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The dental monomer 2-hydroxyethyl methacrylate (HEMA) causes transcriptionally regulated adaptation partially initiated by electrophilic stress

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ABSTRACT

Objectives. Cellular responses including cell death are induced by *in vitro* exposure to the un-polymerized dental monomer 2-hydroxyethyl methacrylate (HEMA). Activation of the Nrf2/ARE signaling pathway has been suggested to mediate the cellular responses. Activation of this pathway may occur either indirectly through generation of increased oxidative stress or through direct binding to cysteine thiols due to the electrophilic properties of HEMA. The objective of this study was to elucidate the potential mechanism of Nrf2/ARE pathway activation after HEMA exposure.

Methods. Global gene expression was investigated after exposure of the human bronchial epithelial cell line BEAS–2B to 2 mM HEMA for 4 h. After exposure to 0.5, 1 or 2 mM HEMA for up to 24 h, western analysis was performed for selected proteins. Finally, the levels of the same proteins were determined after treatment with either the antioxidants Vitamin C, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or BSO (L-buthioninesulfoximine), an inhibitor of GSH formation.

Results. Several of the 25 genes with the highest increase in gene transcription are related to oxidative stress responses. Increased levels of 5 corresponding proteins (HO-1, GCLC, GCLM, NQO1 and SQSTM1) were observed. Antioxidant treatment as well as inhibition of GSH did not affect upregulation of these proteins. Thus, increased ROS or reduced GSH levels appear to be of limited importance in the observed HEMA-induced changes.

Significance. Knowledge of the cellular responses to HEMA is important to evaluate the safety of HEMA-containing biomaterials. The results support that HEMA activates the Nrf2-ARE transcriptional pathway directly through its electrophilic properties.

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1. Introduction

Several studies have described *in vitro* cytotoxic effects after exposure to relatively high concentrations of the commonly used resin monomer 2-hydroxyethyl methacrylate (HEMA) [1–4]. HEMA is water-soluble and identified as a leachable from a range of polymer-based restorative materials [5,6]. The molecular mechanisms involved in the adaptive and cytotoxic effects of HEMA have not been fully elucidated although altered cell signaling, including responses to oxidative stress, activation of MAP-kinases as well as caspases has been shown at high concentrations [2,7–9]. Experiments have also indicated that monomers are capable of inducing DNA damage [1,9–11]. The reported conjugation of HEMA with glutathione (GSH) [8] could impair cellular protection against oxidative damage due to increased levels of reactive oxygen species (ROS).

A major mechanism in the cellular defense against oxidative and electrophilic stress involves activation of the Nrf2/ARE (Nuclear factor erythroid 2-related factor/Antioxidant Response Element) signaling pathway. In the absence of pathway activation, Nrf2 is ubiquitinated by a Keap1 containing complex. Modifications of specific cysteine thiol groups in Keap1 as a response to oxidative and electrophilic stress disrupt this degradation mechanism. This enables Nrf2 to translocate to the nucleus and bind to the ARE sequences of gene promoter regions. Ultimately, this induces transcription of a number of defense genes including genes encoding antioxidants and phase II biotransformation enzymes [12–15].

The Nrf2/ARE signaling pathway has been implied to play a role in protecting cells against resin monomer exposure. Gallorini et al. showed that pretreatment with an Nrf2 activator caused a small but significant protection against HEMA-induced loss of viability in a mouse macrophage cell line [16]. Moreover, both methyl methacrylate (MMA) and HEMA was shown to activate the glutathione S-transferase alpha 1 (GSTA1) gene promoter through ARE *in vitro* [17]. Resin monomers are suggested to modify Keap1 either indirectly by oxidative stress due to GSH depletion [16] or by direct conjugation with cysteine residues on Keap1 due to their electrophilic properties [17]. Recent studies have shown that HEMA induced ARE-mediated transcription at lower concentrations than MMA [17,18]. HEMA has also been reported as more electronegative than MMA, possibly through introduction of a hydroxyl group in the HEMA molecule [18]. Thus, the more pronounced cytotoxic effect of HEMA on ARE activity may correlate with its higher electrophilic reactivity [18,19].

The objective of the present study was to elucidate further whether HEMA mediates its effects primarily through GSH-depletion and increased levels of reactive oxygen species (ROS) or if HEMA may function as an electrophile directly activating components of the Nrf2-ARE transcriptional pathway. To give support for any of these possible mechanisms, we mapped the initial transcriptional changes in a bronchial epithelial cell line exposed to HEMA. The recorded changes were compared with the effects of inhibiting GSH synthesis and attempted counteracted by antioxidants.

2. Material and methods

2.1. Chemicals and reagents

Lechner and La Veck (LHC9) medium was purchased from GIBCO (Life Technologies, Foster City, CA, USA). Collagen (PureCol) was purchased from Inamed Biomaterials (Fremont, CA, USA). 2-Hydroxyethyl methacrylate (HEMA) (CAS no. 868-77-9), purity ≥97%, was from Fluka Chemie (Buchs, Switzerland). L-buthioninesulfoximine (BSO), monobromobimane (mBrB), 2′, 7′-dichlorofluorescein diacetate (DCFH-DA), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), Hoechst 33342, propidium iodide (PI) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma–Aldrich (St Louis, MO, USA). Nuclear isolation and staining solution was purchased from NPE systems (Pembroke Pines, FL, USA).

