Developmental Effects of Chlorpyrifos Extend Beyond Neurotoxicity: Critical Periods for Immediate and Delayed-Onset Effects on Cardiac and Hepatic Cell Signaling

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The fetal and neonatal neurotoxicity of chlorpyrifos (CPF) and related insecticides is a major concern. Developmental effects of CPF involve mechanisms over and above cholinesterase inhibition, notably events in cell signaling that are shared by nonneural targets. In the present study, we evaluated the immediate and long-term effects of CPF exposure of rats during different developmental windows [gestational days (GD) 9–12 or 17–20, postnatal days (PN) 1–4 or 11–14] on the adenylyl cyclase (AC) signaling cascade in the heart and liver. In addition to basal AC activity, we assessed the responses to direct AC stimulants (forskolin, Mn^{2+}); to isoproterenol and glucagon, which activate signaling through specific membrane receptors; and to sodium fluoride, which activates the G-proteins that couple the receptors to AC. Few immediate effects on AC were apparent when CPF doses remained below the threshold for systemic toxicity. Nevertheless, CPF exposures on GD9–12, GD17–20, or PN1–4 elicited sex-selective effects that emerged by adulthood (PN60), whereas later exposure (PN11–14) elicited smaller, nonsignificant effects, indicative of closure of the window of vulnerability. Most of the effects were heterologous, involving signaling elements downstream from the receptors, and thus were shared by multiple inputs; superimposed on this basic pattern, there were also selective alterations in receptor-mediated responses. These results suggest that the developmental toxicity of CPF extends beyond the nervous system, to include cell signaling cascades that are vital to cardiac and hepatic homeostasis. Future work needs to address the potential implications of these effects for cardiovascular and metabolic disorders that may emerge long after the end of CPF exposure. ***Key words:*** **adenylyl cyclase, β-adrenoceptor, chlorpyrifos, critical developmental periods, heart development, liver development, organophosphate insecticides.** ***Environ Health Perspect*** **112:170–178 (2004). doi:10.1289/ehp.6690 available via** ***http://dx.doi.org/*** **[Online 3 November 2003]**

The potential for organophosphate insecticides, notably chlorpyrifos (CPF), to elicit developmental neurotoxicity has led to increasing concern and restricted use [Barone et al. 2000; Landrigan 2001; Landrigan et al. 1999; May 2000; Physicians for Social Responsibility 1995; Pope 1999; Rice and Barone 2000; Slotkin 1999, In press a; U.S. Environmental Protection Agency (EPA) 2000a, 2000b]. The systemic toxicity of organophosphates reflects their ability to inhibit cholinesterase, but it is now evident that other mechanisms are at least equally, if not more, important in determining the long-term liability of fetal or neonatal CPF exposure (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999, In press a). The cyclic AMP (cAMP) signaling pathway has received particular attention because this second messenger coordinates the critical transition from cell replication to cell differentiation in virtually all prokaryotic and eukaryotic cells (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Van Wijk et al. 1973). A number of studies in the developing brain have centered on the effects of CPF on the receptors and signaling proteins that control the activity of adenylyl cyclase (AC), the enzyme that synthesizes cAMP, as well as on the downstream elements that are targets for cAMP (Crumpton et al. 2000; Garcia et al. 2001; Meyer et al. 2003; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Zhang et al. 2002).

Recent attention has begun to explore the contributions of fetal factors and chemical exposures to the later emergence of cardiovascular and metabolic diseases. The "Barker Hypothesis" originally drew a connection between fetal growth retardation and the subsequent incidence of coronary artery disease and diabetes (Barker 2003; Phillips 2002), and there is also significant literature on the long-term consequences of prenatal stress and the role of glucocorticoid hormones (Dodic et al. 1999, 2001; Nyirenda and Seckl 1998). More recently, there are suggestions that environmental toxicants may play an important contributory role in such disorders as hypertension, diabetes, and obesity, beyond neural contributions (Power and Jefferis 2002; Slikker and Schwetz 2003; Toschke et al. 2002). Thus, although most studies on the developmental effects of CPF are appropriately directed toward neurotoxicity, the present work instead takes a similar approach with regard to cell signaling in the liver and heart, concentrating on the cAMP cascade and its responses to some of the major inputs that control that pathway, β-adrenoceptors (βARs), and glucagon receptors (Figure 1). CPF is concentrated in developing peripheral tissues, especially the liver (Hunter et al. 1998), and high doses can be hepatotoxic (Goel et al. 2000). Significant effects in the immature liver or heart are elicited at exposures below the threshold for systemic toxicity, and these include effects on signal transduction (Auman et al. 2000; Song et al. 1997) and cell number and size (Qiao et al. 2002). The present study addresses several essential questions: What is the critical period for effects of CPF on signal transduction in the liver and heart? Are CPF-induced signaling abnormalities present in adulthood? If so, do the adult effects represent persistence of alterations that were elicited during the initial CPF exposure, or alternatively, does CPF disrupt the programming of cell signaling so that defects arise only after a delay?

