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Identification of Atherosclerosis Related Gene Expression Profiles by Treatment of Benzo(a)pyrene in Human Umbilical Vein Endothelial Cells

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Abstract

Benzo(a)pyrene (BaP) is a persistent environmental contaminant and is present in tobacco smoke. BaP is considered a major contributor of cardiovascular disease. While the activation of endothelial cells by stimuli including tobacco smoke and air pollution contributes importantly to cardiovascular disease, the nature of BaP's mechanism is unclear. In this study, gene expression profiles were investigated in BaPtreated human umbilical vein endothelial cells (HUVECs). Various atherosclerosis related genes could be up- and down-regulated more than 2-fold by BaP, and mRNA levels of atherosclerosis related genes encoding apolipoproteinC III, TLR 2, ICAM 1 and exportin 4 were significantly increased by BaP. Our data suggest that BaP-mediated changes in gene expression contribute to the progression of cardiovascular disease.

Keywords: Benzo(a)pyrene, Cardiovascular disease, Endothelial cells, Gene expression

Tobacco smoking is a hazardous addiction that commonly causes a wide variety of diseases. Tobacco smoke contains approximately 4,000 species of toxic and chemical substances[1,2] that are harmful to the human body as, for example, as a major cause of cardiovascular disease[3-5]. The polycyclic aromatic hydrocarbon (PAH) benzo(a)pyrene (BaP) exists in tobacco smoke, environment pollution, various types of processed foods and all kind of organic matter that can be metabolically activated in mammalian cells[6-8].

In smoke, BaP has been associated with endothelial damage in blood vessels and atherosclerotic plaque formation. Furthermore, BaP can change the structure of DNA adducts[7,8]. Cardiovascular diseases such as atherosclerosis are multicellular in nature[9]. Atherosclerosis is induced by an oxidation of lipids and resulting accumulation of extracellular matrix in the intima of arteries[10]. This injury occurs as the result of continuous or repeated endothelial damage due to ailments that include homocystinemia, diabetes, and hypertension[11]. Vascular endothelial dysfunction is a key initiating event in atherosclerosis[12]. Atherosclerosis also results from toxic injury to vessel walls[13]. Vessel wall damage by BaP exacerbates the process, inducing and/or accelerating atherosclerosis[3].

BaP levels are increased in vascular disease such as atherosclerosis[14,15]. But, the effect on transcriptional change induced by BaP is poorly understood in endothelial cells. In this study, we analyzed gene expression profiles in human umbilical vein endothelial cells (HUVECs) to investigate that BaP may induce endothelial dysfunction via change of atherosclerosis related gene.

Cytotoxicity of BaP in HUVECs

Relative survival of HUVECs upon a 1 h exposure to 10 μM BaP was determined by a standard colorimetric assay involving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The survival percentage relative to solvent control (dimethylsulfoxide, DMSO) was determined by a percentage decrease in the optical density following treatment. As shown in Figure 1, 10 μM BaP was not toxic to HUVECs. This BaP concentration was used for all subsequent experiments.

Gene Expression Profiles Analysis

The expression of the battery of genes contained in the 24 k whole human genome microarray (Nimblegen, Madison, WI) was determined prior to and after treatment of HUVECs with 10 μM BaP for 1 h. Hierarchical clustering was used to correlate expression patterns

with BaP exposure. In the results shown in Figure 2, the red color indicates over-expression and the green color indicates reduced expression. The expression of genes related to atherosclerosis were up-regulated more than 2-fold (n=65) or down-regulated more than 2-fold (n=40) in the presence of BaP (Tables 2 and 3). The responsive genes were determined to be involved in various functional categories including signal transduction, apoptosis, cell cycle, cell adhesion, inflammatory response, and transport. Genes found to be up-regulated included HMOX 1, CCL23, SART1, MAP3K10, RUTBC3, LGR5, PCDHGC5, IL-10, and RIMS4. Genes that were down-regulated genes included FASLG, ASAH2, AIF1, GJA4, PPM1L, GNB3, and CD38.

mRNA Level of Several Gene in BaP Treated HUVECs

To confirm the microarray results, reverse transcription-polymerase chain reaction (RT-PCR) was carried out on four selected atherosclerosis related genes that were up-regulated: apolipoproteinC III (ApoC III), Toll-like receptor 2 (TLR 2), Intercellular adhesion molecule 1 (ICAM 1), and exportin 4. We extracted total RNA from BaP-treated HUVECs and determined mRNA levels of each gene using RT-PCR (the primers used are summarized in Table 1). The expression level of all four genes was obtained from BaP-treated HUVECs was higher than the untreated control

