ERJ Express. Published on February 2, 2006 as doi: 10.1183/09031936.06.00012805

Regulation of human lung epithelial cell numbers by diesel exhaust particles

Hasan Bayram[1,2], Kazuhiro Ito[1], Razao Issa[1], Misako Ito[1], Maria Sukkar[1], Kian Fan Chung[1].

[1]Thoracic Medicine, National Heart & Lung Institute, Imperial College, London, UK. [2]Department of Respiratory Medicine, School of Medicine, Dicle University, Diyarbakir, Turkey.

Running title: DEP and cell proliferation and death

(3395 words)

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Correspondence address:

Professor K F Chung.

National Heart & Lung Institute

Imperial College

London SW3 6LY

UK

Tel. +44 207 351 8995

Fax. +44 207 351 8126

E-mail: f.chung@imperial.ac.uk

Abstract (198 words)

Particulate air pollution is associated with respiratory morbidity and has cytotoxic and proinflammatory effects. We examined the effects of diesel exhaust particles (DEP) on proliferation and apoptosis of A549 lung epithelial cells.

When deprived of serum (serum starvation), epithelial cell numbers fell, but DEP (5-200μg/ml) prevented this. Using flow cytometric analysis of propidium iodide (PI) staining, DEP (10μg/ml) increased cells in S phase of cell cycle from 12.85% to 18.75% ($p<0.0001$) after 48hrs, reversing serum starvation-induced $G_{0/1}$ arrest. DEP also reduced the increase in apoptotic cells, as defined by double-expression of annexinV/PI, observed after serum starvation (from 28.35 to 15.46%; $p<0.05$). The anti-oxidants, *N*-acetylcysteine (NAC, 33 mM) and AEOL10113 (10-100μM), the N-terminal c-jun kinase (JNK) inhibitor, SP600125 (33μM), and NF-κB inhibitor, SN50 (33μM), inhibited DEP-induced cell number increase. NAC inhibited DEP-induced reduction of $G_{0/1}$ and increase in cells in the S and G_2/M phases. Expression of $p21^{CIP1/WAF1}$ mRNA and protein seen with serum-starvation was reduced by DEP.

DEP prevented serum-starvation-led decreases in A549 epithelial cells by inducing cell cycle progression and preventing apoptosis, processes involving oxidative stress, inhibition of $p21^{CIP1/WAF1}$ expression and stimulation of JNK and NF-κB. Therefore, low-dose DEP exposure may lead to lung epithelial cell hyperplasia.

Key words: Diesel exhaust particles; apoptosis, cell proliferation, anti-oxidants; nuclear factor-κB

Introduction

With the increasing use of diesel powered engines, particulate air pollution is increasingly being recognised as a major public health hazard and as contributing to the burden of pulmonary and cardiovascular diseases. Thus, there is a strong association between particulate air pollution and impaired lung function, deficits in lung function growth, worsening of asthmatic symptoms, and increased emergency room visits for asthma and COPD [1 2]. In addition, a relationship between all cause mortality, cardiopulmonary deaths and lung cancer mortality in adults living in metropolitan areas and the level of particulate air pollution has been reported [3 4 5].

The mechanisms underlying the deleterious effects of particulates on the lung are unclear. On exposure to particulates, airway epithelial cells, which form the first line of innate immune defence against particles, produce inflammatory mediators such as interleukin (IL)-8, granulocyte macrophage–colony stimulating factor (GM-CSF), regulated on activation, normal T-cell expressed and secreted (RANTES) and soluble intercellular adhesion molecule (sICAM)-1 [6 7] and reactive oxygen species, hydrogen peroxide (H_2O_2) and superoxide (O_2^-) [8;9] that can activate the transcription factors, nuclear factor-κB (NF-κB) and activator protein (AP)-1 pathways [10;11]. In addition, particulates such as those of 10 μM in diameter (PM_{10}) can also cause cell death by necrosis, and by the process of apoptosis [12 13]. A dose-dependent increase of murine airway epithelial cell proliferation *in vivo* has also been reported [14]. The process of proliferation and of apoptosis may occur simultaneously [15]. The effects of particulates on regulation of cell numbers may be related to specific cell types, to different concentrations of particulates and to species differences, and could also result from a balance of effects of particulates on signal transduction pathways that regulate both cell apoptosis and proliferation.

The molecular mechanisms of survival or proliferation or cell death of airway epithelial cells induced by particulates are also unclear. NF-κB activation, which is a cellular effect of particulate exposure, has been associated with cell survival and also with induction of apoptosis [16]. In some models of oxidative stress, activation of the mitogen-activated protein kinase (MAPK) pathway such as the c-jun N-terminal kinase (JNK) has been associated with programmed cell death or apoptosis [17;18]. Under certain circumstances, JNK may also increase cell survival and play a role in cell proliferation [19;20]. Finally, the final key determinant of cell cycle, growth arrest and differentiation is the cyclin-dependent kinase inhibitor, $p21^{CIP1/WAF1}$, [21] which is highly responsive to oxidative stress [22 23], but the effect of particulates on $p21^{CIP1/WAF1}$ expression in lung epithelial cells is unknown.

The purpose of our current investigation is to improve our understanding of the cellular molecular mechanisms by which particulates cause damage of the airway epithelium. In order to address this, we have investigated the effect of diesel exhaust particulates (DEP) on the viability, proliferation and death of the human epithelial A549 cell line. In this *in-vitro* model, the survival and proliferative status of these cells are dependent on the presence of serum which keeps the cells in a proliferating state, while its removal leads to cell death [24]. This model may reflect the effect of extravasated plasma during airway inflammation; under normal circumstances, the airway epithelium is not in contact with serum factors. We have hypothesised that the effects of DEP would depend on whether serum were present or not since the presence of serum determines the basal cell cycle status. We also hypothesised that the outcome of the cell cycle depended on the activation of JNK and NF-κB and the expression of $p21^{CIP1/WAF1}$ in these processes.

We found that DEP at a low concentration inhibited cell apoptosis present when serum was removed and caused proliferation of airway epithelial cells, effects that were mediated through the induction of oxidative stress with activation of the JNK and NF-κB.

These mechanisms indicate that in a normal non-inflamed epithelium, low dose exposure to DEP causes increased epithelial cell survival by preventing apoptosis.

Methods

Preparation of diesel exhaust particle suspension

Diesel exhaust particles (DEP) were a gift from Dr. H. Takano (National Institute for Environmental Studies, Tsukuba, Japan). They were collected from a 4JB1-type, light-duty, four-cylinder, 2.74 L, Isuzu diesel engine (Isuzu Automobile Co., Tokyo, Japan) operated by using standard diesel fuel at a speed of 1,500 rpm under a load of 10 torque (kg/m). DEP were collected as previously described [8], and the mean diameter of the particles was 0.4 μM [25]. The purified DEP were suspended in colourless DMEM medium (Gibco, Invitrogen, Paisley, UK) containing 2mM L-glutamine and 1mM sodium pyruvate (Sigma Chemical Company, Poole, Dorset, UK) (serum free medium, SF) at concentrations of 1 to 200 μg/ml as previously described [7].

