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Proteomic profiling of rat lung epithelial cells induced by acrolein

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Abstract

Aims—Acrolein is a highly toxic unsaturated aldehyde and is also an endogenous byproduct produced from lipid peroxidation. It can be formed from the breakdown of certain pollutants in outdoor air or from burning tobacco or gasoline. Inhalation and dermal exposure to acrolein are extremely toxic to human tissue. Although it is known that acrolein is toxic to lung tissue, no studies have attempted to address the changes induced by acrolein on a global scale.

Main methods—In the present study we have attempted to address the changes in global protein expression induced by acrolein using proteomics analysis in rat lung epithelial cells.

Key findings—Our analysis reveals a comprehensive profiling of the proteins that includes a heterogeneous class of proteins and this compels one to consider that the toxic response to acrolein is very complex. There were 34 proteins that showed changes between the control cells and after acrolein treatment. The expression of 18 proteins was increased and the expression of 16 proteins was decreased following exposure to acrolein. We have further validated two differentially expressed proteins namely annexin II (ANXII) and prohibitin (PHB) in lung epithelial cells treated with acrolein.

Significance—Based on the results of the overall proteomic analysis, acrolein appears to induce changes in a diverse range of proteins suggesting a complex mechanism of acrolein-induced toxicity in lung epithelial cells.

Keywords

Acrolein; Lung epithelial cells; Proteomics; Two dimensional gel electrophoresis

Introduction

Acrolein is a highly toxic three-carbon unsaturated aldehyde produced during the incomplete combustion of organic matter (Hartzell, 1996). Acrolein, a highly reactive α β -unsaturated aldehyde, is a ubiquitous environmental pollutant. It is one of the end products of endogenous lipid peroxidation reactions (Adams and Klaidman, 1993), and is very effective in binding to DNA leading to adduct formation. (Uchida K 1999). Acrolein is also present in tobacco smoke and automobile exhaust emissions linking it to respiratory injury by suppressing host defense mechanisms. Further, acrolein is a biotransformation product of allyl compounds and the

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anticancer drug cyclophosphamide (Uchida K 1999). Human exposure to acrolein may occur as a result of environmental exposure or as a result of endogenous reactions.

Acrolein is also a strong alkylating agent and modifies cysteine and arginine residues of proteins, leading to nonfunctional proteins (Uchida et al. 1998a). Acrolein contained in cigarette smoke induces inflammatory responses in lung (Kehrer and Biswal, 2000). Toxicity studies with acrolein have shown increased apoptosis of alveolar macrophages (Li et al. 1997), inhibition of neutrophil apoptosis (Finkelstein et al. 2001), increased mucus secretion (Borchers et al. 1999), increased pulmonary edema (Hales et al. 1989; Kutzman et al. 1985), and increased bronchial responsiveness (Ben-Jebria et al. 1994; Leikauf et al. 1989). In addition, acrolein induces oxidative modification of proteins which either results in de-regulation of specific signaling pathways or protein inactivation (Uchida et al. 1998 b). We have shown earlier that acrolein induces cyclooxygenase-2 (COX-2) in lung epithelial cells and that this induction was mediated by NF κ B through the involvement of Ca⁺² (Sarkar and Hayes, 2007). Together, these reports indicate that acrolein may cause more harm as a toxicant to lung than any other target tissue because when it is airborne it can easily interact with the respiratory tract to elicit inflammation and induce apoptosis as reported by a number of investigators (Tanel and Averill-Bates, 2007).

Very recently a microarray study in A549, human adenocarcinoma lung cells showed that acrolein treatment produced significant changes in mRNA expression of proteins coding for major pathways like those involved in apoptosis, cell cycle control, transcription, cell signaling, and protein biosynthesis (Thompson and Burcham, 2008). Interestingly, a global cellular protein or gene expression profiling with acrolein has rarely been performed. However, studies in an Alzheimer's disease model have shown extensive carbonylation of proteins in synaptosomes by acrolein (Mello et al. 2007). Moreover, a proteomics approach in oxidative stress resistant cell lines (OC14 cells) showed the enzyme aldolase reductase (AR) to be 4 fold more abundant than its parent line when treated with acrolein (Keightley et al. 2004). A proteomics approach that can provide expression profiling as well as information about modification of proteins is an effective method to globally study changes in cellular proteins following exposure to acrolein. In the present study we used rat lung epithelial cells and exposed them to acrolein to study changes in protein expression by 2 dimensional (2-D) proteomics and the proteins were specifically identified by MALDI-TOF/TOF.

