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

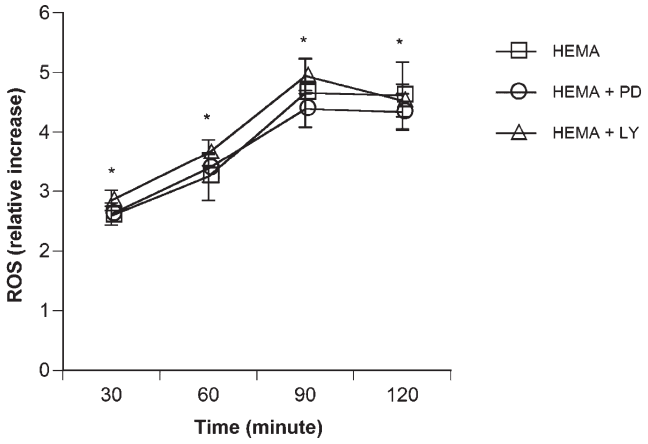

Figure 1. Induction of ROS levels in HPCs exposed to 10 mmol/L HEMA in the presence or absence of LY and PD. ROS were calculated as –fold increase in fluorescence compared with that of untreated cells. ROS levels in the control group were arbitrarily assigned a fluorescence value of 1 ($n = 4$). *Significant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

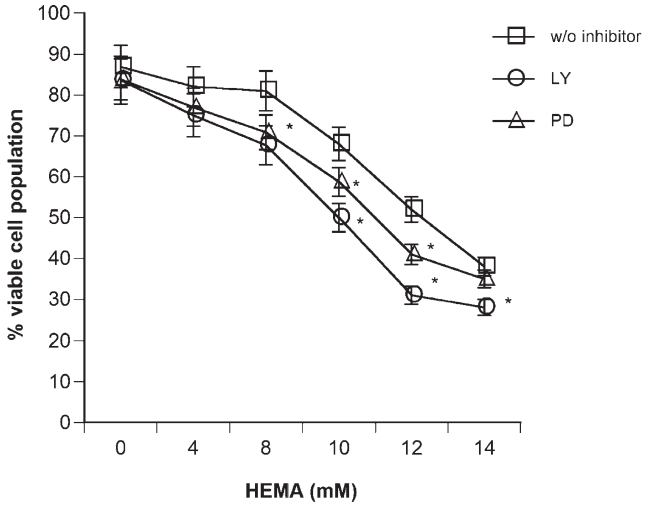

Figure 2. Effects of HEMA on human pulp cell viability in the presence or absence of LY and PD. Cell viability was detected and quantified by flow cytometry as described in "Materials and Methods" ($n = 4$). *Significant differences between cell cultures treated by HEMA with and without inhibitors, $p < .05$.

addition, 10 mmol/L HEMA considerably decreased the levels of phosphorylated PI3K/AKT (Fig. 3).

Discussion

In vitro studies showed that HEMA is released into the adjacent aqueous phase from a broad range of resin-based bonding, cementing, and direct filling materials (4). Different concentrations of this compound are released from clinically used amounts during the first few days after placement in *in vitro* model systems (21). It has been shown that HEMA might diffuse through dentin in concentrations able to cause cellular damage (5). It has been estimated that the concentrations of HEMA from dentinal adhesives would be in the millimolar range after diffusion through the dentin layer (5, 22) . Hence, different ranges of HEMA concentration can reach pulp tissue, and in clinical trials the toxic concentrations depend on the procedures used or on the remaining dentin thickness (6, 23, 24). As a consequence, these concentrations might be high enough to cause detrimental effects, such as modification of the normal cellular responses or homeostasis of the pulpal tissues. Here, as expected, the reduction of human pulp cell viability and the increase in cytotoxicity caused by HEMA was dose-dependent.

In general, cell death and life are controlled by a number of factors within the cell, including a balance between ROS production and the antioxidant system, as well as a balance between pro-death and pro-survival factors. ROS are involved in regulating cell growth, cell differentiation, and cell death. ROS might directly modulate signaling cascades by activating or inhibiting various transcription factors or more indirectly affecting such signaling by changing the cellular redox status (ie, glutathione and thioredoxin) (25, 26). If not counteracted by cellular antioxidants, high ROS levels cause acute injury and damage to cellular proteins, lipids, and DNA, leading to cell death (27, 28). Recently, it has been demonstrated that HEMA causes an increase in ROS levels in different cell lines (7) including HPC (12). Here, in line with the previous studies, we showed high ROS levels after HEMA exposure in HPCs. The intracellular source of increased amounts of ROS in the presence of dental resin monomers is as yet unknown. Elevated ROS levels are produced as a result, for instance, of reduced nicotinamide adenine dinucleotide phosphate oxidase, cytochrome P450, and other enzyme activities (25). Our results do not indicate that the cell survival pathways, ERK and AKT, are directly involved in the control of enzymatic ROS production induced by HEMA. We showed that the specific inhibitors PD98059 and LY294002 did not influence ROS levels in human pulp cells under the current experimental conditions.

