Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R

Ulrich Sydlik,1 Katrin Bierhals,1 Maria Soufi,1 Josef Abel, Roel P. F. Schins,2 and Klaus Unfried1

1Toxicology and 2Particle Research, Institut für umweltmedizinische Forschung an der Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

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Sydlik, Ulrich, Katrin Bierhals, Maria Soufi, Josef Abel, Roel P. F. Schins, and Klaus Unfried. Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R. Am J Physiol Lung Cell Mol Physiol 291: L725–L733, 2006. First published June 2, 2006; doi:10.1152/ajplung.00131.2006.—Apoloptosis and proliferation are important causes of adverse health effects induced by inhaled ultrafine particles. The molecular mechanisms of particle cell interactions mediating these end points are therefore a major topic of current particle toxicology and molecular preventive medicine. Initial studies revealed that ultrafine particles induce apoptosis and proliferation in parallel in rat lung epithelial cells, dependent on time and dosage. With these end points, two antagonistic reactions seem to be induced by the same extracellular stimulus. It was therefore investigated whether proliferation is induced directly by the particles or as a compensation of particle-induced cell death. Experimental conditions excluding compensatory proliferation demonstrated that both end points are induced independently by specific signaling pathways. Events eliciting signaling cascades leading to apoptosis and proliferation were studied with specific inhibitors of membrane receptors. Epidermal growth factor receptor (EGF-R) kinase activity was identified as essential for apoptosis as well as for proliferation. As ultrafine particle-induced proliferation alone was dependent on the activation of β1-integrins, these membrane receptors are suggested to mediate the specificity of EGF-R signaling concerning the decision as to whether apoptosis or proliferation is triggered. Accordingly, MAP kinase signaling downstream of EGF-R showed comparable specificity with regard to receptor-dependent induction of apoptosis and proliferation. As key mediators of signaling cascades, the activation of extracellular signal-regulated kinases 1 and 2 proved to be specific for proliferation in a β1-integrin-dependent manner, whereas phosphorylation of c-Jun NH2-terminal kinases 1 and 2 was correlated with the induction of apoptosis.

n nanoparticles; JNK1/2; ERK1/2; integrins

ULTRAFINE COMBUSTION-DERIVED PARTICLES have been identified as toxicologically important components of inhalable particulate matter (PM). Epidemiologic studies report correlations between exposure to this PM fraction and diseases of the lung and the cardiovascular system (reviewed in Ref. 3). As PM consists of a complex mixture of particles from different sources, combinations of particles with hazardous chemicals like polyaromatic hydrocarbons or reactive metals must be considered as responsible for these effects. It is therefore important to identify the impact of each of these particle groups in a defined experimental system. So far, mechanistic studies have been performed mostly with occupationally or environmentally relevant particle mixtures like diesel exhaust particles (DEP) or PM. The role of the carbonaceous particle core of combustion-derived nanoparticles in this scenario can be investigated with ultrafine carbon black (ufCB), which is a manufactured form of elementary carbon with defined size distribution. In animal experiments, ultrafine particles such as ufCB have been demonstrated to induce end points such as inflammation, proliferation, and lung cancer (3, 16, 19).

Besides their effects on inflammatory cells, particles directly induce reactions in lung epithelial cells that are relevant for pathological outcomes. On one hand, epithelial cells may be stimulated by particles to release proinflammatory factors driving or modulating neutrophilic inflammation (10). On the other hand, effects of particles on epithelial tissue may affect processes like airway remodeling and tumor promotion (1), both of which are linked to disturbed tissue homeostasis. The role of ultrafine particles in the deregulation of the balance between cell death and cell growth is therefore a topic of current particle toxicology. In experiments with a mouse lung epithelial cell line, PM 2.5 as well as ufCB have been described to drive cells into S phase and to increase total cell numbers (22). Analyses of mRNA levels in response to particle treatment revealed dose-specific changes of expression of jun and fos genes as well as genes encoding proteins involved in receptor-mediated apoptosis. In line with these results is the observation that ufCB in human primary epithelial cells induces proliferation via signaling pathways upstream of AP-1 (21). Using pharmacological inhibitors, this study demonstrates that proliferation is dependent on epidermal growth factor receptor (EGF-R) and the mitogen-activated protein (MAP) kinase pathway via extracellular signal-regulated kinases 1 and 2 (ERK1/2).