Materials and Methods

Animal treatments. All experiments using live animals were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996) as adopted and promulgated by the National Institutes of Health. Timed-pregnant Sprague–Dawley rats were housed in breeding cages, with a 12-hr light/dark cycle and with free access to food and water. CPF was dissolved in DMSO to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight; control animals received vehicle (DMSO) injections on the same schedules. For exposure on gestational days (GD) 9–12, dams were injected daily with CPF at 1, 2, or 5 mg/kg body weight, and for later gestational exposure (GD17–20), dams were given CPF daily over a range of 1–40 mg/kg; tissues were harvested

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on GD21. These doses span the threshold for inhibition of fetal brain cholinesterase activity, fetal growth impairment, or reduced maternal weight gain, all of which become evident at about 5 mg/kg (Garcia et al. 2003; Qiao et al. 2002). Additional dams were treated on GD9–12 or GD17–20 with 1 or 5 mg/kg for measurements in the offspring when they reached adulthood [postnatal day (PN) 60]. On the day of birth, all pups were randomized within their respective treatment groups and redistributed to the dams with a litter size of 10 to maintain a standard nutritional status. Randomization was repeated at intervals of several days; in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Offspring were weaned on PN21.

For studies of CPF effects in the first few days after birth, animals were subcutaneously injected with 1 mg/kg daily on PN1–4; for studies in older animals, which tolerate higher doses (Campbell et al. 1997; Pope and Chakraborti 1992; Pope et al. 1991; Whitney et al. 1995), animals were treated daily with 5 mg/kg on PN11–14. The same randomization procedure was followed. Neither regimen evokes weight loss or mortality (Campbell et al. 1997; Dam et al. 1998; Johnson et al. 1998; Song et al. 1997), and in the present study we did not observe any changes in suckling or maternal caretaking. Animals were selected 24 hr and 6 days after the last CPF injection, and at PN60.

These prenatal and postnatal CPF regimens have been previously shown to alter brain development without eliciting overt systemic toxicity (Slotkin 1999, In press a, In press b). Behavioral differences remain apparent, or may first emerge, after weaning (Dam et al. 2000; Levin et al. 2001, 2002).

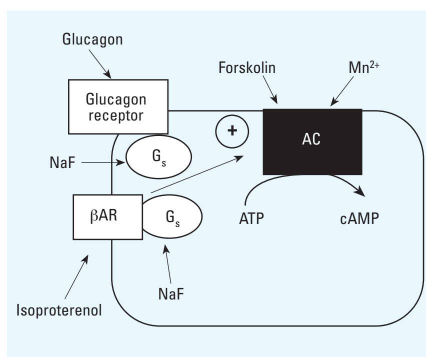

Figure 1. Mechanisms controlling AC activity in developing rat cardiac and hepatic cells. Both βARs and glucagon receptors enhance AC activity through the stimulatory G-protein, G_s. Each step in the pathway can be probed with the appropriate stimulant: isoproterenol for βARs, glucagon for the glucagon receptor, NaF for the G-proteins, and forskolin and Mn^{2+} for AC itself. The attachment of G_s to AC (+) enhances the response to forskolin while suppressing the response to Mn^{2+}; in addition, different AC isoforms show preferential responses to forskolin versus Mn^{2+}. When alterations involve signaling elements downstream from the receptors (G_s, AC), effects will be shared by multiple receptors and will therefore be "heterologous."

For each experiment, animals were decapitated and the tissues were frozen with liquid nitrogen and stored at –45°C. Preliminary experiments showed no changes in any of the measures as a result of freezing and storage.

Membrane preparation. Tissues were thawed and homogenized (Polytron; Brinkmann Instruments, Westbury, NY) in 39 volumes of ice-cold buffer containing 145 mM sodium chloride, 2 mM magnesium chloride, and 20 mM Tris (pH 7.5), strained through several layers of cheesecloth to remove connective tissue, and the homogenates were sedimented at 40,000 × *g* for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer followed by resedimentation and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in a buffer consisting of 250 mM sucrose, 2 mM $MgCl_2$, and 50 mM Tris.

Assays. To evaluate βAR binding, aliquots of membrane preparation were incubated with [^{125}I]iodopindolol (final concentration, 67 pM) in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM sodium ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 µL. Incubations were stopped by dilution with 3 mL ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters, which were then washed with additional buffer and counted by liquid scintillation spectrometry. Nonspecific binding was assessed by displacement with 100 µM isoproterenol.

For assessment of AC activity, aliquots of the same membrane preparation were incubated for 30 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and a creatine phosphokinase–ATP–regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/mL phosphocreatine kinase, with 10 µM GTP in a total volume of 250 µL. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3,000 × *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal, and, in particular, higher concentrations of GTP produced no further augmentation of activity.