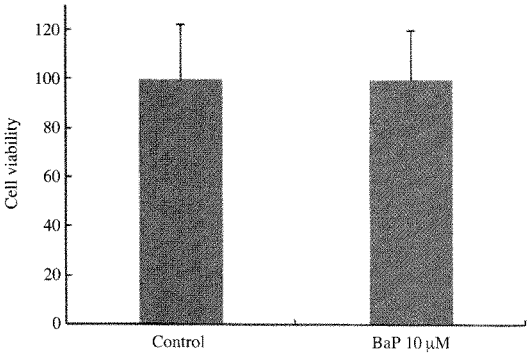

Figure 1. Effect of benzo(a)pyrene (BaP) on HUVECs cell viability. HUVECs were exposed to 10 μM BaP for 1 h. Untreated cells were used as the control. Values represent the percentage of the control. Data are the mean ± SD of triplicate experiments.

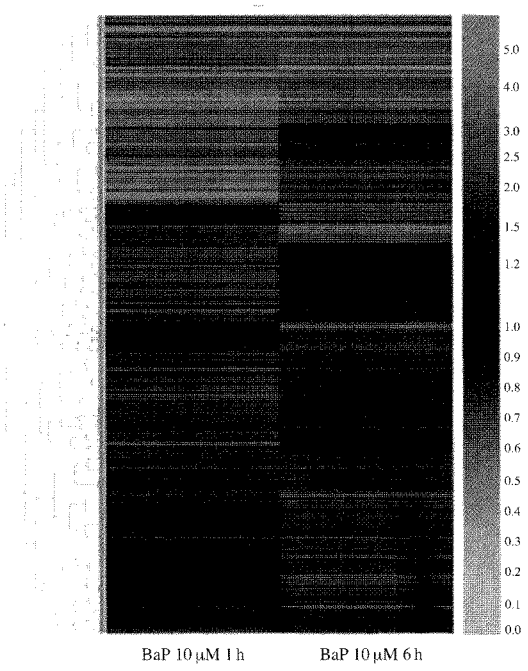

Figure 2. Hierarchical cluster image showing the differential gene expression profiles in BaP-treated HUVECs.

Table 1. Primers design.

Gene symbol	Gene name	Primer	
TLR2	Toll-like receptor 2	F	GATGCCTACTGGGTGGAGAA
		R	CGCAGCTCTCAGATTTACCC
ICAM1	Intercellular adhesion molecule 1 (CD54)	F	CAAGGCCTCAGTCAGTGTGA
		R	CATTATGACTGCGGCTGCTA
APOCIII	ApolipoproteinC III	F	AGCTGGCATAGCAGAGGTGT
		R	CCACACCACCCTCTCAACTT
XPO4	Exportin 4	F	GCTGTTGTCTGCCATTCTCA
		R	CCTCTGCACAGGACTTGACA
Beta-actin	Beta-actin	F	GTGGGGCGCCCCAGGCACCAGGGC
		R	CTCCTTAATGTCACGCACGATTTC

Tabel 2. List of up-regulated atherosclerosis related gene by BaP in HUVECs.

