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# Smoking-Related Alterations in Epidermal Growth Factor and Insulin Receptors in Human Placenta

S.-L. WANG, G. W. LUCIER, R. B. EVERSON,[1] G. I. SUNAHARA,[2] and K. T. SHIVERICK

*Department of Pharmacology and Experimental Therapeutics, University of Florida, Gainesville, Florida 32610, (S.-L.W., K.T.S.) and Biochemical Risk Assessment (G.W.L., G.I.S.) and Epidemiology (R.B.E.) Branches, National Institutes of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

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**SUMMARY**

Studies characterized insulin and EGF receptors in human placental tissue from smokers and nonsmokers. Specific binding of $^{125}$I-labeled insulin and EGF to placental membranes was not different for nonsmokers compared with smokers. EGF and insulin receptor kinases were further studied using a wheat germ agglutinin-purified preparation of solubilized placental membrane proteins. In extracts from the nonsmoker group, EGF stimulated the active phosphorylation of $M_r$ 170,000 and 140,000 protein bands, which was half-maximal ($EC_{50}$) at $5 \times 10^{-8}$ M. In extracts from the smokers group, however, phosphorylation of these two protein bands was barely detectable over a range of 0 to $10^{-6}$ M EGF. Thus, EGF-stimulated phosphorylation of the 170,000 and 140,000 bands was markedly decreased in placental membranes from smokers. In contrast, insulin stimulated the phosphorylation of a 95,000 protein that was immunoprecipitated with anti-insulin receptor antiserum in membrane preparations from both nonsmokers and smokers. Dose-response curves for autophosphorylation indicate that $EC_{50}$ values were 2.6 and 7.0 nM insulin for nonsmokers and smokers, respectively. Laser densitometry scan of the 95,000 band on autoradiograms further showed that maximal $^{32}$P incorporation was 30% greater in smokers compared with nonsmokers. Analysis of the insulin-dependent phosphorylation of an exogenous substrate, poly(Glu,Tyr) (4:1), showed a similar pattern of values for nonsmokers versus smokers. These results indicate that insulin receptor autophosphorylation and tyrosine kinase activity were normal or increased, whereas EGF-stimulated kinase activity was markedly decreased in placental membrane proteins from smokers. Western blot analysis using an antiserum to the EGF receptor showed the presence of immunoreactive bands of 126,000 and 150,000–170,000 in receptor preparations from nonsmokers, whereas only the 126,000 protein was detected in preparations from smokers. Thus, the smoking-related deficiency in EGF receptor autophosphorylation appeared to be due to the absence of a 150,000–170,000 receptor protein. In conclusion, maternal cigarette smoking is associated with selective alterations in two major receptor-mediated pathways thought to be involved in cell growth and differentiation in human placenta.

Maternal cigarette smoking during pregnancy has been associated with fetal growth retardation (1), altered placental morphology (2), and increased risk of premature delivery and spontaneous abortion (3). The mechanism of these developmental effects associated with human cigarette smoking is still unclear. Insulin and EGF have been reported to play an important role in coordinating cell growth and division, as well as in regulation of fetal development during late gestation (4–6). Cell surface receptors for insulin and EGF are integral membrane glycoproteins that share protein sequence homology as members of the src family of tyrosine specific protein kinases (7). Human placenta is a rich source of insulin and EGF receptors (8) and several studies have examined the relationship between exposure to environmental chemicals and biochemical alteration of these receptors (9, 10). In a study of women who were exposed to PCB-contaminated rice oil, Sunahara *et al.* (9) found that placental membranes showed a decrease in EGF-stimulated protein tyrosine kinase activity in the absence of any change in ligand binding. Placental EGF receptor phosphorylation levels were significantly correlated with PCB concentrations and low birth weight. In another study, maternal cigarette smoking was associated with altered high affinity sites for EGF binding to human placental membranes (10). The present study further investigated the effects of maternal cigarette smoking

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[1] Present address: Epidemiology Branch, Health Effects Research Laboratory, US EPA, Research Triangle Park, NC.

[2] Present address: Nestle Research Center, BP353, Vevey Ch-1800, Switzerland.

**ABBREVIATIONS:** EGF, epidermal growth factor; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; poly(Glu$_4$,Tyr), copolymer of glutamate-tyrosine, 4:1; PMSF, phenylmethyl sulfonylfluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PCB, polychlorinated biphenyl; WGA, wheat germ agglutinin; BSA, bovine serum albumin.

on insulin and EGF receptors in human placenta. Insulin and EGF receptor protein kinase activities were compared using WGA-purified preparations of solubilized placental membranes from smokers and nonsmokers.

## Materials and Methods

**Chemicals.** BSA, fraction V, was purchased from United States Biochemical Co. (Cleveland, OH). Porcine insulin, PMSF, aprotinin, leupeptin, adenosine 5′-triphosphate, cytidine 5′-triphosphate, poly-(Glu$_4$,Tyr),*N*-acetyl-D-glucosamine, and SDS-PAGE protein standard kit were from Sigma Chemical Co. (St. Louis, MO). Chemicals for SDS-PAGE and Bio-Rad Immun-Blot assay kit were purchased from Bio-Rad (Hempstead, NY). Agarose-bound wheat germ agglutinin was obtained from Vector Laboratories, Inc. (Burlingame, CA). $^{125}$I-Human insulin labeled by tyrosine B-26 (specific activity, 2000 Ci/mmol), $^{125}$I-EGF (specific activity, 610 Ci/mmole), and [$\gamma$-$^{32}$P]ATP (specific activity, 3000 Ci/mmol) were purchased from Amersham Co. (Arlington Heights, IL). Anti-insulin receptor antiserum ($\alpha$-IR-1) was kindly supplied by Dr. S. Jacobs, Burroughs Wellcome Corp., Research Triangle Park, NC. EGF receptor antiserum (A310) was a generous gift from Dr. G. Carpenter, Vanderbilt University (Nashville, TN). Mouse EGF was purified from submaxillary glands as described by Savage and Cohen (11).