Therefore, at low HEMA concentrations cell defense mechanisms might be induced to restore cellular homeostasis, which in turn limits or counteracts subsequent cell damage. At medium or higher HEMA concentrations the cells' protective mechanisms might be defeated, resulting in the induction of cell death. Thus, diverse cellular responses might be generated, depending on the levels and the extent of cellular stress triggered by HEMA or depending on the strength of cellular defenses including the activation of related pro-survival factors and pathways. In this study, we investigated the effect of HEMA on 2 major survival pathways signaling through the MAPK ERK1/2 and AKT in HPCs.

Activation of MAPKs induces a variety of cell responses, such as activation of gene expression, cell proliferation, cell differentiation, cell cycle arrest, or apoptosis. The activation of extracellular signal-regulated kinase (ERK1/2) is a well-studied MAPK pathway and is fundamental for the regulation of cell survival and apoptosis (14–17). The activation of ERK1/2 by HEMA was recently reported after a long exposure period in rat submandibular salivary gland acinar cells (10). Here we showed that HEMA induces early activation of the ERK1/2 pathway in primary HPCs. It has been reported that the phosphorylation of ERK1/2 might be related to increased ROS levels (25). In our experimental conditions, HEMA caused an increase in human pulp cell ROS levels, but the possible connection between HEMA/ROS and the regulation of the ERK1/2 response remains to be established. Previous studies have shown that activation of ERK1/2 can confer protection from the detrimental effects of ROS (29, 30). Here we treated pulp cells with the MEK inhibitor PD98059 to explore the link between ERK1/2 phosphorylation and HEMA cytotoxicity in our system. Treatment with PD 98059 resulted in a slight but statistically significant decrease in viable pulp cells after exposure to HEMA. Thus, our results suggest and substantiate the pro-survival role of ERK1/2 in HEMA-induced cytotoxicity reported previ-

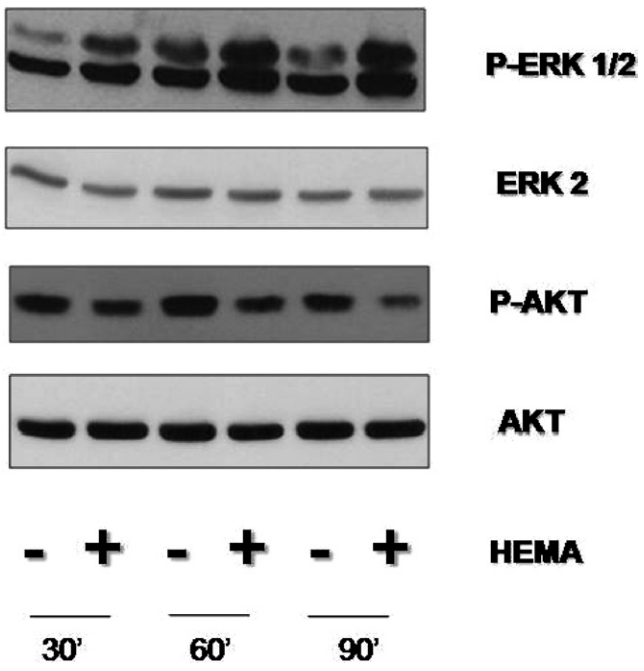

Figure 3. Effect of HEMA on ERK1/2 and AKT pathways. Human pulp cells were treated with 10 mmol/L HEMA for different lengths of time; then cell lysates were separated on SDS-PAGE and immunoblotted with anti-P-AKT or anti-P-ERK1/2. Western blots are representative of at least 4 independent experiments.

ously (10). In our previous study we did not show the involvement of ERK1/2 in the response of primary HPCs to TEGDMA after short exposure (8). However, differences in the sensitivity of ERK1/2 to TEGDMA and HEMA in HPCs could explain the observed differences in signal pattern responses in these 2 studies.

The PI3K/AKT signal transduction pathway has emerged as one of the main signal routes that coordinate complex events leading to changes in cell metabolism, cell growth, cell movement, and cell survival. To date, it appears that PI3K/AKT might negatively regulate proteins that promote the expression of death (31, 32). In our study, the very short exposure of HPCs to HEMA showed an inhibitory effect on activation of PI3K/AKT after 30 minutes. To explore the significance of the PI3K/AKT pathway, we used a specific inhibitor LY294002, which increased HPC cell damage in the presence of HEMA after a long exposure period. In our previous study we provided similar results, showing both that the activation of AKT was blocked by TEGDMA and that LY294002 increased the monomer toxicity in HPC (13). Combining the 2 studies suggests that the cytotoxicity of both TEGDMA and HEMA in HPC cells is mediated, at least in part, through an inhibition of AKT pathways. Some of the protective effects of PI3K/AKT on cell proliferation and survival might be explained by a PI3K/AKT-dependent inhibition of apoptosis through direct phosphorylation and inactivation of pro-apoptotic proteins (eg, BAD) or through anti-apoptotic signaling via activation of the IkB kinase/NF-κB pathway (33) . Interestingly, we previously showed that NF-κB is activated in response to HEMA cytotoxicity (8), whereas here we showed an inhibition of AKT pathways. These 2 findings could suggest, in our experimental model, that NF-κB responses to HEMA might be modulated at least in part by ROS and not by AKT.