Gene symbol	Description	Gene bank No.	Increase
Signal transduction			
HMOX1	heme oxygenase (decycling) 1	NM_002133	3.00
TLR2	toll-like receptor 2	NM_003264	2.78
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5	NM_003667	12.35
PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B (dopamine and cAMP regulated phosphoprotein, DARPP-32)	NM_181505	2.34
CCL23	chemokine (C-C motif) ligand 23	NM_145898	3.73
ARHGDIG	Rho GDP dissociation inhibitor (GDI) gamma	NM_001176	3.31
OR2T11	olfactory receptor, family 2, subfamily T, member 11	NM_001001964	3.16
SSTR4	somatostatin receptor 4	NM_001052	3.07
VAV2	vav 2 oncogene	NM_003371	3.07
GTPBP1	GTP binding protein 1	NM_004286	3.04
Apoptosis			
SART1	squamous cell carcinoma antigen recognized by T cells	NM_005146	2.94
FASTK	Fas-activated serine/threonine kinase	NM_006712	2.01
TNFRSF6B	tumor necrosis factor receptor superfamily, member 6b, decoy	NM_032945	6.49
GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX)	NM_033423	5.48
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	NM_021127	4.34
MAP3K10	mitogen-activated protein kinase kinase kinase 10	NM_002446	4.09
CIDEA	cell death-inducing DFFA-like effector a	NM_198289	4.07
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_000211	3.85
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	NM_003841	2.81
Cell cycle			
RUTBC3	RUN and TBC1 domain containing 3	NM_015705	4.35
KATNB1	katanin p80 (WD repeat containing) subunit B 1	NM_005886	3.84
CCNB2	cyclin B2	NM_004701	3.26
CDT1	chromatin licensing and DNA replication factor 1	NM_030928	3.10
TBC1D24	TBC1 domain family, member 24	NM_020705	2.82
ESCO2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	NM_001017420	2.53
E2F1	E2F transcription factor 1	NM_005225	2.35
MAPK3	mitogen-activated protein kinase 3	NM_002746	4.00
TMEM58	transmembrane protein 58	NM_198149	2.18
EID1	EP300 interacting inhibitor of differentiation 1	NM_014335	2.18
NDE1	nudE nuclear distribution gene E homolog 1 (A. nidulans)	NM_017668	2.17
Cell adhesion			
SELE	selectin E (endothelial adhesion molecule 1)	NM_000450	2.13
PCDHGC5	protocadherin gamma subfamily C, 5	NM_032407	6.00
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	NM_002204	4.84
STAB2	stabilin 2	NM_017564	4.54
ICAM5	intercellular adhesion molecule 5, telencephalin	NM_003259	4.24
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_000211	3.85
PCDH8	protocadherin 8	NM_002590	3.58
PKP3	plakophilin 3	NM_007183	3.46
CDH16	cadherin 16, KSP-cadherin	NM_004062	3.38
ICAM4	intercellular adhesion molecule 4 (Landsteiner-Wiener blood group)	NM_022377	2.79
Inflammatory response			
IL10	interleukin 10	NM_000572	10.57
CDO1	cysteine dioxygenase, type I	NM_001801	3.26
KLRG1	killer cell lectin-like receptor subfamily G, member 1	NM_005810	2.41
LY75	lymphocyte antigen 75	NM_002349	2.36
CCL17	chemokine (C-C motif) ligand 17	NM_002987	2.17
HDAC9	histone deacetylase 9	NM_014707	2.16
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	NM_173163	2.06
IL8RA	interleukin 8 receptor, alpha	NM_000634	2.04
Transport			
XPO4	exportin 4	NM_022459	22.38
RIMS4	regulating synaptic membrane exocytosis 4	NM_182970	3.40

Tabel 2. Continued.

Gene symbol	Description	Gene bank No.	Increase
SLC16A8	solute carrier family 16, member 8 (monocarboxylic acid transporter 3)	NM_013356	3.36
LIN7B	lin-7 homolog B (C. elegans)	NM_022165	3.26
AQP12A	aquaporin 12A	NM_198998	3.15
SLC1A7	solute carrier family 1 (glutamate transporter), member 7	NM_006671	3.07
TMED8	transmembrane emp24 protein transport domain containing 8	NM_213601	3.05
RHBG	Rh family, B glycoprotein	NM_020407	2.98
SNX27	sorting nexin family member 27	NM_030918	2.93
SLC37A1	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	NM_018964	2.91
SHKBP1	SH3KBP1 binding protein 1	NM_138392	2.88

Table 3. List of down-regulated atherosclerosis related gene by BaP in HUVECs.

