TIME- AND CONCENTRATION-DEPENDENT EFFECTS OF CIGARETTE SMOKE ON MONOCYTIC CELL TRANSCRIPTOME

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Introduction

Atherosclerosis is a chronic inflammatory process characterized by a series of biological processes resulting in plaque formation in the vascular wall. Atherosclerotic plaques develop slowly over time, progressing from fatty streaks to complicated lesions, which can lead to life-threatening clinical events such as myocardial infarction and stroke. Numerous studies indicate that the monocyte/macrophage might be an important key player in the initiation and progression of plaque formation [**1**]. It has been shown that exposure to cigarette smoke condensate increases the adhesion of monocytes to endothelial cells *in vitro*, a critical step in the initiation of atherosclerosis [2].

The purpose of this study was to investigate the transcriptional response of monocytic cells exposed to cigarette smoke (CS) over concentration and time.

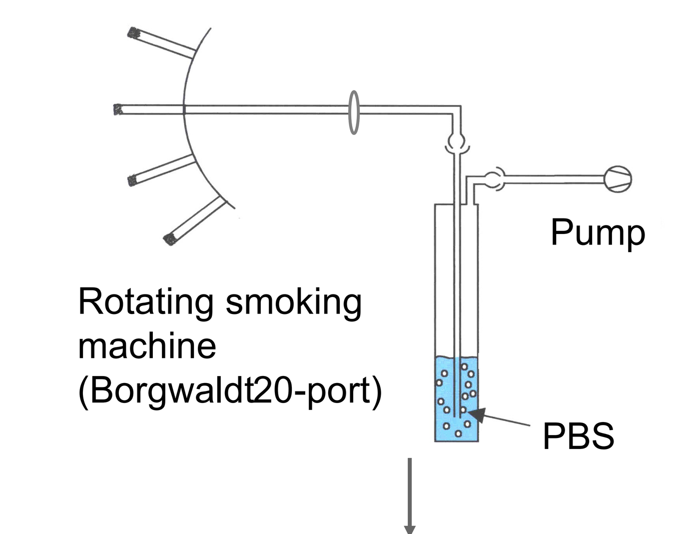
Keywords

Mono Mac 6 cells (MM6): Human monocytic cell line with several features of mature blood monocytes; e.g. CD14 antigen expression, phagocytotic ability, and functional ability to produce cytokines. These cells are often used as an *in vitro* model to demonstrate the actions of monocytes.

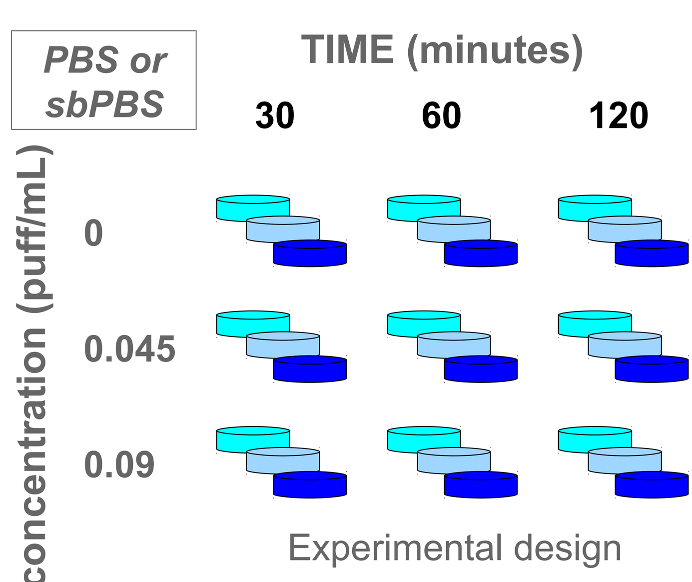
Atherosclerosis: A progressive vascular disease characterized by accumulation of cholesterol in the intima of large arteries. The primary drivers affecting rates of atherosclerotic plaque progression include enhanced cholesterol influx to the vessel wall and accumulation and retention of lipid in the intima due to local and systemic inflammation.