Apoptosis induced by xenobiotics can be considered as a process contributing to the initiation of cell proliferation (15). Compensatory proliferation may occur in those cells of a tissue or cell culture that do not undergo apoptosis and that are stimulated to divide in order to repair tissue damage. In the presence of DNA-damaging compounds this process is relevant for neoplastic transformation. So far, it is not clear whether proliferation induced by ultrafine carbon particles in lung cells is triggered by direct interaction of the particles with target cells or whether it is a consequence of particle-elicited apoptosis.

In this study, we performed systematic experiments to investigate the linkage of apoptosis and proliferation induced by ultrafine particles. Rat lung epithelial cells (RLE-6TN; Ref. 9), as a sensitive model system for pathogenic particle effects,

Address for reprint requests and other correspondence: K. Unfried, Institut für umweltmedizinische Forschung, Au f m Hennekamp 50, 40225 Düsseldorf, Germany (e-mail: klaus.unfried@uni-duesseldorf.de).

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were treated with noncytotoxic dosages of ultrafine particles as well as with bigger carbonaceous particles as a control for ultrafine specificity. As these results revealed a strong indication for independent induction of these end points, the signaling processes relevant for these outcomes were investigated.

MATERIALS AND METHODS

Particles and particle preparations. ufCB 14 nm in diameter (Printex 90) was obtained from Degussa (Frankfurt, Germany), and carbon black (CB) 260 nm in diameter was from H. Haeflener (Chepstow, UK; as Huber 990). Amorphous silica 14 nm in diameter was purchased from Sigma (St. Louis, MO). Stock suspensions (1 mg/ml) of particles were prepared in PBS by sonication for 60 min at 50–60 Hz and 120 W. Particle suspensions were diluted in PBS and used at end concentrations from 1 to 10 µg/cm².

Cell culture and treatment. The rat lung epithelial cell line RLE-6TN (9) was purchased from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C and 5% CO2 in Ham’s F-12 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), t-glutamine (2 mM), and 5% heat-inactivated fetal calf serum (FCS; Sigma). For experiments, cells were seeded at a density of 3 × 10⁴/cm² and grown to 80% confluence. After the cells were washed twice with PBS, the medium was replaced by Ham’s F-12 with 0.5% FCS and incubated for 24 h. Cells were treated with particles for up to 48 h in the absence or presence of inhibitors. Lactate dehydrogenase was determined in supernatants to ensure that particle treatment as well as pretreatment with inhibitors did not induce cytotoxicity and cell death. Inhibitors specific for signaling steps were added to the cells 1 h before particle treatment. Concentrations of inhibitors are indicated in the respective figures. Peptides were dissolved in sterile H2O, 5% acetic acid, or PBS according to the instructions of the manufacturer. The integrin-blocking antibody was diluted in PBS. Pharmacological inhibitors were dissolved in DMSO. In detail, the following inhibitors were used: β₁-integrin blocking antibody Ha2/5 (anti-CD29; BD PharMingen, San Diego, CA); integrin-blocking peptide RGDS (H-Arg-Gly-Asp-Ser-0H) (both Calbiochem); c-Jun NH₂-terminal kinase (JNK) inhibitor 1 L-stereoisomer (GRKKRRQRRR-PP-RPKRPT-Axxora, San Diego, CA); SP-1-integrin blocking antibody Ha2/5 (anti-CD29; BD Phar-Mingen) according to the instructions of the manufacturer. As an early marker of cells undergoing apoptosis, caspase-3 cleavage with a caspase-3 antibody (8G10; Cell Signaling Technology, Danvers, MA) in Western blots (see below). As additional assay, monitoring of late events of apoptosis with transferase-mediated dUTP nick end labeling (TUNEL) assay was performed with the In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the instructions of the manufacturer. For this purpose RLE-6TN cells were cultured on microscopic slides with cell densities and treatments similar to those described above. Quantification of the apoptotic DNA cleavage was determined by fluorescence measurement of four representative sectors of each sample with a charge-couple device-equipped fluorescent microscope and analySIS 3.2 software (Soft Imaging System).

