Aryl Hydrocarbon Receptor Is an Ozone Sensor in Human Skin

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Ozone, one of the main components of photochemical smog, represents an important source of environmental oxidative stress to which the skin is exposed, especially during smoggy and ozone-alert days. However, very little is known about the effects of ozone exposure on human skin. Here, we used normal human epidermal keratinocytes (NHEKs) to determine the effects of attainable levels of ozone exposure on the family of cytochrome P450 (CYP) isoforms, which plays a determinant role in the biotransformation of many environmental pollutants. NHEK exposure to ozone (0.3 ppm) resulted in an increase in protein and messenger RNA (mRNA) expression of CYP1A1, CYP1A2, and CYP1B1. NHEK exposure to ozone also resulted in nuclear translocation of the aryl hydrocarbon receptor (AhR) and in phosphorylation of epidermal growth factor receptor (EGFR). The effect of ozone on events downstream of EGFR was an increased activation of phosphoinositide 3-kinase and phosphorylation of protein kinase B and mitogen-activated protein kinases. We found that AhR silencing by small interfering RNA abolished the capacity of these cells to increase the protein and mRNA expression of CYPs on ozone exposure. Thus, AhR signaling is an integral part of the induction of CYPs by ozone. These studies strongly suggest that there are toxicological consequences of ozone to human skin.

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INTRODUCTION

Ozone is a pollutant of the urban environment and one of the main components of photochemical smog to which the skin is exposed, especially during smoggy and ozone-alert days (Mustafa, 1990; Baudouin *et al.*, 2002; Bell *et al.*, 2004; Valacchi *et al.*, 2004). Increased numbers of motor vehicles on the road have resulted in an increase in the level of ozone, because vehicle emissions are a main source of precursor hydrocarbons and nitrogen oxides. Typical levels of ozone that are recorded in urban environments can range from 0.2 to 1.2 ppm (Mustafa, 1990; Gruber *et al.*, 2005). Further increases in ozone levels in this century will have an adverse effect on skin that is in direct contact with the environment (Sitch *et al.*, 2007). The adverse effects of ozone on respiratory tract tissue have been well characterized, but the skin is also exposed to tropospheric ozone. As a potent oxidant, ozone is capable of reacting with a variety of extracellular and intracellular biomolecules (Pryor, 1979; Thiele *et al.*, 1997a, b; Fortino *et al.*, 2007) and is also capable of damaging the barrier function of the epidermis (Thiele *et al.*, 1997b). The cytotoxicity of ozone is due largely to antioxidant depletion (Thiele *et al.*, 1997c, d; Valacchi *et al.*, 2000) and its interaction with unsaturated lipids that generate damaging free radicals or toxic intermediate products (Pryor and Church, 1991). Ozone exposure not only affects antioxidant levels and oxidation markers in the outermost stratum corneum layer (Valacchi *et al.*, 2002; He *et al.*, 2006) but also induces cellular stress responses in the deeper cellular layers of the skin (Valacchi *et al.*, 2003).

Cytochrome P450s (CYPs) are important heme-containing enzymes that play crucial roles in the metabolism of xenobiotics and endogenous compounds. The oxidative metabolism of drugs, environmental chemicals, hormones, and fatty acids by CYP enzymes is a critical pathway aiding in their excretion from the body, but in some cases metabolism may lead to bioactivation and enhanced toxicity (Cavalieri *et al.*, 2000; Ahmad and Mukhtar, 2004; Nebert and Dalton, 2006). Most xenobiotic procarcinogens are hydrophobic and are substrates of CYP1 enzymes, such as CYP1A1, CYP1A2, and CYP1B1 (Nebert *et al.*, 2004). These three CYP1 enzymes are imperative for metabolically activating a variety of environmental procarcinogens in the aryl- and alkyl-amine, heterocyclic amine, polycyclic aromatic hydrocarbon, and polyhalogenated aromatic hydrocarbon categories (Ahmad

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Abbreviations: Ab, antibody; AhR, aryl hydrocarbon receptor; Akt, protein kinase B; CYP, cytochrome P-450; D-PBS, Dulbecco's phosphate-buffered saline; ERK, extracellular signal-regulated kinases; EGFR, epidermal growth factor receptor; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NHEK, normal human epidermal keratinocyte; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA

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and Mukhtar, 2004; Nebert *et al.*, 2004; Nebert and Dalton, 2006).

The aryl hydrocarbon receptor (AhR) was discovered as a cytosolic, ligand-dependent receptor that binds exogenous ligands, including carcinogenic and/or teratogenic aromatic and halogenated hydrocarbons present in air pollution, cigarette smoke, or foods (for example, dioxin or benzo[*a*]-pyrene) (Poland and Knutson, 1982). Studies have shown that exposure to dioxin stimulates translocation of AhR from the cytosol to the nucleus, where it heterodimerizes with the AhR nuclear translocator and regulates the expression of many genes, including several that encode xenobiotic/carcinogen-metabolizing enzymes, such as CYP1A1, CYP1A2, and CYP1B1 (Nebert and Karp, 2008).