**Human subjects.** Term placentas were obtained from healthy volunteer women aged 18 to 44 at the University of North Carolina Memorial Hospital. Placentas were frozen at −70° within 30–60 min after delivery. Smoking histories were obtained from all subjects and the data gathered included numbers of cigarettes consumed and hours per week of exposure to environmental tobacco smoke (under circumstances that the subject could see and smell the smoke) during each trimester of pregnancy. Questionnaires indicated that there was no recognized chemical exposure other than smoking. Blood samples obtained during the seventh month of pregnancy were assayed for cotinine levels by methods described by Haley *et al.* (12).

**Placental membrane preparation.** Placental membranes were prepared by a modification of the method of Hock and Hollenberg (13). Tissue from individual placentas (50 g) was thawed in ice-cold buffer (0.25 M sucrose with 25 mM Tris·HCl buffer, pH 7.4, containing 1 mM PMSF, 100 KU/ml aprotinin, and 1 $\mu$g/ml leupeptin) for 1 hr before membrane preparation. The minced tissue was repetitively rinsed and homogenized in the same buffer (1:1.5, w/v). Homogenization was carried out, in an ice-chilled beaker, with a Ultra-Turrax homogenizer for 30 sec. The homogenate was centrifuged at 600 × *g* for 15 min, and the supernatant was filtered through double-layered gauze to remove debris before recentrifugation at 10,000 × *g* for 30 min. The resulting supernatant was adjusted to 0.1 M NaCl and 0.2 mM $MgSO_4$ and centrifuged at 40,000 × *g* for 40 min. The pellet was washed with 50 mM Tris·HCl buffer, pH 7.6, containing 1 mM PMSF, 100 KU/ml aprotonin, and 1 $\mu$g/ml leupeptin. The final pellet was resuspended in the same buffer and stored at −70° in 0.2-ml aliquots until analysis. Protein concentration of microsomal membrane preparation was measured by the method of Lowry *et al.* (14) with BSA as a standard.

**$^{125}$I-Insulin binding assay.** $^{125}$I-Insulin binding assay was conducted under equilibrium conditions as described elsewhere (15). Briefly, microsomal membranes (250 $\mu$g of protein in 50 $\mu$l) were incubated in triplicate with $^{125}$I-insulin (40,000 cpm, 0.05 nM), and 0.1 M sodium phosphate buffer, pH 7.5, containing 1.0% BSA (w/v) (buffer A) in a final reaction volume of 200 $\mu$l, for 30 min at 25°, for the measurement of total binding. Nonspecific binding was determined in the presence of 2.5 $\mu$M unlabeled porcine insulin. The reaction was terminated by addition of 1.0 ml of ice-cold buffer A. Membrane-bound $^{125}$I-insulin was separated immediately by centrifugation at 10,000 × *g* for 3 min in an Eppendorf microcentrifuge, followed by aspiration of the supernatant; the pellet was washed with another 1.0 ml of ice-cold buffer A and recentrifuged for 1 min and the supernatant was aspirated. The resultant pellets were counted in a Beckman Gamma 5500 counter. Specific binding was obtained by subtracting the radioactivity of nonspecific binding from total binding and is expressed as a ratio of the specific binding versus unbound $^{125}$I-insulin (bound/free or *B*/*F*).

**$^{125}$I-EGF binding assay.** $^{125}$I-EGF binding assay was conducted under equilibrium conditions by a modified method of Rao *et al.* (16). Microsomal membranes (250 $\mu$g of protein in 50 $\mu$l) were incubated with $^{125}$I-EGF (40,000 cpm, 0.18 nM), and 5 mM Tris·HCl, pH 7.0, containing 125 mM sucrose, 75 mM NaCl, 0.5 mM $Ca^{2+}$, and 0.5% BSA (w/v), in a final reaction volume of 200 $\mu$l, for 30 min at 25°, for the measurement of total binding. Nonspecific binding was determined in the presence of 1.0 $\mu$M unlabeled EGF from mouse submaxillary gland. The reaction was terminated by addition of 1.0 ml of ice-cold 10 mM Tris·HCl, pH 7.0, containing 0.5% BSA. Membrane-bound $^{125}$I-EGF was separated immediately as described in the $^{125}$I-insulin binding. Specific binding is obtained by subtracting the radioactivity of nonspecific binding from total binding and is expressed as a ratio of the specific binding versus unbound $^{125}$I-EGF.

**WGA chromatography.** Glycoprotein receptors were purified from solubilized placental membranes by WGA chromatography (17). Individual membrane preparations from placentas of smokers and nonsmokers were pooled and solubilized in 50 mM HEPES, 10 mM $MgSO_4$, 1 mM PMSF, 1% Triton X-100, and 10% glycerol, pH 7.6, at 4°. It was necessary to pool smoker and nonsmoker membrane preparations to obtain sufficient protein for lectin affinity chromatography purification. The solubilized proteins (20–30 mg) were recycled three times over a column containing 3.5 ml of agarose-bound WGA at 4°. The column was washed with 40 ml of washing buffer (50 mM HEPES, 150 mM NaCl, and 0.1% Triton X-100, pH 7.6) and the receptors were eluted from the column with 10 ml of the same buffer containing 0.3 M *N*-acetyl-D-glucosamine and collected, 1.0 ml per fraction. The eluates were monitored for protein concentration by UV spectrometry at 280 nm. Three eluates with highest absorbance at 280 nm were combined and protein concentration was determined using the Bio-Rad microassay (manual of Bio-Rad Protein Kit). $^{125}$I-Insulin binding to the solubilized and lectin-purified placental membrane was conducted as described by Hedo *et al.* (17).