Materials and methods

Cell culture and treatment

Rat lung epithelial cells (ATCC catalog number CRL-10354), were obtained from ATCC and were propagated in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 100 μ g/ml streptomycin. Cells were grown in a 100 mm tissue culture dish and allowed to adhere overnight followed by serum starvation for a period of 18 hours. Quiescent cells were then treated with 20 μ M acrolein for an additional 24 hours in 10% FBS with penicillin and streptomycin antibiotics. Control cells were cultured in parallel under similar conditions in the absence of acrolein. A single set of experiments was performed in triplicate and two such independent experiments were also performed. Two dimensional gel electrophoresis was performed in these samples in a set of six, with two independent gel runs, in order to ensure the reliability of data and to exclude experimental variation.

Sample Preparation

Rat lung epithelial cells (LE) cells were washed with phosphate buffered saline (PBS) and harvested after 24 hours of acrolein treatment. The cells were centrifuged at 5,000 RPM at 4°C for 10 minutes to obtain a cell pellet. The cell pellet was suspended in 0.5 ml of isoelectric

focusing buffer containing 20 mM Tris, 7M urea, 2M thiourea, 4% CHAPS, 50mM 1,4-dithioerythritol (DTT), 0.2% ampholyte (3-10, Bio-Rad) and a mixture of protease inhibitors (Roche Diagnostics). The cell suspension was sonicated for approximately 30 seconds and centrifuged at 16,000xg for 30 minutes. The cell supernatant was processed through a Ready Prep 2-D Cleanup kit from Bio-Rad (catalog number 163-2130) according to the manufacturer's instructions. The protein content in the supernatant was determined using the Bradford method (Bio-Rad).

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The 2-D gel electrophoresis, gel analysis and spot picking were performed in the RCM Proteomics Core Facility at Texas Southern University (TSU), Houston, TX. The 2-D gel electrophoresis was performed as previously reported (Sarkar et al. 2006a) with minor modifications. In brief, total cellular protein extracts (150 µg protein) were applied on immobilized pH 3-10 gradient strips (IPG 11 cm, Bio-Rad). Isoelectric focusing was run at 250V followed by a gradual increase to 8000V at 3V/min and kept constant for an additional 6 hours in a Protean IEF unit (Bio-Rad). At the end of first dimension the focused strips were equilibrated for 15 min in 50mM Tris-HCL (pH 8.8), 7M urea, 2M thiourea, 30% glycerol containing 10mg/ml DTT followed by 25mg/ml iodoacetamide in the equilibration buffer for 15min at room temperature. The second-dimensional separation was performed on 12.5% homogeneous SDS-polyacrylamide gels of 13.3×8.7cm (W×L) dimension (Criterion gels, catalog number 345-0102, Bio-Rad). These gels were run at a constant volt of 200V, in a Dodeca 2-D running apparatus (Bio-Rad). Following the completion of the gel run, the 2-D gels were fixed with 40% methanol, containing 10% acetic acid for 2 hours and then stained with Sypro Ruby for 12 hours in the dark with gentle shaking. The gels were destained with 10% methanol and 7% acetic acid with three subsequent changes of the destaining solution in the dark. Gels were scanned on a Molecular Fx imager (Bio-Rad) and processed using assisted software. The images were captured as 16 bit files and further analyzed using PD Quest software from Bio-Rad. To ensure the reliability of data and exclude experimental variation, protein expression data were obtained from three biological triplicate samples.

Image Analysis

The image analysis was performed using PD Quest software (Version 7.0; Bio-Rad, Hercules, CA) and the quantity of protein in each spot was normalized by the total valid spot intensity according to the manufacturer's instructions. Spots that showed a greater than 2- fold change in expression compared to control were selected for pick up by a Bio-Rad spot picker (RCM Proteomics Core Facility, TSU).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/TOF-MS)