In this study, we tested the hypothesis that the exposure of human skin to ozone will enhance known upstream regulators of the toxicological metabolism of other environmental pollutants with which the skin comes in contact. To test this hypothesis, we used normal human epidermal keratinocytes (NHEKs) to determine the effects of attainable levels of ozone exposure to human skin on the CYP1 isoform family, which plays an important role in the biotransformation of many environmental pollutants. Because AhR induces the transcription of *CYP1* genes, we determined the effect of ozone exposure on AhR. We also determined the effect of ozone on epidermal growth factor receptor (EGFR) and its downstream events, such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinases (MAPKs). Our results suggest that exposure of NHEKs to ozone resulted in an increased expression of CYP1 isoforms, activation of AhR, and phosphorylation of EGFR with concomitant activation of PI3K/Akt and phosphorylation of MAPK. In addition, we provide evidence that AhR plays a crucial role in the induction of CYP1 isoforms in NHEKs by ozone exposure.

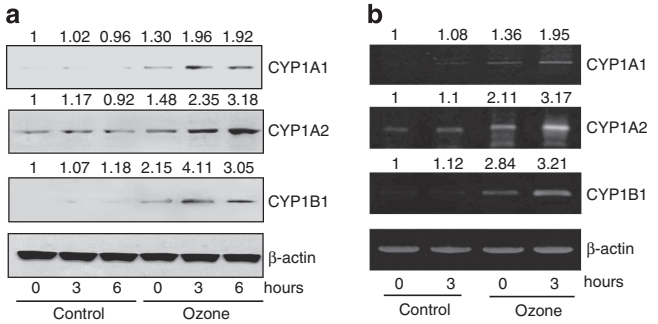

Figure 1. Effect of ozone exposure on the protein and mRNA expression of CYP1A1, CYP1A2, and CYP1B1 in normal human epidermal keratinocytes. Near-confluent keratinocytes in Dulbecco's phosphate-buffered saline (D-PBS) were exposed to 0.3 ppm ozone. This dose was obtained over 20 minutes of exposure to ozone in a closed chamber. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested immediately after 20 minutes (considered as the 0 time point) and 3 and 6 hours after ozone exposure for protein and RNA isolation. (**a**) Western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. (**b**) Reverse transcriptase–PCR. mRNAs were reverse transcribed using the SuperScript III first-strand synthesis system and FailSafe PCR kit. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

RESULTS

Ozone induces CYP protein and mRNA expression in NHEKs

The primary role of CYPs is to participate in the biotransformation of endogenous substrates and xenobiotic materials, such as environmental pollutants, to which the human skin is either deliberately or inadvertently exposed (Ahmad and Mukhtar, 2004). In the present study, we determined the effects of ozone exposure on CYP1 isozymes (such as CYP1A1, CYP1A2, and CYP1B1) at the protein and mRNA levels in NHEKs. To accomplish this, near-confluent keratinocytes were treated in the absence or presence of 0.3 ppm ozone. This dose was obtained over 20 minutes of exposure to ozone in a closed chamber (Valentine, 1985). The ozone level was measured with a Dasibi 1008-PC instrument (Dasibi Environmental Corp., Glendale, CA), interfaced to an ozone chamber (Valentine, 1985). The cells were harvested 20 minutes (considered as the 0 time point) and 3 and 6 hours after ozone exposure, followed by protein and RNA isolation for western blot and reverse transcriptase–PCR analyses, respectively. As shown in Figure 1a, ozone exposure to NHEKs resulted in a marked increase in the protein expression of CYP1A1, CYP1A2, and CYP1B1 compared with that in their respective controls. Increased protein expression of CYP1A1 and CYP1B1 was more pronounced 3 hours after ozone exposure; however, for CYP1A2, maximum induction was observed at 6 hours. Next, we determined the effect of ozone exposure on the mRNA expression of these CYPs by reverse transcriptase–PCR. We found that ozone exposure resulted in an increased mRNA expression of CYP1A1, CYP1A2, and CYP1B1 (Figure 1b).

Ozone induces AhR mRNA expression and causes nuclear translocation of AhR in NHEKs

AhR is a ligand-activated transcription factor that can be activated by numerous structurally diverse synthetic and naturally occurring compounds. It is known to mediate the toxic effects of numerous environmental contaminants (Nebert *et al.*, 2004). To examine whether ozone exposure, similar to known AhR ligands, translocates AhR from the cytoplasm into the nuclear compartment, cells were exposed to ozone (0.3 ppm), and at different time points cytoplasmic and nuclear lysates were prepared for western blot analysis. We found that ozone exposure resulted in decreased expression of the AhR protein in the cytosolic fraction at all time points compared with the respective control. We also observed that there was an increased nuclear translocation of AhR at all time points after ozone exposure compared with their respective controls (Figure 2a). These data suggest that NHEK exposure to ozone, similar to known AhR ligands, translocates AhR from the cytoplasm into the nuclear compartment. Our data also showed that ozone exposure resulted in an increased mRNA expression of AhR (Figure 2b).