Immunoprecipitation. EGF-R was precipitated from cell lysates [harvested in IP buffer: 30 mM Tris·HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1% (w/vol) Triton X-100, 10 mM NaF, 5 mM Na3VO4] of treated or untreated cells by incubation at 4°C first with anti-EGF-R antibody (Upstate Biotechnology, Lake Placid, NY) for 20 h and subsequently with protein A agarose beads (Upstate Biotechnology) for 4 h. Precipitates were then collected and washed with IP buffer; afterwards 20 µg of protein was subjected to SDS-PAGE.

Western blotting. After exposure, cells were washed twice with ice-cold PBS and lysed in modified radiolabeled immunoprecipitation assay buffer [25 mM Tris·Cl pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate, 0.025% NaN₃, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail (both inhibitor cocktails from Sigma)]. Aliquots (5 µg) of total cell protein were separated by SDS-PAGE (10%) and transferred onto polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences). The phosphorylation status of EGF-R was detected with a phosphotyrosine-specific antibody (P-Tyr-100; Cell Signaling, Beverly, MA). The activation of the MAP kinases ERK1/2 and JNK1/2 was determined by their phosphorylation status detected with phospho-p44/42 MAPK (Thr202/Tyr204) antibodies and phospho-stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185) antibodies. Total ERK1/2 and JNK1/2 proteins were monitored with p44/42 MAPK antibodies and SAPK/JNK antibodies (detecting predominantly the 57-kDa protein), respectively. All antibodies were from Cell Signaling Technology. Signal strength was detected with the ECL plus Western Blotting Technology, Danvers, MA). As an example, the phosphorylation status of the MAP kinases ERK1/2 and JNK1/2 was determined by their phosphorylation status detected with phospho-p44/42 MAPK antibodies [Harvested in IP buffer: 30 mM Tris·HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA, 1% (w/vol) Triton X-100, 10 mM NaF, 5 mM Na3VO4] of treated or untreated cells by incubation at 4°C first with anti-EGF-R antibody (Upstate Biotechnology, Lake Placid, NY) for 20 h and subsequently with protein A agarose beads (Upstate Biotechnology) for 4 h. Precipitates were then collected and washed with IP buffer; afterwards 20 µg of protein was subjected to SDS-PAGE.

Statistical analysis. All experiments were repeated independently at least three times. Mean values were compared statistically with Student’s t-test. Differences were assessed as significant when P ≤ 0.05.

RESULTS

Induction of proliferation and apoptosis by ultrafine particles. Ultrafine particles may have an impact on the regulation of the cell cycle by direct interaction with the target cells. To investigate proliferative as well as apoptotic effects of ultrafine carbon particles rat lung epithelial cells were treated in vitro with 10 µg/cm² ufCB. Proliferation was determined and quantified by BrdU incorporation in a time-course experiment up to 48 h (Fig. 1A). Ultrafine particle-induced DNA synthesis was moderately but significantly increased after 24 h of particle treatment. The proliferative effect of ufCB proved to be reproducible at the same time point in a second independent assay detecting PCNA, which accumulates in cells during S phase (Fig. 1B).

