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Effect of water-soluble fraction of cigarette smoke on human aortic endothelial cells – a proteomic approach

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Abstract

Proteomic analysis is an important investigative tool used to systematically explore cellular proteins that are responsive to adverse environmental challenges. Tobacco smoking is the second major cause of death in the world. In this study, we utilized two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) technologies to explore protein changes in human aortic endothelial cells (HAECs) in response to cigarette smoke extracts (CSE). Among 389 individual proteins resolved using 2-DE, 43 had a 2- to 3-fold change in levels as measured by spot intensity and 32 had more than a 3-fold change. Sixteen of the 32 spots with sufficient amount of proteins were excised for identification by performing matrix-assisted laser desorption/ionization (MALDI)-MS analysis. Using a peptide mass fingerprinting (PMF) to search the nrNCBI database, we identified all these 16 proteins, which were either increased ($n = 9$) or decreased ($n = 7$) after CSE treatment. All these proteins have known functions, however, none have been reported to be altered after CSE treatment. The findings from our study suggest that utilizing a systemic investigative tool, such as the proteomic approach using 2-DE, may play an important role in discovering novel molecular mechanisms for cigarette smoking-induced pathological changes. Further investigation following the systemic discoveries must be further examined as they may potentially lead to new therapeutic approaches to smoking-induced diseases – a health issue affecting everyone in the world.

Keywords

cigarette smoke; 2-DE; endothelial cells; proteomics; nitric oxide synthase

Introduction

Tobacco smoking is the second major cause of death in the world. It is currently responsible for the death of one in ten adults, worldwide (about 5 million deaths each year). If the current smoking trend continues, it will cause approximately 10 million deaths per year by 2025. The lives of half of the current smokers ($n = 650$ million) will eventually be taken by tobacco smoking. The economic costs associated with tobacco consumption are astronomical. In addition to the high public health costs of treating tobacco-caused diseases, tobacco smoking kill people at the peak of their productivity, robs families of breadwinners and deprives the nation of a healthy workforce. Due to increased illness, tobacco smokers are also less productive while they are alive. In a 1994 report, it was estimated that the use of tobacco resulted in an annual global net loss of US \$20 billion (<http://www.who.int/tobacco/about/en/>).

Although the impact of tobacco smoking on human health and economy has been well established, little is known about the molecular or cellular mechanisms by which tobacco smoke exerts its disastrous effects. Smoking is associated with an increased incidence of a variety of disorders in which cardiovascular diseases, lung diseases and cancers are the dominant presenting illnesses. Cigarette smoke can cause DNA single strand breaks *in vitro*, which is relevant to carcinogenesis (Borish et al., 1985; Leanderson et al., 1992). *In vitro* experiments showed that cigarette smoke contents like benzo(a)pyrene or its metabolite benzo(a)pyrene diol peroxide alter the gene transcription efficiency (Wang et al., 2003). With more than 4000 chemicals generated, tobacco smoke can affect post-translational modifications that may result in significant alteration in protein functions. Therefore, it is important to explore the protein responses to tobacco smoke in addition to changes at the mRNA levels.

Previous studies (Ma et al., 1999; Wang et al., 2001) exploring smoking effects have focused on a few selected proteins. This approach is mostly based on known biological functions of the proteins and is likely to miss out on some novel mechanisms triggered by a complex mixture of over 4000 chemicals generated by the cigarette smoking. On the other hand proteomic analysis using two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) provides a method for systemic screening of proteins responsive to the challenge of cigarette smoking (Celis et al., 1998; Hochstrasser, 1998). The integration of 2-DE with MS and bioinformatics not only allows rapid and reliable protein separation and identification (Henzel et al., 1993), but also offers information about post-translational modifications, subcellular localizations, and protein-protein interactions (Yanagida, 2002). This approach was recently demonstrated by Gao et al., who analyzed the effect of a single tobacco constituent benzo(a)pyrene using the proteomic tool (Gao et al., 2004). Because tobacco smoke is a complex mixture of more than 4000 compounds, which are generally inhaled together by smokers, a single tobacco smoke constituent may produce biological effects different from the whole smoke in real life. Therefore, in this study, we investigated the proteomic profiles in human aortic endothelial cells (HAECs) treated with total cigarette smoke extract (CSE).