A549 cell culture and cell viability measurement

A549 cells (ATCC, LGC Promochem, Middx, UK) were cultured using phenol red (-) DMEM (Gibco, Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS) and 2mM L-glutamine (Sigma Chemical Company, Poole, Dorset, UK) in 12-well plates (Falcon, BD Biosciences, Cowley, Oxford, UK) for 72hrs, until 70-80% confluence has been reached, when the culture medium was replaced with SF medium. Twenty-four hours later, DEP suspension (0, 5, 10, 50, 100 & 200 μg/ml) was added for 24, 48 and 72 hrs in the absence or presence of study drugs including an anti-oxidant, *N*-acetylcysteine (NAC), a catalytic antioxidant and a superoxide mimetic, AEOL 10113 [26], an inhibitor of c-jun N-terminal kinase (JNK), SP600125 [27], and an inhibitor of the translocation of the p50 subunit of the transcription factor NF-κB, SN50 [28]. As a positive control, A549 cells were left to culture in medium containing 10% FCS. The measurement of alive cell numbers was based upon the reduction of the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan dye by mitochondrial enzymes associated with

metabolic activity, indicating living cells. Therefore the test can be used to detect cytotoxicity and proliferation of cells [29]. The culture medium was replaced with SF medium containing 1mg/ml MTT (Sigma Chemical Company, Poole, Dorset, UK) and incubated for 15 min at 37°C. MTT solution was removed and replaced with DMSO, and the change in colour read at 550nm using a colorimetric plate reader.

Measurement of cell cycle progression and apoptosis

Cell cycle progression was measured by fluorescence-activated cell sorter analysis using propidium iodide (PI)-stained cells as described previously [30]. Annexin V and PI binding was determined using an apoptosis detection kit according to the manufacturer's instruction (Becton Dickinson Pharmingen, UK). To distinguish between apoptosis and necrosis, cells were double-stained with annexin V (green fluorescence) and PI (red fluorescence). Briefly, cells (100,000 cells/sample) were washed in cold PBS twice and suspended in binding buffer containing FITC-conjugated annexin V (10 μg/ml) and PI (10 μg/ml). The cell suspension was incubated in the dark for 15 min and then signals acquired using a Becton-Dickinson FACScan flow cytometer (Oxford, UK). A total of 10,000 events was analysed for each sample with Cell Quest software.

Western Blotting

Cells were rinsed with ice-cold PBS containing protease inhibitors (200μM Na_3VO_4, 2mM phenylmethylsulphonyl fluoride) and lysed in radioimmunoprecipitation assay (RIPA) buffer (PBS containing 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Igepal, and complete protease inhibitor cocktail tablet (Roche Diagnostics, Lewes, UK). Cells were scraped off the flasks and solubilized by sonication followed by centrifugation (10,000 x g, 4°C, 4 min). Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hertfordshire, UK). Lysates were boiled for 5 min and total protein extracts (20μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (SDS-Page)

on a 16% Tris-SDS polyacrilamide precast gel (Novex, Invitrogen, Paisley, UK). The separated proteins were electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Amersham, UK) as previously described [31], and rabbit anti-human $p21^{CIP1/WAF1}$ antibody (Santa Cruz Biotechnology, Calne, UK) was used in a dilution of 1:200. Bands were visualized by enhanced chemiluminescence as recommended by the manufacturer (Hybond ECL, Amersham Pharmacia Biotech, Little Chalfont, UK) and quantified using a densitometer with Grab-It and GelWorks software (UVP, Cambridge, UK). The individual band optical density values for each lane of $p21^{CIP1/WAF1}$ were expressed as the ratio with the corresponding internal control-β-actin optical density values.

Quantitative Reverse Transcription-Polymerase Chain Reaction for $p21^{CIP1/WAF1}$

Cells were harvested for total RNA isolation. Commercially available kits were used to extract total cellular RNA (RNeasy, Qiagen, Crawley, U.K.) and to perform reverse transcription (Omniscript RT, Qiagen). Gene transcript level of $p21^{CIP1/WAF1}$ and the housekeeping gene GAPDH were quantified by real-time PCR using a Taqman system (Applied Bioscience) on a Rotor-Gene 3000 PCR apparatus (Corbett Research, N.S.W., Australia). The primer pair of GAPDH was purchased from Applied Bioscience, the primer pair of $p21^{CIP1/WAF1}$ was designed below. (F) 5′-CAGACCAGCATGACAGATTTC, (R) 3′-GGCTTCCTCTTGGAGAAGAT, (Taqman-probe) 5′-FAM-TACCACTCCAAACGCCGGCT-TAMRA. Variation in cDNA concentration in different samples was corrected for GAPDH expression in each cDNA sample by dividing the calculated value for the gene of interest by the housekeeping gene value.

Study Drugs

N-acetylcysteine (NAC, Sigma, UK), and SN50 (an inhibitor of NF-κB; Calbiochem, Nottingham, UK) were dissolved in DMEM and further diluted to the desired-working concentrations in the same medium. A catalytic antioxidant, AEOL 10113, chemical name

Manganese (III) *Meso*-Tetrakis-(*N*-Methylpyridinium-2-yl) porphyrin (a gift from Dr J D Crapo, National Jewish Medical and Research Centre, Denver, CO, USA) was dissolved in DMEM. SP600125, a JNK inhibitor, a gift from Dr Brydon Bennett, Celgene, San Diego, CA, USA) was dissolved in a stock concentration of 50mM DMSO, and then diluted to the desired concentration in SF medium. The final concentration of DMSO was no more than 0.33%.

Statistical Analysis

Data were analysed using unpaired *t*- test or one-way ANOVA/Bonferroni multiple comparison test. Results are expressed as mean ± SEM. *P* values of less than 0.05 were considered to be significant.

Results

Effect of DEP on A549 cell numbers, cell cycle and apoptosis

A549 cells showed time-dependent growth in the presence of 10% FCS, with confluence achieved by 48 hrs. In the presence of FCS (1, 3.3 and 10%), DEP at 10μg/ml had no effect on cell numbers up to 48 hours. In the absence of serum, the number of cells decreased after 48 and 72 hrs (Table 1 and Fig 1). As indicated by MTT staining, incubation of A549 cells with 5-200 μg/ml DEP did not affect their viability up to 24 hours; at 48 hours, DEP prevented the reduction in cells numbers caused by removal of serum with a maximum effect at 10μg/ml ($p<0.0001$; Table 1 and Fig 1). Similar results were obtained at 72 hours. In order to confirm that DEP (10 μg/ml) induces proliferation of A549 cells, the experiments were repeated and A549 cells in the wells were counted directly using a hemocytometer. As shown in Fig 1D, there was a reduction in cell numbers in the absence of FCS, but DEP induced an increase in cell numbers at 48 and 72 hours of incubation. In the presence of FCS alone, there was an increase in A549 cells but without any further increase in the presence of DEP. These results confirm the data obtained using the MTT assay.

Next, the effect of DEP (10 μg/ml) on cell cycle and apoptosis was examined. When cells were serum-starved, cells in the $G_{0/1}$ phase increased while those in the S and G_2/M phase decreased in comparison with cells left in 10% FCS. DEP (10μg/ml) increased the percentage of serum-starved A549 cells in the S phase (18.8%, ± 0.46%; $p<0.0001$) as compared to untreated serum-starved cells (12.9% ± 0.66%) after 48 hours, with a reduction in the percentage of cells in $G_{0/1}$ (73.2% ± 0.16%; $p<0.0001$) and G_2/M (8.1% ± 0.42%; $p<0.02$) phases as compared to serum-starved cells ($G_{0/1}$: 77.4 ± 0.66% and G_2/M: 9.75 ± 0.37%) after 48hours (Fig 2A). Serum-starvation of cells for 48 hours increased annexin V- and PI-positive apoptotic cells in comparison with cells left in 10% FCS (SF: 28.35 ± 2.03 vs FCS: 13.94 ± 3.95; $p<0.05$), but did not induce any change in PI-positive annexin V-negative

cells, representing necrotic cells. Therefore, DEP prevented the increase in apoptotic cells seen with serum starvation, as indicated by positive staining with Annexin V and PI (15.46% ± 2.79% vs 28.35% ± 2.03%; $p<0.03$; Fig 2B).