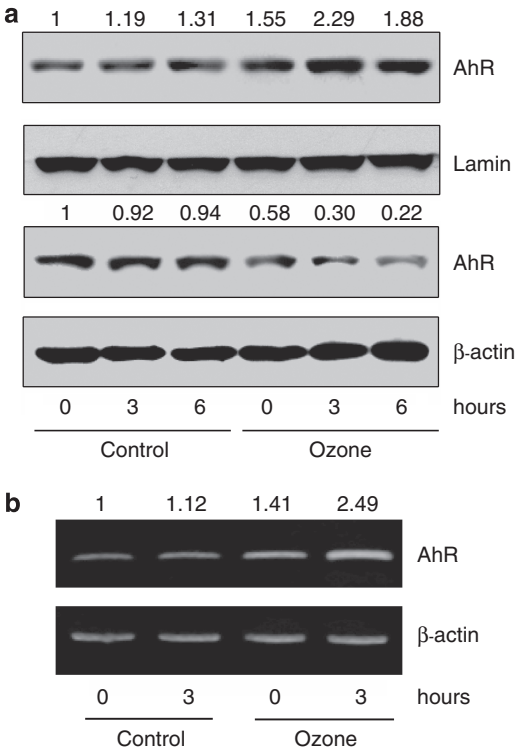

Figure 2. Effect of ozone exposure on the protein and mRNA expression of AhR in normal human epidermal keratinocytes. Near-confluent keratinocytes in Dulbecco's phosphate-buffered saline (D-PBS) were exposed to 0.3 ppm ozone. This dose was obtained over 20 minutes of exposure to ozone in a closed chamber. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested immediately after 20 minutes (considered as the 0 time point) and 3 and 6 hours after ozone exposure for protein and RNA isolation. (**a**) Western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin (cytosolic) and lamin (nuclear). The values above the figures represent relative density of the bands normalized to lamin. (**b**) Reverse transcriptase–PCR. mRNAs were reverse transcribed using the SuperScript III first strand synthesis system and a FailSafe PCR kit. The values above the figures represent relative density of the bands normalized to β-actin. AhR, aryl hydrocarbon receptor. The data shown here are from a representative experiment repeated three times with similar results.

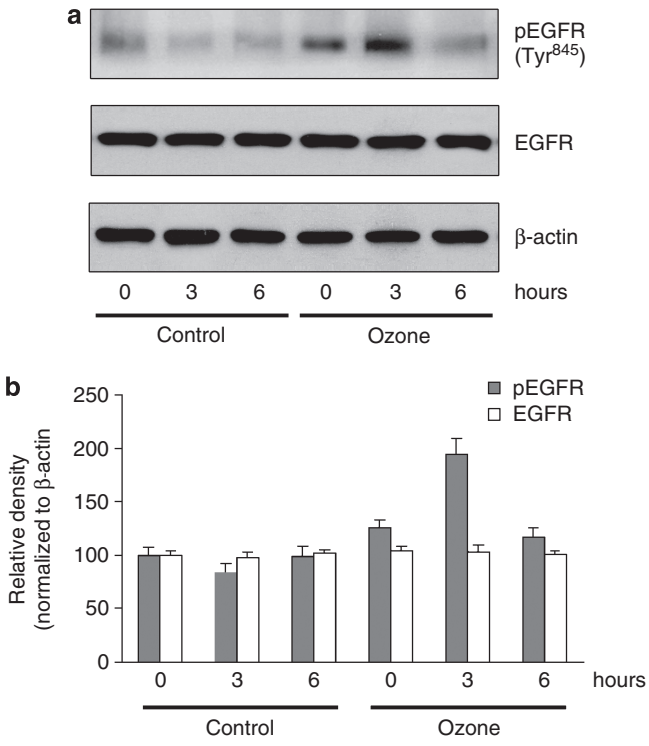

Figure 3. Effect of ozone exposure on the phosphorylation of EGFR in normal human epidermal keratinocytes. Near-confluent keratinocytes in Dulbecco's phosphate-buffered saline (D-PBS) were exposed to 0.3 ppm ozone. This dose was obtained over 20 minutes of exposure to ozone in a closed chamber. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested immediately after 20 minutes (considered as the 0 time point) and 3 and 6 hours after ozone exposure and cell lysates were prepared. (**a**) Western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. (**b**) Relative density of the bands normalized to β-actin. pEGFR, phosphorylated EGFR. The data shown here are from a representative experiment repeated three times with similar results.

Ozone induces phosphorylation of EGFR in NHEKs

Studies have shown that AhR ''cross-talks'' with EGFR by inducing the expression of epidermal growth factor, which is important for EGFR signaling. Therefore, we assessed whether ozone exposure would induce the expression and/or phosphorylation of EGFR. Western blot analysis and relative density data showed that ozone exposure resulted in an increased phosphorylation of EGFR at Tyr^{845} (Figure 3a and b). However, there was no change in the protein expression of total EGFR.

Ozone induces activation of PI3K and phosphorylation of Akt in NHEKs

The signaling pathway induced by activated EGFR also includes phosphorylation of Akt and the activation of catalytic (p110) and regulatory (p85) subunits of PI3K, which play an important role in cell survival. Therefore, we examined the effects of ozone exposure on the phosphorylation of Akt and activation of PI3K. Our data indicate that ozone exposure to NHEKs resulted in increased phosphorylation of Akt at Thr^{308} and in the activation of p110 (catalytic subunit) and p85 (regulatory subunit), as determined by western blot analysis and the relative density of the bands (Figure 4a and b).

