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Title

In vitro effects of low-level aldehyde exposures on human umbilical vein endothelial cells

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Abstract

Tobacco use is associated with heart and respiratory diseases and also with a number of types of cancer. Tobacco smoke contains more than 6000 chemicals and of the most abundant ones are the aldehydes. Aldehydes have been previously shown in *in vitro* studies to induce intracellular oxidative stress and activation of stress signaling pathways, which are associated with cardiovascular disease such as atherosclerosis. Also, aldehydes form one of the toxicant groups recommended for future tobacco product regulation due to its harmful effect. However, the *in vitro* effect of low levels aldehyde exposure has not been established. In this study, we determined the gene expression effects of aldehydes commonly found in tobacco smoke by exposing *in vitro* human umbilical vein epithelial cells (HUVEC). The most relevant aldehydes are used: formaldehyde, acetaldehyde, acrolein, propionaldehyde, crotonaldehyde and butyraldehyde. Sub-cytotoxic exposure levels of the different aldehydes were tested regarding cell proliferation, gene expression changes, oxidative stress responses, and DNA damage. Genes associated with cardiovascular disease development such as DEPP, ARID5B, DKK1, EGR1 and IER3 were found to be dysregulated. Gene expression responses were not related to measurements of oxidative stress or DNA damage using comet assay. These findings suggest that the exposure of low-level aldehydes from tobacco smoke needs to be controlled due to its effect on genes associated with cardiovascular disease.

Introduction

Aldehydes form one of the major classes of chemicals produced by tobacco combustion. Cigarette smoke is the main source of aldehydes, which are generated from thermal decomposition during the burning or smoking process of cigarettes [1]. Other sources of aldehyde can be found in environmental air, cooking oils, paint, and furniture or are formed endogenously through lipid peroxidation[2-4]. As aldehydes are so ubiquitous, it is necessary to manage aldehyde exposure especially in situation where it's preventable. During the production process of cigarettes glycols and sugars are added to tobacco, possibly increasing the amounts of aldehydes produced during tobacco burning[4-6]. The concentration of aldehydes in smoke of cigarettes ranges between 1.32 to 113.82 μM[7, 8-9] . Acrolein, formaldehyde, and acetaldehyde form the major bulk of the aldehyde presence in cigarette smoke, followed by crotonaldehyde, propionaldehyde and butyraldehyde [8]. Interest in the level of aldehydes and its effects on the human body has been mounting since the World Health Organisation Framework Convention on Tobacco Control (WHO FCTC) guidelines has expressed its intention to include aldehydes in the regulation of tobacco products [10]. Aldehydes are reactive compounds and can easily form adducts [11] with cellular protein and DNA causing toxicity to cells. Reactive aldehydes such as acrolein and crotonaldehyde, which are strong electrophiles, capable of binding to proteins causing vascular lesions such as atherosclerotic lesions [12] and oxidative stress [13]. Major endpoints of tobacco smoking are lung cancer and lung disease such as chronic obstructive pulmonary disease (COPD). Previously, gene expression profiling on A549 lung epithelial cells [14] found evidence that gene expression responses to aldehydes are primarily indicative for genotoxicity and oxidative stress. These toxicity mechanisms are linked to lung cancer and COPD, respectively. Besides these lung diseases, tobacco use has also been associated with cardiovascular disease such as atherosclerosis [15 16-18], hypertension [19] and cardiopulmonary dysfunction [20]. Aldehydes, present in abundance in tobacco smoke, have been linked to the development of these diseases. Therefore, we set out to examine gene expression responses to tobacco smoke aldehydes in a cell line representing the cardiovascular system.

Most published studies on *in vitro* responses are conducted on either high concentration of aldehydes [21, 22] or long duration i.e. more than 10 hours [23] or both [24-27]. In the case of tobacco smoke exposure, however, it occurs as repeated acute (short-term) exposures, i.e. every time when a smoker inhales.

It is thus worthwhile to study the chemical effects of smoke aldehydes *in vitro* to cells in an acute exposure situation at physiologically relevant levels i.e. a concentration where it is not yet cytotoxic to the cells.

To enable us to study the effects of aldehyde exposure we performed *in vitro* gene expression profiling on an endothelial cell line. Endothelial dysfunction is associated with inflammation and atherosclerosis[28, 29]. We exposed human umbilical vein endothelial cells (HUVEC) to aldehydes at sub-cytotoxic levels and gene expression analysis was carried out to determine their responses.

In a previous study using the A549 lung cell line, we found evidence for genotoxicity and oxidative stress. However, we found differences across the A549 responses to the three most abundant aldehydes (acrolein, formaldehyde, acetaldehyde) in that formaldehyde mainly induced gene expression changes related to genotoxicity whereas the response to acrolein was mainly associated with oxidative stress. To improve our understanding, we included additional tobacco smoke aldehydes (crotonaldehyde, propionaldehyde and butyraldehyde) in our attempt to gather further information on potential differences and similarities among aldehydes in the HUVEC responses.