**Insulin and EGF receptor phosphorylation.** Insulin receptor phosphorylation was performed by a modification of the method of Lowe *et al.* (18). Lectin-purified receptor preparations were preincubated in the presence of 0 to 1.0 $\mu$M porcine insulin in a final volume of 75 $\mu$l of 50 mM HEPES buffer, pH 7.6, with 10 mM $MgSO_4$ and 1 mM PMSF. After 30 min at 25°, phosphorylation was initiated by the addition of 25 $\mu$l of reaction mixture to give final concentrations of 5 $\mu$M [$^{32}$P]ATP (specific activity 20 $\mu$Ci/nmol), 1 mM cytidine-5-triphosphate, 3 mM $MnCl_2$, 20 mM $MgCl_2$, and 1 mM sodium vanadate ([$^{32}$P]ATP mixture). After 10 min at 25°, 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. In one experiment, anti-insulin receptor antiserum $\alpha$-IR-1 was added to a final titer of 1:90, and immunoprecipitation was carried out as described previously (18). Aliquots (100 $\mu$l) 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 Instruments, Fullerton, CA). EGF-stimulated autophosphorylation was performed under the same condition as insulin-stimulated autophosphorylation except 0 to 1 $\mu$M of EGF was added and, after the preincubation, the phosphorylation was conducted at 0° for 1 min.

**Artificial substrate phosphorylation.** The experiments were performed using a modified method of Rees-Jones *et al.* (19). Lectin-purified receptor preparations (12 $\mu$g) were preincubated in the presence of 0 to 1.0 $\mu$M porcine insulin in a final volume of 100 $\mu$l of 50 mM HEPES buffer, pH 7.6, with 10 mM $MgSO_4$, 1 mM PMSF, and 0.2 mg of poly(Glu$_4$,Tyr). After 30 min at 25°, phosphorylation was initiated by the addition of 20 $\mu$l of [$\gamma$-$^{32}$P]ATP mixture. After 10 min at 25°, the reaction was terminated by spotting 35 $\mu$l of the reaction mixture on 3 MM Whatman filter paper; samples were run in triplicate. The

filter paper was then extensively washed with 10% trichloroacetic acid, containing 10 mM sodium pyrophosphate, followed by 95% ethanol and ether. The filter paper was dried and counted. The counts of $^{32}P$ incorporated in the absence of porcine insulin was subtracted from counts in the presence of porcine insulin to obtain the specific phosphorylation activity. One unit of kinase activity was defined as the incorporation of 1 fmol of phosphate into 1 μg of poly($Glu_4$,Tyr).

**Immunoblot analysis.** EGF receptor protein was examined by SDS-PAGE and immunostaining procedures. Lectin-purified receptor preparations were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose paper, according to the methods of Laemmli (20) and Towbin *et al.* (21), respectively. The blotted nitrocellulose sheet was washed in 20 mM Tris, containing 500 mM NaCl, pH 7.5 (TBS), on a rocker platform for 10 min followed by 3% gelatin for 25 min. The nitrocellulose paper was further washed twice with TBS containing 0.05% Tween-20 (TTBS) and incubated with rabbit EGF receptor antiserum (A310) at 1:200 dilution in TBS for 2 hr at room temperature. The blotted nitrocellulose paper was washed twice with TTBS and then incubated with goat-anti-rabbit IgG-horseradish peroxidase conjugate for 1 hr. Immunoreactive proteins were visualized by incubation for 15–45 min with 3-amino-9-ethylcarbazole in the presence of 0.015% hydrogen peroxide.

## Results

**Clinical Findings.** Six women who reported not smoking cigarettes during their pregnancy and having minimal exposure to environmental tobacco smoke (averaging less than 8 hr per week) are included in this report as nonsmokers. The smoker group included five women who reported smoking an average of 20 cigarettes per day during each trimester of pregnancy and one woman who reported relatively heavy exposure to environmental tobacco smoke (32 hr per week). Although data are incomplete, serum cotinine values among six nonsmokers ranged from 0 to 2 ng/ml and for three smokers from 51 to 314 ng/ml. These values are in agreement with reported levels of cotinine for smokers and nonsmokers (10, 22).

**Insulin and EGF Binding.** The specific binding of $^{125}I$-insulin and $^{125}I$-EGF to membranes from human placenta was dependent on time and protein concentration. Nonspecific binding in all experiments was less than 10% of the total binding. Radioligand degradation was determined in each experiment to correct total cpm in the reaction mixture. Data in Table 1 show the specific binding of $^{125}I$-insulin and $^{125}I$-EGF to 250 μg of membrane protein prepared from individual placentas of nonsmokers and smokers. There was no significant difference in specific binding of $^{125}I$-insulin and $^{125}I$-EGF to the placental membranes between nonsmokers and smokers.

In subsequent experiments, membrane preparations from smokers and nonsmokers were pooled, solubilized, and partially purified by WGA affinity chromatography. Table 2 presents data on the purification and recovery of $^{125}I$-insulin binding after lectin chromatography. Insulin binding was purified 7-fold with 36 and 48% recoveries from solubilized placental membrane preparations from smokers and nonsmokers, respectively. As has been reported by other studies (8, 23), it was not possible to obtain a direct measurement of EGF binding in solubilized preparations. Although the detergent sensitivity of the binding assay for EGF receptors in human placenta has been well described, recovery of EGF- and insulin-stimulated kinase activities in the lectin preparation is evidence that the EGF receptor glycoprotein is co-purified with insulin receptors.

