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ACCELERATED COMMUNICATION

Benzo(*a*)pyrene Inhibits Epidermal Growth Factor Binding and Receptor Autophosphorylation in Human Placental Cell Cultures

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SUMMARY

Studies investigated the effects of benzo(*a*)pyrene (BP) treatment on epidermal growth factor (EGF) receptor binding and kinase activity in human placental cell cultures. Specific binding of ^{125}I-EGF to cells from early gestation placentae was significantly decreased by 37 and 60% following exposure to 1 and 10 μM BP, respectively, for 24 hr. In contrast, cells cultured from term placentae showed no inhibitory effect of either concentration of BP. Specific binding of ^{125}I-labeled insulin and insulin-like growth factors-I and -II to early gestation cells was decreased only 15–18% at 10 μM BP, which indicates that loss of membrane receptors appears to be selective for EGF. Scatchard analysis of early gestation cells revealed that BP was associated with a dose-dependent loss in the number of high affinity EGF binding sites. Evidence from cross-linking and autophosphorylation experiments confirmed that the M_r 170,000 binding protein was decreased in a dose-dependent manner following BP treatment. In comparison, term placental cells exhibit a 26% loss of EGF receptor autophosphorylation without alteration in binding following exposure to 10 μM BP. Thus, early gestation cells exhibit a BP-related down-regulation of EGF receptors, whereas term placental cells show receptor desensitization. No adverse effect of BP treatment was observed on the incorporation of [^{35}S] methionine into proteins secreted by early gestation cells. Further experiments compared the effects of BP with the related polycyclic compounds β-naphthoflavone, α-naphthoflavone, and 3-methylcholanthrene. In early gestation cells, EGF binding and receptor autophosphorylation were measurably decreased at 10 μM concentrations of these polycyclic compounds, but to a lesser extent than observed with BP. In term placental cells, however, EGF binding was unchanged or increased, whereas receptor autophosphorylation was decreased 10–26%. Thus, exposure of term placental cells to these polycyclic compounds leads to a dissociation between EGF binding and receptor protein kinase activity. Finally, aryl hydrocarbon hydroxylase activity was induced 20- to 200-fold in early placental cells exposed to BP, β-naphthoflavone, and 3-methylcholanthrene. In summary, the direct effects of BP and related compounds observed on placental EGF receptors may indicate altered function of EGF in the regulation of cell growth and differentiation in the human placenta.

BP is a potent PAH carcinogen that is a major constituent present in cigarette smoke (1, 2). Previous studies have demonstrated that BP and other PAH inducers of cytochrome P-450IA1 inhibit binding of EGF to cell surface receptors in cultured rodent cell lines (3, 4). Human placentae from cigarette smokers (5, 6) and from women exposed environmentally to polychlorinated biphenyl-contaminated rice oil (5, 7) have been found to have biochemical alterations in EGF receptors. Wang *et al.* (6) further reported that EGF-stimulated kinase activity was markedly decreased in placental membrane proteins from smokers, whereas insulin receptor phosphorylation was normal or increased. Moreover, the smoking-related deficiency in EGF receptor autophosphorylation appeared to be due to the absence

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ABBREVIATIONS: BP, benzo(*a*)pyrene; PAH, polycyclic aromatic hydrocarbon; EGF, epidermal growth factor; IGF, insulin-like growth factor; 3-MC, 3-methylcholanthrene; α-NF, 7,8-benzoflavone; β-NF, 5,6-benzoflavone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AHH, aryl hydrocarbon hydroxylase; HPLC, high pressure liquid chromatography.

of a M_r 170,000 receptor protein. These data suggest that the association of maternal cigarette smoking with fetal growth retardation (8) and altered placental morphology (9) may involve a selective alteration in EGF receptor-mediated effects during gestation. Human placenta contains a large number of EGF receptors throughout gestation (10–14), and EGF has been shown to enhance the release of human chorionic gonadotrophin and human placental lactogen (11, 15, 16) as well as to enhance glucose and amino acid uptake in cultured placental cells (17–19).

The present study investigated the effect of BP on EGF receptor binding and autophosphorylation events in placental cells cultured from early gestation and term placentae. Further studies characterized the effects of BP on the binding of other growth factor ligands and then compared BP with a group of related polycyclic compounds.