Methods

A- Preparation of smoke-bubbled (sb) PBS



B- In Vitro assay with MM6 cells



MM6 cells

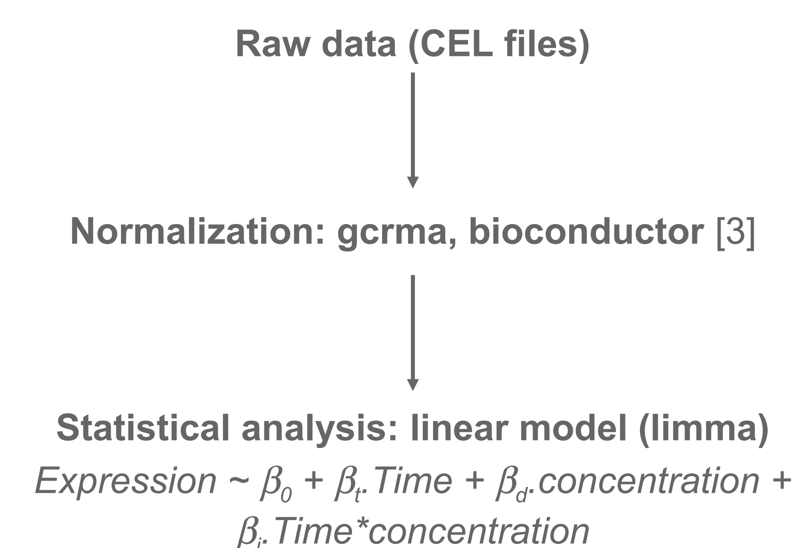
RNA extraction and processing

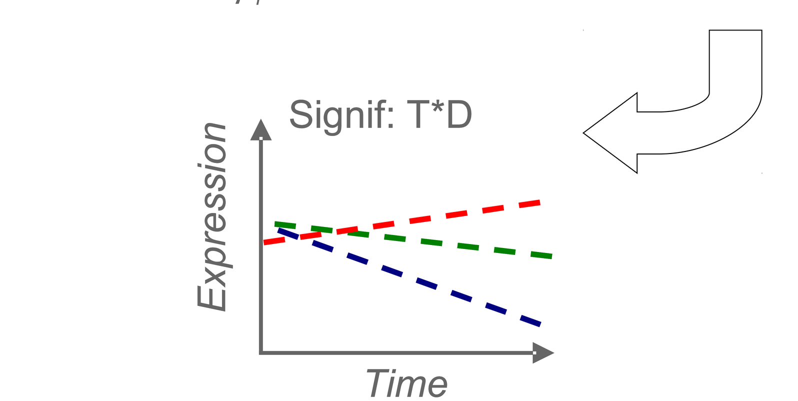
C- Generation of Microarray

Human Genome U133 Plus 2.0 Affymetrix® chip

Figure 1. *Description of the overall experiment.* Human Mono Mac 6 cells were treated with different concentrations (0, 0.045 and 0.09 puff/ml) of CS that had been bubbled through phosphate buffered saline (PBS) or with control PBS (A) for 30, 60, and 120 minutes (B). At each time point, cells were harvested and RNA from different samples was extracted for further transcriptomic analysis using the Human Genome U133 Plus 2.0 Affymetrix® gene expression platform (C). The different blue colors represent 3 independent replications of the same experiment. In total, 27 arrays were performed.