TABLE 1

**Specific binding of $^{125}I$-insulin and $^{125}I$-EGF to placental membranes from smokers and nonsmokers**

Total binding was measured with $^{125}I$-labeled insulin (0.05 nM) or EGF (0.18 nM) in the presence of 250 μg membrane protein. Nonspecific binding was measured in the presence of 2.5 μM insulin or 1.0 μM EGF. Data are expressed as mean ± standard error of six placentas from smokers and nonsmokers.

| | Insulin | EGF |
|---|---|---|
| | % *B/F* | |
| Nonsmoker (6) | 10.8 ± 2.0 | 31.0 ± 4.0 |
| Smoker (6) | 8.9 ± 0.9 | 31.8 ± 5.0 |

TABLE 2

**Purification of placental receptors from nonsmokers and smokers**

Total binding was measured with 0.05 nM $^{125}I$-insulin using 40 μg of solubilized or 12 μg of WGA-purified membrane protein. Nonspecific binding was measured in the presence of 2.5 μM insulin. Protein-bound ligand was precipitated by polyethylene glycol and counted. All samples were run in triplicate. EGF binding was not detectable (ND) in detergent-solubilized membrane preparations.

| | Total protein | $^{125}I$-Insulin bound | $^{125}I$-EGF bound | Recovery |
|---|---|---|---|---|
| | *mg* | *fmol/mg of protein* | | % |
| Triton X-100 extracts | | | | |
| Nonsmoker | 17 | 4.3 | ND | 100 |
| Smoker | 11 | 4.4 | ND | 100 |
| WGA chromatography | | | | |
| Nonsmoker | 0.9 | 30 | ND | 36 |
| Smoker | 0.9 | 26 | ND | 48 |

**Insulin- and EGF-stimulated kinase activities.** EGF receptor kinase activity was assayed at 0° in the presence of 0 to $10^{-6}$ M EGF using 12 μg of lectin-purified membrane glycoprotein. In extracts from nonsmokers, EGF stimulated the active phosphorylation of $M_r$ 170,000 and 140,000 protein bands (Fig. 1). Laser densitometry scans of the autoradiogram show that EGF-stimulated autophosphorylation was half-maximal ($EC_{50}$) at $5 \times 10^{-8}$ M. In extracts from smokers, however, $^{32}P$-incorporation into these two protein bands was barely detectable even at 1 μM EGF. Similar patterns of autophosphorylation were observed in three separate experiments using different samples of placental tissue from smokers and nonsmokers. Thus, EGF-stimulated phosphorylation of the 170,000 and 140,000 bands was markedly decreased in placental membranes from smokers.

Parallel experiments characterized insulin receptor kinase activity at 25°. Fig. 2 demonstrates that insulin stimulated the phosphorylation of a 95,000 protein in preparations from both nonsmokers and smokers. In data not shown, the 95,000 protein was immunoprecipitated with an antiserum to the insulin receptor. Dose-response curves for autophosphorylation indicate that $EC_{50}$ values were 2.6 and 7.0 nM insulin for nonsmokers and smokers, respectively. Laser densitometry scan of the 95,000 band on autoradiograms further showed that maximal $^{32}P$ incorporation was 30% greater in smokers compared with nonsmokers. Fig. 2 also demonstrates the phosphorylation of several protein bands with molecular weights of 170,000, 140,000, 120,000, and 66,000, which was not stimulated by insulin. $^{32}P$-incorporation into 170,000 and 140,000 proteins at 25° appears to reflect basal phosphorylation of the EGF receptor, which was markedly decreased in the receptor preparation from smokers. In contrast, basal phosphorylation of the 120,000 and 66,000 bands was comparable for receptor preparation from smokers and nonsmokers. Similar results were obtained in three separate experiments using different samples of placental tissue from smokers and nonsmokers.