Applied antibodies were; GCLC (PA5-19702) and GCLM (PA5-26111) from Thermo Fisher Scientific (Waltham, MA, USA), NQO1 (A180; #3187S), HO-1 (P249; #5061), TXNRD1 (C63C6; #2429) and AKR1C2 (#13035) from Cell Signaling Technology (Danvers, MA, USA), SQSTM1 (GP62-C) from Progen Biotechnik (Maaßstraße, Heidelberg, Germany), Nrf2 (C20; sc-722), OKL38 (G-16; sc-82002) and SRXN1 (B-10; sc-166786) from Santa Cruz Biotechnology (Dallas, Texas, USA), GAPDH (ab184578) and FTH1 (ab75972) from Abcam (Cambridge, UK), and ALDH3A2 (HPA014769) from Atlas antibodies. Applied secondary antibodies were: IRDye® 680RD Goat anti-Rabbit IgG (H + L) (926-68071), IRDye® 800CW Goat anti-Rabbit IgG (H + L) (926-32211), IRDye® 800CW Goat anti-Mouse IgG (H + L) (926-32210), IRDye® 680RD Donkey anti-Guinea Pig IgG (H + L) (926-68077) and IRDye® 800CW Donkey anti-Goat IgG (H + L) (926-32214) from (LI-COR Biotechnology, Lincoln, NE, USA). All other chemicals were purchased from commercial sources.

2.2. Cell cultures and exposure to HEMA, antioxidants and BSO

The human lung bronchial epithelial cell line BEAS-2B (ECACC) was grown in serum-free Lechner and LaVeck (LHC-9) medium. Culture flasks and plates (Costar, Corning, NY, USA) were pre-coated in HEPES-buffered saline (Lonza, Basel, Switzerland) containing 30 μg/mL collagen. Cells were detached at approximately 85% confluency by using 1 mL 0.25% trypsin-EDTA solution. When detached, 5 mL medium was added for enzyme inhibition before centrifugation and re-seeding (30,000 cells/cm^2). All incubations were performed at 37 °C and 5% CO_2. For exposure experiments, cells were seeded on flat bottom plates (6 and 24 well plates) or in single dishes (21 cm^2) 24 h before exposure to HEMA (0–8 mM).

To study the impact of HEMA and antioxidants on protein levels (Western analysis) the cell cultures were either exposed to HEMA 0.5, 1 or 2 mM for 24 h or pre-treated with either the hydrophilic antioxidant vitamin C (100 μM) or the lipophilic antioxidant Trolox (100 μM) for 1 h, or BSO (0–100 μM) for 8 h followed by exposure to 1 and 2 mM HEMA for 24 h. BSO, an inhibitor of the rate limiting step of GSH synthesis, was used to assess the effect of cellular GSH depletion on cell viability, ROS levels, and protein levels.

To assess adaptive responses, the cells were pre-incubated for 24 h with a (non-lethal) dose of HEMA (1 mM) before exposing the cells to zero to 8 mM HEMA for 24 h for viability analysis.

2.3. Viability

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the cellular viability. Briefly, cells were seeded at a concentration of $25 \times 10^3/cm^2$ on a 24-well plate and incubated for 24 h. The medium was then removed and the cells were incubated with and without 0.5 to 8 mM HEMA for 24 h, before the medium was removed and MTT was added to a final concentration of 0.5 mg/mL in PBS to each well. After 1 h incubation, the MTT solution was removed and 400 μL DMSO was added to each well and incubated for 30 min before absorbance was measured at 570 nm using Synergy H1 Hybrid reader (BioTek Instruments, Winooski, VT, USA).

2.4. Cellular glutathione levels

The relative concentrations of intracellular glutathione were determined according to a previously described assay using monobromobimane (mBrB) [20]. It has been showed that mBrB staining is the method of choice for measuring GSH [21]. A stock solution of 40 mM mBrB in methanol was diluted with PBS to a final concentration of 40 μM. After exposures, the cells were trypsinized and incubated with mBrB for 10 min. The total amount of fluorescence per cell was measured using flow cytometry (Cell Lab Quanta SC; Beckman Coulter, Brea, CA, USA; excitation filter of 350 nm, emission filter of 465 nm). At least 5000 cells were recorded in each sample, and the mean fluorescence intensity was used as a measure of the GSH level in the cell population.

2.5. Intracellular ROS levels

ROS measurements were performed by means of dichlorofluorescein (DCF) assays [22]. The principle of the test is based on the diffusion of Dichlorofluorescin Diacetate (DCFH-DA) into cells, where it is hydrolyzed to non-fluorescent 2, 7-dichlorofluorescin (DCFH). Intracellular ROS subsequently leads to oxidation of DCFH to a measurable fluorescent product, DCF. Original tube of DCFH-DA was dissolved in dimethyl sulfoxide to a final concentration of 3 mM. After 4 h exposure, DCFH-DA was added to a final concentration of 3 μM and placed in the incubator for 15 min. Excess DCFH-DA and media was subsequently removed and PBS added. Afterwards, fluorescence was measured directly from cells on the plates using a Synergy H1 Hybrid reader with an excitation filter set at 485 nm and emission filter set at 529 nm. The fluorescence from each well was captured as mean relative fluorescence unit (RFU).