The collected spots were sent to the University of Texas Medical Branch (UTMB) at Galveston, TX, Proteomics core facility for MALDI-TOF/TOF analysis. Protein gel spots were excised and prepared for Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry analyses. Gel pieces were incubated with trypsin (20 µg/ml in 25 mM ammonium bicarbonate, pH 8.0; Promega Corp.) at 37°C for 6 hours. One microliter of matrix (alpha-cyano-4-hydroxycinnamic acid; Aldrich Chemical Co.), was then applied on the sample spot and allowed to dry. MALDI-TOF/TOF MS was performed using an Applied Biosystems model 4700 Proteomics Analyzer for peptide mass fingerprinting and MS/MS analysis. Following MALDI MS analysis, MALDI MS/MS was performed on several ions from each sample spot. Applied Biosystems GPS Explorer TM Software (v.3.0) was used in conjunction with MASCOT to search the NCBI databases utilizing both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or

MASCOT protein scores. Search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one missed trypsin cleavage, and 50 ppm mass accuracy. The peptide masses were compared with the theoretical peptide masses of all available proteins from all species. All the unmatched peptides or miscleavage sites were excluded for protein identification.

Immunoblotting

Immunoblotting was carried out as previously described (Sarkar et al. 2006). Briefly, LE cells were cultured with 5, 10, 20, 30 and 40 μ M concentrations of acrolein for prohibitin (PHB) and at varying time intervals for ANXII and PHB. Cells were lysed and immunoblotting was performed as described earlier (Sarkar et al. 2006; Sarkar and Hayes, 2007). Briefly, after acrolein treatment, cells were washed twice in ice-cold phosphate buffered saline (PBS) and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% Nonidet P-40, 0.05mM pepstatin A, and 0.2 mM leupeptin. Protein concentrations were analyzed using Bradford reagent from Bio-Rad. (catalog number; 500-0201). Approximately 50 μ g protein was resolved on 10% SDS-PAGE gels, followed by transfer to a nitrocellulose membrane using Trans-Blot SD semidry transfer cell (Bio-Rad). The membranes were blocked one hour in TBST buffer (150 mM NaCl, 20 mM Tris-Cl, and 0.05% Tween 20 [pH 7.4] containing 5% nonfat milk). Membranes were then incubated with either PHB; Santa Cruz Biotechnology, catalog number SC-28259 or ANXII; Santa Cruz Biotechnology, catalog number SC-9061 antibody at a dilution recommended by the manufacturer. The membrane was washed and incubated with horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. Immunoreactivity was detected using enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc., catalog number SC-2048) following the manufacturer's instructions. The intensity of bands was analyzed by Image J software from the National Institutes of Health.

Quantitative PCR

PHB and ANXII mRNA expression was analyzed by real time PCR. The primer sequences for PHB amplification were forward primer: 5'-GGCAGCCTGA GTAGACCTTG-3', and reverse primer 5'-TCACGGTTAAGAGGGAATGG-3' and for ANXII the forward primer was 5'-CATTCTGACTAACCGCAGCA-3' and reverse primer: 5'-CGGTAAATCTCCTGCAGCTC-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control and was amplified by the PCR primer sequence for forward 5'-GTATTGGGCGCCTGGTCACC-3', and 5'-CGCTCCTGGAAGATGGTGATGG-3' for reverse. Real-time PCR was performed using Bio-Rad iCyclerIQ in the presence of SYBR-green. Single-step RT-PCR amplification was performed in 96-well optical reaction plates using the iCycler real-time PCR machine (Bio-Rad Laboratories, Hercules, CA). The optimized concentrations of reaction mixture for best results were a total reaction volume of 10 μ l containing 25 pmoles of each (forward and reverse) primer, 5 μ l (1X final concentration) of 2X SYBR Green RT-PCR reaction mix, 2 μ l of iScript Reverse Transcriptase and 200 ng of total RNA. Using these optimal concentrations of PCR reaction mixture constituents, the amplification reaction for each sample was performed in triplicate using a PCR condition of reverse transcription at 50°C for 15 min, denaturation and RT enzyme inactivation at 95°C for 5 min, followed by 40 cycles of 10 second denaturation at 95°C and 30 second annealing and extension at 60°C. A non-template control was also included in each experiment. Threshold cycle number (Ct value) was analyzed using an iCycler iQ optical system software (Bio-Rad, version 3.0a). Quantitative PCR was normalized to the Ct value of GAPDH from the same sample and the fold change in the expression of each gene was calculated by using the delta-delta Ct method (Livak and Schmittgen, 2001).