Further experiments investigated the insulin-dependent

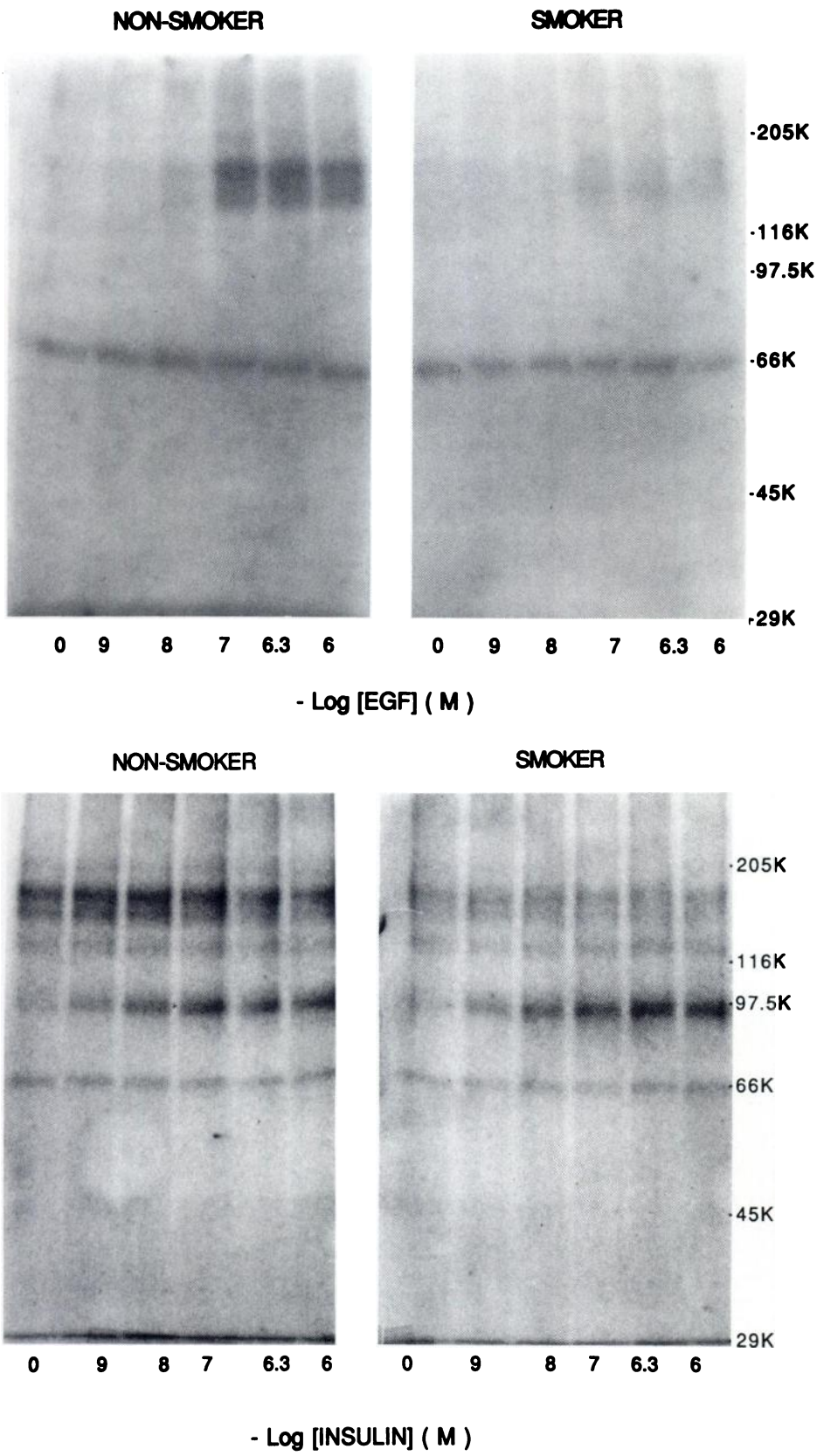


**Fig. 1.** Autoradiograms of SDS-polyacrylamide gels show EGF-stimulated phosphorylation of proteins in solubilized, lectin-purified preparations of placental membranes (12 μg of protein) from nonsmokers and smokers. Phosphorylation assays were conducted in the presence of 0 to 1.0 μM of EGF at 0° for 1 min. The placental membrane protein in each group was pooled from six placentas.

**Fig. 2.** Autoradiograms of SDS-polyacrylamide gels show insulin-stimulated phosphorylation of proteins in solubilized, lectin-purified preparations of placental membranes (12 μg of protein) from nonsmokers and smokers. Phosphorylation assays were conducted in the presence of 0 to 0.1 μM of insulin at 25° for 10 min. The placental membrane protein in each group was pooled from six placentas.

phosphorylation of an exogenous substrate, poly($Glu_4$,Tyr). Fig. 3 shows that insulin-stimulated tyrosine kinase activity was similar for preparations from nonsmokers versus smokers, with $EC_{50}$ values ranging from 9.5 to 19 nM insulin, and maximal $^{32}P$ incorporation of 146 ± 18 and 192 ± 16 fmol/μg of (Glu,Tyr)/10 min, respectively.

**Immunoblot analysis.** Subsequent experiments investigated whether the smoking-related deficiency in EGF receptor

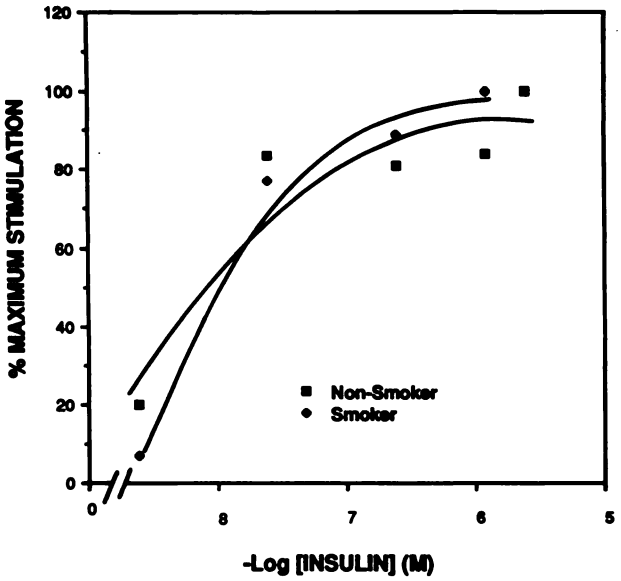


**Fig. 3.** The kinase activity of solubilized, lectin-purified membrane proteins was assayed in the presence of poly(Glu$_4$,Tyr) substrate. The incorporation of $^{32}$P was measured as that stimulated by 0 to 1 $\mu$M insulin in the presence of 3.5 $\mu$g purified proteins during a 10-min incubation. Per cent maximum stimulation was calculated for each point by division of the highest values. Values represent the mean of triplicate samples.

kinase activity was due to inactive receptors or, alternatively, the absence of EGF receptor protein in lectin-purified preparations. Placental membrane glycoproteins were analyzed by SDS-PAGE and immunoblot techniques using an antiserum (A-310) prepared against denatured human EGF receptor protein (24). Data in Fig. 4A show that two immunoreactive bands of comparable intensity were detected in preparations from nonsmokers, a broad 150,000–170,000 band as well as a 126,000 protein. In contrast, receptor preparations from smokers showed only a single major 126,000 immunoreactive protein with very intense staining. Insofar as immunostaining in the 150,000–170,000 region was extremely faint, these data provide evidence that native EGF receptor was not detectable in lectin-purified membrane glycoproteins prepared from placentas of smokers.