A similar time-course experiment was performed to test the impact of ufCB on caspase-3 activity. This apoptotic marker showed elevated levels between 4 and 24 h of treatment, with
Fig. 1. Induction of proliferation and apoptosis in RLE-6TN cells treated with 10 μg/cm² ultrafine carbon black (ufCB). A: time course of 5-bromo-2'-deoxyuridine (BrdU) incorporation. Gray bars, treatment with ufCB; open bars, respective PBS controls. B: quantification and representative Western blot of proliferating cell nuclear antigen (PCNA). Gray bars, treatment with ufCB; open bars, respective PBS controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined as loading control. C: time course of caspase-3 activity. Gray bars, treatment with ufCB; open bars, respective PBS controls. D: quantification and representative Western blot of cleaved caspase-3 (19 kDa) in cells treated with 10 μg/cm² ufCB for 8 h or staurosporine and/or pretreatment with DEVD-CHO as caspase-3 inhibitor. Total extracellular signal-regulated kinase (ERK)1/2 was detected as loading control. E: quantification of transferase-mediated dUTP nick end labeling (TUNEL) assay fluorescence signals from cells treated for 12 h (4 sectors of each sample). Means and SD were determined from at least 3 independent experiments. *Significantly different from respective controls; †significantly different from treatment with 10 μg/cm² ufCB alone.
a maximum after 8 h (Fig. 1C). This effect could also be demonstrated on the level of caspase-3 cleavage (Fig. 1D). The specificity of the caspase-3 activation for ufCB-induced apoptosis was tested after treatment with different dosages by measuring apoptotic DNA fragmentation with the TUNEL assay after 12-h treatment (Fig. 1E). A clear dose response in fluorescent staining could be observed. Moreover, pretreatment with a caspase-3 inhibitor (DEVD-CHO) resulted in a reduction of the number of ufCB-induced apoptotic cells, indicating the validity of the caspase-3 assay for monitoring the apoptotic properties of ultrafine particles.

The results of the time-course experiments (Fig. 1, A and C) with apoptosis beginning after 4 h of treatment and a later-occurring proliferation, at a first glance, could be interpreted as an indication for compensatory proliferation to replace apoptotic cells. However, comparison of different particles in dose-response experiments revealed converse results (Fig. 2, A and B). Two ultrafine particles (ufCB, amorphous silica) with the same median diameter but with different physicochemical characteristics both induced apoptosis and proliferation in a dose-dependent manner. At the highest dosages (10 μg/cm²) significant differences between ufCB and amorphous silica were observed. Whereas ufCB, with regard to mass, seemed to be a stronger inducer of apoptosis (Fig. 2B), amorphous silica induced a higher proliferation rate than ufCB (Fig. 2A). Both differences proved to be statistically significant (P < 0.05). CB with a median diameter of 260 nm was not able at any of the tested dosages to induce either DNA synthesis or caspase-3 activity, indicating a clear size dependence of these particle effects.

The induction of proliferation independent from apoptosis by ufCB was confirmed in a second set of experiments with additional substances inducing or suppressing apoptosis. Figure 2C demonstrates that ufCB is able to induce proliferation when apoptosis is suppressed. Pretreatment of cells with the caspase-3 inhibitor (DEVD-CHO), which blocks particle-induced apoptosis in RLE-6TN cells (Fig. 2D), had no effect on the proliferation levels induced by ufCB (Fig. 2C). The induction of apoptosis with staurosporine in a dose range (10 nM) leading to effects comparable to the ufCB effects (Fig. 2D) also did not induce compensatory proliferation (Fig. 2C). These data indicate that under the chosen experimental conditions proliferation cannot be a consequence of apoptosis and that specific mechanisms and signaling cascades must be responsible for ufCB-induced proliferation.