Effect of *N*-acetylcysteine

N-acetylcysteine (NAC) (3.3 and 10mM) had no effect on cell numbers in SF medium but at 33 mM, there was a significant increase in cell numbers, indicating that oxidative mechanisms were important in cell death induced by serum starvation. In contrast, there was inhibition of the increase in cells induced by DEP (10 μg/ml) with 10 and 33 mM NAC by 27% and 35% respectively at 48 hours (Fig. 3A). This indicates that oxidants may mediate ~~in~~ the increase in cell numbers induced by DEP.

NAC (10 mM) inhibited the DEP-induced increase in A549 cell numbers in the S phase ($p<0.0001$) while leading to an increase in cells in $G_{0/1}$ phase ($p<0.001$). The percentage of cells in G_2/M phase was further decreased by 10mM NAC as compared to DEP alone ($p<0.0001$) (Fig. 4A).

Effect of AEOL10113

AEOL 10113 caused a dose-dependent decrease in the number of cells in the presence of DEP (10 μg/ml), with significant effects at 10μM and 100μM. At these concentrations, there was also a significant increase in cell numbers when A549 cells were serum-starved alone (Fig 3B). Incubation of A549 cells with AEOL10113 (10μM) also led to a significant decrease in the percentage of S phase cells induced by 10μg/ml DEP ($p<0.05$) (Fig 4B).

Effect of JNK inhibitor

While the JNK inhibitor (SP600125) had no effect at either 3.3μM or 10μM, at 33 μM it increased the number of cells in SF medium alone (mean OD from 0.033 ± 0.003 to 0.219 ± 0.015; $p<0.0002$) after 48 hrs (Fig. 5A). By contrast, SP600125 (33 μM) caused a decrease in the number of cells induced by 10μg/ml DEP (Fig. 5A).

Effect of NF-κB inhibitor

SN50 had no effect on cell numbers in SF medium, but dose-dependently inhibited the enhancement of A549 cell numbers induced by DEP (10μg/ml), with significant effect at 33μM (decrease in mean OD from 1.319 ± 0.102 to 0.595 ± 0.004; $p<0.01$; Fig 5B).

Effect of DEP on $p21^{CIP1/WAF1}$ Expression

$p21^{CIP1/WAF1}$ protein expression increased following 48 hours of serum starvation in comparison with cells continuously incubated with 10% FCS ($p<0.01$). DEP added to serum-free treated cells dose-dependently decreased $p21^{CIP1/WAF1}$ protein expression ($p<0.05$) (Fig 6A and B). Similarly, the mRNA expression of $p21^{CIP1/WAF1}$ mRNA was decreased by DEP at 10μg/ml ($p<0.05$) (Fig. 6C).

Discussion

Under *in vitro* conditions of cell culture, with withdrawal of serum factors, A549 cells undergo programmed cell death, with a reduction in viable cell numbers within 48 hours. Under these conditions, we found that DEP improved cell viability but not in a dose-dependent fashion with a maximum effect at a concentration of 10 μg/ml. The increase in cell death found with serum withdrawal was inhibited by low-dose DEP by at least 50%. DEP also prevented $G_{0/1}$ cell arrest, while elevating the percentage of cells in S phase of cell cycle under serum-free conditions. DEP reduced apoptosis of A549 cells, as indicated by decreased number of cells double-stained with annexin V and PI following 48 hours' incubation; the percentage of viable cells (annexin V/PI negative) was also increased. Thus, DEP protected against the reduction in cell numbers caused by serum withdrawal; in other words, it induced a proliferative response and inhibited apoptosis that occurred under conditions of serum starvation. Although there are limitations to this data obtained in vitro on an alveolar epithelial cell line when extrapolated to the in vivo situation, these data would indicate that DEP exposure at a relatively low concentration would cause proliferation of the normal surface epithelium that is not in a state of inflammation. This may in turn lead to an increase in the epithelial layer and possibly to metaplastic changes.

The effect of the antioxidants, *N*-acetylcysteine and AEOL10113, on serum-starved A549 cells was to increase their survival, which was particularly effective with the SOD-mimetic, AEOL10113. On the other hand, the DEP-induced effects on viability and cell cycle of A549 cells were inhibited to a certain extent by the antioxidants. NAC inhibits activation of JNK, p38-MAP kinase and redox-sensitive AP-1 and NF-κB transcription factor activities which regulate the expression of numerous genes [32]. Additionally, NAC prevents apoptosis and promote survival by activating extracellular signal-regulated kinase pathway. The effect of these antioxidants was to decrease cells in the S phase, thus arresting DNA replication.

Therefore, products of oxidative stress were responsible for the effect of DEP on inhibition of apoptosis and on stimulation of cell growth in serum-starved cells. By contrast, on serum-starved cells not exposed to DEP, products of oxidative stress were involved in cell apoptosis and reduced cell growth. These data would indicate that antioxidants may be useful in counteracting the proliferative effect of DEP exposure on the airway epithelium; however, the inhibitory effects of the antioxidants were only partial.

To further elucidate the potential signalling pathways by which DEP effects cell survival, we examined the effects of SP600125, a selective inhibitor of the c-jun N-terminal kinase. SP600125 also inhibited DEP-induced increase in cell viability, and similar to the effect of the antioxidants, increased cell viability in serum-starved cells. Thus, DEP-activation of JNK and of oxidative stress was associated with increases in cell viability, while serum-starvation was also associated with activation of JNK and oxidative stress but with a reduction in cell viability, through the induction of apoptosis. SP600125 has been shown to inhibit proliferation of an adenocarcinoma cell line, KB-3, with a similar dose-dependence. Similar effects were obtained when JNK was inhibited with antisense oligonucleotides to JNK2, indicating that the effect of SP600125 was through selective inhibition of JNK activation [19]. JNK activation has been shown in serum-starved A549 cells, coincident with the initiation of apoptosis [24]. The JNK pathway may therefore be implicated in both apoptosis and survival signalling [20].

SN50, which prevents the nuclear translocation of NF-κB, inhibited DEP-induced cell viability but not the effects of serum-starvation alone, indicating the importance of NF-κB activation in DEP-induced inhibition of apoptosis. NF-κB transcriptionally activates genes for cytokine, chemokine and adhesion molecules that participate in inflammatory reactions in the lung. The DEP-induced release of cytokines such as IL-8, GM-CSF and RANTES, and the adhesion molecule sICAM-1 from human bronchial epithelial cells may be secondary to

the activation of NF-κB [6;7]. Exposure of epithelial cells to DEP also leads to the increased expression and secretion of amphiregulin, a ligand of epidermal growth factor receptor, which in turn induces the secretion of GM-CSF [33]. However, it is also possible that amphiregulin directly modulates apoptosis [34].