2.6. RNA extraction

For the transcriptome studies $1{,}9 \times 10^6$ cells were seeded in T75 flasks and grown for 24 h before the medium was removed and 15 mL of fresh medium containing 2 mM HEMA or medium without HEMA was added. The cells were further incubated for 4 h before the cells were harvested and centrifuged at $300 \times g$ at 22 °C for 5 min. Total RNA was extracted using the RNAeasy mini kit (QIAGEN, Hilden, Germany) following the manufacturers' protocol. To remove any DNA left under the RNA extraction, a DNA clean-up was performed using the RNAse-Free DNase kit (QIAGEN, Hilden, Germany).

2.7. Microarray analysis

Verification of RNA integrity using Bioanalyzer (Agilent Technologies, USA), synthesis of cRNA and hybridization assays were performed at the Genomics Core Facility, Oslo University Hospital and Helse Sør-Øst in Oslo, Norway. Briefly, biotin-labeled cRNA was synthesized from 500 ng total RNA using Illumina Totalprep RNA amplification kit. A total amount of 750 ng cRNA was used for hybridization onto Illumina HumanHT-12 v4 Expression BeadChip, and scanned with Illumina bead array reader. Results were imported; quantile normalized and imputed using GenomeStudio.

2.8. Western analysis

BEAS 2B cells were seeded in 21 cm^2 culture dishes at a density of $25 \times 10^3/cm^2$. After exposure (as described in Section 2.2), the dishes were washed twice with PBS, and the cells were lysed directly in 200 μl sample buffer (12% SDS, 150 mM Trizma® base, 25.5% glycerol, pH adjusted to 7 with HCl). Subsequently, 10 μl of each sample was electrophoresed by standard SDS-PAGE [23], blotted onto nitrocellulose filters, and analyzed using specific antibodies [24]. To confirm equal levels of protein in each well, staining with Ponceau and GAPDH were used as loading controls. Filters were blocked with 3% Bovine Serum Albumin (BSA) or 5% skimmed milk in tris buffered saline (TBS)-Tween (0.1% [vol/vol] Tween 20) and then incubated with primary antibodies diluted in TBS-Tween containing 1% (wt/vol) BSA or 5% skimmed milk, depending on the manufacturers antibody instructions. Working dilutions of 1:2500 for most investigated proteins, and 1:5000 for GAPDH and antibodies from Santa Cruz Biotechnology were found appropriate for immunoblotting. Immunoreactive protein was detected and quantified using the Odyssey CLx imaging system (LI-COR Biotechnology, Lincoln, NE, USA) after incubation with a fluorocrome-conjugated secondary antibody.

2.9. Statistical analysis

Analysis of the microarray data was performed using the J-Express software, where differential gene expression was analyzed using Rank Product [25,26]. A q-value (adjusted p-value) of 0.05 was used as cut-off for up and down regulated genes. Student t-test for pairwise comparison was used for the evaluation of antioxidant effects on ROS levels. For the remaining data, statistical significance of treatment effects was evaluated using one- or two-way ANOVA followed by Bonferroni's test for multiple comparisons. The t-test and ANOVA analyses were performed using the statistical software GRAPHPAD PRISM 6.0 (GraphPad Software, San Diego, CA, USA). In the figures, data are presented as mean ± SD of

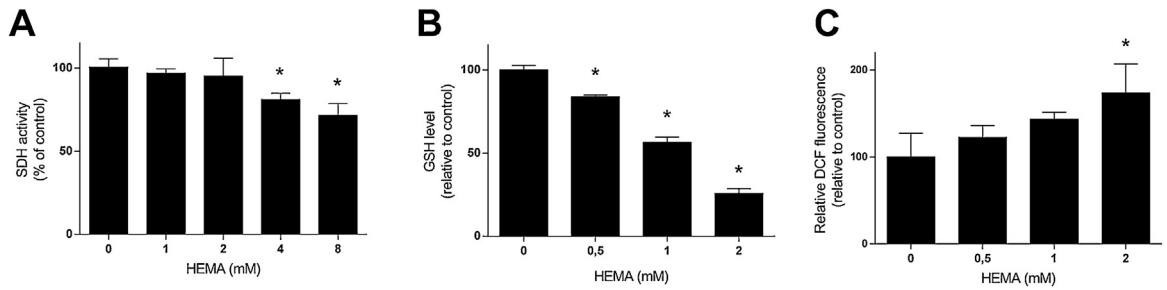

Fig. 1 – a–c: (a) Dose dependent effects of increasing concentrations of HEMA on cell viability measured with the MTT test. (b) Dose dependent effects of increasing concentrations of the HEMA on GSH levels. (c) Dose dependent effects of increasing HEMA concentrations on ROS levels. *Significantly different from the respective control groups, $p < 0.05$, $n = 3–5$.

at least 3 independent experiments. $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Effects of HEMA on cell viability, GSH- and ROS levels*

Twenty four hours exposure to HEMA concentrations below 4 mM did not reduce cell viability as measured by the MTT test (Fig. 1a). However, 4 h exposure to up to 2 mM HEMA resulted in a concentration-dependent GSH-depletion (Fig. 1b) and a concentration-dependent increase in ROS levels (significant at 2 mM; Fig. 1c). Therefore, concentrations up to 2 mM HEMA were used to subsequently assess transcriptional activation and protein expression.