Role of EGF-R and β1-integrins. To identify signaling pathways responsible for the end points induced by ufCB membrane receptors likely to be involved in early signaling steps were investigated. EGF-R is considered as an important signaling factor activated by ultrafine particles (4, 21). Phosphorylation of EGF-R was therefore investigated in RLE-6TN cells treated with 10 μg/cm² ufCB. Phosphotyrosine was detected in Western blot assays with a specific antibody. This probably early event of signaling was measured in time-course experiments between 2 and 480 min (Fig. 3A). After correction for differences in efficiency of immunoprecipitation, significant phosphorylation induced by ufCB was observed. Interestingly, a typical phosphorylation pattern occurred with two distinct time frames. Whereas a singular very early signal appeared after 2-min treatment, a second more persistent signal was observed from 120 up to 480 min.

The functional relevance of the EGF-R activation was tested in a second set of experiments by inhibition of kinase activity by tyrphostin AG-1478. This inhibitor was tested for both end points, apoptosis and proliferation. Pretreatment with tyrphostin AG-1478 led to a decrease of ufCB-induced DNA synthesis and caspase-3 activity to control levels (Fig. 3, B and C). Under the chosen experimental cell-starving conditions no effect of the EGF-R inhibitor on background proliferation was observed. As both signaling pathways induced by ultrafine par-

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**Fig. 2.** Independent induction of apoptosis and proliferation by ultrafine particles in RLE-6TN cells. A: dose response of BrdU incorporation after 24-h treatment. B: caspase-3 activity after 8-h treatment. C: BrdU incorporation in cells pretreated with DEVD-CHO or staurosporine. D: caspase-3 activity in cells pretreated with DEVD-CHO or staurosporine. Dark gray bars, ufCB; light gray bars, amorphous silica; filled bars, carbon black; open bars, PBS controls. *Significantly different from PBS control; †significantly different between ufCB and amorphous silica; ‡significantly different from treatment with ufCB alone.
icles leading to the opposed end points seem to make use of EGF-R, we investigated further membrane receptors possibly involved in eliciting MAP kinase signaling pathways.

In earlier studies (2) we demonstrated the involvement of integrins in particle-induced signaling. Figure 3, D and E, show experiments in which integrin signaling was blocked. The peptide RGDS is known to inhibit signaling via integrin dimers that bind to a specific amino acid sequence (RGD) in extracellular matrix proteins (17). Treatment of the cells with this peptide, before particle treatment, resulted in a dose-dependent reduction of the particle-specific proliferation (Fig. 3D). The specificity of this reaction is confirmed, as the control peptide containing amino acids in a nonfunctional sequence had no effect. Moreover, pretreatment with a β1-integrin-inactivating antibody (anti-CD29) had the same effect, indicating the importance of β1-containing integrin receptors for particle-induced proliferation. Interestingly, this linkage to integrins can be observed only for proliferation, but not for apoptosis, as none of the inhibitors had significant effects on ultrafine particle-induced caspase-3 activation (Fig. 3E). Integrin-mediated signaling therefore seems to be specific for ultrafine particle-induced proliferation.

MAP kinase activation by ufCB. To investigate signaling cascades downstream of membrane receptors, we analyzed MAP kinases ERK1/2 and JNK1/2 for their activation by measuring phosphorylation status. Specific time courses of
MAP kinase activation were observed in Western blots with phospho-specific antibodies after treatment of RLE-6TN cells with a dosage of 5 μg/cm² ufCB, which was demonstrated to induce apoptosis and proliferation (Figs. 2, A and B). ERK1/2 phosphorylation was significantly increased in treated cells compared with sham-treated controls 8 h after particle application (Fig. 4A). JNK1/2 phosphorylation, however, was already increased significantly 2 h after particle addition and persisted up to 16 h (Fig. 4B). The differences in signal strength of JNK1/2 phosphorylation after 4, 8, and 16 h proved not to be significant when normalized to PBS controls. After 16 h, a reproducible increase in background activation of both MAP kinases associated with cell starvation was observed. This nonspecific phosphorylation was described earlier as not associated with proliferation (2). The differences in the time course of these MAP kinases may represent the specificity of these signaling pathways for the end points of apoptosis and proliferation.