Endothelial cells are the first line of defense in vasculature against cigarette smoking-induced damages. Smokers often have dysfunctional endothelial cells, which lead to vascular diseases including atherosclerosis and aneurysms. There is strong evidence that endothelial derived nitric oxide (NO), mainly produced by endothelial nitric oxide synthase (eNOS) plays an essential role in endothelial functional integrity and survival. Adequate NO production appears to be essential for endothelial cells to survive the attacks by adverse factors. Indeed, studies demonstrate that cigarette smoking can specifically repress eNOS expression and activity, hence results in endothelial dysfunction (Andersen et al., 2004; Barua et al., 2002; Sarkar et al., 1999; Su et al., 1998; Wang et al., 2000). It is not clear, however, what other functional profiles may also be disrupted by cigarette smoking in addition to or following eNOS disruption. In the current study, we studied the protein expression profiles in endothelial cells exposed to the water-soluble fraction of the cigarette smoking. In addition, we also pre-activated eNOS using L-arginine – a well-known substrate of eNOS, or pre-inhibited eNOS using N-omega-nitro-L-arginine methyl ester (L-NAME) – a non-specific NOS inhibitor, before the endothelial cells were challenged with CSE. We wish to determine whether L-arginine or L-NAME pretreatment would have significant impacts on CSE-induced proteomic profiles.

Materials and methods

Cell culture

HAECs were purchased from Cell applications, Inc (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO), unless indicated otherwise. HAECs were cultured in F-12K media (ATCC, Manassas, VA) containing bovine endothelial cell growth supplement (45 µg/ml), heparin (100 µg/ml, Acros, NJ), penicillin (50 IU/L), streptomycin (50 µg/L,

Cellgro, Herndon, VA) and 20% fetal calf serum (Invitrogen corporation, Carlsbad, CA) in a 5% CO₂/air atmosphere. Only cells within 3–5 passages were used for the experiments. At 70–80% confluence, cells were treated with CSE (0.01 cigarette equivalent unit/ml) for 4 h in the media described above. In the pretreatment experiments, the HAECs were incubated with 200 µmol/L of L-arginine or 400 µmol/L of L-NAME for 30 min before exposed to 0.01 CSE for 4 h by replacing the medium with the fresh media containing 0.01 CSE. For maximum effects on endothelial functional changes, we chose the CSE dose at 0.01 unit and treatment for 4 h. Higher dose or longer duration would induce significant cell death (See Results). Experiments were repeated three times for each experimental condition (described below).

Apoptosis detection

HAEC cultured on gelatin-coated petri dishes (1×10^6 cells) were treated with CSE. At the end of the experimental period, floating and adherent cells were collected and centrifuged for 5 min at 1000g. The cells with apoptotic changes were measured using In Situ Cell Death Detection Kit, Fluorescein (Roche, Indianapolis, IN), which detects DNA fragmentation during apoptosis using a FACScan Becton Dickinson Instrument and the Cell-quest software (Franklin Lakes, NJ). The degree of apoptosis was determined by the percentage of apoptotic cells over total cells.

HAECs treated with CSE

CSE was prepared as described previously (Wang et al., 2001). Briefly, a research cigarette (2R4F, from Tobacco Health Research, University of Kentucky) was inserted into a 10-cm length of plastic tube that was attached to a tube submerged in a flask containing 20 ml of cell culture medium. The ignited cigarette was completely consumed in puffs over 5 min period. During this time the cigarette smoke was bubbled through the medium, collecting the water-soluble components of the cigarette smoke. The pH of the CSE was between 7.4 and 7.5. Since there is no standardized way to express the quantity of CSE, the concentration of CSE was calculated in arbitrary units as cigarette equivalents per milliliter of the medium. CSE was used to treat the cells within 30 min of extraction. Cigarette 2R4F contains 11.70 mg total particulate matter, 9.70 mg tar and 0.85 mg nicotine per cigarette. Although we do not know the exact chemical components of the CSE in our preparation, it is likely that all water soluble contents – particulate matter and gas phase should be dissolved in the culture medium solution. Toxic chemicals in CSE include tobacco specific nitrosamines, alkaloid, ammonia, carbon monoxide, chlorinated dioxins and furans, and a large collection of pyridine derivatives including nicotine (Ji et al., 2002).

Following CSE-treatment, the HAECs were then harvested for protein extraction. Cells were rinsed three times with cold PBS before they were lysed with osmotic lysis buffer (10 mmol/L Tris, PH 7.4, 9.3% SDS, 5 mmol/L MgCl₂, 50 µg/ml RNase, 50 µg/ml DNase) for 30 min on ice. Protein concentration was determined using BCA method (Smith et al., 1985).