A final study reexamined crude membrane preparations to determine whether increased levels of the 126,000 immunoreactive species of the EGF receptor were detectable in placentas from smokers. Immunoblot data in Fig. 4B show that two proteins of 116,000 and 126,000 were readily detectable in crude membrane preparations from smokers, but not from nonsmokers. It warrants note that it was not possible to detect 170,000 receptor in crude membranes using immunoblot analysis due to limitations on the amount of protein that could be applied to polyacrylamide gels, as well as the sensitivity of the antibody. In data not shown, both 116,000 and 126,000 immunoreactive proteins were readily solubilized from crude membranes with Triton X-100, whereas only the 126,000 species bound to the WGA affinity column. These results provide evidence that the presence of lower molecular weight immunoreactive proteins in placental preparations from smokers was not the result of EGF receptor degradation during solubilization and lectin purification.

## Discussion

A number of growth factor receptors with intrinsic protein tyrosine kinase activity have been implicated in the transmembrane signaling processes essential for DNA replication and cell proliferation (7, 25). Lucier *et al.* (10) have reported that placental membranes from smokers showed an alteration in binding properties of the high affinity site of the EGF receptor, as well as a decrease in EGF-stimulated autophosphorylation activity. In the present study, insulin and EGF receptors were solubilized from human placental membranes and partially purified by lectin chromatography to yield a preparation of membrane glycoproteins that was substantially enriched in both insulin and EGF-stimulated kinase activities. $EC_{50}$ values for autophosphorylation of insulin and EGF receptors in preparations from nonsmokers were in agreement with values reported for human placenta (8). In contrast, EGF receptor autophosphorylation was minimally detectable in membrane glycoprotein preparations from smokers, despite the presence of normal or increased insulin kinase activities. Insofar as the binding of EGF, as well as insulin, to crude placental membranes was not different for smokers compared with nonsmokers, data indicate that maternal smoking was associated with a selective loss of EGF receptor autophosphorylation. Although a dissociation between EGF receptor binding and receptor autophosphorylation has also been observed in human placentas from women exposed to PCBs (9), it is not known whether insulin receptor function was altered. In the present study, immunochemical evidence further shows that the smoking-related deficiency in EGF receptor kinase activity in puri-

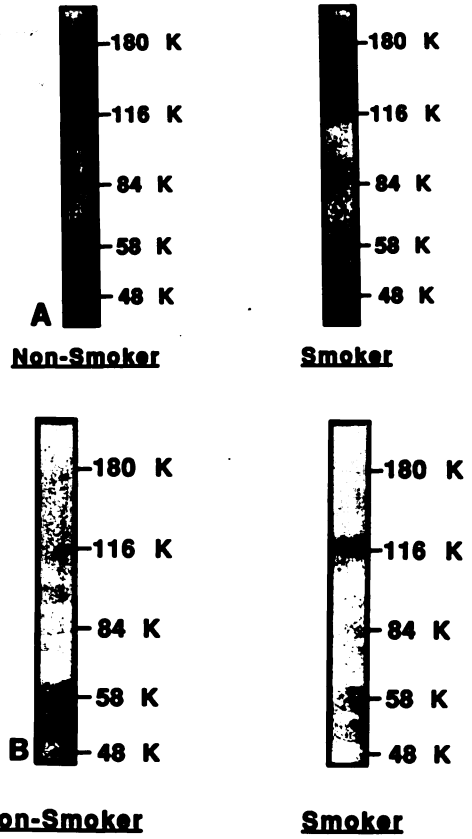


**Fig. 4.** A, Immunoblot of lectin-purified EGF receptor protein prepared from placentas of nonsmokers and smokers. Samples (30 $\mu$g of protein) were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunostained using rabbit anti-EGF receptor antiserum (1:200) as described in Materials and Methods. B, Immunoblot of crude membrane preparations (100 $\mu$g of protein) prepared from placentas of nonsmokers and smokers.

fied receptor preparation was due to the absence of native 170,000 protein. In this regard, the nature and source of the 116,000 and 126,000 species of immunoreactive protein detected in membrane preparations from smokers remain to be determined.

Cigarette smoke contains a complex mixture of chemicals including polyaromatic hydrocarbons, carbon monoxide, cyanide, and nicotine. Studies of placental tissue from women who smoke have shown the induction of aryl hydrocarbon hydroxylase activity, which was associated with the covalent binding of polyaromatic hydrocarbons to DNA *in vitro* (26–28); Everson *et al.* (22) recently reported the presence of smoking-related DNA adducts in human placenta *in vivo*. In the present study, the differential loss of EGF receptor autophosphorylation in the presence of normal insulin receptor kinase activity would argue against generalized effects of smoking on membrane structure or on membrane-associated kinases and phosphatases. The selective effect on EGF receptors provides further evidence against nonspecific changes due to decreased placental oxygenation and hypoxia caused by carbon monoxide, cyanide, or nicotine.