Materials and Methods

Materials

HUVEC (Product Code C-003-5C) were purchased from Cascade Biologics. The following chemicals were purchased from Sigma-Aldrich: Acrolein, Formaldehyde, Acetaldehyde, Butyraldehyde, Propionaldehyde and Crotonaldehyde. Dulbecco's Phosphated Buffered Saline (DPBS) [-]$CaCl_2$ and [-]$MgCl_2$, 0.05% Trypsin-EDTA, Fungizone, Penicillin and Streptomycin (with 5000 Units/mL Penicillin and 5000 µg/mL Streptomycin) were purchased from Gibco/Invitrogen; Medium 200 and LSGS (Low Serum Growth Supplement) were purchased from Cascade Biologics. Trypan Blue Stain 0.4% Membrane filtered, prepared in 0.85% saline was from Invitrogen Corporation. Cell Proliferation Reagent (WST-1) was from Roche Diagnostics GmbH (Mannheim, Germany). Each chemical was prepared first in phosphated buffered saline solution followed by dilution to the desired concentration with complete medium.

Cell culture and exposure levels

Complete medium consisted of Medium 200 supplemented with 2% LSGS, Fungizone (2.5 µg/mL) and 1% Penicillin and Streptomycin antibiotics. HUVEC were grown in the prepared complete medium. Cells were kept in a humidified atmosphere at 37 ˚C and 5% CO_2. Upon reaching approximately 80% confluence, cells were passaged using trypsin solution. Cells were passaged twice a week. Cells were seeded in a flat bottom 96-wells plate at a concentration of 1.5 x 10^4 cells/well with complete medium for 24 h before exposure to chemicals. Passages 8 to 12 were used in this whole study.

The cell proliferation after exposure to the 7 chemicals was evaluated using the WST-1 assay. Cell proliferation was assessed at three time-points (1, 2, 4h) by incubating HUVEC with increasing concentrations of each aldehyde.

The dose range was chosen around the corresponding inhaled concentrations based on intense smoking regime collected from a smoking machine as previously described [14]. Compound testing concentration ranges were selected with half-log steps between doses.
The concentrations of each chemical that resulted in 20% loss in cell proliferation (CV80) were determined using PROAST software (www.rivm.nl/proast) [30]. For all calculations, we used at least three independent experiments with four replicate measurement wells per dose per experiment.

Cell exposure concentrations for microarray analysis were based on these CV80 concentrations at up to 4 h except where they exceeded 1 mM. In the latter case the concentration was not considered physiologically relevant and a concentration of 1 mM was used for microarray profiling. The same conditions were used for HMOX1 and Comet assays.

Microarray analysis

Based on the cell proliferation ~~viability~~ studies, aldehyde exposure concentrations in gene expression study were as follows: Formaldehyde 4.016 µM, Acrolein 0.534 µM, Crotonaldehyde 5.206 µM, Acetaldehyde 1000 µM, Butyraldehyde 1000 µM, Propionaldehyde 1000 µM. The concentrations of each aldehyde that resulted in 20% loss in cell proliferation study, ~~viability~~ were determined using PROAST software (www.rivm.nl/proast).[30]. CV80 concentrations for transcriptomics experiments were calculated for each compound on the basis of the model curve fit. For all calculations, we used at least three independent experiments with four replicate measurement wells per dose per experiment.

Cells were exposed to compounds or PBS vehicle for 2 h. After that, cells were detached from 12-well plates with RNA protect buffer. RNA protect buffer (Qiagen, Venlo, the Netherlands) was added to the cells. Cells were homogenized and total RNA was extracted using the RNeasy kit (Qiagen). RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA quality was determined using automated gel electrophoresis (Bioanalyzer 2100; Agilent Technologies, Amstelveen, the Netherlands). All RNA samples passed RNA integrity number QC, having RIN values > 7.

Gene expression profiling was performed at the MicroArray Department of the University of Amsterdam, the Netherlands, using the same procedures as for our previous study [14]. Test samples were labeled with Cy3 and a Reference sample (made by pooling equimolar amounts of RNA from test samples) was labeled with Cy5, followed by hybridisation to Nimblegen 12 x 135 k Homo sapiens HG18 microarrays. Each microarray corresponded to labeled RNA from one individual sample.

Raw microarray signal data were normalized in R statistical software (www.r-project.org), as described previously [14 31]. Normalized data for the resulting 45,033 probes were further analyzed in R and Microsoft Excel.