2-D electrophoresis

The cellular proteins prepared above were first lyophilized before redissolved in 1.0 mg/ml SDS boiling buffer (5% SDS, 5% β-mercaptoethanol, 10% glycerol and 60 mmol/L Tris, pH 6.8) and placed in boiling water bath for 3 min before they were loaded in a 2-D gel. A 50 µl protein solution (containing 50 µg extracted protein) was loaded in each well. The 2-D electrophoresis was performed according to the method of O'Farrell (1975). Briefly, isoelectric focusing was carried out in a glass tube of inner diameter 2.0 mm using 2.0% pH 3.5–10 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ) for 9600 volts/h. One µg of an IEF internal standard, tropomyosin was added to the sample. This protein migrates as a doublet with lower polypeptide spot of MW 33 000 and pI 5.2. The enclosed tube gel pH gradient plot for this set of ampholines was determined with a surface pH electrode.

After equilibration for 10 min in a buffer containing 10% glycerol, 50 mmol/L dithiothreitol, 2.3% SDS and 0.0625 mol/L Tris (pH 6.8) the tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel. The following proteins were added as molecular weight markers to a well in the agarose that sealed the tube gel to the slab gel: myosin (220 000), phosphorylase A (94 000), catalase (60 000), actin (43 000), carbonic anhydrase (29 000) and lysozyme (14 000).

Gel visualization, image analysis and protein identification

The gel was silver-stained and scanned with a laser densitometer (Model PDSI, Molecular Dynamics, Inc, Sunnyvale, CA). The general method of computerized analysis for the paired experimental and control gels included automatic spot finding and quantification, automatic background subtraction (mode of non-spot) and automatic spot matching in conjunction with detailed manual checking of the spot finding and matching functions. Spot percentage is equal to spot integrated density (volume) expressed as a percentage of total density of all spots measured. Difference is defined by the fold-change of the percentage of a spot to the matched counterpart in the control gel. The molecular weight and pI values for each spot were determined from algorithms applied to the reference image. For protein identification, the protein spots were cut off the silver-stained 2-D gel before digested with trypsin. The resulting peptide mixture was analyzed by MALDI-MS, the profile of the peptide masses obtained by MS were matched with the profile of known proteins in the database (<http://www.expasy.ch>) (Cottrell, 1994; Henzel et al., 1993; Mann et al., 1993; Shevchenko et al., 1996).

Results

Defining CSE doses and treatment durations

First, we investigated dose and time-dependent induction of HAEC apoptosis by CSE. Cells were treated with CSE from 0.001 to 0.02 units for 4 h. CSE dose-dependently induced endothelial apoptosis, in which 0, 0.001, 0.005, 0.01 and 0.02 U CSE treatment for 4 h caused endothelial cell death $3.8\% \pm 0.5\%$, $18.3\% \pm 1.1\%$, $23.1\% \pm 0.5\%$, $30.5\% \pm 1.0\%$ and $65.5\% \pm 2.5\%$, respectively. Although induction of apoptosis was evident at 0.001 CSE, the maximal effect was observed at 0.02. We also tested a higher CSE dose (0.10 unit), at which majority of the cells died early on (1–2 h) of the treatment (data not shown). CSE at the dose of 0.01 units also induced time-dependent endothelial apoptosis over 24 h. The CSE-induced apoptosis started at 4 h and peaked at 24 h. Thus, for proteomic analyses, we used 0.01 unit of CSE for 4 h to achieve the maximal effects on protein expression but minimal effects on cell death.

Proteomic analyses in HAEC after CSE treatment

After spot detection, background subtraction and volume normalization, 389 protein spots were detected in untreated HAECs. The normalized volumes of all protein spots are shown in Table 1 with the reference spot numbering, isoelectric point (pI) and molecular weight (MW) for each spots analyzed in the samples. The fold increase or decrease (difference) of the separated protein spots for comparisons between groups were also shown in Table 1. The differences were calculated from spot percentages (individual spot density divided by total density of all measured spots). Protein spots absent in CSE-treated HAECs are indicated by –; and protein spots present only in CSE-treated cells are indicated by +++. Decreased protein spots with a fold difference of 3 or more are highlighted in bold while increased spots with a fold difference of 3 or more are highlighted in bold italic.

Effects of CSE treatment on protein levels in HAECs

By spot fold change analysis, the proteins with a fold difference of 3 or more (CSE treated cells Vs untreated cells) are shown in a representative gel image from the control groups with silver stain (Figure 1). The spot no. 389 (blue downward arrow) was completely gone in CSE-treated HAECs, which was later identified as prolyl 4 hydroxylase α (P4H α) (Raveendran et al., 2004). CSE treatment decreased 14 protein spots (Spot nos. 87, 89, 110, 132, 133, 174, 177, 181, 296, 297, 300, 305, 307 and 308, yellow downward arrow) when compared to untreated cells. On the other hand, levels of certain proteins were increased by CSE treatment (spot nos. 3, 5, 28, 53, 178, 188, 204 and 266, red upward arrow) when compared to control. As shown in Figure 2, two proteins (spot nos. 37 and 315, white box) were only present in CSE-treated cells.