Materials and Methods

Chemicals. EGF was either purified from mouse submaxillary glands as described by Savage and Cohen (20) for phosphorylation experiments or purchased from Sigma (St. Louis, MO) for binding studies. Porcine insulin (24.5 units/mg) was a gift of Connaught Laboratories (Willowdale, Ontario, Canada). Recombinant [Met_{56}]-IGF-I was a generous gift from Dr. J. Nueesch (Ciba-Geigy, Basel, Switzerland) and Dr. W. Rutter (Chiron Corp., Emeryville, CA). IGF-II was purified by HPLC from acid-ethanol extracts of Cohn fraction IV-4 as previously described (21). BP was purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-MC, β-NF, α-NF, BSA (fraction V), SDS-PAGE protein standard kit, HEPES, PMSF, adenosine 5′-triphosphate, and cytidine 5′-triphosphate were from Sigma. [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol) was obtained from Amersham Co. (Arlington Heights, IL). Materials for cell culture include trypsin (hog pancreas) from ICN Chemicals (Montreal, Quebec, Canada), Ham's F-10 medium from Microbiological Associates (Johns Scientific, Toronto, Ontario, Canada), and fetal bovine serum from GIBCO (Grand Island, NY).

Cell cultures. Placentae were obtained from early gestation pregnancies (6 to 14.5 weeks) following elective abortion and from term pregnancies (38 to 40 weeks) delivered by elective Caesarian section. Protocols for obtaining materials were approved in accordance with the guidelines provided by the Medical Research Council of Canada. Placental cells were cultured as previously described (11, 17, 22). Briefly, tissue was dispersed with 0.25% trypsin and cells were plated onto 35- or 100-mm tissue culture dishes. Cells were cultured as monolayers at 37° with 95% air/5% CO_2 in 3 to 5 ml of Ham's F-10 medium with 10% fetal bovine serum and antibiotics [penicillin G (200 IU/ml), gentamycin G (40 μg/ml), and amphotericin B (5 μg/ml)]. On day 3, the medium was replaced and the chemical to be studied was added in a volume of 15 μl of dimethyl sulfoxide to obtain a final concentration of 1 to 10 μM. Control cultures received 15 μl of dimethyl sulfoxide. The cultures were incubated at 37° and 5% CO_2 for 24 hr before the binding assay was conducted.

Binding assay. Iodinations with Na^{125}I (NEN Corp.) were performed by the chloramine T method for EGF, insulin, IGF-I, and IGF-II, as previously described (23). The specific activity of the ligands was approximately 220–265 mCi/μg of EGF, 200–250 for insulin, and 150–200 for the IGFs. Assays of binding of ^{125}I-labeled EGF, insulin, and IGFs to placental cells were conducted at 4° for 20 hr as previously described (10, 11, 17, 22). Briefly, after exposure to polycyclic chemicals for 24 hr, cell cultures were washed four times in cold phosphate-buffered saline. Cells were removed from culture dishes with a rubber policeman and resuspended in cold phosphate-buffered saline. Cell suspensions were pelleted at 4° and viability was assessed by trypan blue exclusion. The binding assay buffer mixture consisted of 0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, 0.1% BSA, 4–6 × 10^4 cpm of ^{125}I-ligand, and 100 μg of cell protein, in the presence or absence of unlabeled growth factor, in a final volume of 0.5 ml. Assays were terminated after 18–20 hr with the addition of 3.0 ml of cold washing buffer, 0.1 M HEPES, pH 7.5, 0.1% BSA, and centrifugation at 2000 × g for 30 min at 4°. Total binding is calculated as the ratio of cpm bound/cpm ligand added × 100, which is the percentage of total radioactivity in the reaction mixture. Specific binding was expressed as the difference between radioactivity bound in the absence (total binding) and presence (nonspecific binding) of excess unlabeled ligand (1 μg of EGF and insulin, 200 ng of IGF-I and IGF-II) and was calculated from duplicate or triplicate determinations.

Affinity labeling. Covalent cross-linking of ^{125}I-labeled ligands to cellular binding proteins was performed as previously described (23). Samples were run on SDS-polyacrylamide gels with a 5 to 15% gradient. The gels were dried and exposed to X-ray film for 5–14 days at −70°. Laser densitometry was performed on the identified radioactive peaks or bands.

Protein synthesis. In some experiments, cells were incubated with 10 μM BP for 24 hr, washed, and further incubated with 50 μCi of [^{35}S]methionine in methionine-free medium for 3 hr at 37°. Following incubation, culture medium was dialysed extensively (3500 molecular weight cut-off), lyophilized, and resuspended in electrophoresis buffer. Two-dimensional SDS-PAGE of the samples was performed according to the methods described by Roberts *et al.* (24).