We have shown that DEP inhibits serum-withdrawal-induced increase in expression of $p21^{CIP1/WAF1}$ mRNA and protein. DEP may exert, at least in part, its preventive effect on apoptosis of A549 cells through inhibition of $p21^{CIP1/WAF1}$, since it is one of key proteins regulating cell cycle, growth arrest and differentiation [35], and is responsive to oxidative stress [23]. Therefore, DEP inhibition of $p21^{CIP1/WAF1}$ expression may prevent $G_{0/1}$ cell arrest, which was induced by serum removal. A549 cells in the presence of serum starvation expressed high levels of $p21^{CIP1/WAF1}$ while those cultured in 10% foetal calf serum had lower levels, and under these conditions, modulation of $p21^{CIP1/WAF1}$ is likely to control the cell cycle since it binds to and inactivates cyclin D/Cdk complexes.

The adverse effects of DEP on different lung cell types are known to involve oxidative stress pathways [10 36;37]. Thus, DEP and their organic extracts induce the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in macrophages and human bronchial epithelial cells [13;38 39], in turn activating transcription factors such as NF-κB and activator protein (AP)-1 [10;11]. Oxidative stress can initiate pro-inflammmatory effects in macrophages and bronchial epithelial cells [6;7;13;40;41], mediated by phosphorylation-dependent cell signalling pathways, including activation of the mitogen-activated protein kinase pathways. Our data indicate that there is a direct effect of DEP partly through oxidative stress to increase cell numbers by direct inhibition of apoptosis occurring under conditions of serum starvation, with activation of JNK and NF-κB under these conditions.

Our observations may have several implications of clinical relevance. Under the normal situation of an intact epithelium in the absence of serum with no inflammatory response, the epithelial cells are under a balanced turnover of proliferating and apoptotic cells. Our data would indicate that, at low levels of exposure, DEP may induce hyperplasia of a normal epithelium by preventing cell apoptosis, perhaps forming the basis for a metaplastic epithelium. However, in the presence of inflammation with serum extravasation, the effects of DEP on cell numbers are masked by those of serum which itself induces proliferation. Oxidative stress factors may underlie the DEP-induced prevention of apoptosis of epithelial cells and antioxidants can protect against DEP-induced increase in epithelial cells. Inhibitors of NF-κB and JNK pathways may also be useful.

Acknowledgements

HB was supported by a European Respiratory Society Long-Term Research Fellowship. We thank Dr James Crapo for the kind provision of AEOL 10113 and Dr H Takano for the supply of diesel exhaust particles.

References

1. McConnell R, Berhane K, Gilliland F, Molitor J, Thomas D, Lurmann F *et al*. Prospective Study of Air Pollution and Bronchitic Symptoms in Children with Asthma. *Am.J Respir.Crit Care Med* 2003;**168**:790-7.

2. Atkinson RW, Anderson HR, Sunyer J, Ayres J, Baccini M, Vonk JM *et al*. Acute effects of particulate air pollution on respiratory admissions: results from APHEA 2 project. Air Pollution and Health: a European Approach. *Am.J Respir.Crit Care Med* 2001;**164**:1860-6.

3. Kunzli N, Kaiser R, Medina S, Studnicka M, Chanel O, Filliger P *et al*. Public-health impact of outdoor and traffic-related air pollution: a European assessment. *Lancet* 2000;**356**:795-801.

4. Samet JM, Dominici F, Curriero FC, Coursac I, Zeger SL. Fine particulate air pollution and mortality in 20 U.S. cities, 1987-1994. *N.Engl.J Med* 2000;**343**:1742-9.

5. Pope CA3, Thun MJ, Namboodiri MM, Dockery DW, Evans JS, Speizer *et al*. Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. *American Journal of Respiratory & Critical Care Medicine* 1995;**151**:669-74.

6. Bayram H, Devalia JL, Khair OA, Abdelaziz MM, Sapsford RJ, Sagai M *et al*. Comparison of ciliary activity and inflammatory mediator release from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients and the effect of diesel exhaust particles in vitro. *J Allergy Clin.Immunol.* 1998;**102**:771-82.

7. Bayram H, Devalia JL, Sapsford RJ, Ohtoshi T, Miyabara Y, Sagai M *et al*. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells in vitro. *Am.J Respir.Cell Mol.Biol.* 1998;**18**:441-8.

8. Sagai M, Saito H, Ichinose T, Kodama M, Mori Y. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radic.Biol.Med* 1993;**14**:37-47.

9. Hiura TS, Kaszubowski MP, Li N, Nel AE. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J Immunol.* 1999;**163**:5582-91.

10. Nel AE, Diaz-Sanchez D, Li N. The role of particulate pollutants in pulmonary inflammation and asthma: evidence for the involvement of organic chemicals and oxidative stress. *Curr.Opin.Pulm.Med* 2001;**7**:20-6.

11. Jimenez LA, Thompson J, Brown DA, Rahman I, Antonicelli F, Duffin R *et al*. Activation of NF-kappaB by PM(10) occurs via an iron-mediated mechanism in the absence of IkappaB degradation. *Toxicol.Appl.Pharmacol* 2000;**166**:101-10.

12. Alfaro-Moreno E, Martinez L, Garcia-Cuellar C, Bonner JC, Murray JC, Rosas I *et al*. Biologic effects induced in vitro by PM10 from three different zones of Mexico City. *Environ.Health Perspect.* 2002;**110**:715-20.

13. Li N, Wang M, Oberley TD, Sempf JM, Nel AE. Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. *J Immunol.* 2002;**169**:4531-41.

14. Ichinose T, Takano H, Miyabara Y, Sagai M. Long-term exposure to diesel exhaust enhances antigen-induced eosinophilic inflammation and epithelial damage in the murine airway. *Toxicol.Sci.* 1998;**44**:70-9.

15. Timblin CR, Shukla A, Berlanger I, BeruBe KA, Churg A, Mossman BT. Ultrafine airborne particles cause increases in protooncogene expression and proliferation in alveolar epithelial cells. *Toxicol.Appl.Pharmacol* 2002;**179**:98-104.

16. Barkett M,.Gilmore TD. Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 1999;**18**:6910-24.

17. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;**270**:1326-31.

18. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A *et al*. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 2000;**288**:870-4.

19. Du L, Lyle CS, Obey TB, Gaarde WA, Muir JA, Bennett BL *et al*. Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: evidence that mitotic Bcl-2 phosphorylation is JNK-independent. *J Biol.Chem.* 2004;**279**:11957-66.

20. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;**103**:239-52.

21. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nat.* 1993;**366**:701-4.

22. Esposito F, Cuccovillo F, Vanoni M, Cimino F, Anderson CW, Appella E *et al*. Redox-mediated regulation of p21(waf1/cip1) expression involves a post-transcriptional mechanism and activation of the mitogen-activated protein kinase pathway. *Eur.J Biochem.* 1997;**245**:730-7.

23. Yin Y, Solomon G, Deng C, Barrett JC. Differential regulation of p21 by p53 and Rb in cellular response to oxidative stress. *Mol.Carcinog.* 1999;**24**:15-24.

24. Huang Y, Chan AM, Liu Y, Wang X, Holbrook NJ. Serum withdrawal and etoposide induce apoptosis in human lung carcinoma cell line A549 via distinct pathways. *Apoptosis.* 1997;**2**:199-206.