Effects of L-arginine and L-NAME pretreatment on CSE-treated HAECs

Protein spots with a fold difference of ≥ 3 are shown in L-arginine or L-NAME pretreatment experiments (Figure 3). As shown in the Figure 3, L-arginine + CSE treated cells had increased protein levels in 4 spots (spot nos. 133, 163, 282 and 308, red upward arrow) as compared to CSE only treated cells. Spot nos. 20, 28, 129 and 195 (yellow downward arrow) were decreased in L-arginine + CSE treated cells when compared to CSE-treated cells. L-arginine pretreatment itself had no effect on the protein expression. Figure 4 showed 3 protein spots (spot no. 3, 39 and 308, red upward arrow) increased by L-NAME + CSE treatment when compared to CSE-treated cells. Spots 158 and 208 (yellow downward arrow) were decreased in L-NAME + CSE treated cells, and L-NAME itself has no effect on the protein expression.

Identification of selected proteins by MALDI-MS

Protein spots with altered expression of more than 3-fold, which also had sufficient absolute quantity ($n = 16$) were excised from corresponding 2-DE gels followed by in-gel digestion with trypsin and identified using MALDI (matrix-assisted laser desorption ionization)-MS analysis. Using the peptide mass fingerprint (PMF), all 16 proteins were successfully identified including 5 heat shock proteins, 2 members of calcium regulating proteins, a translation initiation factor, and other proteins that were not previously connected with smoking. Table 2 lists the protein spots digested with trypsin and analyzed by the MALDI-MS along with their molecular weights.

Discussion

Proteomic analysis is a powerful technique used to characterize protein expression profile and post-translational modifications. Using 2-DE, 389 protein spots are detectable in HAECs cultured *in vitro*. Of those proteins, we identified 16 which have more than a 3-fold change. In the identified proteins, several categories of proteins were of particular interest in the cellular responses to CSE exposure in HAECs. CSE altered the heat shock proteins (Hsp) which was previously observed (Ryder et al, 2004; Vayssier et al, 1998a,b). CSE also increased the calcium regulating proteins, annexin VI and annexin II, which were not reported earlier in smoking related changes. Only annexin I was reported as decreased in the bronchoalveolar lavage fluids from healthy smokers (Vishwanatha et al., 1998). Myosin was decreased in CSE-treated HAECs, which was correlated with earlier observation in nicotine toxicity (Li et al., 2004). The effects of CSE on α -actinin, moesin, laminin binding protein, P4H α , eukaryotic translation initiation factor 4A and α enolase have not been previously reported.

In the present study we observed an increased expression of Hsp-70, Hsp-27 and gp96. All these Hsps can protect cells from the potentially fatal consequences of adverse environmental, physical, or chemical stresses, such as cigarette smoking (Beere, 2001; Caudill et al., 2001; Li et al., 2002; Xanthoudakis et al., 2000). However, the 6- to 8-fold increases in Hsp expression

could still not save the CSE-insulted endothelial cells from death. It is well known that cellular stress can engage in two fundamental responses: signal transduction pathways leading cell death, such as apoptosis, or stress response that functions to sustain survival by limiting cellular damage and accelerating recovery. Coordinated interaction between these two functionally opposing pathways, apoptosis and the stress response, determine the cellular susceptibility to damaging stresses. Although Hsps, such as Hsp-70, may be able to prevent CSE-induced endothelial necrosis, studies by others and our lab indicated that overexpressed Hsps were not sufficient to prevent cells to undergo apoptotic pathway (Vayssier et al., 1998a, Vayssier-Taussat et al., 2001).

We have further shown that up-regulated annexin family members (2 and 6) may inhibit neutrophil migration and blood coagulation, and profibrinolytic (Gerke et al., 2002; Kim et al., 2002; Sacre et al., 2002). They may also be relevant to fibrosis and remodeling of cardiovascular system (Benevolensky et al., 2000; Trouve et al., 1999). Although on the surface this may appear to be contradictory to the global pro-thrombotic effects by the cigarettes, one must bear in mind that annexin expression in endothelial cells only represents one small aspect of the entire thrombotic cascades. The final direction of pro- or anti-thrombotic action is determined by many other factors and systems such as platelet activation. However, if CSE indeed promotes endothelium-mediated fibrinolysis, it may be an additional mechanism for vulnerable plaque formation in smokers.