EGF receptor phosphorylation. Monolayers of placental cells were solubilized in 0.3 ml of TGP buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES, 1.0 mM PMSF, pH 7.4) on ice for 20 min. Extracts were combined, centrifuged at 100,000 × g for 30 min at 4°, and stored at −70°. EGF receptor phosphorylation was performed by a modification of the method of Lowe *et al.* (25). Solubilized cell homogenate, 40–60 μg of protein, was preincubated in the absence or presence of 1.0 μM EGF in a final volume of 75 μl of 50 mM HEPES buffer, pH 7.6, with 10 mM $MgSO_4$ and 1 mM PMSF. After 30 min at 22°, samples were placed on ice for 5 min and phosphorylation was then initiated by the addition of 25 μl of reaction mixture to give final concentrations of 5 μM [^{32}P]ATP (specific activity, 20 μCi/nmol), 1 mM cytidine-5-triphosphate, 3 mM $MnCl_2$, 20 mM $MgCl_2$, and 1 mM sodium vanadate ([^{32}P]ATP-mixture). After 1 min at 0°, the reaction was terminated by the addition of an equal volume of stopping solution (0.17 M Tris·HCl, 10% SDS, and 100 mM dithiothreitol, pH 6.8) and heating at 100° for 10 min. Aliquots (100 μl) were analyzed by SDS-PAGE followed by autoradiography. The intensity of ^{32}P-labeled protein bands shown on X-ray film was quantitated by Soft-Laser densitometer, model SF-TRFF (Biomed Instrument, Inc., Fullerton, CA).

AHH activity. AHH activity was measured in cells as previously described by Manchester *et al.* (26). After extensive washing, cells were pooled from five dishes in each treatment group and homogenized in 2 ml of buffer. Protein concentration was measured according to the method of Lowry *et al.*. Cell homogenates (500 μl) were incubated in duplicate with BP substrate for 30 min. Blank values for BP-treated cells were determined from samples with zero incubation time and samples incubated without substrate for 30 min. Enzyme activity was determined as the amount of fluorescent metabolites formed using a 3-hydroxy-benzopyrene reference standard (27).

Data analysis. All experiments were performed using at least two different placental culture preparations. Statistical analyses were performed with Student's t test. Scatchard analysis of binding data was performed using a computer-based program for a two site model (28).

Results

Effects of BP on EGF binding. As has been previously reported (11), the specific binding of ^{125}I-EGF to cultured human placental cells was maximal at 4° over 20 hr, reversible, and proportional to cell protein and ligand concentrations. The percentage of specific binding in term placental cells was 22.8 ± 5.2% (mean ± SE, three placentae, six replicates) compared

with 19.1 ± 2.3% (seven placentae, four replicates) in early gestation cells. Nonspecific binding in all experiments was less than 2–7% of the total binding.

Data in Fig. 1 show the effect of 24-hr exposure to BP on the binding of ^{125}I-EGF to cultured placental cells. Specific binding to early gestation placental cells was significantly decreased from control by 37% at 1 μM BP ($p < 0.01$) and by 60% at 10 μM BP ($p < 0.001$). In distinct contrast, cells cultured from term placentae showed no loss of EGF binding following exposure to either concentration of BP. The loss of EGF binding in early gestation cells was not apparent after only 6-hr exposure to BP and appeared to be time dependent. Scatchard plots of ^{125}I-EGF binding to early gestation cells are shown in Fig. 2. A marked decrease in the number of high affinity EGF binding sites is evident, with a 50% decrease at 1 μM BP and a 73% decrease at 10 μM, whereas little effect was observed on receptor affinity.

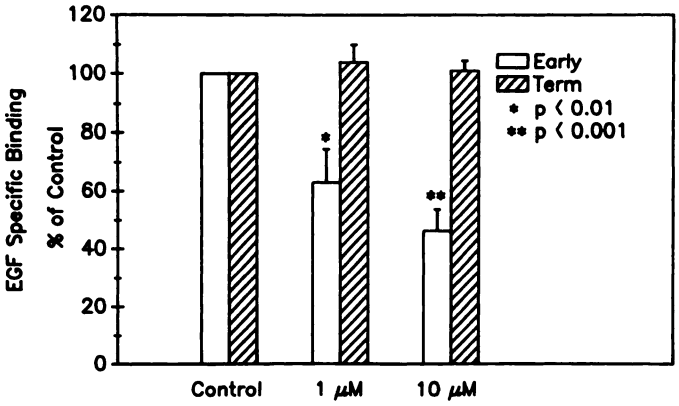

Fig. 1. Comparison of the effect of BP treatment on ^{125}I-EGF binding to cultured cells from early gestation and term placentae. Cultured cells were incubated with 0, 1, or 10 μM BP for 24 hr at 37°. After washing, the binding of ^{125}I-EGF to intact cells (100 μg of cell protein) was assayed at 4° for 20 hr. In a typical experiment for control term cells, 99,735 cpm of ligand were added, total binding was 22,341 cpm (22.4% total binding) and nonspecific binding in the presence of excess unlabeled EGF was 2,293 cpm (2.3% nonspecific binding). In order to compare data from repeated experiments, the percentage of specific binding has been normalized to 100% for control cells. Data are presented as the mean ± standard error of replicate cultures from seven early gestation (8.5–14.5 weeks) and three term placentae.