End points of JNK1/2 and ERK1/2 signaling. The relevance of these signaling pathways for the induction of apoptosis and proliferation was tested with specific pharmacological inhibitors. The MEK1/2 inhibitor PD-98059 is accepted to block specifically the ERK1/2 pathway. Pretreatment with this inhibitor resulted in a significant reduction of ultrafine particle-induced DNA synthesis but not caspase-3 activation (Fig. 5, A and B). This inhibitor also showed no effect on background proliferation under the experimental conditions. The JNK blocking peptide L-JNKI1 as well as the pharmacological JNK inhibitor SP-600125 had no effect on ufCB-induced proliferation (Fig. 5C). Particle-dependent apoptosis, however, was significantly reduced by L-JNKI1 (Fig. 5D), demonstrating the importance of JNK1/2 in ufCB-dependent apoptosis.

Receptor-mediated signaling. The linkage of JNK1/2 and ERK1/2 signaling with the membrane receptors EGF-R and β₁-integrin was investigated with the respective specific inhibitors (Fig. 6). ufCB-induced phosphorylation of both MAP kinases was significantly reduced by the EGF-R inhibitor tyrphostin AG-1478 (Fig. 6A), demonstrating that both signaling cascades make use of EGF-R when elicited by ultrafine particles. Inhibitors of integrins, however, only had an effect on particle-induced ERK1/2 activation and not JNK1/2 activation (Fig. 6B). The integrin-blocking peptide (RGDS) as well as the β₁-integrin blocking antibody (anti-CD29) significantly reduced particle-induced ERK1/2 phosphorylation, whereas the phosphorylation of JNK1/2 in treated cells remained unchanged by these two inhibitors. It is therefore likely that β₁-integrin-containing dimers are the key actors mediating particle-induced ERK1/2 activation.

DISCUSSION

Using a model system of rat lung epithelial cells treated with ultrafine particles, we were able to demonstrate that cell proliferation as well as apoptosis, each measured by two independent assays, are induced as a specific response to ultrafine particle treatment. As proliferation in the literature has sometimes been discussed as compensation of particle-induced apoptosis (1), here such a link between these end points was investigated in depth. A first indication that these end points are induced independently is the distinct responses to the different ultrafine particles ufCB and amorphous silica. If proliferation were a direct consequence of particle-induced apoptosis one would expect higher proliferation rates for ufCB, which induced significantly more apoptosis than amorphous silica. Blocking of apoptosis with a specific caspase-3 inhibitor did not lead to any changes in particle-induced proliferation rates. As a positive control, staurosporine, which induces apoptosis by blocking of protein kinases, was used and also showed no effect on proliferation. This observation, however, may be due to the kinase-blocking effect of this inhibitor. Together, these experiments allow us to conclude that compensation of cell death is not the trigger for ultrafine particle-induced proliferation.

The initial events as well as the relevant signaling cascades inducing and mediating these pathologically relevant end points are of special interest because this knowledge would be helpful for risk assessment of PM as well as for preventive medical approaches, e.g., for susceptible groups of humans.