Regulated genes for the various compounds were identified by using a one-way ANOVA. Obtained p-values were corrected for multiple testing by calculating the false discovery rate (FDR). Probes with a false discovery rate <5% and absolute ratio >1.5 (between compound and vehicle control) were considered significant. When multiple

probes corresponding to the same gene were significant, their data were averaged to remove redundancy in further analysis.

Similarities across compound gene expression profiles were determined using the cosine correlation coefficients across the set of regulated genes. This type of correlation coefficient looks at overall similarities between gene expression fold changes patterns that are normalized against their overall effect size compared to the non-treated (i.e. PBS) exposure.

Functional and Gene Ontology (GO) term Annotation were determined using the DAVID web tool (http://david.abcc.ncifcrf.gov) [32] in combination with CoPub text mining (http://services. nbic.nl/cgi-bin/copub/CoPub.pl) [33] and the Comparative Toxicogenomics Database (http://www.ctdbase.org) [34].

Determination of HMOX1 response

Heme oxgyenase-1 (HMOX1) is an antioxidant enzyme, which plays an important role in the protection of cells against oxidative stress due to chemical insult. We examined the effects of low-level aldehyde exposure on HMOX1 expression in HUVECs at 2-hour exposure. The exposure for the HMOX1 determination is performed in 12- well plates based on the 2-hour cell proliferation concentrations of the aldehydes. After exposure, cells were washed in PBS, followed by incubation with PBS+0.5% Triton-X100 for 10 min at 0 °C. To measure HMOX1 in the cell lysates, the DuoSet IC ELISA kit from R&D Systems (USA) was used. For the determination of the amount HMOX1 in the cell lysates, measurement of the total protein is needed. This was measured using the BCA Protein Assay Kit (Pierce Biotechnology). The HMOX1 determination is prepared in accordance to the supplier's protocol. One-way ANOVA was used for the analysis of HMOX1 results.

Comet Assay

The method used for the modified comet assay was described earlier[35]. Briefly, the alkaline comet assay was conducted under normal conditions (without pre-incubation with Fpg) and with a pre-incubation with Fpg. The normal comet assay measures DNA strand breaks and alkali labile sites. In the Fpg comet assay, kindly provided by Gunnar Brunborg (NIPH, Oslo, Norway), samples were treated with the enzyme Fpg, which is a glycosylase that recognizes oxidatively damaged DNA (especially 8-oxo-dG and ring-opened formamidopyrimidine lesions) and cuts the DNA at these sites. This will increase the overall number of DNA fragments, and the difference between these 2 measurements (i.e. with *vs* without Fpg) is usually used as an indication for oxidative stress induced DNA lesions. However, this does not hold for aldehydes, because aldehydes are able to form DNA-protein crosslinks. These crosslinks inhibit the migration of DNA in the COMET assay and therefore a smaller difference between the 2 measurements (with *vs* without Fpg) is indicative for more crosslinks. Taken together, the modified comet assay (computed using gene expression data) can therefore provide different kinds of data on aldehyde effects on DNA. Comet assay data were compared to gene expression responses by determining the mutual correlation between genes and comet assay parameters. Additionally, comet assay data were compared between the various exposures using a one-way ANOVA.

Results

Cytotoxic effects of aldehydes on HUVEC

HUVEC were exposed to a range of aldehyde concentrations for 1, 2 , and 4 h. For each of the aldehyde exposures, at the different time points, concentrations were determined that led to a 20% loss in cell proliferation study, as described in the Materials and Methods section. These CV80 concentrations are given in Table 1 and Figure 1. When taken together, 4 h seems to be the most sensitive time point regarding cytotoxicity. To examine the gene expression responses in the period leading up to this time point, the 2 h time point was chosen for microarray gene expression analysis.

Gene expression responses

To determine HUVEC gene expression responses for the various aldehyde exposures, RNA isolated from exposed cells was used for microarray analysis. Using the same statistical stringencies as used in our A549 study [14] (FDR 5% and absolute ratio to PBS control > 1.5) we found 17 genes differentially expressed by at least one aldehyde (Table 2).

The magnitudes of the response differed across the various aldehydes but were most pronounced for propionaldehyde and butyraldehyde. Overall responses to aldehydes differed in (quantitative) magnitude, but were mostly similar in their (qualitative) nature. This is also illustrated by the cosine correlations, which is a measure on overall similarity of the compound responses across the dysregulated genes, with a value of 1.0 (exactly similar) to 0 (no similarity), Correlation values between the compound profiles which were all > 0.5 (Table 3) indicating there is a positive similarity across the affected genes by each aldehyde. Correlation values were especially high for propionaldehyde and butyraldehyde. These findings all points towards common transcriptomic responses of HUVEC to the different aldehydes.