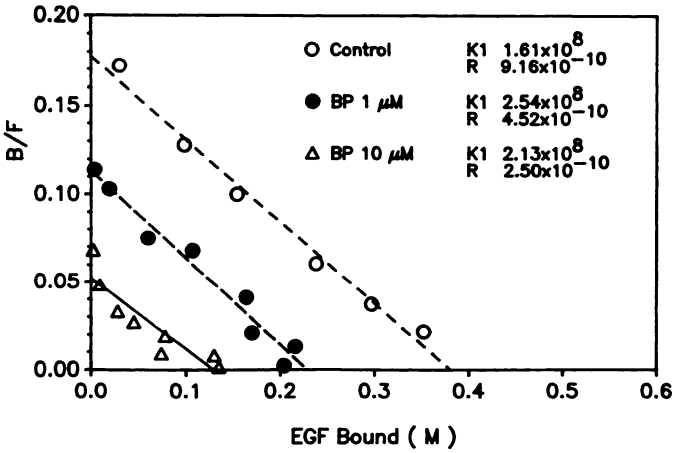

Fig. 2. Scatchard analysis of ^{125}I-EGF binding to cultured early gestation placental cells following treatment with the indicated concentrations of BP. Values represent the mean of replicate cultures from three placentae of 12–13 weeks gestation. K_1 values are the calculated high affinity binding constants and R is the number of high affinity binding sites, expressed as pmol/mg of protein.

The effects of BP treatment on early gestation cells were further examined by covalent cross-linking of ^{125}I-EGF to binding proteins in homogenates of placental cells. Analysis by SDS-PAGE and autoradiography, shown in Fig. 3, revealed that the major EGF receptor species with M_r 170,000 showed a dose-dependent decrease following exposure to BP and this band was barely detectable at the 10 μM concentrations. The identity and significance of the M_r 93,000 and 45,000 species were not apparent, but these bands were also decreased following exposure to BP. Insofar as cellular homogenates rather than intact cells were cross-linked in these experiments, the lower M_r species may reflect EGF-binding proteins reported to be present in various intracellular organelles (11, 29).

Effects of BP on insulin, IGF-I and IGF-II binding. The specific binding of ^{125}I-labeled insulin, IGF-I, and IGF-II to placental cells was reversible and dependent on time and cellular protein concentrations (22). Table 1 shows that treatment of early gestation cells with 1 μM BP is associated with a 7–13% decrease in the binding of insulin, IGF-I, and IGF-II, compared with a 37% loss in EGF binding. BP at 10 μM was associated with only a 15–18% reduction in the binding of the insulin-related ligands, whereas EGF binding decreased 60%. Subsequent affinity labeling experiments revealed little effect of BP on either the M_r 130,000 Type-I IGF receptor species or the M_r 260,000 Type-II IGF receptor (data not shown). Thus, the dose-dependent loss of placental membrane receptors following BP exposure appears to be selective for EGF.

Effects of BP on protein synthesis. Our studies have used protein content as the binding assay unit rather than cell number, due to the heterogeneity in cell size obtained from syncytiotrophoblast dispersion. In this regard, treatment of early gestation cells with 10 μM BP was not associated with visible toxicity or alterations in protein content. In further experiments, control and BP-treated cells were incubated with

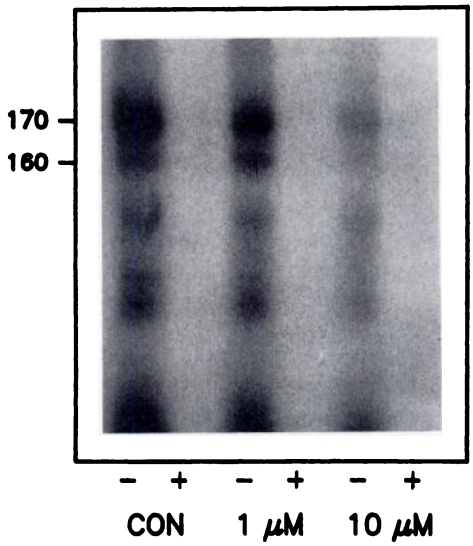

Fig. 3. Autoradiogram of SDS-polyacrylamide gels, which shows the covalent cross-linking of ^{125}I-EGF to membrane proteins following treatment of cultured early gestation placental cells with the indicated concentrations of BP. After treatment, cells were exposed to ^{125}I-EGF in the presence (+) or absence (−) of unlabeled EGF (10 μg/ml) and affinity labeled with disuccinimide suberate. *Con*, control.