The significant increase on α -actinin and moesin may be relevant to endothelial permeability through their effects on cytoskeletal-plasma membrane interaction and cell surface-extracellular matrix interaction (Bretscher et al., 2000; Louvet-Vallee, 2000; Lum et al., 1994, 1996). Overexpression of moesin may promote monocyte adhesions to endothelial cells through its functional interaction with adhesion molecules such as ICAM-1, VCAM-1 and L-selectin (Barreiro et al., 2002; Berryman et al., 1993; Ivetic et al., 2002). Among all highly regulated proteins, moesin appears to be the only one being restored by the L-Arg preconditioning (Table 2). It is not clear how this restoration could be associated with reduced apoptosis by L-Arg treatment before CSE exposure.

Among those down-regulated proteins, α -tubulin may reflect the apoptotic changes induced by CSE in endothelial cells. Microtubules are formed by association of protofilaments that are themselves made up of alternating α and β tubulin monomers. Microtubules are involved in many essential functions throughout the cell cycle and their degradations are one of the hallmark phenomena in the apoptotic process (Atencia et al., 2000; Downing, 2000; Wang et al., 1999). Our experiments have also shown a significant reduction in α -enolase. The α -enolase is a multifunctional protein located in both cytoplasm and cell surface, and is primarily one of the key glycolytic enzymes (Pancholi, 2001). It can function as a surface receptor for the binding of plasminogen, acts as a nuclear Myc-binding protein, endothelial hypoxic stress protein and Hsp (Pancholi, 2001). While we are not clear how α -enolase reduction could be associated with toxic effect of CSE, its primary function as a glycolytic enzyme could mediate the association between cigarette smoking and insulin resistance and diabetes.

While protein changes documented in our experiments are likely to occur *in vivo*, one of the main limitations is that neither protein changes nor the smoking components documented in our study are inclusive for all changes in the real life smoking environment. Firstly, it is the technical limitation of 2-D gel electrophoresis. As reported by others, one of the major shortfalls in 2-D approach is that it will frequently miss out on small molecular weight and less abundant proteins, which may include important transcription factors. Although our analyses were able to reveal nearly 400 protein spots in a single gel, they are not inclusive. Together with the relative inaccuracy in quantitative analyses of spot density, it is certain that some important regulatory proteins responsive to cigarette smoking may not be discovered with the current

technique. Secondly, CSE is an artefactual preparation of cigarette smoking *in vitro*. CSE may represent the closest to the *in vivo* smoking with regard to water soluble particulate and gas phase chemicals, but it still lacks direct interactions with *in vivo* biological environment such as blood constituents. Furthermore, we used MALDI-MS for protein identifications only on the spots with sufficient amount of proteins and more than 3-fold changes. There is no doubt that many of the protein spots with less than 3-fold changes or less abundant could also be functionally important for cigarette smoking-induced cellular changes. However, it is not cost-effective to go through the entire list of separated protein spots for MALDI-MS identification. With limited resources, one has to work with proteins that have the most dramatic changes.

In summary, it is clear that the unbiased systemic approach will provide for a better understanding into the mechanisms mediating the biological effects by tobacco smoking. Using proteomic analysis, we found the various intracellular proteins, which have not been previously connected to cellular response to genotoxic stress, were significantly affected by CSE treatment. While our findings did not reveal any mechanisms involved in CSE-induced pathological changes, they do provide target pathways for further mechanism investigations.

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Figure 1.

Cellular proteins from cultured human aortic endothelial cells (HAECs) identified by 2-D gel electrophoresis. HAECs were treated with or without 0.01 unit CSE for 4 h and cell protein extracts were separated on a pH 3.5–10 ampholines, followed by a 10.0% SDS-PAGE and silver staining. The number around the spot was the index number in the gel for identification purpose. Blue arrow indicates the spot (P4H α) that was completely disappeared in CSE-treated HAECs. The spots with decreased intensity in CSE-treated HAECs were indicated by yellow arrows and spots with increased intensities were shown by red arrow. Brown arrow indicates IEF internal standard which has a MW of 33 000 and pI 5.2.

Figure 2.

CSE treatment specifically induced the expression of two proteins in human aortic endothelial cells. 2-DE was carried out as described in the Method section. The protein spot no. 37 and 315 were detectable only in CSE-treated HAECs. These spots were indicated by the white box with their spot identification number. Brown arrow indicates IEF internal standard which has a MW of 33 000 and pI 5.2.

Figure 3.