Ozone induces phosphorylation of MAPKs in NHEKs

Given that ozone induced phosphorylation of EGFR, we evaluated the effect of ozone exposure on the signaling cascade triggered by this receptor. The signaling pathway induced by activated EGFR includes MAPKs (such as extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1/2), and p38), which play an important role in the mitogenic, cell survival, and inflammatory responses mediated by EGFR. We found that ozone exposure to NHEKs resulted in an increased phosphorylation of ERK1/2, JNK1/2, and p38, as determined by western blot analysis and the relative density of the bands (Figure 5a and b).

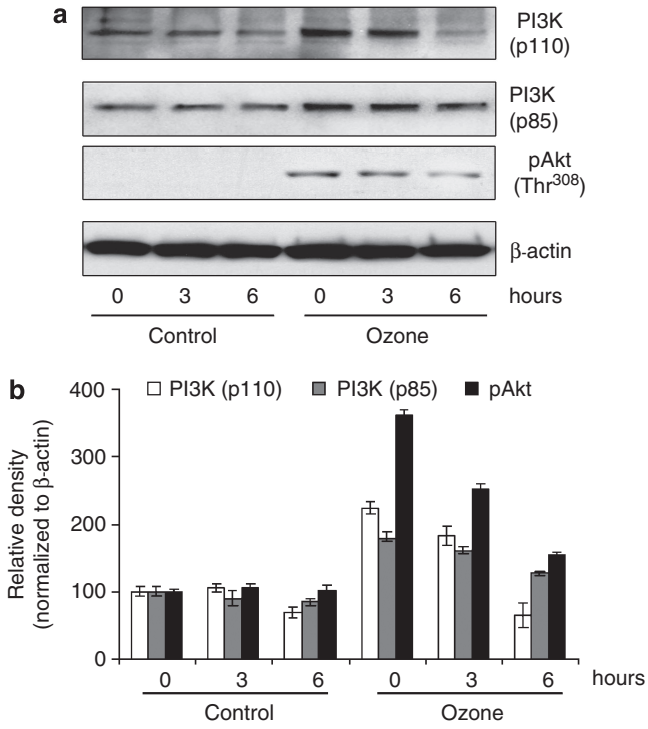

Figure 4. Effect of ozone on the protein expression of phosphoinositide 3-kinase (PI3K) and phosphorylation of protein kinase B (Akt) in normal human epidermal keratinocytes. Near-confluent keratinocytes in Dulbecco's phosphate-buffered saline (D-PBS) were exposed to 0.3 ppm ozone. This dose was obtained over 20 minutes of exposure to ozone in a closed chamber. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested immediately after 20 minutes (considered as the 0 time point) and 3 and 6 hours after ozone exposure and cell lysates were prepared. (**a**) Western blot analysis. (**b**) Relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

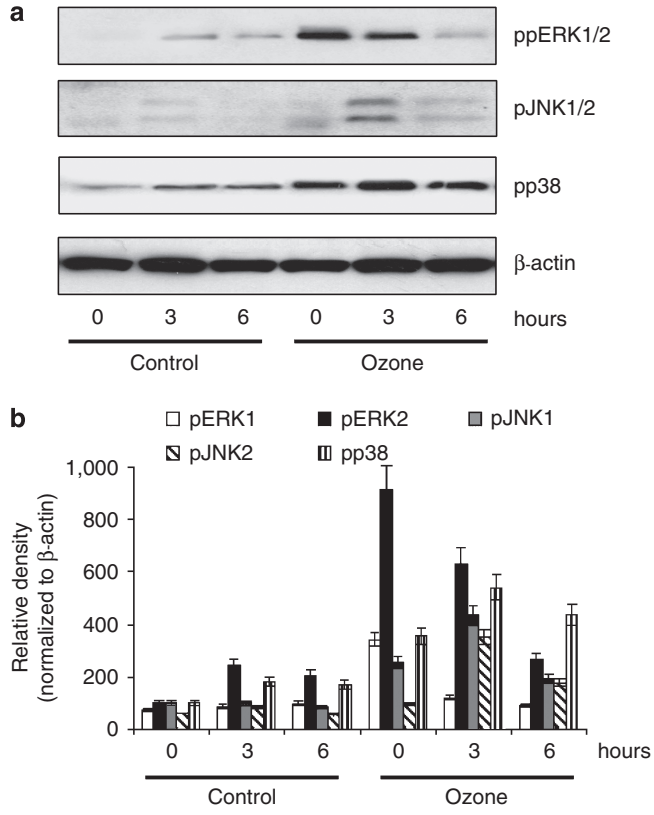

Figure 5. Effect of ozone exposure on the phosphorylation of mitogen-activated protein kinases in normal human epidermal keratinocytes. Near-confluent keratinocytes in Dulbecco's phosphate-buffered saline (D-PBS) were exposed to 0.3 ppm ozone. This dose was obtained over 20 minutes of exposure to ozone in a closed chamber. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested immediately after 20 minutes (considered as the 0 time point) and 3 and 6 hours after ozone exposure and cell lysates were prepared. (**a**) Western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. (**b**) Relative density of the bands normalized to β-actin. pERK, phosphorylated extracellular signal-regulated kinase; pJNK, phosphorylated c-jun N-terminal kinase. The data shown here are from a representative experiment repeated three times with similar results.