TABLE 1

Effect of BP on percentage of specific binding of ^{125}I-labeled growth factors to cultured early gestation placental cells

Cells were incubated with BP as described in Fig. 1. After washing, cells were exposed to ^{125}I-labeled EGF (40,000 cpm), insulin (40,000 cpm), IGF-I, or IGF-II (20–30,000 cpm) at 4° for 20 hr. Binding is expressed as the percentage of total cpm in the reaction mixture. Nonspecific binding, measured in the presence of excess unlabeled ligand, was 2–7%. Placental cells from early gestation placenta were cultured in one to three separate experiments (Exp no.). Each percentage of specific binding value represents the average of two or three replicates. The percentage of specific binding obtained for each experiment in the absence of BP is indicated as 100% control.

Ligands	Exp no.	Binding				
		Control cells	BP, 1 μM		BP, 10 μM	
		% of specific binding	% of specific binding	% of control	% of specific binding	% of control
EGF	1	14.7	10.3	70.0	6.4	43.3
	2	20.1	15.7	78.0	10.4	51.5
	3	22.6	9.1	40.0	6.0	26.6
	Mean			63.0		40.5
IGF-1	1	5.1	5.0	97.7	4.7	91.2
	2	2.0	1.6	78.6	1.5	72.1
	Mean			88.2		81.7
IGF-2	1	3.9	3.6	93.6	3.1	80.4
	2	4.8	4.4	92.3	4.0	83.8
	Mean			93.0		82.1
Insulin	2	3.5	3.1	87.3	3.0	84.7

[^{35}S]methionine for 3 hr and the radiolabeled proteins secreted into the culture media were characterized by two-dimensional SDS-PAGE. The fluorograms in Fig. 4 show that BP did not result in apparent changes in the M_r or isoelectric point of the major secreted proteins. If anything, BP was associated with increased secretion of a minor M_r 26,000 protein.

Differential effects of other polycyclic compounds on EGF binding. A group of polycyclic compounds related to BP were further examined for effects on ^{125}I-EGF binding (Table 2). In early gestation cells, specific binding was decreased 32% from control with β-NF (10 μM) and 12% with α-NF (10 μM), compared with a 60% loss following BP (10 μM) exposure. As previously observed with term placental cells, EGF binding was not significantly altered by treatment with BP. Exposure of term cells to 10 μM α-NF, β-NF, and 3-MC, however, was associated with apparent increases in EGF binding, which were significant with α-NF and β-NF. Thus, the effects of this group of polycyclic compounds were markedly different for early gestation versus term placental cells.

Effects on EGF-stimulated kinase activity. EGF receptor kinase activity was assayed using solubilized cell homogenates, which were incubated with [^{32}P]ATP in the presence or absence of EGF. The autoradiograms in Fig. 5 show that EGF stimulated the phosphorylation of a M_r 170,000 band in proteins from early gestation (Fig. 5A) and term (Fig. 5B) cells. Laser densitometry scan of the M_r 170,000 band in Fig. 5A indicates that 10 μM BP treatment of early gestation cells was associated with a 64% decrease in EGF-stimulated ^{32}P incorporation. EGF receptor autophosphorylation was also measurably decreased in early gestation cells at 10 μM α-NF (43%), β-NF (31%), and 3-MC (13%). Although 1 μM BP was not associated with a decrease in the intensity of the M_r 170,000 band, this sample showed the phosphorylation of a unique M_r 100,000 band, which was stimulated by EGF. In comparison, all samples exhibited the phosphorylation of a M_r 50,000 band, which was not stimulated by EGF and which appeared to be comparable in all samples regardless of treatment.

In term placental cells (Fig. 5B), treatment with 10 μM BP, β-NF, and α-NF, respectively, was associated with 10–26% reductions below control in EGF-stimulated phosphorylation of the M_r 170,000 band. Thus, exposure of term cells to these polycyclic compounds is associated with alterations in EGF protein kinase that were not coordinated with changes in binding (Table 2).

AHH in cultured cells. A final experiment examined whether it was possible to induce AHH activity in cultured placental cells. Table 3 shows that exposure of early gestation cells to BP, β-NF, α-NF, and 3-MC at two concentrations did not markedly alter cellular protein content. Treatment with BP, β-NF, and 3-MC was associated with 20- to 200-fold increases in AHH activity, with induced AHH being highest at 10 μM exposure levels. Although a 5-fold increase in AHH activity was observed at 1 μM α-NF, activity at 10 μM was at control levels. Because there was no evidence of α-NF cytotoxicity based upon protein content, this observation likely reflects the known inhibitory effect of α-NF on induced AHH activity (30). In data not shown, AHH activity in term placental cells was also induced 5- to 50-fold following exposure to 3-MC and BP, respectively.

