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Flavin-containing enzymes as a source of reactive oxygen species in HEMA-induced apoptosis

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ABSTRACT

Objective. Oxidative stress induced by compounds of dental composites like 2-hydroxyethyl methacrylate (HEMA) due to excess formation of reactive oxygen species (ROS) disturbs vital cell functions leading to apoptosis. The sources of ROS in cells exposed to resin monomers are unknown. The present study investigates functions of flavin-containing ROS and RNS (reactive nitrogen species) producing enzymes in cells exposed to HEMA.

Methods. The formation of oxidative stress in RAW264.7 mouse macrophages exposed to HEMA (0–6–8 mM) was determined by flow cytometry (FACS) after staining of cells with 2′7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA), dihydroethidium (DHE) or dihydrorhodamine 123 (DHR123). Cells in apoptosis or necrosis were identified by annexin-V-FITC/propidium iodide labeling followed by FACS analysis. Expression of ROS/RNS producing enzymes was analyzed by Western blotting.

Results. DCF fluorescence increased in cells exposed to HEMA for 1 h suggesting the production of hydroxyl radicals, H_2O_2, or nitric oxide and superoxide anions which form peroxynitrite (ONOO-). Increased DHR123 fluorescence after 24 h indicated the formation of mostly H_2O_2. The induction of apoptosis in the presence of HEMA was decreased by low concentrations of diphenylene iodonium (DPI), an inhibitor of flavin-containing enzymes. Expression of $p47^{phox}$, a regulatory subunit of the superoxide producing Nox2, was downregulated, and the expression of NOS which produces nitric oxide (NO) was possibly inhibited by feedback loop mechanisms in HEMA-exposed cultures. Inhibition of HEMA-induced apoptosis by VAS2870 or apocynin further suggested a crucial function of Nox2.

Significance. The present findings show the physiological relevance of flavin-containing enzymes in monomer-induced oxidative stress and apoptosis.

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1. Introduction

Clinically used dental composites release resin monomers as a result of incomplete polymerization processes. Particularly hydrophilic monomers like 2-hydroxyethyl methacrylate (HEMA) are bioactive, and thus capable of interfering with tissues of the oral mucosa or the dental pulp after diffusion through dentin [1,2]. Depending on the remaining dentin thickness, resin monomers like HEMA may be available at biologically relevant concentrations sufficient to influence crucial functions of pulp tissues *in vivo* [3–6]. These compounds, which are used to direct the course of dental therapy, can cause specific stress responses in cell cultures derived from various oral target tissues. Physiologically relevant levels of resin monomers interfere with vital odontoblast cell functions *in vitro* such as matrix mineralizing capability or expression of gene products essential for tertiary dentin formation [7,8]. Monomers may also delay essential functions of targeted cells of the innate immune system like the release of cytokines stimulated by lipopolysaccharide (LPS) released from cariogenic Gram-negative microorganisms [9,10]. Moreover, it is firmly established that resin monomers trigger programmed cell death or apoptosis through the intrinsic pathway as a consequence of oxidative DNA damage as a physiological process [11,12]. These biological effects observed in a variety of eukaryotic cells exposed to dental resin monomers are related to the formation of reactive oxygen species (ROS) beyond the capacities of the cellular antioxidant system causing the phenomenon of oxidative stress [1].

Cellular redox homeostasis is maintained as a strictly controlled balance between the formation of pro-oxidants such as ROS and the activity of non-enzymatic and enzymatic antioxidants [13]. Recently, it has been detected that the resin monomer HEMA induced the activation of a protective network of pathways under the control of the redox-sensitive transcription factor Nrf2 (nuclear factor erythroid 2 [NF-E2]-related factor 2), a major transcriptional activator of numerous genes coding for enzymatic antioxidants. Activation of Nrf2 was identified as a fundamental mechanism that protected cells from monomer-induced apoptosis in particular, although it is certainly involved in other phenomena related to the control of excessive pro-oxidants as well [14]. Yet, the specific nature of pro-oxidants generated in cells exposed to resin monomers like HEMA is still unknown, and the identification of their cellular origin is essential. Insight into these mechanisms will help establish effective strategies for the development of specific means to control adaptive cellular responses associated with oxidative stress in oral tissues exposed to monomers in a clinical situation.