In general, a total of 17 genes were differentially expressed at this short term 2 hour exposure at sub-cytotoxic level, with 13 genes up-regulated and 4 genes down-regulated (Table 2 and Figure 2). Among the regulated genes, two genes showed consistent down-regulation for all aldehyde exposures, namely CXXC5 and SMAD5. Several other genes showed consistent up-regulation (Table 2), most notably C10ORF10 (DEPP), LRIG3, and PNRC1. C10ORF10 (DEPP) gene is the most significantly affected gene with 2.66- and 4.05-fold upregulation after exposure to propionaldehyde and butyraldehyde, respectively.

Functional annotation analysis found no evidence for a significant functional enrichment based on GO terms or other databases. However, literature search and CoPub text mining analysis gave evidence that many of the genes are associated with cardiovascular disease (DEPP, ARID5B, DKK1, EGR1, IER3) and stress response (ERFI1, SMAD6, GADD45A) as well as pathways associated with these processes.

Contrary to our expectations, we did not find a significant effect on the heme oxygenase gene, although aldehyde exposure is associated with generation of oxidative stress and HMOX1 was found to be induced by acrolein in A549 cells [14, 36, 37]. Gene expression ratios for HMOX1 were mostly subtle up to 1.091 (acetaldehyde), with the notable exception of crotonaldehyde, which gave a 2.019 fold induction of HMOX1 expression at 2 h exposure. However, it should be pointed out that this induction did not meet the statistical significance threshold of 5% FDR.

HMOX1

To examine oxidative stress responses in more detail, we determined HMOX1 protein levels upon exposure for 2-h timepoint at levels used for gene expression analysis. HMOX1 levels to the 7 aldehydes studied are given in Figure 3. A one-way ANOVA comparison between these groups have a p-value of 0.015, however, in a post-hoc comparison (Dunnett's test, Bonferroni correction) none of the aldehydes showed a significant difference when compared to the control ($p > 0.1$). Crotonaldehyde was found to affect HMOX levels, followed by butyraldehyde. We found both crotonaldehyde (at 5.2 μM and 2-hour exposure) and butyraldehyde (at 1000 μM), at 2-hour exposure, to increase the level of HMOX1 in HUVEC by 50% and 15% respectively, although this was not statistically significant ($P > 0.1$). The other 4 aldehydes did not show any changes in the expression of HMOX1. The results suggest that formaldehyde and acrolein, although present at levels that weakly affect cell proliferation, are not potent enough to induce elevated levels of HMOX1. Overall, under the present exposure conditions, no significant increase in the cellular oxidative response as measured with HMOX1 was observed.

Comet Assay

In the comet assay experiment, we found less pronounced effects compared to gene expression results (Table 4). For each of the four parameters given in Table 4, a one-way ANOVA comparison across the seven exposures results in a p-value > 0.4. The Fpg comet assay assesses a combined effect of oxidative stress (*i.e.* strand breaks and alkali labile sites that increase migration of DNA in the COMET-assay) and DNA-crosslinks (leading to decreased migration). Since we found no significant oxidative stress related gene-expression changes and Fpg

treated samples that were exposed to aldehydes generally had lower percentage tail-DNA than control cells, we assume that the observed differences are mainly due to aldehydes forming DNA-DNA or DNA-protein crosslinks. The results showed that the DNA damage can be ranked as (smallest to largest damage): propionaldehyde < crotonaldehyde < (formaldehyde, acrolein) < butyraldehyde < acetaldehyde. It is interesting to find the effect of 4.106 μM formaldehyde has the same DNA damage extend to that of acrolein which is at a concentration about 7.6 times lower at 0.534 μM. Similarly, crotonaldehyde, which has the concentration at 5.206 μM, was found to have a stronger effect than propionaldehyde at 1000 μM.

To help understand this finding, we determined the correlations between gene expression and the comet assay difference between samples with and without treatment with FpG (potentially representing crosslinks). Among genes that correlated negatively ($R < -0.8$, $P = 0.031$) with the comet assay difference, we found enrichment for apoptosis and cytoskeleton/kinetochore-associated genes. These findings suggest that the cells are delayed at the cell cycle checkpoint, leading to cell cycle arrest and/or apoptosis.

Discussion

Aldehydes form one of the major groups of chemicals found in cigarette smoke, and accumulating evidence shows its role in the development of tobacco related diseases. To broaden our knowledge on the effects of aldehydes on the cardiovascular system, we exposed human endothelial cells to the most common aldehydes present in tobacco smoke and compared the effect on gene expression level as well as other endpoints.