3.2. *Microarray analysis*

Exposure to 2 mM HEMA for 4 h increased transcription of 25 genes with at least 1.3 fold increase ($p < 0.05$; Table 1). Several of these genes, including the 8 first in Table 1, coded for proteins under transcriptional regulation by the Nrf2/ARE signaling pathway.

3.3. *Effect of HEMA exposure on protein levels*

Based on assumed properties of gene products, western analysis was performed for selected proteins after 24 h HEMA exposure as depicted in Table 1. HEMA increased the levels of GCLC, GCLM, NQO1, SQSTM1 and HO-1 (HMOX1) in a concentration-dependent manner (Fig. 2). The levels of OKL38, TXNRD1, AKR1C2, SRXN1, ALDH3A2 and FTH1 were not significantly affected by HEMA (data not shown). To verify activation of the Nrf2/ARE pathway, Nrf2 levels were assessed after 4 h exposure to 2 mM HEMA and found to be significantly increased (Fig. 2). Based on the assumption that its levels are primarily regulated by degradation rate [15] and not by *de novo* synthesis, a briefer exposure time was chosen.

3.4. *Effects of antioxidant treatments*

To elucidate the role of indirect activation of the Nrf2/ARE pathway in HEMA-induced effects, the effects of antioxidants were tested. Co-incubation with HEMA and Trolox for 4 h did not increase the ROS level compared to Trolox alone, whereas a small, but significant increase was seen after co-incubation with vitamin C and HEMA for 4 h compared to vitamin C alone (Fig. 3).

The involvement of ROS on protein levels in the HEMA exposed cells was assessed by 1 h pre-treatment with antioxidants (100 μM vitamin C or Trolox) followed by 24 h co-incubation with HEMA. This treatment did not significantly reduce the HEMA-induced levels of the antioxidant/defense proteins GCLC, GCLM, NQO1, SQSTM1 and HO-1 (Fig. S1).

3.5. *Effects of inhibition of GSH synthesis*

To further assess the role of ROS caused by GSH depletion in the Nrf2/ARE pathway activation, cells were treated for 24 h (cell viability) and 8 h (GSH analysis) with BSO (up to 50 μM), an inhibitor of glutamate cysteine ligase (GCL) and thereby GSH synthesis. The time dependent effect of BSO on GSH depletion was initially measured (not shown). A significant decrease was delayed compared to that measured after HEMA exposure (data not shown). Thus, the experiments involving BSO treatment were also performed with accordingly increased exposure time. BSO treatment for 24 and 32 h did not significantly affect cell viability (32 h shown) but resulted in a significant decrease in GSH levels at 8 h (Fig. 4a and b). Despite reduced GSH levels, increases in ROS levels were not significant at this time point (data not shown).

To assess the role of reduced GSH levels on the levels of antioxidant/defense protein levels we incubated the cells with various concentrations of BSO. All proteins were measured after 24 and 32 h BSO treatment (32 h shown) except for Nrf2 that was measured after 8 h. BSO treatment at the examined concentrations and exposure time did not alter the protein levels of Nrf2, GCLC, GCLM, NQO1 and SQSTM1, whereas the levels of HO-1 (HMOX1) were significantly increased (Fig. 2S).

3.6. *Pretreatment with HEMA*

To test whether HEMA induced activation of the Nrf2/ARE pathway protect against a consecutive exposure to HEMA, the cells were pretreated with 1 mM HEMA (24 h) before exposure to increasing concentrations of HEMA (0–8 mM). The results showed a dose-response dependent pattern of reduced cell

Table 1 – Increased transcription of genes after 4 h exposure to 2 mM HEMA. The 25 most upregulated (p value less than 0.05 and/or more than 1.25 fold increase) are shown (n = 3).