Silencing of AhR inhibits ozone-induced CYP protein and mRNA expression in NHEKs

AhR is a receptor/transcription factor that regulates *CYP* gene transcription and is activated by environmental carcinogens (Nebert *et al.*, 2004). To assess whether ozone-induced CYP protein and mRNA expression is AhR-dependent, we next examined the effect of AhR silencing on the protein and mRNA expression of CYPs. NHEKs were transfected with either AhR small interfering RNA (siRNA) (100 nmol l^{-1}) or control scrambled siRNA (100 nmol l^{-1}) using the Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD) specific for NHEK transfection. After transfection, the cells were cultured for 24 hours, and then the medium was replaced with Dulbecco's phosphate-buffered saline (D-PBS). Next, the cells were exposed to ozone (0.3 ppm) for 20 minutes and harvested 3 hours later. We confirmed that silencing of AhR by siRNA inhibited the ozone-induced increase in AhR protein and mRNA expression in NHEKs, whereas the control scrambled siRNA cells remained responsive to ozone (Figure 6a and b). Further, we found that AhR knockdown by siRNA abolished the capacity of these cells to increase the protein and mRNA expression of CYP1A1, CYP1A2, and CYP1B1 upon ozone exposure, whereas the control scrambled-transfected cells remained unaffected (Figure 6a and b). These data indicate that ozone-induced CYP protein and mRNA expression is, in part, mediated through activation of AhR. Moreover, we found that silencing of AhR by siRNA inhibited the ozone-induced phosphorylation of EGFR in NHEKs (data not shown).

Silencing of EGFR had no effect on ozone-induced CYP protein and mRNA expression in NHEKs

We next determined whether ozone-induced CYP protein and mRNA expression is EGFR-dependent. For this, cells were transfected with either EGFR siRNA (100 nmol l^{-1}) or control scrambled siRNA (100 nmol l^{-1}) using the Nucleofector Kit specific for NHEK transfection. We verified that suppression of EGFR expression by siRNA inhibited the ozone-induced increase in the protein and mRNA expression of EGFR in NHEKs, whereas the control scrambled siRNA cells remained unaffected (Figure 7a and b). However, EGFR

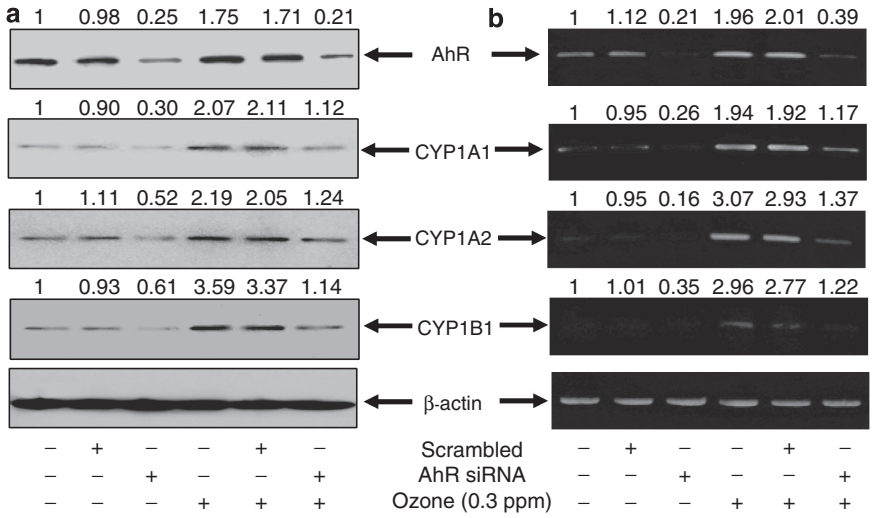

Figure 6. Effect of AhR silencing on ozone-induced cytochrome P450 protein and mRNA expression in normal human epidermal keratinocytes. After transfection, cells were cultured for 24 hours, after which the medium was replaced with Dulbecco's phosphate-buffered saline (D-PBS) and cells were exposed to ozone (0.3 ppm) as detailed in the ''Materials and Methods'' section. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested 3 hours after ozone exposure for protein and RNA isolation. (**a**) Western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. (**b**) Reverse transcriptase–PCR. mRNA was reverse transcribed using the SuperScript III first-strand synthesis system and a FailSafe PCR kit. siRNA, small interfering RNA. The data shown here are from a representative experiment repeated three times with similar results.