Effect of L-arginine pretreatment on CSE-induced changes in human aortic endothelial cells. HAECs were treated with 200 $\mu\text{mol/L}$ of L-arginine – a known substrate for eNOS – for 30 min and the medium was removed. The cells were then treated with 0.01 unit CSE for 4 h. After the experimental period the cells were collected and 2-DE was conducted as described in the Method section. L-arginine pretreatment decreased levels (> 3 -fold) of 4 proteins and increased the levels (> 3 -fold) of 4 other proteins when comparing to HAECs treated with CSE only. Yellow arrow showed the decreased spots and red arrow showed the increased spot with their spot identification numbers. Brown arrow indicates IEF internal standard which has a MW of 33 000 and pI 5.2.

Figure 4.

Effect of L-NAME pretreatment on CSE induced changes in human aortic endothelial cells. HAECs were treated with 400 $\mu\text{mol/L}$ of L-NAME – a known inhibitor of eNOS – for 30 min. The L-NAME containing medium was then removed before the 0.01 unit CSE treatment for 4 h. After the experimental period the cells were collected and 2-DE was carried out as described in the Method section. L-NAME pretreatment significantly decreased levels (> 3 -fold) of 2 proteins and increased levels (> 3 -fold) of other 3 proteins when compared to the cells treated with CSE only. Yellow arrow showed the decreased spots and red arrow showed the increased spots with their spot identification numbers. Brown arrow indicates IEF internal standard which has a MW of 33 000 and pI 5.2.

Table 1

Protein spots identified in 2-D gel with density differences >2 fold*

Reference spot no.	Isoelectric point (pI)	Molecular weight (MW)	(1) Control spot %	(2) CSE (0.01) Spot %	(3) L- Arg +CSE Spot %	(4) L- NAME +CSE Spot %	2 vs 1 difference	3 vs 2 difference	4 vs 2 difference
389	6.25	65 576	0.03	nd	nd	nd	—	nd	nd
87	5.56	62 687	0.37	0.09	0.21	0.17	-4.26	2.48	1.97
89	6.65	63 879	0.11	0.02	0.02	0.06	-4.97	-1.05	2.60
110	7.21	57 534	0.06	0.02	0.04	0.01	-3.12	1.98	-1.43
132	6.31	49 968	0.19	0.04	0.03	0.09	-4.62	-1.32	2.15
133	6.8	50 697	0.14	0.03	0.09	0.03	-5.05	3.30	-1.10
174	7.77	38 603	0.07	0.02	0.03	0.04	-3.00	1.16	1.53
177	5.78	38 081	0.13	0.04	0.04	0.04	-3.52	1.05	1.06
181	5.82	36 573	0.01	0.00	0.01	0.01	-3.20	1.80	1.08
296	8.12	20 698	0.08	0.02	0.02	0.03	-3.75	1.12	1.37
297	5.78	19 700	0.08	0.02	0.05	0.01	-3.71	2.46	-1.47
300	5.67	19 634	0.32	0.10	0.17	0.16	-3.05	1.65	1.56
305	6.5	19 202	0.15	0.04	0.08	0.03	-3.65	1.93	-1.27
307	6.09	18 525	0.43	0.13	0.31	0.29	-3.44	2.48	2.34
308	5.31	17 884	0.38	0.07	0.45	0.39	-5.16	6.03	5.28
163	5.31	39 468	0.12	0.05	0.14	0.04	-2.54	3.12	-1.06
282	4.93	22 937	0.06	0.05	0.14	0.11	-1.32	2.99	2.38
3	5.6	130 478	0.01	0.10	0.22	0.35	14.89	2.28	3.60
39	5.6	75 410	0.02	0.01	0.02	0.05	-1.64	1.39	3.96
37	6.3	76 819	nd	0.08	0.07	0.07	+++	-1.12	-1.03
315	5.94	17 190	nd	0.13	0.23	0.18	+++	1.70	1.37
5	6.39	104 523	0.05	0.39	0.40	0.29	8.56	1.02	-1.35
28	6.82	81 274	0.03	0.18	0.01	0.29	5.99	-19.53	1.59
53	5.92	72 155	0.34	2.34	1.83	2.24	6.97	-1.28	-1.04
178	6.38	38 013	0.03	0.10	0.11	0.15	3.16	1.08	1.52
188	7.85	36 029	0.24	1.23	1.31	1.15	5.18	1.06	-1.07
204	8.31	34 768	0.01	0.06	0.06	0.03	5.62	1.01	-1.77
266	5.79	26 272	0.02	0.16	0.16	0.14	8.30	-1.00	-1.11
20	6.95	87 965	0.10	0.09	0.03	0.06	-1.07	-3.00	-1.54
129	6.82	52 303	0.11	0.05	0.00	0.06	-2.13	-127.05	1.15
195	5.66	35 427	0.08	0.07	0.01	0.05	-1.09	-5.95	-1.44
158	5.18	40 591	0.11	0.21	0.20	0.03	1.83	-1.03	-6.68
208	7.69	34 391	0.02	0.05	0.05	0.01	2.20	-1.06	-4.00
4	5.37	105 108	0.14	0.43	0.82	0.91	2.97	1.92	2.14
6	6.58	112 764	0.09	0.20	0.19	0.27	2.12	-1.06	1.34
7	6.93	109 748	0.17	0.51	0.29	0.25	2.91	-1.73	-2.05
8	7.57	110 565	1.94	1.21	0.59	0.62	-1.60	-2.07	-1.96
15	6.63	90 938	0.43	1.22	1.04	1.12	2.83	-1.17	-1.09
24	5.71	83 546	0.03	0.01	0.01	0.01	-2.66	-1.14	1.08
32	6.46	77 833	0.09	0.04	0.04	0.05	-2.29	1.16	1.37
34	6.23	77 398	0.10	0.04	0.07	0.06	-2.58	1.76	1.39
36	6.64	77 855	0.02	0.05	0.04	0.05	2.42	-1.24	-1.01
42	6.17	75 805	0.10	0.05	0.06	0.06	-2.09	1.26	1.24
47	7.46	76 771	0.04	0.02	0.02	0.02	-2.26	1.32	1.47
49	6.48	74 501	0.04	0.02	0.03	0.03	-2.51	1.85	2.03
51	6.83	75 030	0.02	0.03	0.01	0.02	1.35	-2.22	-1.78
52	6.88	73 989	0.04	0.03	0.01	0.02	-1.16	-2.50	-1.36
59	6.1	71 024	0.24	0.28	0.12	0.25	1.15	-2.28	-1.12
61	6.91	71 462	0.03	0.03	0.02	0.06	-1.17	-1.32	2.13
69	6.45	68 562	0.05	0.02	0.03	0.05	-2.77	1.97	2.93