Pro-oxidants encompass ROS as well as RNS (reactive nitrogen species) including radicals and non-radicals [13,15,16]. ROS such as superoxide anions, hydroxyl or peroxyl radicals may function as oxidizing agents *per se*, or convert into molecules like hypochlorous acid (HOCl) or hydrogen peroxide (H_2O_2). On the other hand, nitric oxide (NO) is a reactive nitrogen species and a radical generated by nitric oxide synthases (NOS). Finally, superoxide anions and NO combine to peroxynitrite ($ONOO^-$) to become the basis of a cascade of reactions yielding numerous derivatives like NO_2, NO_3, or N_2O_3. The formation of $ONOO^-$ is most relevant under physiological conditions since this molecule is extremely potent in the oxidation of cellular macromolecules [13,15,16].

Considering the vast number of bioactive derivatives with distinct physiological functions, the identification of the sources of ROS, and possibly RNS, which generate oxidative stress in monomer-exposed cells is extremely challenging and complex. So far, the analysis of resin monomer-induced oxidative stress has focused primarily on the use of $DCFH_2$-DA (2′,7′-dichlorfluorescein-diacetate) as a redox-sensitive fluorescent dye [17–21]. Dihydroethidium (DHE) was used to specifically indicate the formation of superoxide anions due to its oxidation into 2-hydroxyethidium, and dihydrorhodamine 123 (DHR123) allowed for the analysis of hydrogen peroxide (H_2O_2) formation [19,22–24].

We assume that a plethora of ROS and RNS possibly generated in monomer-exposed cells might originate from the activity of flavin-containing enzymes such as NADPH oxidases (Nox), xanthine oxidoreductase (XOR), or nitric oxide synthases (NOS). NADPH oxidases as flavoenzymes transport electrons across cell membranes and reduce molecular oxygen to generate superoxide anions. Nox enzyme activities have been described as essential for cell proliferation, signaling, apoptosis or cell response to environmental stresses caused, for instance, by pathogenic microorganisms [25]. Xanthine oxidoreductase as a molybdoflavinenzyme catalyzes purine degradation thereby reducing molecular oxygen to superoxide. The enzyme exists in two forms, xanthine reductase (XR) and xanthine oxidase (XO), depending on the cellular redox status [26]. Finally, nitric oxide (NO) as a signaling molecule is produced by three isoforms of nitric oxide synthases identified so far. The inducible NOS (iNOS) is expressed in macrophages after physiological activation through pro-inflammatory stimuli [27].

We hypothesize that pharmacological inhibition of these various flavoenzymes in cells exposed to the resin monomer HEMA might be a valuable strategy for gaining insight into the multitude of ROS or RNS generated. Moreover, both the levels and quality of ROS and RNS could vary depending on exposure periods. Since the induction of apoptosis by a resin monomer has been causally related to the formation of oxidative stress [28], we also presumed that the inhibition of Nox, XOR, or NOS activities would indicate the origin of ROS or RNS responsible for monomer-induced apoptosis. Finally, the analyses of the expression of Nox, XOR, or NOS, and proteins directly related to the regulation of redox homeostasis under the control of Nrf2, would add even more evidence to the identification of the intracellular sources of monomer-induced oxidative stress. For this purpose, we used RAW264.7 mouse macrophages as a suitable model cell line of the innate immune system and we analyzed HEMA concentrations which induce concentration-dependent effects in these cells as repeatedly established in related recent projects [12,14]. Conclusively, the findings of these analyses will encourage the creation of novel approaches for dental treatment strategies to protect human pulp tissues from oxidative stress and support a beneficial environment for tissue repair [29].