	Gene		Fold change	p
1	HMOX1[a]	Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA.	4.44	0.0
2	OKL38[a]	Homo sapiens pregnancy-induced growth inhibitor (OKL38), transcript variant 1, mRNA.	2.96	0.0
3	TXNRD1[a]	Homo sapiens thioredoxin reductase 1 (TXNRD1), transcript variant 4, mRNA.	2.06	0.0
4	GCLC[a]	Homo sapiens glutamate-cysteine ligase, catalytic subunit (GCLC), mRNA.	1.70	0.0
5	GCLM[a]	Homo sapiens glutamate-cysteine ligase, modifier subunit (GCLM), mRNA.	1.70	0.0
6	HSPA1A[a]	Homo sapiens heat shock 70 kDa protein 1A (HSPA1A), mRNA.	1.67	0.0
7	SRXN1[a]	Homo sapiens sulfiredoxin 1 homolog (S. cerevisiae) (SRXN1), mRNA.	1.62	0.0
8	NQO1[a]	Homo sapiens NAD(P)H dehydrogenase, quinone 1 (NQO1), transcript variant 1, mRNA.	1.47	3E-4
9	LOC644132	PREDICTED: Homo sapiens misc_RNA (LOC644132), miscRNA.	1.43	3E-4
10	FTH1[a]	Homo sapiens ferritin, heavy polypeptide 1 (FTH1), mRNA.	1.40	0.004
11	FBXO30	Homo sapiens F-box protein 30 (FBXO30), mRNA.	1.35	0.006
12	MAP3K14	Homo sapiens mitogen-activated protein kinase kinase kinase 14 (MAP3K14), mRNA.	1.36	0.006
13	UGDH	Homo sapiens UDP-glucose dehydrogenase (UGDH), mRNA.	1.35	0.012
14	HSPA1B[a]	Homo sapiens heat shock 70 kDa protein 1 B (HSPA1B), mRNA.	1.34	0.014
15	ALDH3A2[a]	Homo sapiens aldehyde dehydrogenase 3 family, member A2 (ALDH3A2), transcript variant 2, mRNA.	1.33	0.016
16	AKR1C3	Homo sapiens aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) (AKR1C3), mRNA.	1.33	0.030
17	LOC729009	PREDICTED: Homo sapiens misc_RNA (LOC729009), miscRNA.	1.37	0.030
18	AKR1C2[a]	Homo sapiens aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III) (AKR1C2), transcript variant 3, mRNA.	1.33	0.030
19	ADM	Homo sapiens adrenomedullin (ADM), mRNA.	1.31	0.038
20	LOC340357	Homo sapiens hypothetical LOC340357 (LOC340357), non-coding RNA.	1.31	0.038
21	DNAJB4	Homo sapiens DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4), mRNA.	1.31	0.038
22	MRPL19	Homo sapiens mitochondrial ribosomal protein L19 (MRPL19), nuclear gene encoding mitochondrial protein, mRNA.	1.33	0.039
23	SQSTM1[a]	Homo sapiens sequestosome 1 (SQSTM1), mRNA.	1.29	0.039
24	SPSB1	Homo sapiens splA/ryanodine receptor domain and SOCS box containing 1 (SPSB1), mRNA.	1.28	0.041
25	FTHL8	Homo sapiens ferritin, heavy polypeptide-like 8 (FTHL8) on chromosome X.	1.33	0.049

[a] Show for which genes western analysis of protein expression were performed.

viability similar to that seen for HEMA exposure without pre-treatment (Fig. 5).

4. Discussion

In the present study, we measured a dose-dependent GSH-depletion as well as increased ROS levels in cells exposed to HEMA, which is in agreement with previous studies [2,8]. These changes have been assumed key events in the onset of HEMA-induced toxicity *in vitro*. This view, however, has been challenged by studies that describe GSH- and ROS-independent contributions to HEMA toxicity [17,18,27]. At higher doses of HEMA, such responses may be difficult to investigate due to extensive cell damage and the onset of cell death signaling. At lower concentrations, however, cells may promote survival by adjusting to the increased stress situation. Analysis of these adaptive responses may shed light on the molecular interactions of HEMA in living cells, both dependent and independent of cellular ROS and GSH levels.

The airways of dental personnel can be exposed to low concentrations of HEMA on a daily basis [28,29]. Thus, we aimed to explore altered gene expression in a bronchial epithelial cell line after exposure to "non-lethal" concentrations of HEMA, demonstrated to be 2 mM or below in the current model system. We show that the expression of a number of genes was altered in HEMA exposed cells. Some cellular functions possibly affected by the genes with increased expression are antioxidant capacity (*HMOX-1, NQO1*), thiol-metabolism (*GCLC/M, TXNRD1*), ER-stress/selective autophagy (*HSPA1A/B, SQSTM1*) and capacity to handle lipid peroxidation products (*OKL38, ALDH3A2*). Western blotting revealed significantly increased levels of several of the corresponding proteins (HO-1, NQO1, GCLC, GCLM and SQSTM1/p62). Fig. 6 summarizes how these cellular pathways may be involved in activation of Nrf/ARE and AP-1.

Nrf2 regulates many of the genes that showed the highest increase in gene transcription [13,30,31]. Accordingly, HEMA exposure led to increased protein levels of Nrf2. This suggests increased transcription of Nrf2/ARE regulated genes in the cellular response to HEMA exposure. However, the total ALDH3A2 and OKL38 protein levels did not increase significantly in cells exposed to HEMA for 24 h. It could be that the levels of these proteins, which play important roles in the detoxification of lipid peroxidation products [32,33], increases transiently or only in local cellular compartments. A local increase may not be detectable at the cellular level by western blotting. Since Trolox has been reported to be a lipophilic antioxidant [34], an initial lipid damage could explain the observation that HEMA induced ROS was counteracted by Trolox only.

The level and nuclear translocation of Nrf2 is partly repressed by interaction with the cytosolic protein Keap1, a cellular sensor for electrophiles and oxidants [31,35,36]. Modification of specific cysteine thiol groups in Keap1 is an