Reference spot no.	Isoelectric point (pI)	Molecular weight (MW)	(1) Control spot %	(2) CSE (0.01) Spot %	(3) L-Arg +CSE Spot %	(4) L-NAME +CSE Spot %	2 vs 1 difference	3 vs 2 difference	4 vs 2 difference
74	6.8	67 745	0.16	0.06	0.10	0.08	-2.85	1.77	1.35
80	6.46	65 085	0.04	0.05	0.10	0.10	1.14	1.11	2.30
81	6.8	65 515	0.11	0.04	0.09	0.05	-2.92	2.35	1.37
95	8.13	61 071	0.07	0.08	0.08	0.03	1.12	1.05	-2.40
104	6.37	58 602	0.13	0.05	0.12	0.10	-2.67	2.20	1.13
105	6.87	58 727	0.34	0.16	0.12	0.18	-2.14	-1.31	1.19
111	7.05	57 400	0.14	0.05	0.06	0.06	-2.62	1.11	1.25
115	6.77	56 452	0.08	0.09	0.04	0.12	1.14	-2.22	1.25
116	7.21	56 327	0.05	0.02	0.04	0.02	-2.23	1.75	-1.20
121	6.82	54 712	0.43	0.20	0.18	0.19	-2.12	-1.11	-1.08
123	5.45	52 981	0.08	0.03	0.02	0.03	-2.43	-1.77	1.06
126	7.03	52 975	0.10	0.05	0.05	0.05	-2.04	1.07	1.03
134	6.46	49 829	0.30	0.11	0.10	0.15	-2.85	-1.10	1.36
136	5.73	49 164	0.01	0.02	0.02	0.03	2.59	1.12	2.08
139	6.54	50 028	0.18	0.07	0.05	0.06	-2.64	-1.40	-1.06
145	6.69	48 556	0.31	0.14	0.14	0.15	-2.31	-1.01	1.10
146	6.39	47 462	0.11	0.08	0.03	0.11	-1.45	-2.59	1.46
150	5.22	45 873	0.15	0.11	0.22	0.09	-1.41	2.03	-1.16
157	5.36	41 881	0.45	0.45	0.41	0.22	1.01	-1.10	-2.07
162	6.06	39 935	0.17	0.06	0.04	0.10	-2.85	-1.61	1.77
169	7.64	38 954	0.14	0.06	0.12	0.09	-2.22	1.94	1.39
170	6.8	39 037	0.08	0.08	0.04	0.10	1.08	-2.13	1.13
173	5.57	38 383	0.15	0.11	0.28	0.07	-1.33	2.51	-1.72
176	5.44	38 262	0.10	0.22	0.12	0.15	2.17	-1.79	-1.43
200	5.24	35 125	0.05	0.06	0.02	0.04	1.12	-2.60	-1.63
201	5.53	35 065	0.66	1.34	1.46	1.40	2.02	1.09	1.04
210	6.24	33 807	0.05	0.02	0.01	0.01	-2.94	-1.61	-1.50
219	8.32	32 846	0.17	0.07	0.07	0.05	-2.49	-1.02	-1.27
221	7.41	33 332	0.05	0.07	0.04	0.12	1.61	-2.03	1.59
223	6.35	32 665	0.06	0.11	0.04	0.06	1.70	-2.76	-1.79
228	5.31	31 265	0.04	0.03	0.01	0.05	-1.30	-2.63	1.87
229	5.88	31 205	0.24	0.10	0.06	0.08	-2.44	-1.75	-1.26
230	5.94	31 084	0.05	0.06	0.02	0.05	1.17	-2.85	-1.19
231	5.17	30 001	0.55	0.39	0.97	0.28	-1.43	2.50	-1.37
232	7.45	31 523	0.13	0.08	0.10	0.17	-1.72	1.39	2.24
243	5.67	30 961	0.26	0.33	0.21	0.80	1.25	-1.56	2.43
249	6.89	28 504	0.12	0.06	0.08	0.06	-2.06	1.30	-1.05
250	5.61	28 187	0.24	0.12	0.11	0.14	-2.05	-1.05	1.18
254	8.91	27 868	0.09	0.09	0.05	0.03	-1.05	-1.81	-2.71
257	7.37	27 926	0.04	0.10	0.08	0.10	2.47	-1.35	-1.04
264	6.83	27 035	0.48	0.21	0.20	0.26	-2.36	-1.01	-1.06
271	8.18	25 666	0.31	0.69	0.47	0.65	2.20	-1.47	-2.08
278	6.38	24 264	0.35	0.12	0.11	0.25	-2.92	-1.07	2.08
279	8.31	24 157	0.11	0.12	0.07	0.05	1.07	-1.71	-2.45
298	5.16	19 181	0.06	0.02	0.06	0.04	-2.44	2.53	1.60
299	8.86	20 035	0.48	0.17	0.12	0.12	-2.83	-1.42	-1.49
304	5.53	18 875	0.21	0.08	0.04	0.10	-2.50	-2.37	1.25
314	5	17 188	0.82	0.39	0.85	0.63	-2.13	2.21	1.64
316	4.68	16 698	0.32	0.19	0.17	0.40	-1.71	-1.11	2.15
317	8.8	17 392	0.68	0.49	0.64	1.02	-1.39	1.31	2.08
319	7.42	17 152	0.32	0.20	0.48	0.56	-1.59	2.39	2.75
321	5.06	14 706	0.20	0.13	0.28	0.27	-1.53	2.11	2.02

