by one-way analysis of variance followed by Bonferroni for multiple comparisons. The level of significance was set at $p < .05$.

Results

The results showed a time-dependent increase in ROS formation after exposure to HEMA for 30–120 minutes (Fig. 1). A significant level of ROS production was observed at all HEMA concentrations (data not shown). In HPCs, 10 mmol/L HEMA caused a significant increase in ROS after 30-minute exposure, reaching a more than 4-fold increase compared with control cells after 2 hours (Fig. 1).

HEMA caused a dose-dependent decrease in HPC viability after a 24-hour incubation period (Fig. 2). A concentration of 10 mmol/L HEMA caused a significant reduction in cell viability compared with control cells (Fig. 2).

Therefore, we treated cells with ERK specific inhibitor PD98059 and the PI3K/AKT inhibitor LY294002. Although after 24-hour treatment the percentage of the viable cell population was not significantly affected by inhibitors alone, this population decreased with increasing HEMA concentration ($p < .05$) (Fig. 2). Ten mmol/L HEMA plus PD98059 caused a slight but significant ($p < .05$) reduction in HPC viability, whereas the reduction was more prominent in the presence of LY294002 (Fig. 2), with a decrease to 50% ($p < .05$). Interestingly, the levels of ROS in dental pulp cells exposed to HEMA were not affected by either inhibitor (Fig. 1).

After exposure to 10 mmol/L HEMA, a marked increase in phosphorylation of ERK1/2 was observed at 30–90 minutes (Fig. 3). In

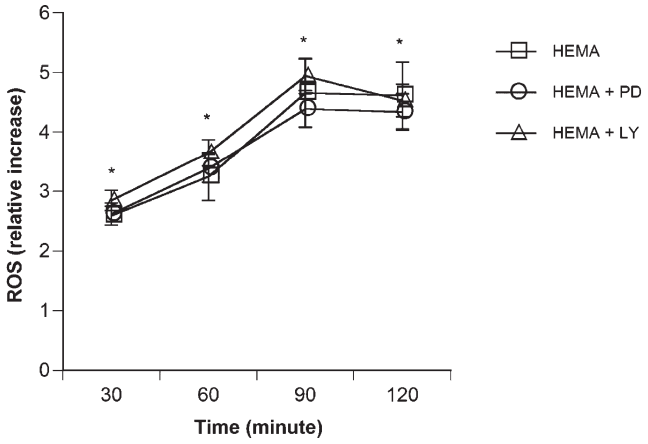

Figure 1. Induction of ROS levels in HPCs exposed to 10 mmol/L HEMA in the presence or absence of LY and PD. ROS were calculated as –fold increase in fluorescence compared with that of untreated cells. ROS levels in the control group were arbitrarily assigned a fluorescence value of 1 ($n = 4$). *Significant differences between treated and untreated cell cultures, $p < .05$.