25. Takano H, Yanagisawa R, Ichinose T, Sadakane K, Yoshino S, Yoshikawa T *et al*. Diesel exhaust particles enhance lung injury related to bacterial endotoxin through expression of proinflammatory cytokines, chemokines, and intercellular adhesion molecule-1. *Am.J Respir.Crit Care Med* 2002;**165**:1329-35.

26. Batinic-Haberle I, Liochev SI, Spasojevic I, Fridovich I. A potent superoxide dismutase mimic: manganese beta-octabromo-meso-tetrakis-(N-methylpyridinium-4-yl) porphyrin. *Arch.Biochem.Biophys.* 1997;**343**:225-33.

27. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W *et al*. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc.Natl.Acad.Sci.U.S.A* 2001;**98**:13681-6.

28. Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J Biol.Chem.* 1995;**270**:14255-8.

29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J.Immunol.Methods* 1983;**65**:55-63.

30. Tomita K, Caramori G, Lim S, Ito K, Hanazawa T, Oates T *et al*. Increased p21(CIP1/WAF1) and B cell lymphoma leukemia-x(L) expression and reduced apoptosis in alveolar macrophages from smokers. *Am.J Respir.Crit Care Med* 2002;**166**:724-31.

31. Ito, K., Lim, S., Caramori, G., Chung, K. F., Barnes, P. J., and Adcock, I. M. Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression and inhibits glucocorticoid actions in alveolar macrophages. FASEB 2001; 15: 1110-2.

32. Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. *Cell Mol.Life Sci.* 2003;**60**:6-20.

33. Blanchet S, Ramgolam K, Baulig A, Marano F, Baeza-Squiban A. Fine particulate matter induces amphiregulin secretion by bronchial epithelial cells. *Am.J Respir.Cell Mol.Biol.* 2004;**30**:421-7.

34. Hurbin A, Dubrez L, Coll JL, Favrot MC. Inhibition of apoptosis by amphiregulin via an insulin-like growth factor-1 receptor-dependent pathway in non-small cell lung cancer cell lines. *J Biol.Chem.* 2002;**277**:49127-33.

35. Xiao H, Hasegawa T, Isobe K. Both Sp1 and Sp3 are responsible for p21waf1 promoter activity induced by histone deacetylase inhibitor in NIH3T3 cells. *J Cell Biochem.* 1999;**73**:291-302.

36. Kumagai Y, Arimoto T, Shinyashiki M, Shimojo N, Nakai Y, Yoshikawa T *et al*. Generation of reactive oxygen species during interaction of diesel exhaust particle

components with NADPH-cytochrome P450 reductase and involvement of the bioactivation in the DNA damage. *Free Radic.Biol.Med* 1997;**22**:479-87.

37. Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro. *Thorax* 1996;**51**:1216-22.

38. Hiura TS, Li N, Kaplan R, Horwitz M, Seagrave JC, Nel AE. The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. *J Immunol.* 2000;**165**:2703-11.

39. Matsuo M, Shimada T, Uenishi R, Sasaki N, Sagai M. Diesel exhaust particle-induced cell death of cultured normal human bronchial epithelial cells. *Biol.Pharm.Bull.* 2003;**26**:438-47.

40. Becker S, Soukup JM, Gilmour MI, Devlin RB. Stimulation of human and rat alveolar macrophages by urban air particulates: effects on oxidant radical generation and cytokine production. *Toxicol.Appl.Pharmacol* 1996;**141**:637-48.

41. Ohtoshi T, Takizawa H, Okazaki H, Kawasaki S, Takeuchi N, Ohta K *et al*. Diesel exhaust particles stimulate human airway epithelial cells to produce cytokines relevant to airway inflammation in vitro. *J Allergy Clin.Immunol.* 1998;**101**:778-85.

Table 1. Effect of diesel exhaust particles (DEP) on A549 epithelial cell numbers at 48 hours

	Optical Density [1]			
DEP	**SF**	**1% FCS**	**3.3% FCS**	**10% FCS**
0 (μg/ml)	0.41 (0.06)	0.92 (0.03)	1.18 (0.06)	1.19 (0.06)
10 (μg/ml)	1.04 (0.06)***	0.90 (0.06)	1.03 (0.07)	1.20 (0.05)

[1]Cell numbers were assayed by the reduction of MTT to an insoluble formazan dye measured at an optical density of 550nM after 48 hours. Results are expressed as mean (SEM) of at least three experiments.

DEP, diesel exhaust particles; SF, serum free medium; FCS, foetal calf serum.

***P<0.0001 vs 0μg/ml DEP.

Legend to Figures:

Figure 1.

Effect of diesel exhaust particles (DEP; 0-200μg/ml) on viability of A549 cells as assessed by MTT assay after 24hrs (Panel A), 48hrs (Panel B) and 72hrs (Panel C) in serum-free medium. Cell numbers grown in the presence of 10% fetal calf serum (FCS) are shown for comparison. Results are expressed as mean ± SEM of at least five experiments. *** $p<0.0001$ vs 0 μg/ml DEP. Panel D shows the effect of DEP (10 μg/ml) in the presence or absence of FCS on cell numbers measured directly by hemocytometry over a 72 hour period. Results expressed as mean ± SEM of at least 3 experiments. **$p<0.01$ compared to FCS-.

Figure 2.

Panel A: Effect of 10μg/ml diesel exhaust particles (DEP) on cell cycle of A549 cells following 48 hours of incubation. Results are expressed as mean ± SEM of at least three experiments. *$P<0.05$, **$P<0.01$, ***$P<0.0001$ vs serum free treatment. FCS: fetal calf serum.

Panel B: Effect of diesel exhaust particles (DEP; 10 μg/ml) on apoptosis or necrosis of A549 cells following 48hrs' incubation with serum-free medium (SF), as assessed by flow cytometric analysis (FACs) of Annexin V (A) and propidium iodide (P). Foetal calf serum, FCS; serum free medium, SF; lower left, LL= live cells; lower right, LR= apoptotic cells; upper right, UR= late apoptotic/early necrotic cells; upper left, UL= necrotic cells. Results are expressed as mean ± SEM of at least three experiments. * $p<0.05$ and ** $p<0.005$ vs SF treated cells.

Figure 3.

Effect of *N*-Acetylcysteine (NAC; Panel A), and AEOL 10113 (AEOL; Panel B) at 48 hours on A549 cell number changes induced by diesel exhaust particles (DEP; 10 μg/ml) added to

cells grown in serum-free medium. Cell numbers grown in the presence of 10% fetal calf serum (FCS) are shown for comparison. Results are expressed as mean ± SEM of at least three experiments. ***p<0.0001 vs SF; #p <0.05 and ##p<0.01 vs DEP.

Figure 4.