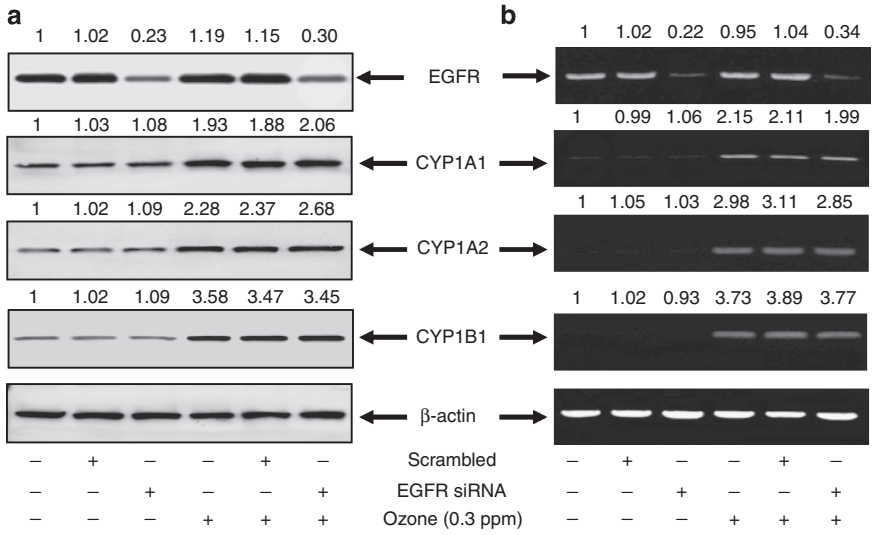

Figure 7. Effect of EGFR silencing on ozone-induced cytochrome P450 protein and mRNA expression in normal human epidermal keratinocytes. After transfection, cells were cultured for 24 hours, after which the medium was replaced with Dulbecco's phosphate-buffered saline (D-PBS) and cells were exposed to ozone (0.3 ppm) as detailed in the ''Materials and Methods'' section. After ozone exposure, the D-PBS was removed, fresh medium was added, and cells were harvested 3 hours after ozone exposure for protein and RNA isolation. (**a**) Western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. (**b**) Reverse transcriptase–PCR. mRNAs were reverse transcribed using the SuperScript III first-strand synthesis system and a FailSafe PCR kit. siRNA, small interfering RNA. The data shown here are from a representative experiment repeated three times with similar results.

knockdown by siRNA did not show any effect on ozone-induced protein or mRNA expression of CYPs. These data indicate that induction of CYPs by ozone is not mediated through the activation of EGFR.

DISCUSSION

Although the consequences of ozone on extracellular and intracellular biomolecules in the skin are well known (Pryor, 1979; Thiele *et al.*, 1997b; Valacchi *et al.*, 2003; He *et al.*, 2006; Fortino *et al.*, 2007), its effects on CYP1 isoforms responsible for the metabolic activation of environmental pollutants have so far been relatively unstudied. Here we show, for the first time, that exposure of NHEKs to ozone resulted in increased protein and mRNA expression of CYP1 isoforms (such as CYP1A1, CYP1A2, and CYP1B1) (Figure 1a and b). We also provide evidence that AhR plays a crucial

role in the induction of CYP1 isoforms in NHEKs by ozone exposure. In general, CYP1 isoforms act by increasing the polarity of their substrates to facilitate the elimination of xenobiotics, and, for this reason, they are often referred to as detoxification enzymes. In many instances, however, CYP-catalyzed metabolic products are more toxic than the parent compounds, which will ultimately enhance the binding of the reactive metabolite to the DNA, which thus implicates them in the enhancement of cutaneous carcinogenesis (Katiyar *et al.*, 2000; Ahmad and Mukhtar, 2004). Most xenobiotic procarcinogens are hydrophobic and are substrates of CYP1 enzymes (such as CYP1A1, CYP1A2, and CYP1B1). These three CYP1 enzymes are imperative for metabolically activating a variety of environmental procarcinogens in the aryl- and alkyl-amine, heterocyclic amine, polycyclic aromatic hydrocarbon, and polyhalogenated aromatic hydrocarbon categories. Several studies have shown that the regulation of CYP1A1 expression plays a decisive role in carcinogenesis, as many chemicals that induce skin CYP1A1 expression are also initiators of skin tumors in man (Falk *et al.*, 1964). Furthermore, CYP1A1-dependent metabolic activation of procarcinogens into reactive carcinogenic metabolites in skin has been reported (Kinoshita and Gelboin, 1972). Therefore, it is warranted to explore the development of inhibitors of ozone-induced CYP expression, which may protect human skin against the adverse effects of ozone exposure.

It has been well documented that on ligand binding, AhR translocates to the nucleus, dimerizes with the AhR nuclear translocator, and induces the transcription of *CYP* genes with a concomitant increase in CYP proteins and their activities (Nebert *et al.* 2004; Fritsche *et al.*, 2007). The AhR–AhR nuclear translocator complex interacts with dioxin-responsive elements and modulates gene expression. In addition, the ligand-activated receptor can affect cellular functions by activating the non-receptor tyrosine kinase, c-Src, which is associated specifically with the AhR complex (Enan and Matsumura, 1996). Dissociation of c-Src from ligand-activated receptor induces c-Src translocation from the cytosol to the cell membrane, where it functions as a co-transducer of transmembrane signals emanating from a variety of polypeptide growth factor receptors, including EGFR (Kohle *et al.*, 1999; Park and Matsumura, 2006). EGFR is activated by one of several structurally related ligands, including EGF and transforming growth factor-alpha (TGF-α) (Gregory, 1975; De Larco *et al.*, 1980). The outcomes of EGFR activation are pleiotropic and can depend on cell type, co-expression of other c-erbB receptor family members, and local environmental signals, including cell–cell and cell–matrix interactions. Typical responses encompass enhanced mediator release, cell migration, proliferation, and differentiation (Polosa *et al.*, 2004). In this study, we found nuclear translocation of AhR (Figure 2a), an increase in AhR mRNA expression (Figure 2b), and phosphorylation of EGFR (Figure 3a and b) in NHEKs after ozone exposure. Our data further show that AhR knockdown by siRNA abolished ozone-induced CYP1 isoform mRNA and protein expression (Figure 6a and b). These data suggest that NHEK exposure to ozone, similar to known AhR ligands, translocates AhR from the cytoplasm into the nuclear compartment, leading to an increased expression of AhR-dependent *CYP1* genes and proteins. These studies decisively show that AhR is involved in ozone-induced CYP1 induction.