Gene symbol	Description	Gene bank No.	Decrease
Signal transduction			
FASLG	Fas ligand (TNF superfamily, member 6)	NM_000639	0.19
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	NM_002546	0.43
GNB3	guanine nucleotide binding protein (G protein), beta polypeptide 3	NM_002075	0.46
OR5K1	olfactory receptor, family 5, subfamily K, member 1	NM_001004736	0.15
IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	NM_002187	0.17
ASAH2	N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2	NM_019893	0.17
PPP1R12B	protein phosphatase 1, regulatory (inhibitor) subunit 12B	NM_032103	0.22
HLA-DRB3	major histocompatibility complex, class II, DR beta 3	NM_022555	0.25
GNG7	guanine nucleotide binding protein (G protein), gamma 7	NM_052847	0.26
Apoptosis			
ASAH2	N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2	NM_019893	0.17
CD38	CD38 molecule	NM_001775	0.45
Cell cycle			
AIF1	allograft inflammatory factor1	NM_004847	0.43
KIF2B	kinesin family member 2B	NM_032559	0.36
GAS1	growth arrest-specific 1	NM_002048	0.44
Cell adhesion			
PPM1L	protein phosphatase 1 (formerly 2C)-like	NM_139245	0.28
ITGA7	integrin, alpha 7	NM_002206	0.34
CDH18	cadherin 18, type 2	NM_004934	0.35
ITGB8	integrin, beta 8	NM_002214	0.38
HES1	hairy and enhancer of split 1, (Drosophila)	NM_005524	0.43
SPAM1	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	NM_153189	0.46
AMIGO2	adhesion molecule with Ig-like domain 2	NM_181847	0.47
C20orf42	chromosome 20 open reading frame 42	NM_017671	0.48
Inflammatory response			
AIF1	allograft inflammatory factor1	NM_004847	0.43
AZU1	azurocidin 1 (cationic antimicrobial protein 37)	NM_001700	0.14
ALOX5AP	arachidonate 5-lipoxygenase-activating protein	NM_001629	0.34
Transport			
GJA4	gap junction protein, alpha 4	NM_002060	0.47
KCNA5	potassium voltage-gated channel, shaker-related subfamily, member 5	NM_002234	0.29
ETFB	electron-transfer-flavoprotein, beta polypeptide	NM_001014763	0.36
SCN10A	sodium channel, voltage-gated, type X, alpha subunit	NM_006514	0.37
COLEC10	collectin sub-family member 10 (C-type lectin)	NM_006438	0.38

also effectively induced by treatment with BaP (Figure 3). These data suggest that BaP may contribute to the progression of atherosclerosis by elevating the mRNA levels of atherosclerosis related genes.

Discussion

Tobacco smoke is a risk factor for cardiovascular

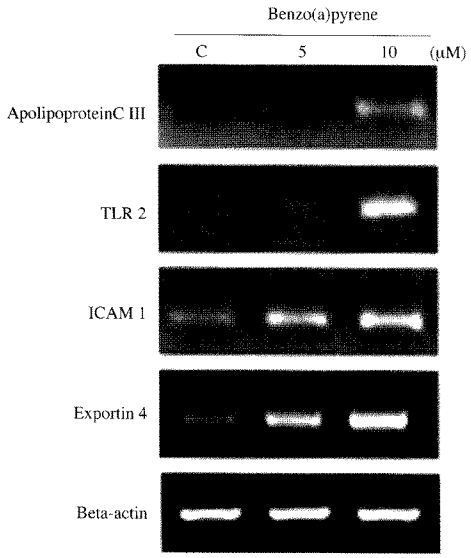

Figure 3. Increased mRNA level following BaP treatment of HUVECs. HUVECs were exposed to 10 μM BaP for 1 h. ApoC III, TLR 2, ICAM 1, exportin 4, and β-actin mRNA levels were measured by RT-PCR. The products were performed on 1.5% agarose gel electrophoresis using β-actin as the loading control.

disease and can damage organ systems[16,17]. BaP is linked with endothelial damage in blood vessels[18]. It is also well-known that endothelial dysfunction preludes cardiovascular diseases such as atherosclerosis[19-21]. In this paper, we determined the influence of BaP on gene expression at a BaP concentration that is non-toxic to the cells in vitro (Figure 1). Our data show that a non-toxic level of BaP induces gene expression changes in endothelial cells.

BaP induced transcriptional change by up-or down-regulation of atherosclerosis related genes (n=65 and 40, respectively) implicated with endothelial dysfunction. Up-regulated genes included HMOX 1, CCL23, SART1, MAP3K10, RUTBC3, LGR5, PCDHGC5, IL-10, and RIMS4, and down-regulated genes included FASLG, ASAH2, AIF1, GJA4, PPM1L, GNB3, and CD38. In particular, we were interested in determining if the up-regulated genes included those encoding ApoC III, TLR 2, ICAM 1, and exportin 4, which are atherosclerosis-related proteins that have signal transduction and transport functions in endothelial cells. ApoC III is a very low density lipoprotein. Through its positive interaction with plasma triglyceride, ApoC III may have a role in triglyceride metabolism[22] and is, therefore, of interest regarding vascular disease. TLR 2 plays a role in the human immune system that leads to cytokine secretion and the inflammatory response[22,23]. ICAM 1, which is induced by interleukin-1, tumor necrosis factor alpha, and the intercellular 1 adhesion molecule, is a ligand for LFA1 (CD11A/CD18) that is expressed on vascular endothelium[24,25]. Finally, exportin 4 is a protein that shuttles between the nucleus and the cytoplasm, whose increased levels induce the atherosclerosis-related proteins plasminogen activator inhibitor-1 (PAI-1) and endothelin-1 (ET-1). As a result, exportin 4 may influence the progression of atherosclerosis[26]. We observed that the mRNA levels of all four of these genes were increased by treatment of BaP (Figure 3).