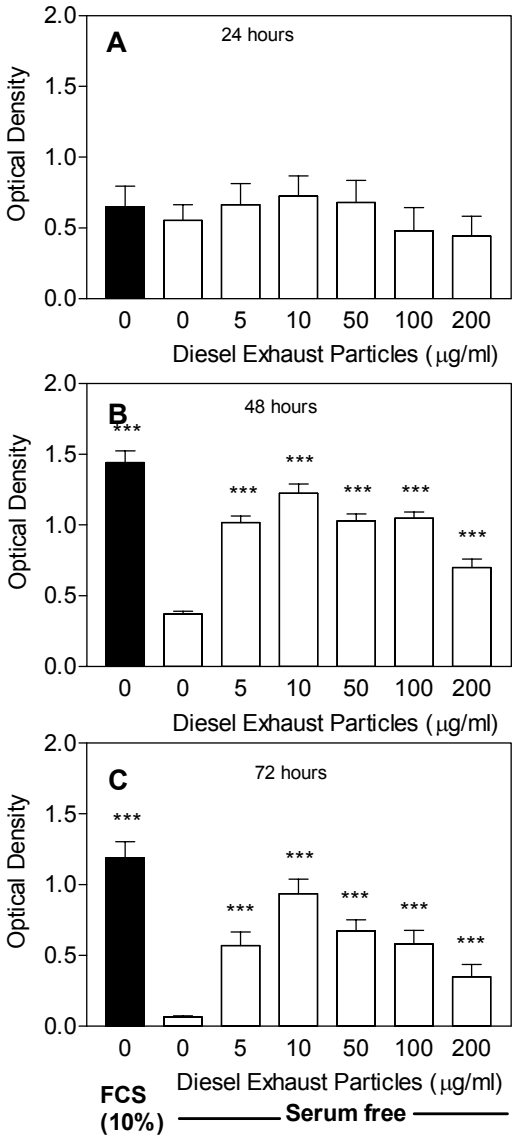
Effect of (a) *N*-Acetylcysteine (NAC) (*p<0.05 and ***p<0.0001 vs SF; ###p<0.0001 vs SF + DEP), and (b) AEOL10113 (10μM) (#p<0.05 vs SF + DEP) on cell cycle progression of A549 cells following 48 hours of incubation with 10μg/ml diesel exhaust particles (DEP). Results are expressed as mean ± SEM of at least two experiments (for NAC: three experiments, for AEOL: two experiments).

Figure 5.

Effects of SP-600125, a JNK inhibitor (Panel A) and of SN50, an NF-κB inhibitor (Panel B), at 48 hours on A549 cell number changes induced by diesel exhaust particles (DEP; 10 μg/ml) added to cells grown in serum-free medium. Cell numbers grown in the presence of 10% foetal calf serum (FCS) are shown for comparison. For Panel A, a control DMSO experiment is shown (DMS) since the SP600125 was dissolved in 0.33% DMSO. Results are expressed as mean ± SEM of at least three experiments; ***P<0.0001 and **P<0.01 vs SF; ##P<0.01 vs DEP; ###P<0.0001 vs 0.33% DMSO + DEP.

Figure 6.

Effect of diesel exhaust particles (DEP; 0, 1 and 10 μg/ml) on $p21^{CIP1/WAF1}$ protein expression measured by Western blot (panels A, B), and mRNA expression (panel C) measured by quantitative PCR of A549 cells following 48 hrs' incubation in serum-free media. Panel A shows representative Western blots. The effect of culture in 10% foetal calf serum alone (FCS) is shown. Results are shown as mean ± SEM of at least three experiments. * p<0.05 and ** p<0.01 vs 0μg/ml DEP treated cells.


A
24 hours
Optical Density
2.0
1.5
1.0
0.5
0.0
0 0 5 10 50 100 200
Diesel Exhaust Particles (μg/ml)
B
48 hours
***
Optical Density
0 0 5 10 50 100 200
Diesel Exhaust Particles (μg/ml)
C
72 hours
***
Optical Density
0 0 5 10 50 100 200
Diesel Exhaust Particles (μg/ml)
FCS (10%)
Serum free

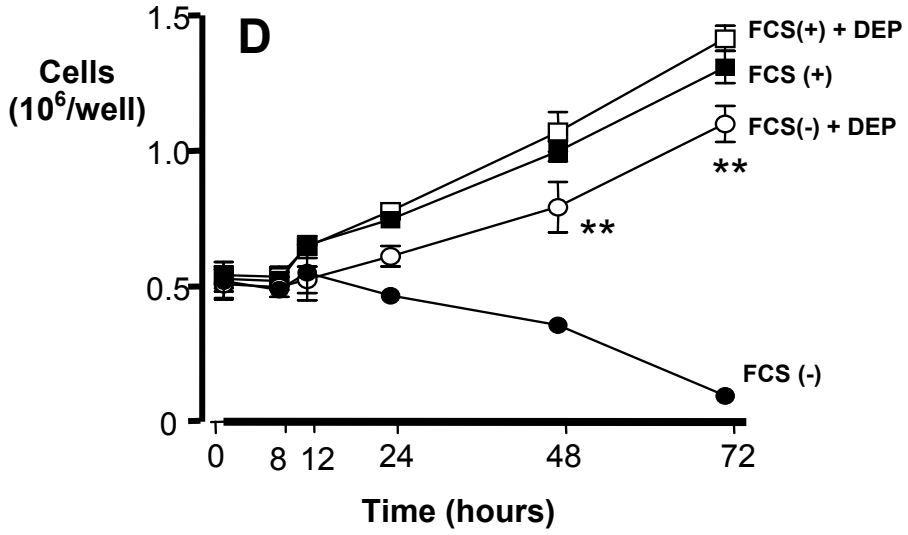

D
Cells
(10^6/well)
1.5
1.0
0.5
0
0
8
12
24
48
72
Time (hours)
FCS(+) + DEP
FCS (+)
FCS(-) + DEP
FCS (-)
**
**

Fig 1.