In the gene expression results, several of the genes found in this study have been associated with cardiovascular disease development, for example, DEPP, ARID5B, DKK1, EGR1 and IER3. C10ORF10 (or DEPP) is expressed in peripheral vascular endothelial cells and the gene was reported as upregulated in neo-vascularization and tumor angiogenesis settings[38]. We found here that propionaldehyde and butyraldehyde significantly upregulated DEPP. Interestingly, this gene is also induced by the other aldehydes in this study, and is one of the most strongly responding genes. This indicates that further investigation into this gene and its response to compound exposure may be warranted to gain understanding of its functionality.

EGR1 functions as transcriptional regulator that responds to a number of stimuli. In a study on cardiovascular damage after ischemia/reperfusion, EGR1 was found to be overexpressed in myocardial tissues (both *in vivo* and *in vitro*) and to be downregulated by cardioprotective calcium channel blockers, indicating a mechanistic role in cardiovascular damage[39]. Other studies found that EGR1 contributes to Ang-1-induced endothelial cell migration and proliferation [40] and that cigarette smoking induces vascular proliferative disease through the activation of Egr1 [41, 42]. Also, EGR1 is involved in vascular cell proliferation[43]. Additionally, EGR1 is upregulated in the thrombus-covered wall of human abdominal aortic aneurysm and contributes to the thrombogenic and inflammatory pathogenesis involved[43, 44].

Pyruvate dehydrogenase kinase-4 (PDK4) is responsible for the regulation of acetyl-CoA where glucose is converted to fatty acids or amino acids. We found upregulation of PDK4 gene in HUVEC upon exposure to butyraldehyde and (albeit not significant) to most other aldehydes. Overexpression of PDK4 is reported in the heart and other tissues of diabetic rodents[45]. Work by Zhao et al [46] found that overexpression of PDK4 is sufficient to cause metabolic imbalances and provoke cardiomyopathy as a result of stress induced pathways.

Gene Ontology annotations indicate that EFNA1 and EFNB2 are tyrosine kinases involved in angiogenesis and cardiac development.

For some genes found dysregulated in our study, a functional connection to cardiovascular disease was found by means of genetic studies.

ARID5B is highly expressed in the cardiovascular system and a study on a Japanese population, found association between genetic variation of ARID5B and susceptibility to coronary atherosclerosis[47].

Other genetic studies have found an association between LRIG3 and risk of incident heart failure in adults of European and African ancestry [48] and between genetic variation of SMAD6 and congenital cardiovascular malfunction[49].

Besides genes involved in cardiovascular (dys)function or development, we found regulation of several genes for which Gene Ontology or text mining indicated a role in stress response, such as ERRFI1, which was found to be upregulated during chronic stress [50, 51]. In addition to this, a gene associated with DNA damage signaling and apoptosis, [52] CXXC5 was significantly upregulated. CXXC5 was found to be dysregulated in butryaldehyde exposure in HUVEC cell lines. The function of this CXXC5 (CXXC finger 5 (CF5)) is not well known but in a recent study, CXXC5 gene has been identified as a critical component in the DNA damage-signaling pathway [52]. Other genes of similar functions, such as GADD45A and IER3 were found to be upregulated rather significantly by butyraldehyde. Several studies found that GADD45A plays an important role in control of cell-cycle checkpoint, DNA repair process and signaling transduction, and DNA damaging agents induces its expression [53, 54]. Upregulation of GADD45A has found to contribute to stress response effect that causes cardiomyopathy in diabetic condition [55] and dysregulated blood pressure in pregnant women[56].

Immediate early response gene 3, IER3 is reported to be involved in various cellular functions and have a role in the physiology of the cardiovascular system. In the hearts from pressure overloaded mice due to aortic constriction, IER3 expression was elevated in the strained myocardium [57], similar to the strain induced IER3 expression in cultured cardiomyocytes [58]. Previous studies by Lee et al [59] and Jeong et al [26] which treated HUVEC with acrolein at 10 μM and 25 μM for 1 hour and 6 hours and crotonaldehyde at 10 μM for 2 and 12 hours found a strong and significant effect on cardiovascular genes. This "no-observed-effect level" as found in this study, may be due to short exposure time coupled with relatively low sub-cytotoxic levels of acrolein and crotonaldehyde exposure (at 0.534 μM and 5.206 μM respectively). This shows that at low level and short exposure interval, there is not yet a detrimental effect on the cells, although this may not be the case if the low level of exposure is repeated over a prolonged period of time through continuous smoking or exposure to this aldehyde.