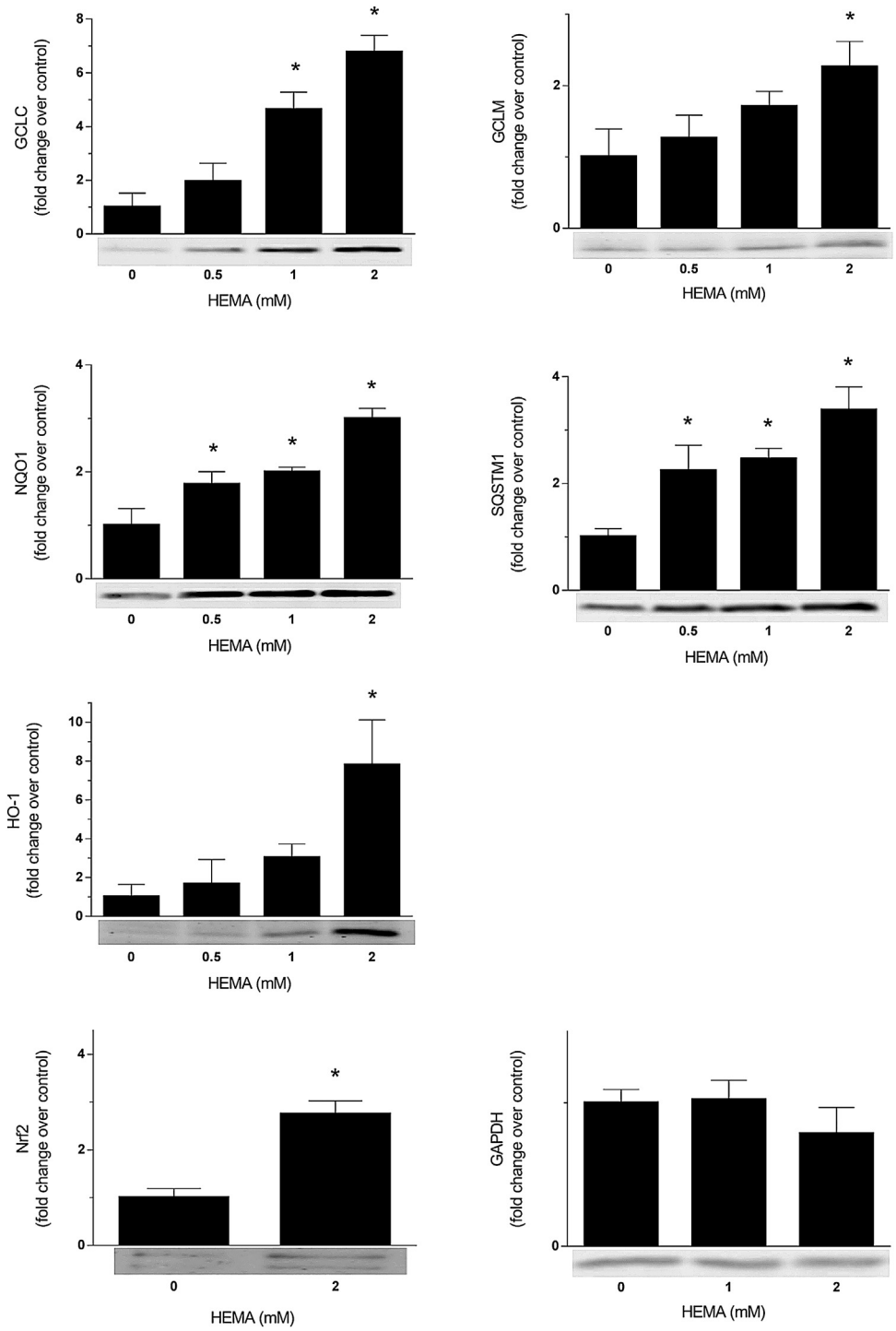

Fig. 2 – Effect of HEMA exposure on protein levels. Western analysis was done on selected proteins among the 25 upregulated at mRNA level as depicted in Table 1 after 24 h HEMA exposure. The blots were quantified and the value representing mean ± SD of n = 3–4 experiments are shown as bars on top of the figure. One representative blot for each experiment is shown. The levels of GAPDH show protein loading in the same range for each concentration of HEMA. The results show a pattern of dose dependent increase in the levels of GCLC, GCLM, HO-1, NQO1, SQSTM1 and Nrf2. *Significantly different from the respective control groups, $p < 0.05$.

important event in canceling this Nrf2 repression. Hence, both thiol-oxidations after a HEMA-induced shift in cellular redox status as well as direct HEMA conjugation as an electrophile to these thiol-groups may account for Nrf2 activation by HEMA (as summarized in Fig. 6). To elucidate a possible indirect activation of the Nrf2/ARE pathway through increased oxidative stress and GSH-depletion, we co-incubated the cells with HEMA and antioxidants or treated the cells with BSO.

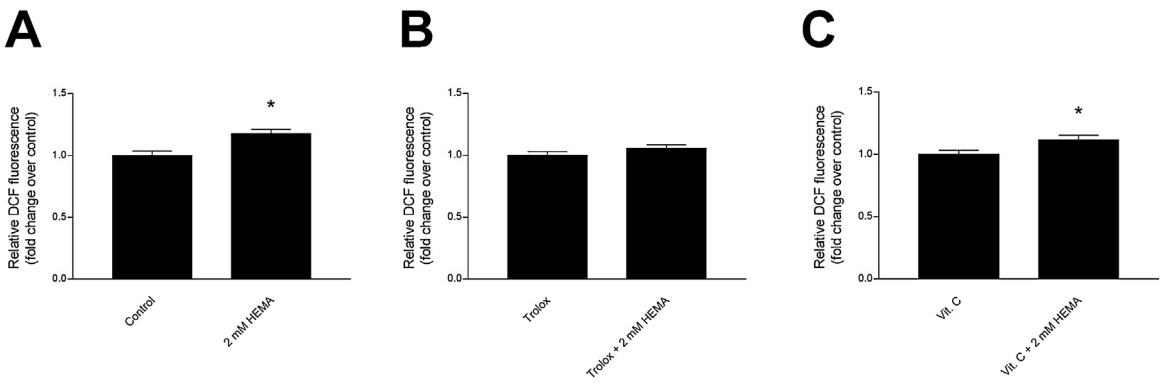

Fig. 3 – a–c. Effects of HEMA (a) or antioxidants Trolox (b) and VitC (c) alone or in combination with HEMA on ROS levels. Cells were pre-incubated with antioxidants for 1 h followed by 4 h HEMA exposure. *Significantly different from the respective control groups, $p < 0.05$, $n = 5$.