The present study indicates that BaP can affect the production of mRNA by genes involved in endothelial dysfunction, presumably by the induction of genes related to atherosclerosis. This finding suggests that BaP, which is a toxic component in tobacco smoke and an environmental contaminant, may play a role in progression of cardiovascular diseases such as atherosclerosis.

Materials & Methods

Materials

Benzo(a)pyrene, dimethylsulphoxide (DMSO) and MTT were obtained from Sigma Aldrich (St. Louis, MO). Primer of apolipoprotienC III, ICAM-1, TLR-2 and exportin-4 were purchased from Bioneer. M-199, Fetal Bovine Serum (FBS) were purchased from WelGENE Inc (South Koera).

Cell Culture

HUVECs were cultured in M199, supplemented with 10% inactivated fetal bovine serum, 1% penicillin, 10 ng/mL human fibroblast growth factor, and 18 mU/mL heparin in an atmosphere of 5% CO_2 at 37°C. The cells was passaged every 2-3 days[13]. HUVECs were cultured to about 80% confluence and further incubated with fresh medium containing the above reagents. Throughout these experiments, the cells were used within passages[4-9].

Viability Assay

Cells (5×10^3 cells/well) were seeded in wells of a 96-well culture plate and were incubated in the presence of M199 medium for 24 h at 37°C. The medium was removed and replaced with serum-free M199. Benzo(a)pyrene was added to a final concentration of 25 μM and incubation was continued for 1 h at 37°C. Twenty microliters of a 5 mg/mL solution of MTT in phosphate buffered saline (PBS) was added to each well and incubation was continued for 4 h at 37°C. At

that time, the MTT solution was removed and 50 μL of a DMSO solution was added to each well. The plate was agitated on a plate shaker for 15-30 min and the absorbance was recorded using a microplate reader at a wavelength of 540-570 nm[27].

RNA Preparation

Cells were seeded in a 100 mm-diameter plate containing M199 medium. After 24 h, cells were washed and the medium was replaced with serum-free M199 medium. After 30 min, BaP was added to a final concentration of 10 μM and incubation was continued for 1 h. RNA was extracted using 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA pellets were washed in 70% ethanol, dried, and dissolved in diethylpyrocarbonate for RNase inhibition. Total RNA was quantified[13] using a ND-100 spectrometer (NanoDrop Technologies, Wilmington, DE).

RT-PCR

After RNA preparation, complementary DNA (cDNA) was synthesized using 2 μg of total RNA. cDNA synthesis was performed in steps of 5 min at 70°C, 10 min at 25°C, 60 min at 42°C, and 5 min at 95°C. For apolipoproteinC III and TLR 2, annealing was done for 30 s at 58°C, and for ICAM 1 and exportin 4 annealing condition was 30 s at 55°C. All reactions were cycled 30-40 times. Oligo-nucleotide primers (Bioneer, Daejon, Korea) are shown in Table 1. The resulting products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide[13].

DNA Microarray Analysis

Gene expression analysis was conducted on the RNA samples using 24 k whole human genome microarray (NimbleGen). Triplicate analyses were simultaneously performed for each chemical. Labeling and hybridization were performed using the platinum Biochip Reagent Kit (Genocheck, Seoul, Korea). Hybridization was performed in a hybridization oven at 62°C for 12 h. After washing (2 X SSC/0.1 % sodium dodecyl sulfate for 2 min at 58°C, 1 X SSC for 2 min at room temperature (RT) and 0.2 X SSC for 3 min at RT), each slide was dried by centrifugation at 800 rpm for 3 min at RT. Hybridized slides were scanned with a GenePix 4000B scanner (Axon Instruments, Sunnyvale, CA) and the scanned images were analyzed using GenePix Pro 5.1 software (Axon Instruments)[28].

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