A- Microarray Data Analysis





B- Functional analysis

- Ingenuity Pathway Analysis (IPA) software [4]
- DAVID database system [5]

Figure 2. *Transcriptomic data analysis.* The data analysis (A) was conducted to identify genes the expression of which is significantly regulated in a time- and concentration-dependent manner by CS. During the statistical analysis step, a linear model was defined and applied on gcrma (GC-Robust Multiarray Averaging)- normalized gene expression matrix using the limma package from the bioconductor [3,6]. Probe sets with a false discovery rate (FDR; Benjamini Hochberg method) $\leq$ 0.01 associated to the β_i coefficient (unit: minute.puff.mL^{-1}; interaction) were selected as significantly time- and concentration-dependent regulated genes as shown in the above figure example. A canonical pathway/ biological function over-representation analysis (B) of the list of genes modulated by CS was conducted using public and commercial softwares to provide a biological interpretation of CS effect on MM6 cells.

Results

A

Name	P-values	Adjusted P-values	Number of genes
Top pathways in IPA			
Oxidative Stress Reponse Mediated by Nrf2	9.0E-04		12
VDR/RXR Activation	1.7E-03		7
Hormone Receptor Regulated Cholesterol Metabolism	1.3E-02		2
p53 Signaling	1.4E-02		6
Anti-Apoptosis	3.2E-02		3
Top KEGG pathways in DAVID			
p53 Signaling	2.4E-03	2.1E-01	7
Prostate cancer	9.0E-03	3.6E-01	7