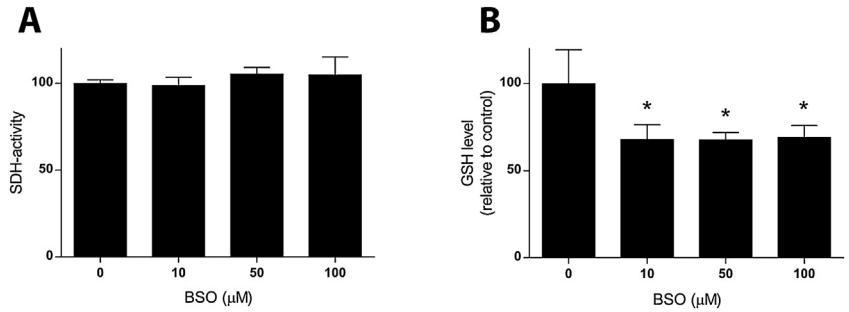

Fig. 4 – a–b. Effect of BSO treatment on cell viability and GSH levels and antioxidant protein levels. (a) Treatment for 24 h with BSO up to 50 μM did not significantly affect cell viability but resulted in a significant decrease in GSH levels after 8 h exposure (b). *Significantly different from the respective control groups, $p < 0.05$, $n = 3$.

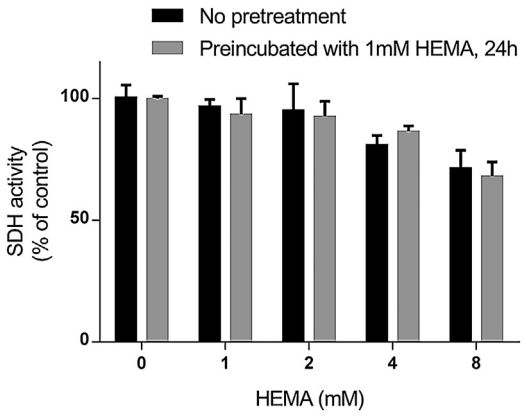

Fig. 5 – Pretreatment for 24 h with 1 mM HEMA before exposure to increasing concentrations of HEMA (0–8 mM). Cell viability measured with the MTT test (n = 5).

The lack of counteracting effects of Trolox and vitamin C on the protein levels of HO-1, GCLC, GCLM, NQO1 and SQSTM1/p62 support ROS independent mechanisms in the HEMA-induced transcriptional changes. The lack of measurable changes in protein levels of Nrf2, GCLC, GCLM, NQO1, and SQSTM1 after BSO treatment also question a mechanism initiated by GSH-depletion.

Although the ROS increase at the time point measured was not significant (data not shown), the effects of BSO on GSH and ROS in BEAS cells have been shown to be significant with longer exposure time [27]. However, the HO-1 protein levels were significantly increased after BSO treatment, suggesting the presence of a link between GSH-depletion and increased HO-1 levels. The *HMOX1* gene regulation is commonly linked to Nrf2/ARE activity, but induction of this gene in Nrf2-null mice point towards other regulation mechanisms, possibly through activating protein 1 (AP1) regulated transcription [37]. The absence of a measurable increase in Nrf2-levels in BSO-treated BEAS cells combined with the increase in HO-1 levels, further support an Nrf2 independent regulation of *HMOX1* in the present study. In addition to AP-1 regulated transcription, regulation at a post-transcriptional level is a possible mechanism for controlling HO-1 protein level.

Although our results point towards a GSH- and ROS-independent Nrf2 activation, there is at least one more possible mechanism of unlocking the Keap1 mediated Nrf2 degradation than by Keap1-HEMA interaction. Sequestome 1 can compete with Nrf2 for binding to Keap1. Hence, increased SQSTM1/p62 levels may result in abolished Nrf2 ubiquitination and thereby increase Nrf2 activation [38–40]. In addition