Immunofluorescence

The immunofluorescence procedure was carried out as detailed by Sarkar et al (2006b). The cells were grown on sterile glass coverslips and then treated with acrolein in the presence of 10% serum for 24 hours. The cells were then washed with chilled PBS and fixed with cold methanol for 20 minutes at -20°C. The cells were washed again with PBS and blocked with 5% normal goat serum, for 1 hour at room temperature. Cultures were then incubated for 1 hour either with PHB antibody; catalog number, SC 28259, or ANXII antibody, catalog number SC - 9061, (Santa Cruz Biotechnology) at a dilution of 1:200 and 1:300 in PBS with 5% goat serum, respectively. The cells were then washed three times with PBS and incubated with Texas red-conjugated secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:500 for 1 hour. Finally, the cells were washed with PBS followed by incubation with 4, 6 diamino-2-phenylindole (DAPI) for 15 min and then thoroughly washed again with PBS. The coverslips with stained cells were mounted on glass slides with antifade mounting medium and viewed under a fluorescence-microscope (Nikon, Japan).

Results

The proteomics analysis of LE cells in control and after acrolein treatment revealed a complex set of changes in protein profile. These changes spanned a wide range of functional proteins involved in various cellular functions. The correlation coefficient between the control and acrolein treated cells was 75% and most of the differentially expressed proteins were located between the pH range of 4.0-7.0 (Fig 1A). The differences in the intensities of protein spots were compared and analyzed by PD Quest software (Bio-Rad) and showed a wide range of proteins with difference in their spot intensities. A total of 34 proteins were found to be altered by acrolein treatment. Of the 34 proteins, 18 proteins were increased by acrolein and 16 proteins were decreased as compared to control (Table 1 and Table 2). The identification of these protein spots was done by MALDI-TOF/TOF, followed by a MASCOT search for matching peptides with sequence coverage of nearly 38%. The list of upregulated and downregulated proteins is shown in Table 1 and Table 2, respectively. Seven proteins showed a more than 2-fold increase compared to control that included mainly structural protein, glucose regulated protein and oxidative stress- related protein. Vimentin, a mesenchymal marker, that is predominantly expressed as an intermediate filament protein in the cytoplasm (Poggi et al. 2001), was increased by acrolein treatment. Vimentin is spot 1802 (Fig. 1 A and Table 2). Rota et al (2001) reported that in human gingival fibroblast (HGF) cells, acrolein and acetaldehyde can bind and interact with cytoskeletal structures to prevent adhesion of cells by disrupting tubulin and vimentin intermediate filaments (VIF). This resulted in a disturbance of the HGF cytoskeleton which included disruption of microtubules and VIF with alterations in cell shape.

Peroxisome-3 (PRDX3) spot 6002, is a unique protein that was increased over 11-fold by acrolein compared to control. PRDX3 is a mitochondrial peroxidase that defends cells against oxidative damage (Chang et al. 2004). Acrolein induces oxidative stress and correlates to a relative increase in this protein as a protective measure against acrolein induced oxidative stress in the cells. Tubulin alpha-6, spot 2701, was increased by 2.65-fold following acrolein treatment. Tubulin alpha-6 belongs to a structural family of proteins and alteration in this protein may be predictive of patient outcomes in non-small cell lung cancer chemotherapy (Seve et al. 2005).

In response to acrolein treatment of LE cells, there were very few proteins that were significantly decreased as compared to control. Protein tyrosine 3-monooxygenase, spot 1101, was decreased by almost 50% by acrolein treatment compared to control. This enzyme also known as tyrosine hydroxylase is a rate-limiting step in the biosynthesis of catecholamines. There was decreased expression of PHB, spot 3101. In recent years, PHB has gained importance because of its diverse function including its role in cell death by apoptosis (Mishra

et al. 2006). Although the role of PHB in acrolein-associated cellular effects has not been exploited, it is interesting that the PHB level is reduced in acrolein treated LE cells. To better understand PHB's role in acrolein treated LE cells, we extended our studies using different experimental methods. The results of these studies are discussed below.