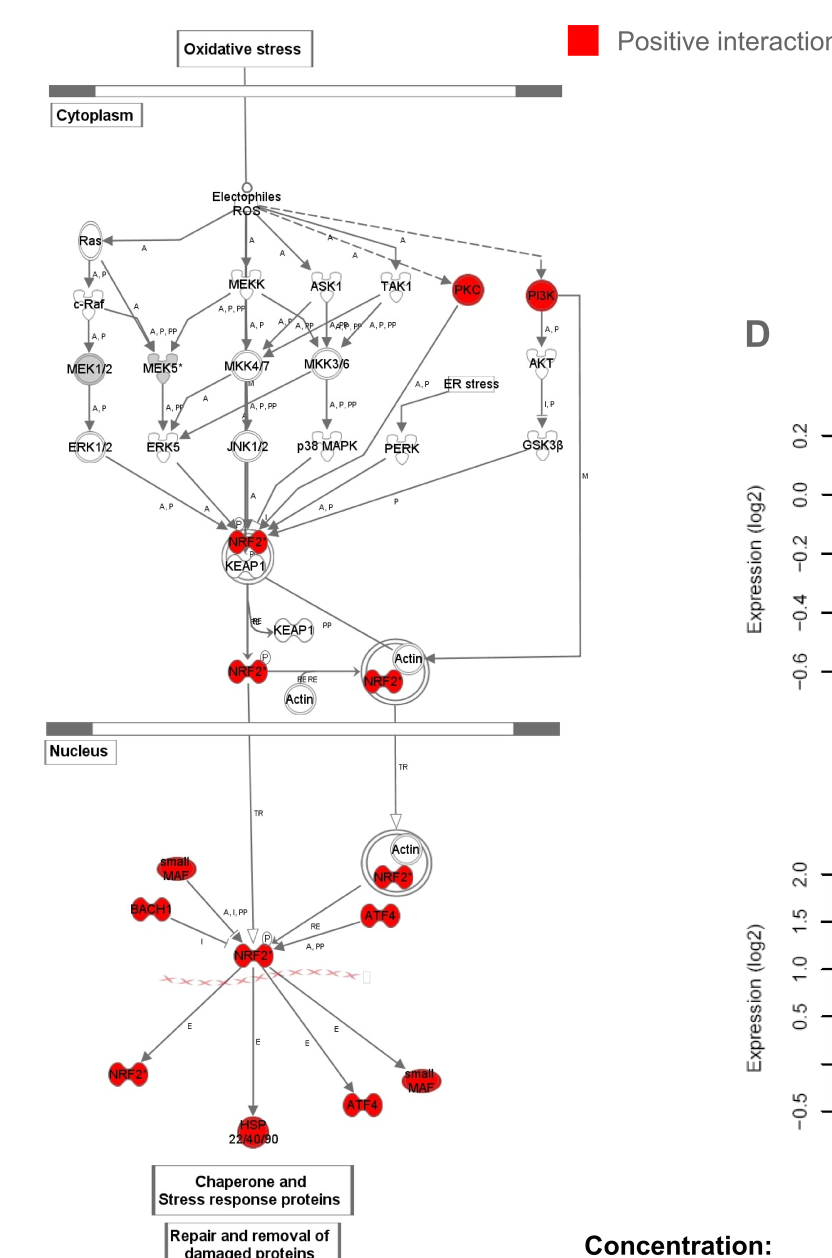
B

Top Biological function clusters in DAVID	Enrichment score
Lumen	12.4
Stress Response (UPR)	8.2
Basic-leucine zipper (bZIP) transcription factor factor	6.0
Transcription regulation (nucleus)	5.4
Nucleolus	4.7
Transcription (repressor activity)	3.8
Chaperone / Heat shock protein	2.8
Apoptosis	2.4

C

Probeset ID	Gene symbol	β_i	FDR
NRF2-mediated oxidative stress response / Unfolded protein response / ER stress			
202672_s_at	ATF3	0.45	4.0E-05
1554980_a_at	ATF3	0.33	6.7E-03
200779_at	ATF4	0.14	3.7E-05
204194_at	BACH1	0.14	2.9E-03
212501_at	CEBPB	0.09	1.1E-04
200880_at	DNAJA1	0.10	2.7E-04
225061_at	DNAJA4	0.14	3.6E-03
200664_s_at	DNAJB1	0.25	4.6E-05
200666_s_at	DNAJB1	0.22	1.6E-03
203811_s_at	DNAJB4	0.21	8.6E-03
202842_s_at	DNAJB9	0.17	5.8E-03
200064_at	HSP90AB1	0.05	8.1E-03
214359_s_at	HSP90AB1	0.06	9.8E-03
210189_at	HSPA1L	0.21	3.6E-05
117_at	HSPA6	0.59	2.5E-04
200806_s_at	HSPD1	0.06	9.6E-03
205133_s_at	HSPE1	0.06	4.4E-03
36711_at	MAFF	0.42	6.7E-06
226206_at	MAFK	0.12	7.4E-03
201146_at	NFE2L2 (NRF2)	0.09	2.2E-03
37028_at	PPP1R15A	0.37	3.3E-07
202014_at	PPP1R15A	0.37	4.9E-07
209685_s_at	PRKCB	0.05	7.5E-03
218145_at	TRIB3	0.22	6.3E-05
1555788_a_at	TRIB3	0.34	5.3E-04
200670_at	XBP1	0.07	9.9E-03
p53 Signaling			
214710_s_at	CCNB1	-0.09	1.1E-04
213523_at	CCNE1	-0.07	8.3E-03
205034_at	CCNE2	-0.18	1.7E-04
211814_s_at	CCNE2	-0.14	4.9E-03
202284_s_at	CDKN1A (p21)	0.15	4.4E-03
203725_at	GADD45A	0.13	7.0E-05
204285_s_at	PMAIP1	0.12	4.5E-03
223195_s_at	SESN2	0.37	3.1E-06
223196_s_at	SESN2	0.45	4.6E-05