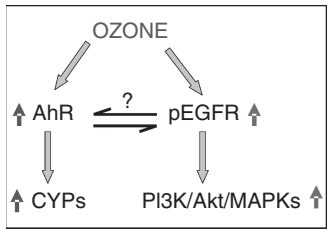

Figure 8. Schematic diagram showing the mechanism of cytochrome P450 (CYP) induction by ozone. AhR, aryl hydrocarbon receptor; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; pEGFR, phosphorylated EGFR; PI3K, phosphoinositide 3-kinase.

The signaling pathways induced by activated EGFR include the PI3K/Akt and MAPKs, both of which play an important role in cell growth, proliferation, and survival (She *et al.*, 2005). PI3K is a dimer composed of an inhibitory/regulatory (p85) and a catalytic (p110) subunit. The p85 subunit anchors to an erbB receptor docking site, and the p110 subunit is responsible for the phosphorylation and activation of the protein serine/threonine kinase Akt (Yarden and Sliwkowski, 2001). Studies have suggested that signaling through the PI3K/Akt pathway plays a role in neoplastic transformation (Hanahan and Weinberg, 2000). We found that exposure of NHEKs to ozone caused an increase in the protein expression of PI3K (p85 and p110) and in the phosphorylation of Akt at Thr^{308} (Figure 4a and b). The MAPKs encompass a large number of serine/threonine kinases and are divided into three multimember subfamilies: ERK1/2, JNK1/2, and p38 kinase (Wada and Penninger, 2004). Studies have shown that MAPK subfamilies are activated in response to oxidant injury and, therefore, could potentially contribute to influencing cell survival (Schütze *et al.*, 2008). Our observations suggested that ozone exposure of NHEKs induced phosphorylation of MAPKs (such as pERK1/2, pJNK1/2, and pp38) (Figure 5a and b).

In conclusion, the results from this study clearly show that exposure of NHEKs to ozone resulted in an increased expression of CYP1 isoforms, activation of AhR, and phosphorylation of EGFR with concomitant activation of PI3K/Akt and phosphorylation of MAPK. Furthermore, these data indicate that induction of CYPs by ozone is mediated through the activation of AhR and not by that of EGFR. Thus, AhR signaling is an integral part of CYP induction by ozone (Figure 8). These studies strongly suggest that there are toxicological consequences of ozone on human skin.

MATERIALS AND METHODS

Antibodies

The antibodies (Abs) for CYP1A1 and CYP1A2 were obtained from Fitzgerald Industrial International (Concord, MA). CYP1B1 Ab was procured from Gentest (Woburn, MA). AhR Ab was purchased from GeneTex, Inc. (San Antonio, TX). The monoclonal and polyclonal

Abs for ERK1/2 (phospho-p44/42, Thr^{202}/Tyr^{204}), JNK1/2 (phospho-p54/46, Thr^{183}/Tyr^{185}), p38 (phospho-p38, Thr^{180}/Tyr^{204}), PI3Kinase (p85), PI3Kinase (p110), and p-Akt (Thr^{308}) were obtained from Cell Signalling Technology, Inc. (Danvers, MA). Anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary Abs were purchased from Amersham Life Science (Arlington Heights, IL). siRNA for AhR and control scrambled siRNA were purchased from Dharmacon (Lafayette, CO). The antibiotics (penicillin and streptomycin) used were obtained from Cellgro Mediatech (Herndon, VA). Protein was estimated using the BCA protein assay kit obtained from Pierce (Rockford, IL).

Cell culture and treatment

NHEKs were obtained from Cascade and grown in EpiLife medium (Portland, OR). The cells were maintained at 37 °C and in 5% CO_2 in a humid environment. Near-confluent keratinocytes in D-PBS (10 mmol l^{-1}, pH 7.4) were exposed to approximately 0.3 ppm ozone. This dose was obtained over a period of 20 minutes exposure to ozone in a closed chamber. After ozone exposure, the D-PBS was removed and fresh medium was added. The cells were harvested immediately after 20 minutes (considered as the 0 time point) and after 3 and 6 hours of ozone exposure for protein and RNA isolation. Typical levels of ozone that are recorded in urban environments can range between 0.2 and 1.2 ppm (Mustafa, 1990; Gruber *et al.*, 2005). The rationale for using 0.3 ppm of ozone is based on the fact that, in Los Angeles, over a 10-year period, the Environmental Protection Agency estimated that the second highest average level of ozone was 0.25 ppm (this number was higher in Mexico City), which indicates that the highest dosages were even greater than 0.25 ppm (CGER, 1991).