As suggested in several studies using diverse particle samples, EGF-R plays an initial role for particle-elicited signaling resulting in ERK1/2 phosphorylation and subsequent activation of the transcription factor AP-1 (1). Interestingly, our
results demonstrate that EGF-R is involved in both signaling pathways leading to apoptosis as well as to proliferation in one cell line in the same growth state. So far it is not clear how this signaling is elicited by ultrafine particles. On one hand, receptor activation may be mediated by direct interaction of the particles with cellular structures like extracellular matrix, cell membrane, or the receptors themselves. On the other hand, there are some indications for indirect receptor activation via particle-induced reactive oxygen species (ROS). Experiments studying the effects of scavengers demonstrate an influence of ultrafine particle-induced ROS on the activation of EGF-R and transcription factor AP-1 (5, 21). Therefore, ROS are likely to be responsible for immediate-early reactions on signaling. For EGF-R, data must be considered that indicate that human bronchial epithelial cells treated with PM 2.5, DEP, or ufCB are stimulated to produce EGF-R ligands like heparin-binding EGF and amphiregulin (4, 21). Both reports discuss these results as a possible autocrine loop for persisting activation of EGF-R-dependent signaling, which may be first initiated by particle-induced ROS. Such effects as well as ultrafine particle-induced cytokines may also be involved in the proliferating effect of ufCB observed after 24-h treatment. The biphasic phosphorylation of EGF-R in cells treated with ufCB may be an indication for an activation pattern based on an early ROS-dependent and a late rather ligand-dependent phosphorylation. However, the influence of such a possible mechanism on proliferation or on earlier-occurring apoptosis remains to be investigated.

The ufCB-dependent trigger of two different outcomes both mediated by EGF-R should result in the differential induction of two separate signal pathways. The inhibitor experiments of the present study demonstrate on the level of signaling as well as through measurement of the end points apoptosis and proliferation that ultrafine particle-induced EGF-R signaling can result in the activation of two separate MAP kinase pathways with specific end points. Whereas ERK1/2 activation seems to result in a proliferative cell reaction, JNK1/2 phosphorylation appears to be responsible for ultrafine particle-induced apoptosis. The role of EGF-R signaling induced by ultrafine particles in processes other than apoptosis and proliferation is not clear. However, Kim et al. (10) demonstrated that the expression of the proinflammatory cytokine IL-8 is dependent on MAP kinase signaling rather than on NF-kB, which was assumed to be the central mediator of particle-induced inflammatory reactions.

The conflicting function of the same receptor (EGF-R) for the two opposing end points, proliferation and apoptosis, raises the question of how this ambivalence is managed mechanistically. As a second type of membrane receptors possibly involved in particle-induced MAP kinase signaling, integrins have been investigated. Integrin blocking resulted in a decrease in particle-induced proliferation as well as specific ERK1/2 phosphorylation. Integrins are heterodimeric membrane receptors that are responsible for signal transduction across the cell membrane (7). During cell detachment and cell adhesion, interactions between EGF-R and integrin receptors have been
described. Under these conditions, both receptors cooperatively regulate the decision between induction of anoikis or cell survival. They modulate the activation of proteins of the Bcl-2 family as well as the induction of MAP kinase pathways relevant for proliferation (6, 18). Here we describe that integrin-mediated MAP kinase signaling is also relevant in cellular response to a xenobiotic stress that is not associated with cell adhesion or anoikis. Further studies must prove whether a cooperative action of these two membrane receptors is necessary for ultrafine particle-induced proliferation.

JNK1/2 activation has been described as an important signaling pathway leading to mitochondrion-mediated apoptosis.
in alveolar epithelial cells (13). These data, however, were obtained in cells treated with bleomycin. Studies investigating effects of particulate xenobiotics in lung cells on JNK1/2 activation mostly concentrated on highly metal-laden particles (11, 20) or used DEP or organic extracts from DEP (14, 23). However, studies investigating lung cells treated with cadmium alone revealed no indication for the importance of JNK1/2 signaling in apoptosis (12). Here we demonstrate for the first time that the carbonaceous particle core alone is also able to induce JNK1/2 activation in an EGF-R-dependent manner. Blocking experiments demonstrated the involvement of this activation in ultrafine particle-induced apoptosis.

With the present study we were able to demonstrate that proliferation as well as apoptosis are induced by ultrafine particles using specific signaling pathways. Both pathways depend on the kinase activity of the EGF-R. Downstream of EGF-R, the MAP kinases ERK1/2 mediate cell proliferation in an integrin-dependent manner. JNK1/2 is suggested as a pathway leading to apoptosis caused by ultrafine carbon particles.

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