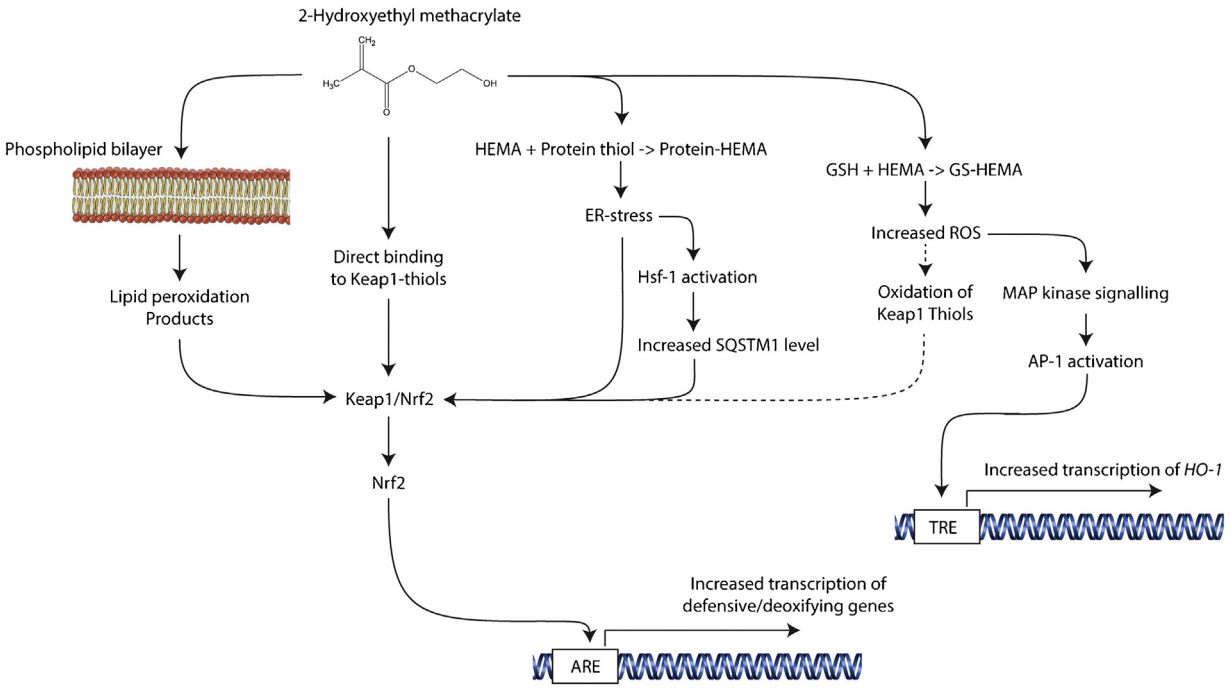

Fig. 6 – Proposed pathways for HEMA induced cellular toxicity. This schematic illustration summarizes the cellular pathways that appear to be activated upon HEMA exposure. The illustration summarizes the Discussion section and is in accordance with the current data, but is not solely based on the data presented in this study.

to Nrf2 regulation, *SQSTM1* transcription can be activated by heat shock factor 1 (HSF1). Thus, HSF1 may indirectly activate Nrf2 through increased SQSTM1/p62 level [41,42]. Upregulation of Nrf2 levels and several Nrf2/ARE regulated mRNAs after 4 h exposure, however, argues against that HEMA activation of the Keap1/Nrf2 pathway is primarily HSF1 dependent since increased SQSTM1/p62 protein levels were not measurable until 16 h after start of exposure.

As for most genes, the regulation of cytoprotective genes can involve complex interplay between different signaling pathways. In addition to altered Nrf2 activity [16,17], HEMA has previously been shown to affect MAP-kinases [2,43] and NFkB [44,45]. Interestingly, AP-1 activity can be modulated by MAP kinases thus linking this signaling pathway to the Nrf2 independent regulation of HMOX1 after BSO treatment. Interplay between the Nrf2 and NFkB pathways has also been described, although fewer details regarding this crosstalk are known [46,47].

Nrf2 activation by tertiary butylhydroquinone (tBHQ) has been shown to protect cells against HEMA-induced toxicity [16]. To explore whether a HEMA-induced activation of Nrf2 would confer similar protection, we pre-incubated the cells for 24 h with 1 mM HEMA prior to a second HEMA exposure. The resulting survival pattern was not significantly different from the pattern of HEMA exposed cells without pretreatment. This indicates that the activation of transcription by Nrf2/ARE is an adaptive response that only partly protects against HEMA-induced stress in BEAS 2B cells. One possible scenario is that cells are protected against toxic byproducts from membrane damage, but not from the membrane damage itself (Fig. 6). The observation of mainly PI-positive cells (permeable membranes) in several studies of HEMA exposed cells support such membrane damage [11,27,48]. In contrast to HEMA, the reported protective role of tBHQ [16] could also involve other Nrf2 independent effects as shown in mouse and human hepatocyte cell lines and cardiomyocytes [49,50].

Overall, our results indicate HEMA to activate the Nrf2-ARE transcriptional pathway and cause increased levels of proteins critical in detoxification and elimination of ROS and electrophiles. Although several studies have indicated mechanisms involving monomer-induced oxidative stress and protection through antioxidants, our results suggest GSH depletion and increased oxidative stress as less important events in BEAS-2B cell response to HEMA. Rather, our results support that HEMA activates the Nrf2-ARE transcriptional pathway by a direct binding to Keap-1. This view is in line with the results of Orimoto et al. [17] who showed that concentrations of HEMA similar to those applied in our study increased ARE activity without reducing intracellular GSH levels. Further supporting involvement of ROS-independent mechanisms, co-treatment with the antioxidant *N*-acetylcysteine did not decrease the activation rate of ARE activity induced by HEMA [17]. Finally, it should be noted that the mechanism behind adaptive and toxic responses to HEMA exposure may vary between cell type investigated and the concentrations of HEMA applied. With this in mind, both ROS-dependent and independent effects should be considered when evaluating the toxic potential of HEMA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dental.2018.11.008.

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