Preparation of cell lysate

After exposure of cells to ozone, the medium was aspirated and the cells were washed twice in D-PBS. The cells were then incubated in 0.4 ml ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% Nonidet P-40, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (pH 7.4)) with a freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA). The cells were then centrifuged at 13,000 *g* for 25 minutes at 4 °C, and the supernatants (cell lysate) were collected, aliquoted, and stored at −80 °C. The protein concentration was determined by the BCA protein assay kit.

Preparation of cytosolic and nuclear lysates

After treatment of cells with ozone for various times, the medium was aspirated and the cells were washed twice in D-PBS. The cells were incubated in 0.4 ml ice-cold lysis buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM, pH 7.9), KCl (10 mM), EDTA (0.1 mM), EGTA (0.1 mM), dithiothreitol (1 mM), and phenylmethylsulfonyl fluoride (1 mM)) with freshly added protease inhibitor cocktail for 15 minutes, after which 12.5 µl of 10% Nonidet P-40 was added. The contents were then mixed and centrifuged for 1 minute (14,000 *g*) at 4 °C. The supernatant was saved as a cytosolic lysate and stored at −80°C. The nuclear pellet was resuspended in 50 µl of an ice-cold nuclear extraction buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (20 mM, pH 7.9), NaCl (0.4 M), EDTA (1 mM), EGTA (1 mM), dithiothreitol (1 mM), and phenylmethylsulfonyl fluoride (1 mM)) with freshly added protease inhibitor cocktail for 30 minutes with intermittent mixing. The tubes were centrifuged for 5 minutes (14,000 *g*) at 4 °C, and the supernatant (nuclear extract) was stored at −80 °C. The protein concentration was determined by the BCA protein assay kit.

Western blot analysis

For western blotting, 25–50 µg protein was resolved over 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The non-specific sites on blots were blocked by incubating in a blocking buffer (5% non-fat dry milk/1% Tween 20 in 20 mmol l^{-1} Tris-buffered saline, pH 7.6) for 1 hour at room temperature, incubated with the appropriate monoclonal primary Ab in blocking buffer for 90 minutes to overnight at 4 °C, followed by incubation with an anti-mouse or anti-rabbit secondary Ab horseradish peroxidase conjugate and detection by chemiluminescence (ECL kit, Amersham Biosciences, Buckinghamshire, UK) and autoradiography, using an XAR-5 film obtained from Eastman Kodak Co. (Rochester, NY). Densitometric measurements of the bands were taken using the digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT).

Reverse transcriptase–PCR

Near-confluent keratinocytes were exposed to ozone and RNA was isolated using an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Santa Clarita, CA). After extraction, mRNAs were reverse transcribed using the SuperScript III first strand synthesis system (Invitrogen Corporation, Carlsbad, CA) and a FailSafe PCR kit (Epicenter Biotechnologies, Madison WI). The following primer sequences were used: CYP1A1, forward: 5′-TCACAGACAGCCTG ATTGAGA-3′ and reverse: 5′-GATGGGTTGACCCATAGCTT-3′; CYP1A2, forward: 5′-TGGTATTCTGGGTGCTCAAGGGTT-3′, reverse: 5′-TGGAGAAGGTGTTGAGGGCATTCT-3′; CYP1B1, forward: 5′-GTATATTGTTGAAGAGACAG-3′, reverse: 5′-AAAGAGGT ACAACATCACCT-3′, AhR, forward: 5′-TCCACCTCAGTTGGCTT TGTTTGC-3′ and reverse: 5′-ATTCGGATATGGGACTCGGCACAA-3′, EGFR, forward, 5′-CTTCTTGCAGCGATACAGCTC-3′, reverse: 5′-ATGCTCCAATAAATTCACTGC-3′, and β-actin, forward: 5′-GTGGGGCGCCCCAGGCACCA-3′, reverse, 5′-CTCCTTAATGT CACGCACGATTTC-3′. PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

Silencing of AhR and EGFR by siRNA

For suppressing AhR and EGFR expression, AhR, EGFR, and control scrambled siRNAs were purchased from Dharmacon. NHEKs were transfected with either AhR siRNA (100 nmol l^{-1}), EGFR siRNA (100 nmol l^{-1}), or control scrambled siRNA (100 nmol l^{-1}) using the Nucleofector Kit specific for NHEK transfection from Amaxa Biosystems. The cells were resuspended in a solution from the Nucleofector Kit following the manufacturer's guidelines. A volume of 100 µl of Nucleofector solution was mixed with 2×10^6 cells and siRNA. It was then transferred to the cuvette provided with the kit and nucleofected with an Amaxa Nucleofector apparatus. The cells were transfected using the U-20 pulsing parameter and transferred into 100-mm plates containing 37 °C prewarmed culture medium. Although keratinocytes are difficult to transfect, an ~80% transfection efficiency of siRNA was achieved by electroporation. After

transfection, the cells were cultured for 24 hours, after which the medium was replaced with D-PBS and the cells exposed to ozone (0.3 ppm). After 20 minutes of ozone exposure, the D-PBS was removed and fresh medium was added. The cells were harvested 3 hours after ozone exposure for protein and RNA isolation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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