Although one or more of the chemicals in cigarette smoke may alter EGF receptor function via phosphorylation (29), the present study presents evidence that cigarette smoke may influence the activity of processes that convert native EGF receptor protein into 116,000 and 126,000 species that are devoid of kinase activity (30). Although our study used a combination of protease inhibitors in all stages of membrane purification, the phosphorylation of a doublet of 170,000 and 140,000 proteins in purified receptor preparations from nonsmokers is evidence for the presence of an endogenous neutral protease (31). It warrants emphasis, however, that we found no evidence of degradation of the insulin receptor $\beta$-subunit undergoing autophosphorylation in receptor preparations from smokers (32). Data from our immunological studies would be consistent with an interpretation that cigarette smoking is associated with a further shift to the predominance of lower molecular weight species of placental EGF receptors. We do not yet know, however, whether smoking alters the intracellular processing of placental EGF receptors *in vivo* (30, 33) or, alternatively, the activity of proteolytic enzymes in placental preparations (31). Evidence that 116,000 and 126,000 immunoreactive species of EGF receptor protein are readily detectable in crude membrane preparations from placentas of smokers argues against increased formation through degradation of the receptor during solubilization and lectin purification procedures. In any event, the processes altered by cigarette smoke appear to be specific for the EGF receptor, compared with the insulin receptor.

The physiological impact of inactive EGF receptors on placental growth and function remains to be determined. Recent studies with mutated EGF receptors provide evidence that tyrosine kinase activity is essential for both the immediate and late actions of EGF, including alterations in intracellular calcium, activation of gene transcription and cell proliferation, and receptor down-regulation (34). Immunochemical studies of human placenta have localized EGF receptors on the syncytiotrophoblast layer in placental villi (35, 36) and in smooth muscle cells of umbilical blood vessels (36). The high level of EGF receptors in human placenta during early and late gestation has been suggested to play an important physiological role in hormone secretion (35, 37), as well as in nutrient transport (37). A recent study by Hofmann *et al.* (38) reported that women delivering growth-retarded infants had significantly lower urinary EGF concentrations. Placentas from smokers have been found to have morphologic signs of trophoblast basement membrane thickening and damage to placental and umbilical vasculature (2, 3). Levels of urinary estrogen and serum placental lactogen were decreased in smokers (39), as is placental amino acid uptake (40). Thus, smoking-related changes in EGF receptors may underlie a number of the biochemical and morphological alterations observed in placentas from women who smoke. A final consideration is that smoking may be associated with EGF receptor changes in fetal tissues, as well as in the placenta. EGF has been shown to promote eyelid opening, tooth eruption, and development of the palate, skin, lung, and gut in various species (5, 6). Thus, research into the biochemical mechanisms that underlie the effects of smoking on placental EGF receptors will provide a better understanding of the susceptibility of the developing fetus to environmental chemicals.

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**References**

1. Pirani, B. B. K. Smoking during pregnancy. *Obstet. Gynecol. Surv.* **33**:1–13 (1978).
2. Asmusen, I. Ultrastructure of the villi and fetal capillaries in placentas from smoking and nonsmoking mothers. *Br. J. Obstet. Gynecol.* **87**:239–246 (1980).
3. Naeye, R. L. Abruptio placentae and placenta previa: frequency, perinatal mortality and cigarette smoking. *Obstet. Gynecol.* **55**:701–704 (1980).
4. Milner, R. D. G., and J. Hill. Fetal growth control: the role of insulin and related peptides. *Clin. Endocrinol.* **21**:415–433 (1984).
5. Cohen, S., and C. R. Savage, Jr. Recent studies on the chemistry and biology of epidermal growth factor, part II. *Recent Prog. Horm. Res.* **30**:551–574 (1974).
6. Tsutsumi, O., and T. Oka. Epidermal growth factor deficiency during pregnancy causes abortion in mice. *Am. J. Obstet. Gynecol.* **156**:241–244 (1987).
7. Ullrich, A., J. R. Bell, E. Y. Chen, R. Herrera, L. M. Petruzzelli, T. J. Dull, A. Gray, L. Coussens, Y. C. Liao, M. Tsubokawa, A. Mason, P. H. Seeburg, C. Grunfeld, O. M. Rosen, and J. Ramachandran. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature (Lond.)* **313**:756–761 (1985).
8. Pike, L. J., E. A. Kuenzel, J. E. Casnelli, and E. G. Krebs. A comparison of the insulin- and epidermal growth factor-stimulated protein kinases from human placenta. *J. Biol. Chem.* **259**:9913–9921 (1984).
9. Sunahara, G. I., K. G. Nelson, T. K. Wong, and G. W. Lucier. Decreased human birth weights after *in utero* exposure to PCBs and PCDFs are associated with decreased placental EGF-stimulated receptor autophosphorylation capacity. *Mol. Pharmacol.* **32**:572–578 (1987).
10. Lucier, G. W., K. G. Nelson, R. B. Everson, T. K. Wong, R. M. Philpot, T. Tiernan, M. Taylor, and G. I. Sunahara. Placental markers of human exposure to polychlorinated biphenyls and polychlorinated dibenzofurans. *Environ. Health Perspect.* **76**:79–87 (1987).
11. Savage, C. R., Jr., and S. Cohen. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* **247**:7609–7621 (1972).
12. Haley, N. J., C. M. Axelrod, and K. A. Tilton. Validation of self-reported smoking behavior: biochemical analysis of cotinine and thiocyanate. *Am. J. Public Health* **73**:1204–1207 (1983).
13. Hock, R., and M. D. Hollenberg. Characterization of the receptor for epidermal growth factor-urogastrone in human placental membrane. *J. Biol. Chem.* **255**:10731–10736 (1980).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
15. Wang, S. L., M. K. Raizada, and K. T. Shiverick. Ontogeny of insulin receptors in the rat hemochorial placenta. *Dev. Biology* **123**:487–493 (1987).
16. Rao, C. V., F. R. Carman, Jr., N. Chegini, and G. S. Schultz. Binding sites for epidermal growth factor in human fetal membranes. *J. Clin. Endocrinol. Metab.* **58**:1034–1042 (1984).
17. Hedo, J. A., L. C. Harrison, and J. Roth. Binding of insulin receptors to lectins: evidence for common carbohydrate determinants on several membrane receptors. *Biochemistry* **20**:3385–3393 (1981).
18. Lowe, W. L., F. T. Boyd, D. W. Clarke, M. K. Raizada, C. Hart, and D. LeRoith. Development of brain insulin receptors: structural and functional studies of insulin receptors from whole brain and primary cell cultures. *Endocrinology* **119**:25–35 (1986).