* We only selectively included the spots with 2 fold or more changes between treatment conditions. The full list of the detected spots can be viewed upon request.

Table 2
Changes in protein levels expressed as % spot of the total blot (fold changes by comparing to control HAECs)

Proteins	Controls	CSE	L-Arg+CSE	L-NAME+CSE
<i>Up-regulated</i>				
HSP-70, Lamin B1	0.02	0.01 (0.5)	0.02 (1)	0.05 (3.96)
HSP-70	0.34	2.34 (6.88)	1.83 (5.38)	2.24 (6.59)
HSP-27	0.02	0.16 (8.30)	0.16 (8.30)	0.14 (7)
Alpha-actinin	0.05	0.39 (8.56)	0.40 (8.7)	0.29 (5.8)
Annexin 6	0 (nd)	0.08	0.07	0.07
Annexin 2	0.24	1.23 (5.18)	1.31 (5.45)	1.15 (4.79)
Moesin	0.03	0.18 (5.99)	0.01 (0.4)	0.29 (9.7)
HSP gp96	0.05	0.39 (7.8)	0.40 (8)	0.29 (5.8)
Laminin-binding protein	0.11	0.21 (1.91)	0.20 (1.82)	0.03 (0.27)
<i>Down-regulated</i>				
Myosin light chain	0.38	0.07 (0.18)	0.45 (1.18)	0.39 (1.01)
Prolyl 4-hydroxylase	0.03	0 (nd)	0 (nd)	0 (nd)
eIF4A	0.19	0.04 (0.21)	0.03 (0.16)	0.09 (0.47)
Alpha enolase	0.14	0.03 (0.21)	0.09 (0.64)	0.03 (0.21)
Alpha enolase	0.11	0.05 (0.45)	0.00 (0.01)	0.06 (0.54)
Alpha tubulin	0.37	0.09 (0.24)	0.21 (0.57)	0.17 (0.46)
Alpha tubulin, glutaminase	0.11	0.02 (0.18)	0.02 (0.18)	0.06 (0.55)