Discussion

Maternal cigarette smoking during pregnancy has been associated with fetal growth retardation, altered placental morphology, and increased risk of premature delivery and spontaneous abortion (8, 9). Mechanisms underlying this developmental toxicity are still unclear, in large part because cigarette smoke contains a complex mixture of chemicals in addition to BP and related PAHs (2). The present study provides the first evidence that BP exposure is directly associated with a dose-dependent loss of EGF receptors in cultured human placental cells over a relatively brief 24-hr period. A striking observation was that the BP-associated loss of EGF receptors was seen with cells from early gestation but not term placentae. Term placentae, however, exhibit loss of EGF receptor autophos-

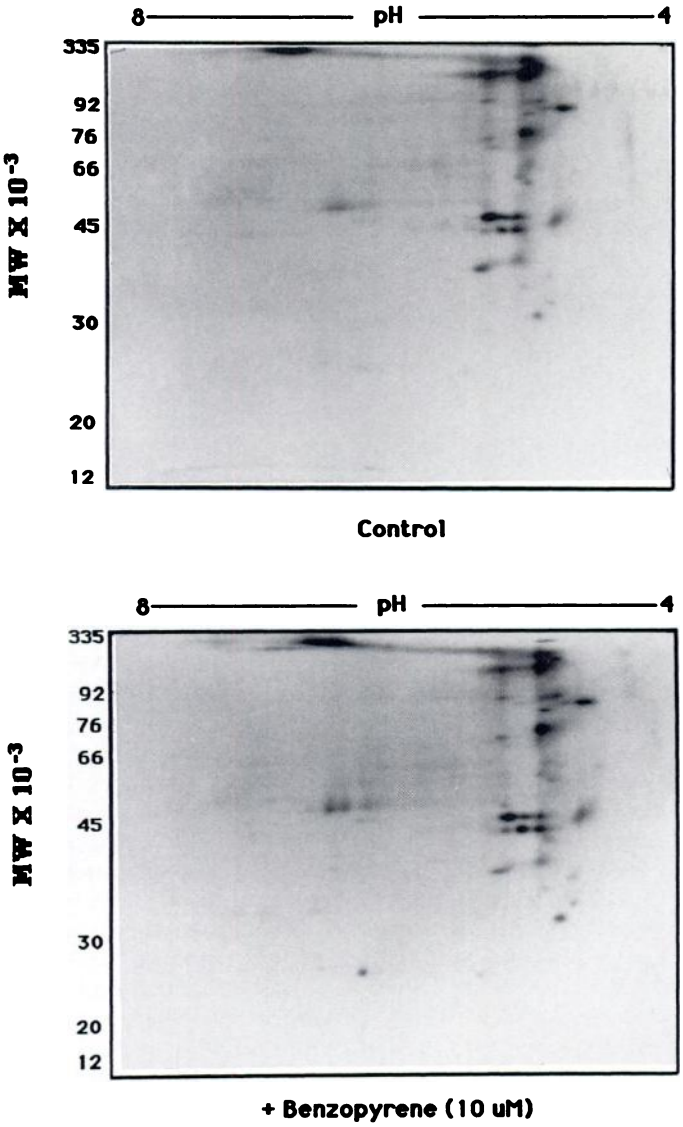

Fig. 4. Representative fluorographs of two-dimensional SDS-polyacrylamide gels of [^{35}S]methionine-labeled proteins secreted by early gestation placental cells. Following treatment with BP for 24 hr, cells were incubated with 50 μCi of [^{35}S]methionine for 3 hr at 37°.

phorylation activity without alteration in binding (receptor desensitization) following BP exposure. A dissociation between EGF receptor binding and receptor protein kinase activity has been previously observed in term human placentae from smokers (5, 6) and from women exposed to polychlorinated biphenyls (5, 7). Of substantial importance is our observation that the major inhibitory effects of BP were seen early in gestation, when major events take place in growth and development within the feto-placental unit and at a time when EGF receptor-mediated biological effects are likely to be greatest (11–16). In addition, the effects of BP on EGF receptors were also seen with related polycyclic compounds tested, suggesting that these compounds may produce similar effects on EGF receptor function.

Down-regulation of EGF binding following exposure to BP and related PAH compounds has been previously observed in studies with cultured mouse fibroblasts (3) and mouse hepatoma cells (4). In the present study, the BP-associated loss of EGF receptors on early placental cells was observed under

TABLE 2

Effects of polycyclic compounds on ^{125}I-EGF binding to early and term gestation cultured placental cells

Cultured cells were incubated for 24 hr at 37° in the presence of polycyclic compounds at the indicated concentrations. After washing, the binding of ^{125}I-EGF to intact cells (100 μg of protein) was assayed at 4° for 20 hr. In a typical experiment for control cells, 40,000 cpm of ligand were added, specific binding was 20.1%, and nonspecific binding was 2.3%. Cells were cultured from *n* (number of placentae, number of replicates) early gestation and term placentae. In order to compare treatment effects, specific binding in untreated cells is normalized to 100% control. Data are expressed as mean ± standard error.