Interestingly annexin 1 (ANXI), spot 7301, and annexin 3 (ANXIII), spot 5201 were decreased while ANXII, spot 9303 was increased by acrolein treatment in LE cells (Fig 1A, Fig. 1B and Fig 1C). ANX II expression was significantly increased to 2.56 ± 1.01 (n=6) in acrolein treated cells compared to control cells. The representative gel showed a 1.22 fold increase as shown in Table 2, while the other replicate gels showed a relative increase as compared to control. Since most of the gels showed a significant increase in fold change, we attempted to validate this protein using a specific experimental approach. Annexins belong to a family of proteins that have diverse functions and are discussed extensively. (Gerke et al. 2005; Wolberg and Rouby 2005; and Kwon et al. 2005). Since ANXII is involved in cell proliferation and a number of other cellular functions, we investigated the effect of acrolein on ANXII. We treated the LE cells with 20 μ M of acrolein and observed an increase in ANXII expression in a time dependent manner. A time dependent increase in ANXII was observed with a maximum increase at 24 hours (Fig. 2B and Fig. 2C). In addition to the increase in ANXII observed by immunoblot analysis, we performed real time PCR and immunofluorescence analysis to validate the observation. (Fig. 2A and Fig. 2D). As shown in figure 2A, ANXII mRNA expression was found to be upregulated at 3 hours and then it was decreased at 6 and 24 hours. The ANXII fluorescence signal was increased after 24 hours of acrolein treatment of LE cells as shown in Figure 2D. These results show that acrolein transcriptionally induced ANXII protein in LE cells. It has been shown that acrolein causes epithelial cells to undergo differentiation and ANXII is involved in cellular differentiation. Therefore, these results suggest that acrolein-induced differentiation may be dependent on ANXII (Grafstrom et al. 1988; Harder et al. 1992).

In addition to ANXII, we also investigated and validated the decrease in PHB which is also involved as an inhibitor of cell proliferation. Moreover, prohibitin also functions as a chaperone protein in the mitochondria (Mishra et al. 2006; Czarnecka et al 2006; Nijtmans et al. 2002). In the present study we found a significant reduction in prohibitin level (0.53 ± 0.2 ; n=6) in acrolein treated LE cells compared to control (Fig. 1A and Fig. 1B). The representative gel shown in Figure 1 showed a 0.83 fold decrease, while other replicate gels showed relative decrease in fold change with a mean of 0.53 ± 0.2 (Table 1). The inset view shown in Figure 1B also showed a relative decrease in acrolein treated cells as compared to control cells. A mass spectra for the peptide fingerprint of PHB is shown in Figure 1C. To further confirm the results obtained from the proteomics study we performed real time PCR, immunoblot analysis, and immunofluorescence analysis in cells treated with acrolein either with different concentrations or with the same concentrations of acrolein at different time intervals of exposure. All of these results are summarized in Figure 3. Transcriptional inactivation was observed by real time PCR and the results showed a time dependent decrease in prohibitin mRNA in LE cells treated with acrolein (Fig. 3A). The decrease in PHB mRNA was observed at 3 hours followed by a time dependent decrease until 24 hours. The transcriptional decrease in PHB mRNA may be due to a decrease in co-activators of this gene. It has been reported that under hypoxic conditions PHB is decreased in rabbit lung and acrolein is known to be involved in inducing hypoxia (Henschke et al. 2006; Zhang et al. 2007). A time course study reveals that as early as 2 hours following acrolein exposure, PHB protein expression decreased up to 24 hours (Fig. 3B). There were no changes compared to control at 1 hour. In addition a dose-dependent decrease in PHB protein expression was also observed by acrolein in LE cells that correlates with the transcriptional decrease (Fig. 3E). We found a correlation with the decrease in prohibitin expression by immunoblot as well as by immunostaining. There was a significant

decrease in prohibitin staining in cells treated with acrolein and the protein was localized in the cytosol (Fig. 3D).

Discussion

Using proteomics technology, we have detected acrolein-induced changes in protein expression in rat lung epithelial cells. The present study was conducted as part of an effort to provide a better understanding of the effects of acrolein on rat lung epithelial cells. In rat lung epithelial cells, acrolein toxicity manifests itself primarily by an inhibition of cell proliferation and eventually, initiation of cell death (Sarkar and Hayes, 2007). The 2-D electrophoresis approach outlined above resulted in the identification of 34 altered protein species with acrolein (Tables 1 and 2). ANXII which was increased by treatment with acrolein has been implicated in a variety of cellular functions including inhibition of cell proliferation (Croxtall and Flower, 1992). Lipocortin-1 (annexin-1) mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line, anti-inflammatory effects (Gerke and Moss, 2002; Flower and Rothwell 1994, Philip et al. 1997), and regulation of cell differentiation (Violette et al. 1990), and membrane trafficking (Diakonova et al. 1997). In addition, ANX1 has been reported to be involved in the inhibition of proinflammatory mediators such as phospholipase A2, cyclooxygenase-2 and inducible nitric oxide; induction of apoptosis in inflammatory cells; and induction of IL-10, an anti-inflammatory cytokine (Parente and Solito, 2004). Acrolein induces inflammation and has been shown to increase cyclooxygenase-2 (Sarkar and Hayes 2007). Acrolein has also been found to be associated with cellular differentiation (Grafstrom et al. 1988), and the increase in annexin II by acrolein might deregulate cellular signaling involved in cellular differentiation.