Earlier studies on HUVEC found evidence of oxidative stress when exposed to acrolein for 0.5 to 1.0 hours above 1 μM[60, 61] and that this effect is restored after 24 hours. Another study [62] found similar effects by crotonaldehyde, another α, β-unsaturated aldehyde, which lead the authors to conclude that HUVEC is capable of maintaining the balance of the redox status via an antioxidant enzymatic process as an adaptive response towards oxidative stress. In our study, perhaps due to shorter exposure or lower dose or both, we did not find significant effects on oxidative stress. Additionally, we did not observe significant effects on several other genes or pathways that might have been expected to respond to aldehyde exposure. These include adhesion molecules and other genes involved in

endothelial function; inflammatory response genes such as cytokines; as well as genes involved in aldehyde metabolism (such as aldehyde dehydrogenases or aldo-keto reductases). For these pathways, the short exposure time and low dose are probably reasons why no significant regulation was found. However, whereas these findings were contrary to our expectations, it is interesting that, under these conditions, we did find effects on gene expression indicating perturbed cardiovascular functionality. These changes warrant further study to determine their relevance for tobacco smoke regulation.

The extent of DNA damage caused by the various aldehyde exposures was assessed using the comet assay [63]. Aldehydes are able to form strand breaks and protein-crosslinks, which may inhibit the migration of DNA in the comet assay; a smaller difference in percentage DNA in the tail between the measurements with Fpg *versus* without Fpg in aldehyde treated cells, when compared to the control sample, is indicative for possible crosslinks caused by these aldehydes. DNA damage was found upon exposure to (in increasing order) formaldehyde, acrolein, crotonaldehyde and propionaldehyde at the onset of cytotoxic (CV 80) concentrations at a short 2-hour exposure. Within the context of the present study, there is only limited DNA damage found in the comet assay. Similarly as for oxidative stress, this can probably be attributed to short exposure time and exposure at the onset of cytotoxic concentration. At higher level of exposure, DNA strand breaks and DNA protein cross links effects were reported on acrolein at 1-hour exposure of above 3μM [64], at mM level for formaldehyde, acrolein and crotonaldehyde [65] and above 100mM for acetaldehyde, propioaldehyde and butyraldehyde [66].

Overall, however, we found no correlation between aldehyde chain length, unsaturated bonds, or concentration, and the degree of DNA crosslinking, nor with gene expression responses of the genes that were specifically found for all aldehydes. Most notably, the two aldehydes that gave the most pronounced gene expression responses (propionaldehyde and butyraldehyde) give the most divergent comet assay results. When correlating gene expression changes with the amount of damage, we find evidence for cell cycle arrest and/or apoptosis for those genes that correlate negatively with the comet assay data. Taken together, the combined comet assay data show limited signs of DNA damage for the exposures used in this study, and the gene expression changes as described in Table 2 are of a different nature than those involving genotoxicity.

Conclusions

To summarize, we found that various aldehydes give qualitatively similar gene expression responses in genes associated with cardiovascular dysfunction. Therefore, we conclude that under the conditions used in this study (relatively short exposures to sub-cytotoxic levels), the responses in HUVEC indicate that aldehydes might influence processes underlying cardiovascular risks. These gene expression responses occur at exposures at which oxidative stress and genotoxicity data do not occur at a statistically significant level. Although a limitation of this study is that only one time-point was studied and not at continuous exposure, this study indicates that exposure to aldehydes, in sub-cytotoxic amount, for instance through smoking, may contribute to the development of vascular diseases. Further work needs to be carried out to understand the continuous exposure of aldehyde *in vivo* (human) related to DNA damage and various toxicological effects in the human biological system.

Conflict of Interest

The authors declare that they have no conflict of interest. The content is solely the responsibility of the authors and does not necessarily represent the views of National Institute of Public Health, The Netherlands (RIVM), Netherlands Food and Consumer Product Safety Authority (NVWA) or Health Sciences Authority (HSA), Singapore.

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Table 1: Calculated concentration (in μM) corresponding to 80% cell proliferation study for the 3 time points studied.

Time Point (hour)	1	2	4
Formaldehyde	61.7	6.8	4.0
Acetaldehyde	> 1000	> 1000	> 1000
Acrolein	12.8	7.3	0.53
Propionaldehyde	> 1000	> 1000	> 1000
Crotonaldehyde	19.4	9.5	5.2
Butyraldehyde	> 1000	> 1000	> 1000