	^{125}I-EGF binding	
	Early gestation	Term gestation
	%	
Control	100.0 (*n* = 7, 4)	100.0 (*n* = 3, 6)
BP		
1 μM	62.7 ± 11.6[a] (*n* = 7, 4)	98.3 ± 1.1 (*n* = 2, 4)
10 μM	40.5 ± 7.3[a] (*n* = 7, 4)	102.0 ± 2.0 (*n* = 3, 6)
α-NF 10 μM	87.7	116.4 ± 8.5[b] (*n* = 2, 4)
β-NF, 10 μM	67.1	110.3 ± 1.8[a] (*n* = 2, 4)
3-MC 10 μM	ND[c]	134.7

[a] $p < 0.005$.
[b] $p < 0.05$.
[c] ND, not determined.

conditions where binding was steady state and where ligand internalization and degradation have been shown to be minimal (11, 17). The effect of BP appeared to be selective for the binding of EGF, compared with insulin, IGF-I, and IGF-II, in early gestation cells. In experiments with mouse hepatoma cells, Karenlampi *et al.* (4) also found a selective inhibitory effect of BP on EGF binding, whereas phorbol ester or insulin binding to treated cells was unaffected. In the present study, the loss of high affinity EGF binding sites following BP treatment was confirmed by affinity labeling and autophosphorylation experiments using preparations of total cellular proteins. These data suggest that down-regulation at higher concentrations of BP may reflect increased receptor degradation without concomitant synthesis of new receptors. In this regard, Wang *et al.* (6) observed that lower molecular weight immunoreactive species of EGF protein were readily detectable in crude membrane preparations from placentae of smokers. Experiments are in progress to determine whether the BP constituent of cigarette smoke may directly alter the intracellular processing and/or biosynthesis of EGF receptors in placental cells.

The present study clearly demonstrates the capacity of cultured human placental cells to respond to BP and related polycyclic compounds directly, with the induction of AHH activity. Manchester *et al.* (26) have previously shown induction of AHH in cultured human umbilical vein endothelial cells exposed to 3-MC. It is noteworthy that the level of induced AHH that we observed in cultured early placental cells is within the range reported in term placental microsomes from smokers (26). In this regard, a preliminary report by Sanyal *et al.* (31) indicated that BP metabolism was increased in first trimester placental tissue from smokers, compared with nonsmokers. A consistent observation in studies of term placental tissue from women who smoke has been the presence of induced AHH, which was also associated with the covalent binding of BP to DNA *in vitro* (32–34). Further investigations now indicate the presence of smoking- and BP-related DNA adducts in term human placenta *in vivo* (35, 36). Most recently, Pasanen *et al.* (37) reported the immunohistochemical detection of human placental cytochrome P-450-associated monooxygenase system