Signaling along the PI3K/AKT pathway might be regulated by phosphatases at several levels (31, 32). In particular, the activation of the pathway might be counteracted by protein tyrosine phosphatases or by lipid phosphatases (PTEN and SHIP), which dephosphorylate the second messenger PIP3. In addition, protein kinase B is activated by phosphorylation of Thr-308 and Ser-473, and dephosphorylation of these residues by Ser/Thr phosphatases would inactivate the kinase. Because all the phosphatases mentioned negatively regulate PI3K-dependent signaling, their oxidative inactivation would result in an activation of the PI3K/AKT cascade. Loss of function of any of these phosphatases would lead to a shift in the phosphorylation/dephosphorylation equilibrium and in turn in the activation/inactivation pathway balance. The inhibitory effect of HEMA treatments on the activation of the AKT pathway might depend on a loss of function of one of these phosphatases.

Moreover, the ERK1/2 and PI3K/AKT pathways interact with each other to regulate cell growth and survival (14–17, 31, 32). Although both of these pathways are commonly thought to have anti-apoptotic and drug resistance effects on cells, they might display different cell lineage specific effects. Generally, the cell's fate is determined by crosstalk between pro-apoptotic and anti-apoptotic signaling pathways, such as AKT versus ASK1 and ERK1/2 versus JNK/p38, which plays a significant role in altering susceptibility to apoptosis (15, 32). Here the inhibition of AKT activation in HPCs might be the result of crosstalk between these 2 protective pathways that try to counteract HEMA cytotoxicity, although further studies are needed to provide experimental evidence. Obviously, this antagonistic machinery composed of many pro-apoptosis and anti-apoptosis stress-responsive kinases is necessary for a variety of cellular outcomes.

Conclusion

Recent progress in the analysis of the cellular toxicology of dental monomers has provided new insights into the cellular responses and intracellular mechanisms that are induced (7, 34). In the present study, HEMA affected the ERK1/2 and AKT pathways of primary HPCs. Our findings demonstrated that quick activation of ERK1/2 is involved in a protective mechanism against cell damage induced by HEMA, whereas the inhibition of AKT activation might be a possible cause of HEMA cytotoxicity (Fig. 4). Moreover, the ERK1/2 and AKT pathways are involved in several physiologic processes such as cell proliferation and differentiation, which play a major role in homeostasis of the dentin pulp complex. Hence, the release of monomers by dental resin materials such as adhesive or bonding systems might impair pulp tissue regeneration and reparation by acting on various cell signaling pathways.

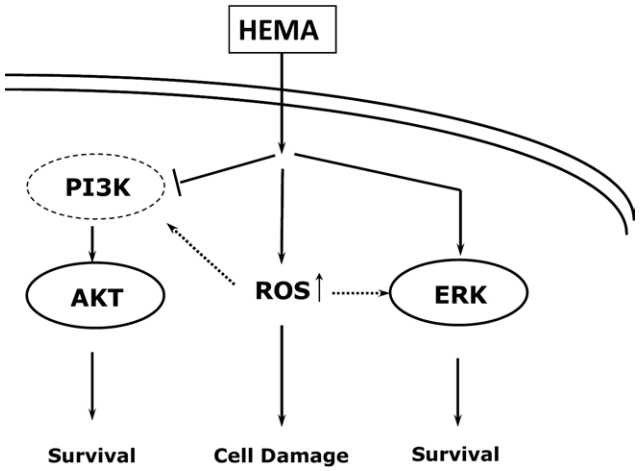

Figure 4. Schematic diagram of HEMA effects on the ERK and AKT survival pathways. Solid lines indicate experimental evidence of pathways and molecules activated by HEMA, whereas dotted lines indicate a model of hypothetical links between cellular effects observed in the presence of HEMA. The exposure of HPC to HEMA induces elevated levels of ROS and triggers cell damage. ROS levels induced by HEMA might modulate the phosphorylation of ERK, which plays a protective role against HEMA cytotoxicity. AKT is a key pathway in the control of cell survival. The possible links to elevated levels of ROS remain to be elucidated. Activation of AKT by PI3K is inhibited in the presence of HEMA.

Acknowledgments

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