Table 2: Ratios to control for significantly regulated genes by at least one compound*

	form	acet	acro	prop	croton	butyr
ARID5B	0.930	1.040	0.942	1.142	1.052	1.631
C10ORF10	1.264	1.158	1.101	2.668	1.167	4.053
CXXC5	0.876	0.932	0.937	0.806	0.834	0.634
DKK1	1.089	1.105	1.009	0.910	1.138	0.533
EFNA1	0.996	1.063	0.981	1.253	0.984	1.543
EFNB2	1.017	1.024	1.012	1.361	1.049	2.021
EGR1	1.052	1.006	1.054	1.236	1.030	1.513
ERRFI1	1.016	0.919	0.998	1.130	1.039	1.659
FAM13A	1.076	1.104	1.004	1.320	1.081	1.681
GADD45A	1.020	1.036	0.957	1.335	1.034	1.933
GBP1	1.004	1.033	1.050	1.321	1.160	1.756
IER3	0.946	1.041	1.039	1.331	1.008	1.715
LRIG3	1.177	1.196	1.097	1.313	1.182	1.535
PDK4	1.074	1.015	1.001	1.340	1.141	1.603
PNRC1	1.028	1.113	1.111	1.431	1.182	1.615
RIMBP3	1.083	1.061	1.088	0.915	0.951	0.662
SMAD6	0.825	0.928	0.934	0.772	0.866	0.637

*Affected genes have more than ±1.50 fold change (i.e. > 1.5 or < 0.67) after 2 hour exposure of HUVEC to the aldehydes relative to PBS as vehicle control.

Table 3: Overall correlation analysis (using the cosine correlation approach) among the aldehydes, which is showing the extent of similarity among the regulated genes for the various exposures

aldehydes	form	acet	acro	prop	croton	butyr
form	1	0.666	0.668	0.616	0.677	0.459
acet		1	0.683	0.700	0.718	0.542
acrolein			1	0.599	0.677	0.452
prop				1	0.742	0.950
croton					1	0.672
butyr						1

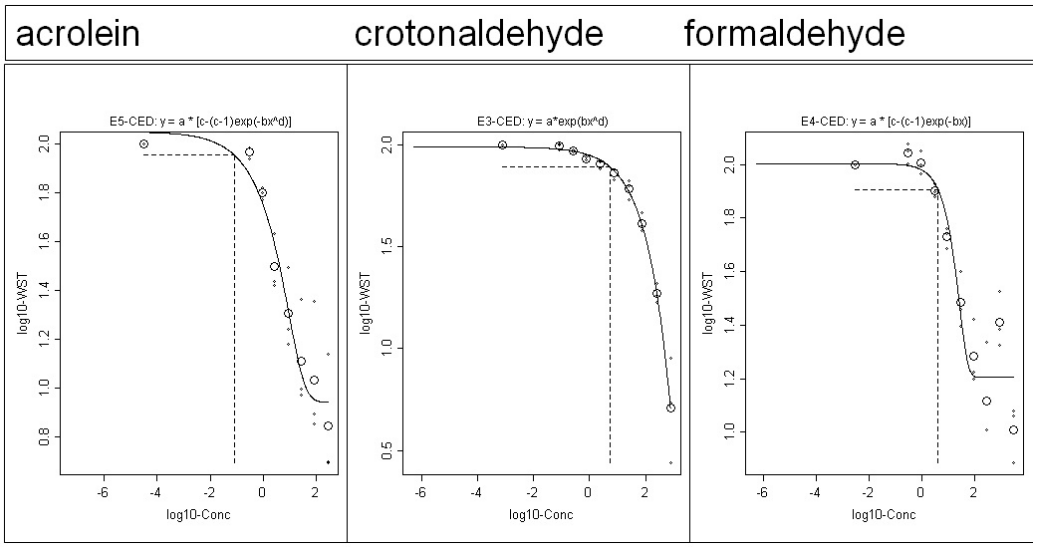
Table 4: HUVEC analyzed with Comet Assay performed with and without pretreatment with Fpg. Cells were exposed for 2 hours at the concentrations used for microarray, relative to DMSO as control.

Chemical	Concentration µM	Average (%DNA in Tail)			
		Without Fpg	With Fpg	Difference (with Fpg minus without Fpg)	Difference vs Control (± SD)
Control	0.00	0.94 ± 0.46*@	3.24 ± 0.18	2.30 ± 0.49	0 (± 0.69)
Formaldehyde	4.00	0.79 ±0.47	2.71 ± 0.19	1.92 ± 0.51	-0.38 (± 0.71)
Acetaldehyde	1000	0.79 ±0.13	1.57 ± 0.17	0.78 ± 0.21	+1.52 (± 0.53)
Acrolein	0.53	1.33 ± 0.22	3.30 ± 1.42	1.97 ± 1.43	-0.33 (± 1.51)
Propionaldehyde	1000	0.60 ± 0.01	3.35 ± 0.98	2.75 ± 0.98	+0.45 (±1.10)
Crotonaldehyde	5.20	1.03 ± 0.09	3.09 ± 0.10	2.06 ± 0.13	-0.24 (± 0.51)
Butyraldehyde	1000	1.51 ± 0.36	2.34 ± 0.01	0.83 ± 0.36	-1.47 (± 0.61)

*%DNA in the tail
@Mean of n=2 analyses

Figures

Figure 1: Cell proliferation and CV80 determination of acrolein, crotonaldehyde and formaldehyde exposure during 4 h:



The three graphs represent concentration dependent effect on cell proliferation (CV80) of acrolein, crotonaldehyde and formaldehyde determined by WST-1 cytotoxicity assay at 4 hour time point on HUVEC.