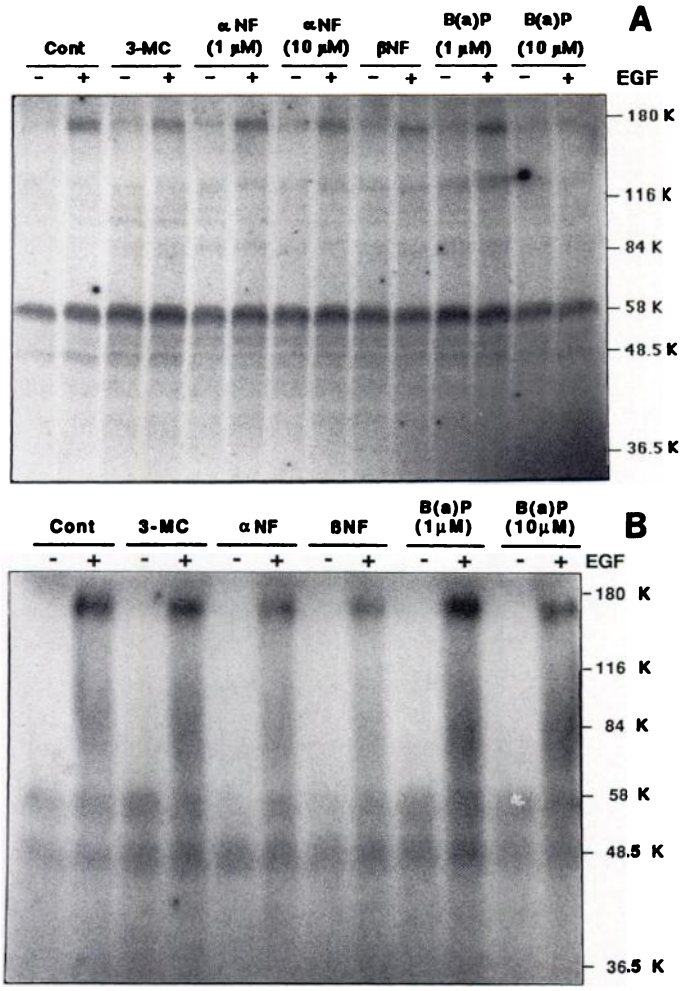

Fig. 5. Audioradiograms of SDS-polyacrylamide gels showing EGF-stimulated phosphorylation of proteins in solubilized preparations of cultured cells from early gestation (A) and term placentae (B). Cells were incubated with BP, α-NF, β-NF, or 3-MC for 24 hr at 37°. Phosphorylation assays were conducted with 60 μg of cell protein and [^{32}P]ATP in the presence of (+) or absence (−) of 1 μM EGF at 0° for 1 min. *Cont*, control.

in both syncytiotrophoblastic and cytotrophoblastic areas in term placentae from smokers. Our observation that substantial AHH induction occurs in cultured cells from early gestation placentae further emphasizes the potential for toxic exposure to environmental chemicals in the early feto-placental unit. The relationship of AHH induction to loss of EGF receptors, however, remains to be established. Karenlampi *et al.* (4) concluded that electrophilic metabolites of PAH, formed by P450IA1 induced during exposure of hepatoma cells to these chemicals, were important in the down-regulation of EGF receptors. In view of the potential association of P450IA1 and cytotoxicity (32–34), our study used several criteria to distinguish whether the effects of BP treatment on placental EGF receptors reflected nonspecific changes due to generalized toxicity. First, the effect of BP appeared to be selective for the binding of EGF compared with insulin, IGF-I, and IGF-II in early gestation placental cells. Second, no adverse effect of BP treatment was apparent on the incorporation of [^{35}S]methionine into proteins secreted by early gestation placental cells over 3 hr in serum-free medium. Third, our observation that down-regulation of EGF receptors with BP was specific for early gestation and not term placental cells suggests that the

TABLE 3

Effect of polycyclic compounds on AHH activity in cultured cells from human placenta

Primary cultures of cells from early gestation placentae were exposed for 24 hr to various concentrations of the respective compounds. After extensive washing, cells were pooled from five dishes in each treatment group and homogenized in 2 ml of buffer. AHH activity was measured in duplicate by fluorometric assay of phenolic metabolites using 3-hydroxy-BP as the reference standard. Blank values for BP-treated cells were zero incubation time and samples incubated without substrate for 30 min.

Treatment	Protein	AHH
	mg/ml	*pmol/mg of protein/min*
Control	2.10	0.03
BP		
1 μM	2.19	0.66
10 μM	1.99	4.92
3-MC		
1 μM	2.28	2.61
10 μM	2.20	3.36
β-NF		
1 μM	2.12	0.74
10 μM	2.19	3.38
α-NF		
1 μM	2.28	0.16
10 μM	1.98	0.05

effect of BP is not due to a general toxic effect of PAHs but rather is due to effects on the EGF receptor that are related to gestational age.

The physiological impact of EGF receptor down-regulation or desensitization on placental cell function remains to be determined. EGF receptors have been immunolocalized on syncytiotrophoblasts at all stages of gestation (12–14) and on selected cytotrophoblasts of first trimester human placenta (14). EGF stimulates human chorionic gonadotrophin and human placental lactogen secretion in both monolayer and explant cultures of early gestation and term placental tissue (11, 15). Under serum-free conditions, EGF effects on peptide hormone secretion were found to be correlated with differentiation of cytotrophoblasts to form syncytiotrophoblasts, rather than cell proliferation (16). In our study, the BP-related loss of EGF receptors in early gestation but not term placental cells may reflect differences in the ratio of cytotrophoblasts to syncytiotrophoblasts (11, 14), as well as the extent of differentiation of cytotrophoblasts to form syncytia during the *in vitro* culture period (38). Conversely, EGF has been reported to exert short term effects on glucose and amino acid uptake in cultured placental cells (17, 18). Our preliminary studies indicate that BP treatment stimulated aminoisobutyric acid uptake in both early and term cells and enhanced the EGF-stimulated amino acid uptake (19). In this regard, Horst and Sastry (39) have reported that amino acid uptake capacity was increased in human placental villi from smokers. The long-term physiological consequences of BP treatment on differentiation and nutrient transport are under investigation. In summary, the direct effects of BP and related polycyclic compounds on placental EGF receptors may indicate altered function of EGF in the regulation of placental growth of smoking mothers.

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