E- *Nrf2-mediated oxidative stress response (IPA)*



D

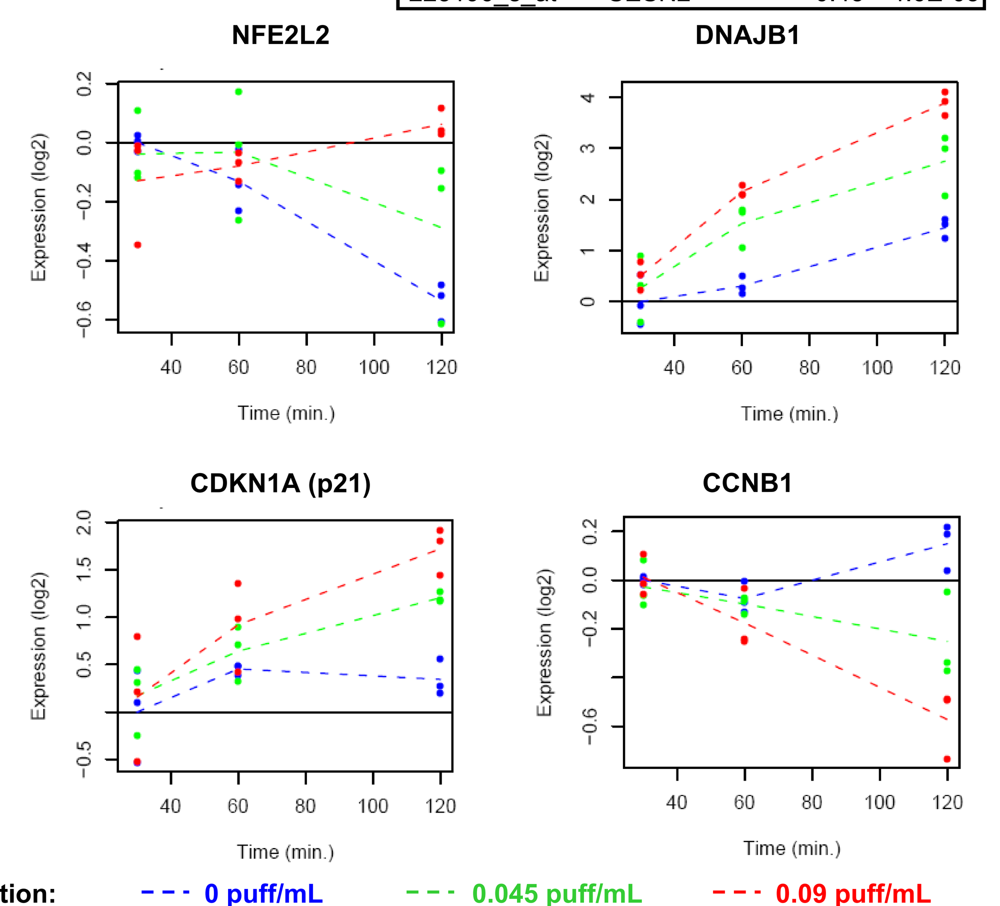


Figure 3. *Functional analysis of the differentially expressed gene list.* The statistical analysis identified 502 probe sets (~428 genes) with a significant interaction (FDR $\leq$ 0.01) between the time and concentration variables. Functional analyses were generated through the use of IPA and DAVID (Database for Annotation, Visualization, and Integrated Discovery). Top canonical pathways are displayed in table A. In IPA, Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. In DAVID, the EASE score ("P-values") corresponds to a modified Fisher Exact P-value (more conservative). A multiple hypothesis testing correction procedure was applied to adjust p-values using the Benjamini-Hochberg method. The functional annotation clustering approach implemented in DAVID allowed to identify top biological function clusters listed in table B. The group enrichment score corresponds to the geometric mean (in –log scale) of member (annotation terms inside each cluster for which the p-value is calculated as previously described)'s p-value in a corresponding cluster. Table C contains genes significantly regulated by CS and over-represented in some biological functions/pathways. Examples of the expression profile over concentration and time of some of these genes are shown in D and mapping of significantly regulated genes in Nrf2-mediated oxidative stress response pathway is displayed in E. It is important to note that these results assume the actions of the vapor components soluble in the PBS, and excludes the effects of particulate phase.

Conclusions

Overall, the results demonstrate a comprehensive picture of the cigarette smoke-specific transcriptional gene expression regulation, particularly stress-response pathways - when focusing on those changes in cigarette smoke-exposed human monocytes that are time- and concentration-related. This approach will enhance the ability to further investigate the complex gene expression changes in monocytes underlying the biological responses that may be involved in the development of atherosclerosis.

References

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