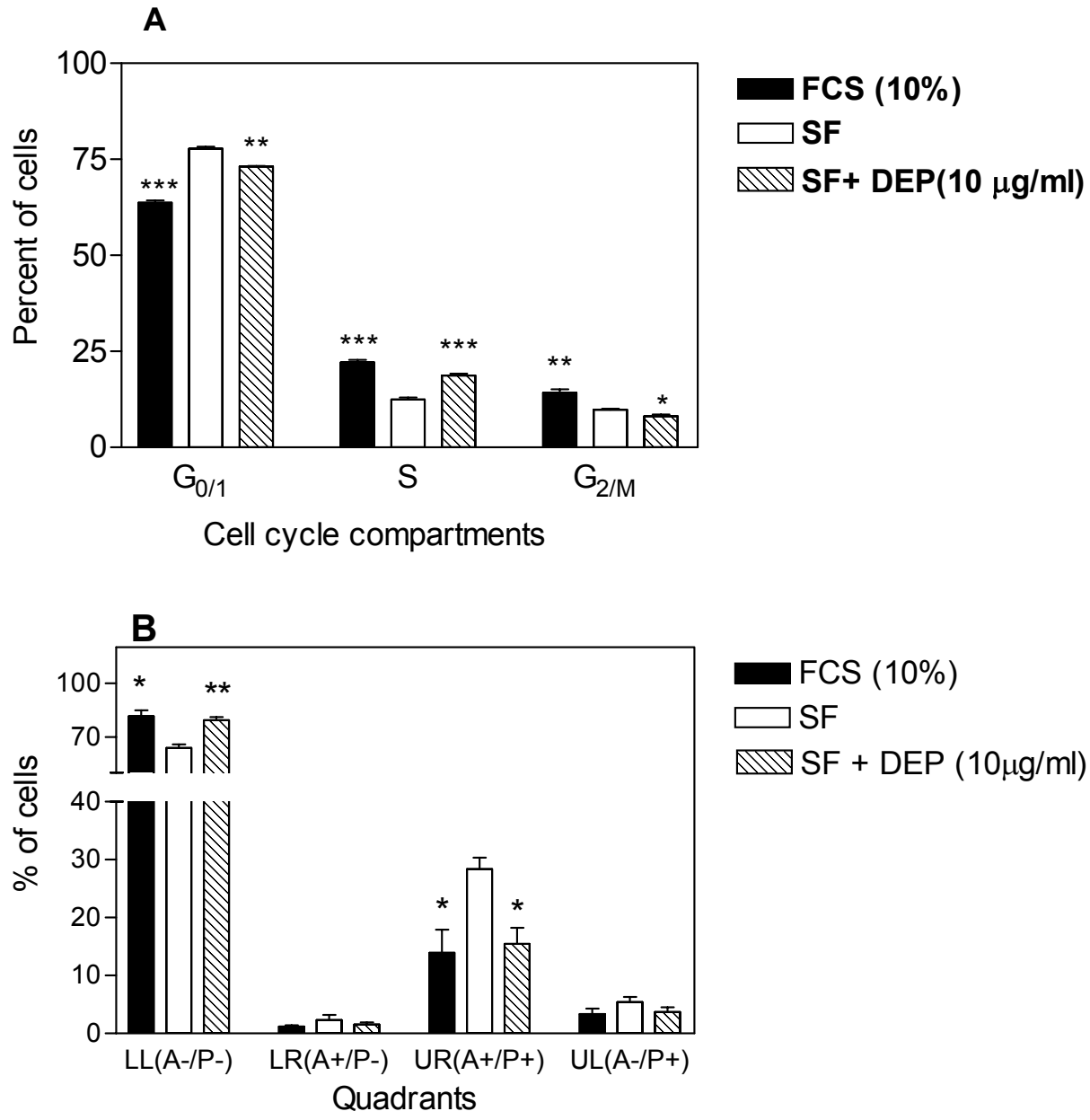

A
100
75
50
25
0
Percent of cells
***
**
***
***
**
*
G0/1
S
G2/M
Cell cycle compartments
FCS (10%)
SF
SF+ DEP(10 μg/ml)
B
100
70
40
30
20
10
0
% of cells
*
**
*
*
LL(A-/P-)
LR(A+/P-)
UR(A+/P+)
UL(A-/P+)
Quadrants
FCS (10%)
SF
SF + DEP (10μg/ml)

Fig 2

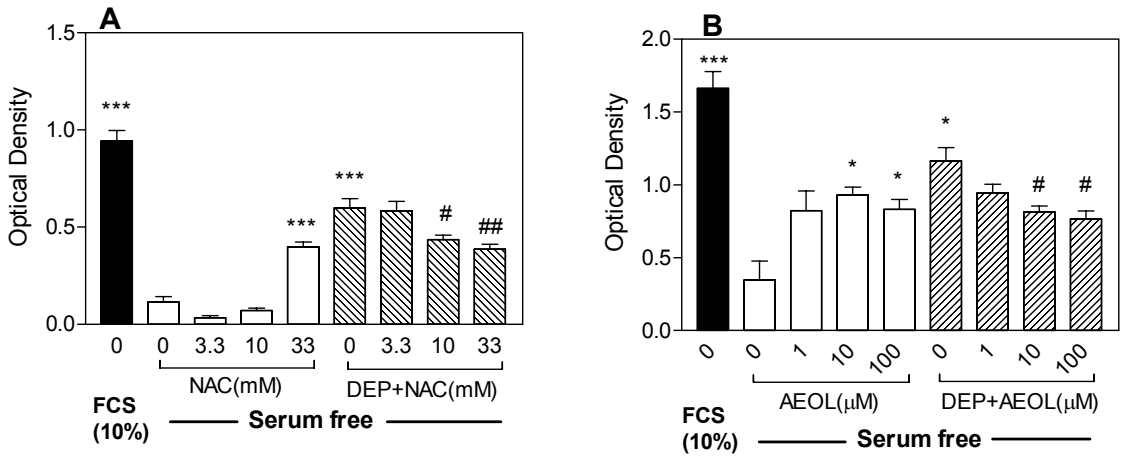

A
Optical Density
1.5
1.0
0.5
0.0
***
***
***
#
##
0
0
3.3
10
33
0
3.3
10
33
NAC(mM)
DEP+NAC(mM)
FCS
(10%)
Serum free
B
Optical Density
2.0
1.5
1.0
0.5
0.0
***
*
*
*
#
#
0
0
1
10
100
0
1
10
100
AEOL(μM)
DEP+AEOL(μM)
FCS
(10%)
Serum free

Fig 3.