Figure 2: Heatmap of gene expression responses of aldehyde exposed compared to PBS control

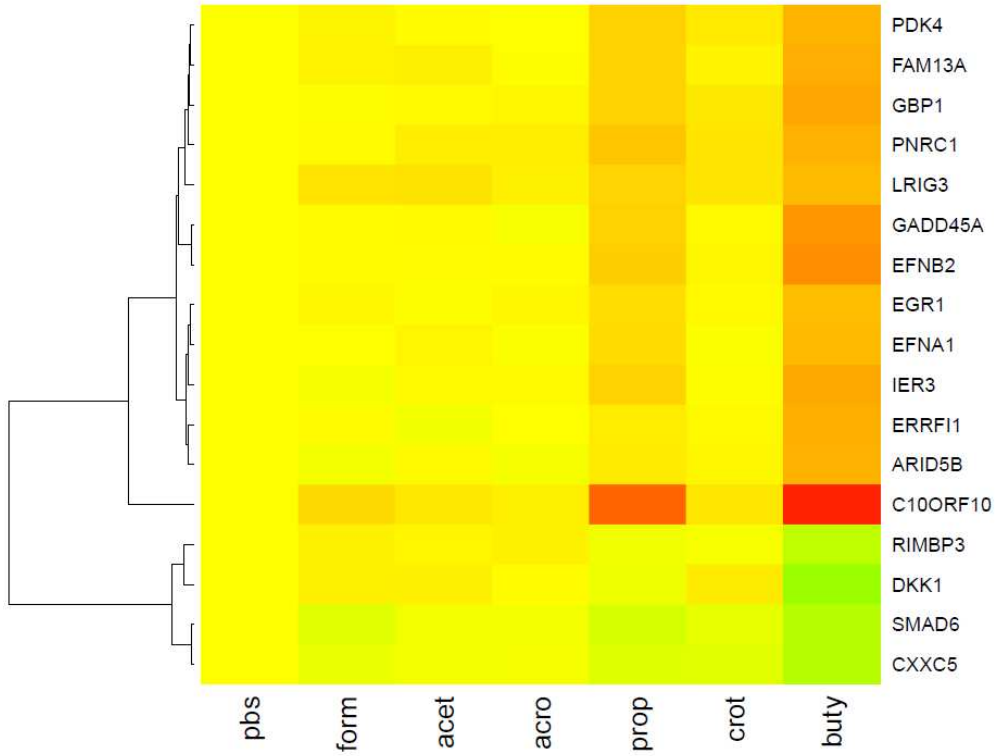


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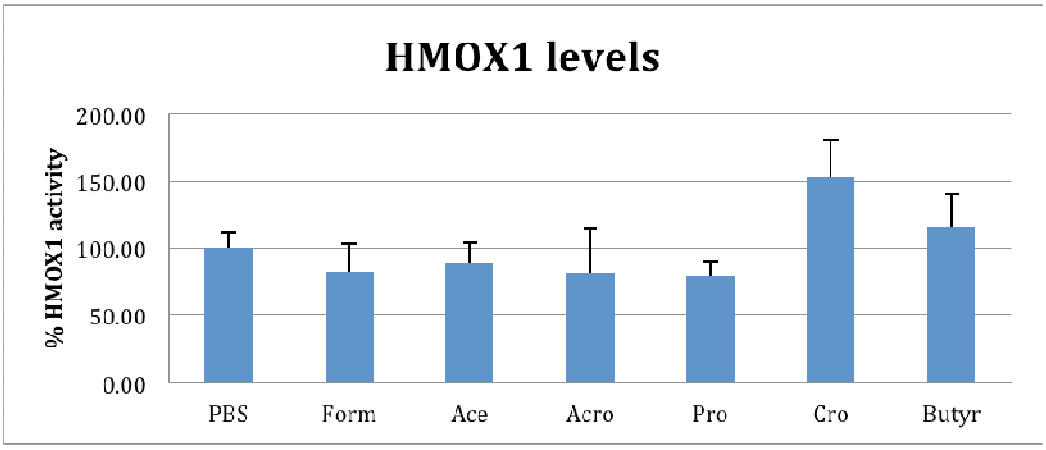
Figure 3: HMOX1 protein levels after 2 hour exposure of the six aldehydes at the concentrations used for microarray, calculated as percentage relative to PBS vehiculum as control



*Mean value is presented with n=3. No significant differences are observed (P>0.1).