19. Rees-Jones, R. W., S. A. Hendricks, M. Quarum, and J. Roth. The insulin receptor of rat brain is coupled to tyrosine kinase activity. *J. Biol. Chem.* **259**:3470–3474 (1984).
20. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. *Nature (Lond.)* **277**:680–685 (1970).
21. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
22. Everson, R. B., E. Randerath, R. M. Santella, R. C. Cefalo, T. A. Auitts, and K. Randerath. Detection of smoking related covalent DNA adducts in human placenta. *Science (Wash. D. C.)* **231**:54–57 (1986).
23. Nexo, E., R. A. Hock, and M. D. Hollenberg. Lectin-agarose immobilization, a new method for detecting soluble membrane receptors. *J. Biol. Chem.* **254**:8740–8743 (1979).
24. Stoscheck, C. M., and G. Carpenter. Characteristics of antibodies to the epidermal growth factor receptor-kinase. *Arch. Biochem. Biophys.* **227**:457–468 (1983).
25. Hunter, T., and J. A. Cooper. Protein tyrosine kinases. *Annu. Rev. Biochem.* **54**:897–930 (1985).
26. Berry, D. L., P. K. Zachariah, T. J. Slaga, and M. R. Juchau. Analysis of the biotransformation of benzo(*a*)pyrene in human fetal and placental tissues with high pressure liquid chromatography. *Eur. J. Cancer* **13**:667–675 (1977).
27. Vaught, J. B., H. L. Gurtoo, N. B. Parker, R. LeBoeuf, and G. Doctor. Effects of smoking on benzo(*a*)pyrene metabolism by human placental microsomes. *Cancer Res.* **39**:3177–3183 (1979).
28. Pelkonen, O., and H. Saarni. Unusual patterns of benzo(*a*)pyrene metabolism and DNA-benzo(*a*)pyrene adducts produced by human placental microsomes *in vitro*. *Chem. Biol. Interact.* **30**:287–296 (1980).
29. Iwashita, S., and C. F. Fox. Epidermal growth factors and potent phorbol tumor promoters induce epidermal growth factor receptor phosphorylation in a similar but distinctly different manner in human epidermoid carcinoma A431 cells. *J. Biol. Chem.* **259**:2559–2567 (1984).
30. Soderquist, A. M., and G. Carpenter. Biosynthesis and metabolic degradation of receptors for epidermal growth factor. *J. Membr. Biol.* **90**:97–105 (1986).
31. Cassel, D., and L. Glaser. Proteolytic cleavage of epidermal growth factor receptor: a $Ca^{2+}$-dependent, sulfhydryl-sensitive proteolytic system in A431 cells. *J. Biol. Chem.* **257**:9845–9848 (1982).
32. Lerea, K. M., and J. N. Livingston. A thiol sensitive degradative process of liver uncouples autophosphorylation of the insulin receptor from insulin binding. *Biochem. J.* **236**:535–542 (1986).
33. Lai, W. H., H. J. Guyda, and J. J. M. Bergeron. Binding and internalization of epidermal growth factor in human term placental cells in culture. *Endocrinology* **118**:413–423 (1986).
34. Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and M. G. Rosenfeld. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature (Lond.)* **328**:820–822 (1987).
35. Maruo, T., H. Matsuo, T. Oishi, M. Hayashi, R. Nishino, and M. Mochizuki. Induction of differentiated trophoblast function by epidermal growth factor: relation of immunohistochemically detected cellular epidermal growth factor receptor levels. *J. Clin. Endocrin. Metab.* **64**:744–750 (1987).
36. Magid, M., L. B. Nanney, C. M. Stoscheck, and K. E. King, Jr. Epidermal growth factor binding and receptor distribution in term human placenta. *Placenta* **6**:519–526 (1985).
37. Lai, W. H., and H. J. Guyda. Characterization and regulation of epidermal growth factor receptors in human placental cell cultures. *J. Clin. Endocrinol. Metab.* **58**:344–352 (1984).
38. Hofmann, G. E., Ch.V. Rao, M. J. Brown, L. F. Murray, G. S. Schultz, and T. A. Siddiqi. Epidermal growth factor in urine of nonpregnant women and pregnant women throughout pregnancy and at delivery. *J. Clin. Endocrin. Metab.* **66**:119–123 (1988).
39. Mochizuki, M., T. Maruo, T. Masuki, and T. Ohtsu. Effects of smoking on fetoplacental-maternal system during pregnancy. *Am. J. Obstet. Gynecol.* **149**:413–420 (1984).
40. Rowell, P. O. The effect of maternal cigarette smoking on the ability of human villi to concentrate $\alpha$-aminoisobutyric acid *in vitro*. *Res. Commun. Subst. Abuse* **2**:253–257 (1981).

**Send reprint requests to:** Dr. Shoou-Lih Wang, Pharmacology Department, Box J-267, JHM Health Center, University of Florida, Gainesville, FL 32610.