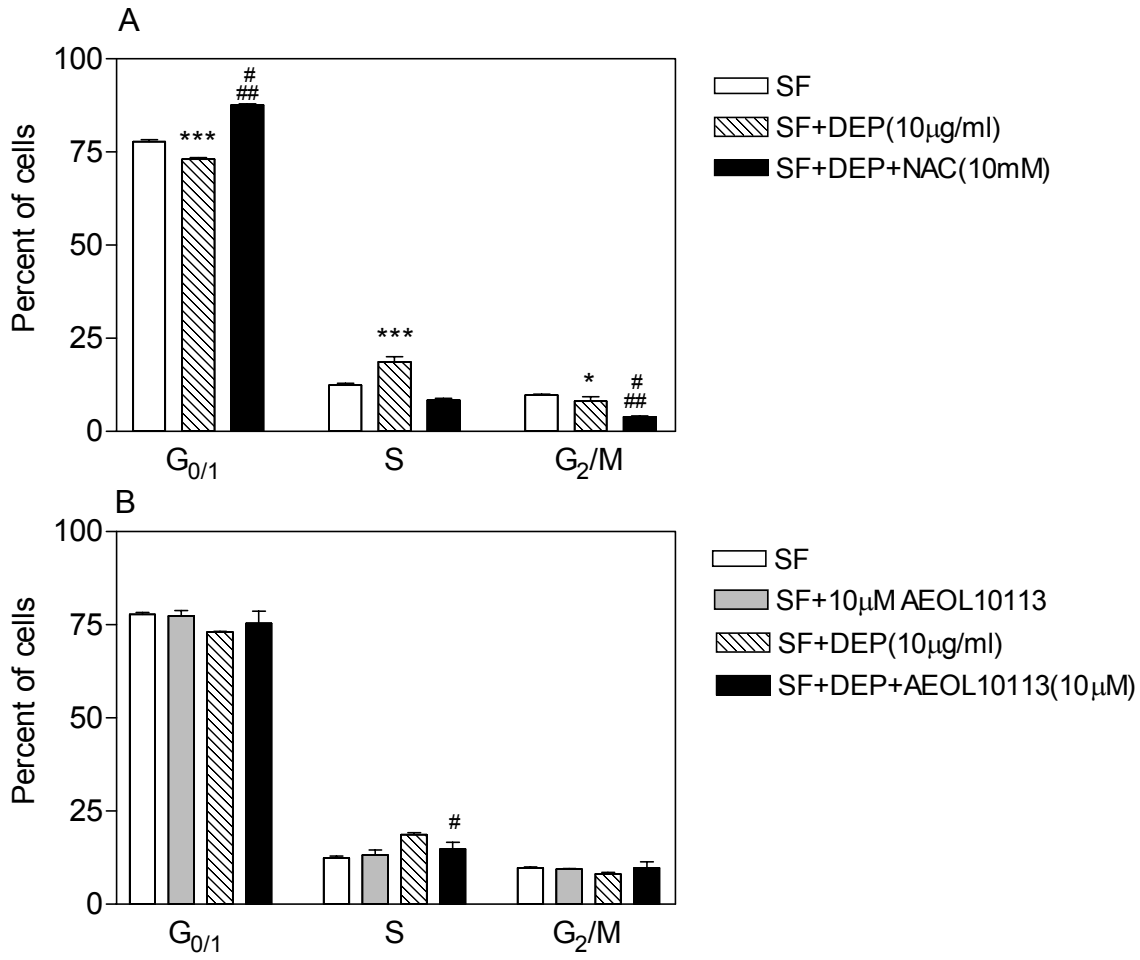

A
100
75
50
25
0
Percent of cells
***
#
##
***
*
#
##
G0/1
S
G2/M
SF
SF+DEP(10μg/ml)
SF+DEP+NAC(10mM)
B
100
75
50
25
0
Percent of cells
#
G0/1
S
G2/M
SF
SF+10μM AEOL10113
SF+DEP(10μg/ml)
SF+DEP+AEOL10113(10μM)

Fig 4.

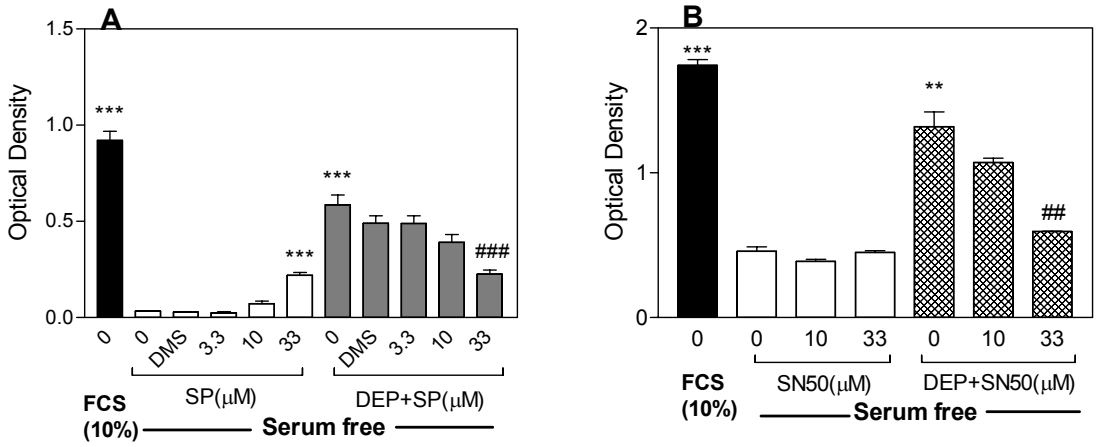

A
Optical Density
1.5
1.0
0.5
0.0
***
***
***
###
0
0
DMS
3.3
10
33
0
DMS
3.3
10
33
FCS
(10%)
SP(μM)
DEP+SP(μM)
Serum free
B
Optical Density
2
1
0
***
**
##
0
0
10
33
0
10
33
FCS
(10%)
SN50(μM)
DEP+SN50(μM)
Serum free

Fig 5.

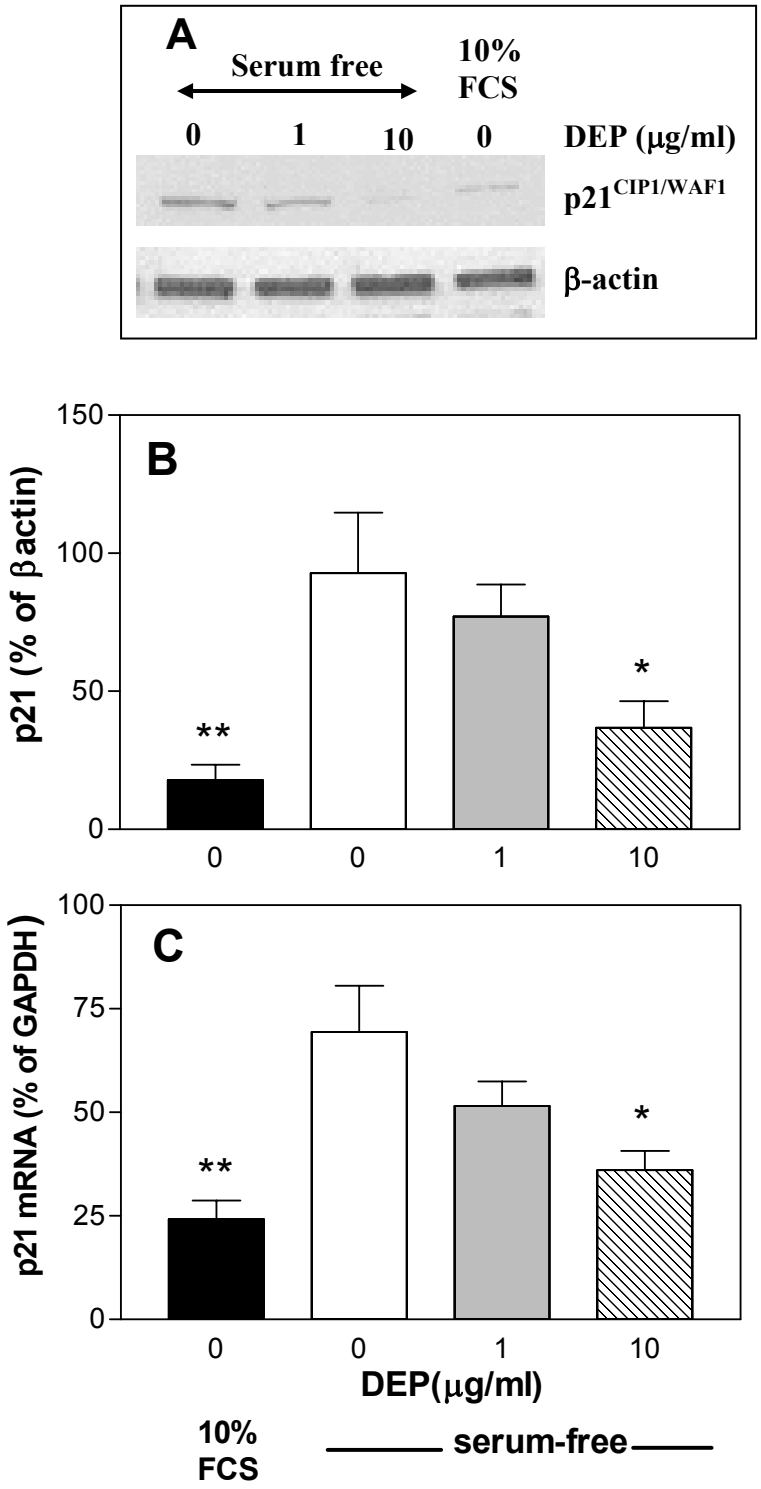

A
Serum free
10% FCS
0
1
10
0
DEP (μg/ml)
p21CIP1/WAF1
β-actin
B
p21 (% of βactin)
150
100
50
0
**
*
0
0
1
10
C
p21 mRNA (% of GAPDH)
100
75
50
25
0
**
*
0
0
1
10
DEP(μg/ml)
10% FCS
serum-free

Fig 6.