PHB was decreased by almost 50% in cells treated with acrolein. PHB, a mitochondrial protein, has been implicated as an important antiproliferative protein inducing apoptosis and inhibiting cell proliferation by blocking the G1/S transition of the cell cycle (McClung et al. 1992). PHB induces apoptosis by interacting with the retinoblastoma protein as well as being involved in the p53 pathway (Wang et al. 1999). Furthermore, PHB has been described as a novel, independent regulator of apoptosis. Acrolein can induce apoptosis through the death receptor pathway (Tanel and Bates, 2007) and directly through the mitochondrial pathway (Tanel and Bates, 2005). In the present study, PHB expression decreased at the level of mRNA and proteins in the cells. These decreases correlated to each other thereby suggesting that acrolein inhibits PHB expression at both the transcriptional and translational levels. The transcriptional expression of PHB is regulated by IL-6 (Theiss et al. 2007), however, in the present study we have not estimated the levels of expression of IL-6 or its receptor expression following exposure to acrolein in LE cells. Additional studies are needed to determine the involvement of IL-6 in prohibitin regulation. It is important to note that acrolein has been shown to decrease expression of IL-2 and IL-1 β (Lambert et al. 2005).

Conclusion

In conclusion, the results of these studies reveal that acrolein induces changes in a diverse range of proteins that may be related to cellular toxicity in LE cells and that toxic effects of acrolein may be due to deregulation of proteins involved in proliferation and apoptosis. The spectrum of proteins that showed changes following acrolein exposure may also have some association with development of certain lung diseases and disorders.

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Fig.1.

2-DE gel protein pattern from control and acrolein treated LE cells (A). Control cells were grown in DMEM with 10% were grown in DMEM with 10% for 24 hours, as described in the materials and methods section. The cell lysate was prepared and then sonicated in 2-DE extraction buffer. Approximately 150 μ g of protein was used for 2-DE and stained with Sypro Ruby. Images were acquired and differentially expressed proteins were identified by PD-Quest software analysis. Expression of protein spots showing significant changes are marked with arrows. The panel shows enlarged regions of the 2-DE protein profile representing changes in protein amounts of PHB, PRDX3 and ANXII in control and acrolein treated LE cells (B). PHB

and ANXII identification was done by peptide fingerprinting. Details are described in materials and methods (C).

Fig. 2.

Real time PCR for ANXII (A). Western blot analysis of ANXII at 0, 3, 6, 12 and 24 hours of treatment with 20 μM acrolein (B). The representation of mean density of bands from three independent experiments. Values are means $\pm$ SD. (C). Immunostaining of ANXII in control and acrolein treated LE cells. Cells were grown on glass coverslips and then treated with 20 μM acrolein for 24 hours. The cells were then washed with chilled PBS and fixed in methanol at -20°C for 15 minutes. This was then followed by immuno-detection by ANXII specific antibody as described in materials and method. Cytosolic staining of ANXII is shown in red and the nucleus is stained in blue (D).

Fig. 3.

Real time PCR for PHB (A). Western blot analysis of PHB in LE cells treated with 20 μM acrolein at different time intervals (B). The representative mean densities of bands are shown from three independent experiments. Values are mean $\pm$ SD (C). Immunostaining of PHB in control and acrolein treated LE cells. Cells were grown and treated as described in Fig. 2 D. Staining of PHB is shown in red and nucleus is in blue (D). Dose response of acrolein at 0 μM to 40 μM of concentration for PHB protein expression (E), representative mean densities of bands from three independent experiments, values are mean $\pm$ SD (F).

Table 1
Proteins decreased by acrolein in rat lung epithelial cells as compared to control.

Spot Number	Protein name	Expectation Value	Accession Number	MW	PI	Score	Fold change
5702	Heterogeneous nuclear ribonucleoprotein H2	1.10E-13	gi62078769	49547.4	5.89	175	0.83
6403	Capping protein (actin filament)	7.00E-15	gi161556900	39059.8	6.11	187	0.92
7301	Annexin I	7.00E-21	gi16978501	39147.1	6.97	247	0.86
401	Predicted:similar to reticulocalbin	5.60E-14	gi134856626	38066.6	4.67	178	0.73
601	Reticulocalbin 2	3.50E-13	gi138303857	37409.9	4.27	170	0.96
5201	Annexin III (Lipocortin III)	3.50E-20	gi151980303	36568.7	5.96	240	0.84
6101	Endoplasmic reticulum protein 29	1.10E-22	gi116758848	28614	6.23	265	0.92
502	Reticulocalbin 3.EF-hand Ca binding protein	1.30E-03	gi153734248	37918.2	4.72	74	0.95
2101	Rho GDP dissociation factor (GDI) alpha	8.80E-19	gi131982030	23449.8	5.12	226	0.84
5701	Aldehyde dehydrogenase	5.60E-21	gi116073616	48638.6	6.06	248	0.76
9101	MGC127753 (Bos taurus)	3.50E-11	gi174354127	24578.7	7.01	150	0.74
2603	Heterogeneous nuclear ribonucleoprotein F	1.40E-11	gi125742579	46043	5.31	154	0.67*
3101	Prohibitin	7.00E-28	gi113937353	29858.9	5.57	317	0.83*
8301	Lipocortin	8.80E-23	gi1235879	39137	6.97	266	0.82
2601	CaBP1	8.80E-41	gi1488838	47589.9	4.95	446	0.74
1101	Tyrosine 3-monoxygenase	2.80E-10	gi19507243	28150.9	4.81	141	0.52*

Trypsin digested proteins were identified by MALDI-TOF/TOF MS.

* Spots statistically significant at P<0.05.

Table 2
Proteins increased by acrolein in rat lung epithelial cells as compared to control.

Spot MW Number	PI	Protein name Score change	Fold	Expectation Value	Accession Number
802	Unnamed protein product			3.50E-24	gi11334284
58060.7	5.35	280	3.64*		
7701	Enolase 1, alpha			2.20E-07	gi159808815
47470.4	6.16	112	1.18		
1802	Vimentin			3.50E-31	gi114389299
53757.1	5.06	350	7.81*		
3401	Farnesyl pyrophosphate synthetase			1.40E-13	gi1120478
41146.1	5.29	174	1.01		
6002	peroxiredoxin 3			1.80E-10	gi111968132
28588.6	7.14	143	11.07*		
2402	Actin,cytoplasmic2			2.20E-38	gi162657474
62571.2	6.97	422	12.22*		
	(Gamma -actin)				
5802	Glucose regulated protein,58kDa			2.80E-10	gi138382858
57043.9	5.88	141	2.56*		
5704	Unknown (protein for IMAGE: 7107492)			2.20E-08	gi160688498
50856.1	7.53	122	1.06		
3503	beta actin (Homo sapiens)			2.80E-07	gi14501885
42051.9	5.29	111	1.72*		
7602	Eef1g protein			5.60E-21	gi151261278
50371.3	6.31	248	1.16		
9303	annexin A2			1.80E-48	gi19845234
38938.9	7.55	523	1.22*		
5801	Glucose regulated protein,58kDa			7.00E-23	gi138382858
57043.9	5.88	267	1.07		
6704	Enolase 1, alpha			2.20E-31	gi16978809
47428.3	6.16	352	1.16		

Spot MW Number	PI	Protein name Score change	Fold	Expectation Value	Accession Number
102	tyrosine 3/tryptophan 5-monooxygenase				
29326.5	4.63	281	1.03	2.80E-24	gi15803225
8003	activation protein. epsilon polypeptide expressed in non-metastatic cells 2				
17385.9	6.92	319	1.24	4.40E-28	gi155926145
1801	iodothyronine 5' monodeiodinase				
54375.4	4.87	517	1.18	7.00E-48	gi1202549
3803	heat shock protein 1(chaperonin)				
61098.5	5.91	273	2.31*	1.80E-23	gi111560024
2701	Tubulin alpha 6 (predicted)				
50589.7	4.96	310	2.65*	3.50E-27	gi150927313

Trypsin digested proteins were identified by MALDI-TOF/TOF MS.

* Spots statistically significant at P<0.05.