AC activity was evaluated in several ways (Figure 1). First, we measured basal AC activity without addition of any stimulants. Next, we compared the responses to activation of two stimulatory G-protein (G_s)-coupled receptors, the βAR, and glucagon receptor, using 100 µM isoproterenol or 3 µM glucagon, respectively. Third, we evaluated the effects of global activation of all G-proteins elicited by sodium fluoride (10 mM). Finally, we compared the responses of two direct AC stimulants, forskolin (100 µM) and Mn^{2+} (10 mM); these discriminate the effects of G_s–AC association, which selectively enhances the forskolin response (Limbird and Macmillan 1981), as well as allowing for detection of shifts in the AC isoform (Zeiders et al. 1999b). The concentrations of each stimulant produce maximal responses, as assessed in earlier studies (Auman et al. 2000, 2001; Zeiders et al. 1997, 1999a).

Data analysis. For treatments given to dams, only one fetus was used from each dam, so the number of determinations represents the number of dams. The fetuses were derived from the same litters as those used in two earlier studies on cell damage and cholinergic biomarkers (Garcia et al. 2002; Qiao et al. 2002), and effects on cholinesterase activity, maternal and fetal body weights, and other litter characteristics appear in those publications. For postnatal determinations, each litter contributed no more than one male and one female for a given set of determinations.

Data are presented as means and SEs. For convenience, some results are given as the percent change from control values, but statistical evaluations were always conducted on the original data. To establish treatment differences in receptor binding or AC activity, a global analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted across the *in vivo* treatment groups, ages, sexes, tissues, and the measurements made on the membranes (βAR binding, AC activity under six different conditions); the latter were considered to be repeated measures because each membrane preparation was used for the multiple conditions under which AC was determined. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC measures that differed from control values; these were conducted by lower-order ANOVAs, followed, where appropriate, by Fisher's protected least significant difference test to identify individual values for which the CPF groups differed from the corresponding control. For all tests, significance for main treatment effects was assumed at $p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran 1967).

For presentation, control values were combined across the multiple cohorts (controls used for administration on GD9–12,

GD17–20, PN1–4, PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Materials. Animals were purchased from Zivic Laboratories (Pittsburgh, PA, USA). CPF was obtained from Chem Service (West Chester, PA, USA). [^{125}I]Iodopindolol (specific activity, 2,200 Ci/mmol) was from Perkin-Elmer Life Sciences (Boston, MA, USA), and cAMP radioimmunoassay kits were purchased from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were bought from Sigma Chemical Company (St. Louis, MO, USA).

Results

Development of βAR binding and AC in controls. In accordance with earlier results (Auman et al. 2000; McMillian et al. 1983; Navarro et al. 1991), large ontogenetic changes were observed in the development of βAR binding and AC responses to different stimulants in heart and liver over the course from late gestation to adulthood (Table 1). Cardiac βARs decreased about 50% over that period, whereas the liver showed a much more profound decrease that was paralleled by a loss of basal AC activity. The tissues also displayed large disparities in AC activities measured in the presence of specific stimulants. In the heart, activity with addition of isoproterenol, glucagon, NaF, and forskolin all increased with age, but for Mn^{2+}, the effect was diminished in adulthood. In the liver, only the forskolin-stimulated activity was higher in adulthood than in the fetus, and all other measures showed a decrease in AC. There were only minor sex differences in adulthood, with females showing slightly higher hepatic βAR binding and slightly higher isoproterenol-stimulated cardiac AC.

Because the stimulant-associated AC activities were superimposed on large ontogenetic changes in basal AC, the values were reassessed as the specific response, the ratio of AC with stimulant to basal AC. Despite the ontogenetic decline in βAR binding, the cardiac AC response to isoproterenol doubled between GD21 and adulthood, paralleled by a similar rise in the response to glucagon (Figure 2A). In contrast, the hepatic AC response to isoproterenol declined significantly in adulthood, whereas the response to glucagon was markedly higher than in the fetus. Disparities between the two tissues were similarly displayed by the response to direct AC stimulants (Figure 2B). In the heart on GD21, the response to forskolin was nearly double that to Mn^{2+}, and the forskolin response increased with age, whereas that to Mn^{2+} did not. Accordingly, the forskolin:Mn^{2+} preference ratio doubled over the course of development. In the liver, the initial response to Mn^{2+} was greater than that to forskolin; although both stimulants showed an increased response in adulthood, the preferential effect for Mn^{2+} was lost, such that nearly equivalent responses were seen for the two stimulants. Accordingly, both tissues showed ontogenetic changes in the forskolin:Mn^{2+} preference ratio suggestive of an isoform shift (Zeiders et al. 1999b).

Systemic toxicity of CPF. In keeping with earlier reports (Garcia et al. 2002; Qiao et al. 2002), CPF treatment on GD9–12 impaired maternal weight with a threshold at 5 mg/kg, displaying small, transient deficits that resolved completely by parturition (data not shown). Similarly, as found before (Garcia et al. 2002; Qiao et al. 2002), the GD17–20 CPF regimen had a similar threshold, with progressively larger deficits as the dose was raised to 10, 20, or 40 mg/kg (data not shown). With either regimen, GD21 fetal heart weights remained normal at all CPF doses, but liver weights were reduced in animals receiving doses above 5 mg/kg; the effects on tissue weights have already been reported (Garcia et al. 2002; Qiao et al. 2002). Neither regimen affected the number of fetuses or fetal viability, nor were there effects on the number of pups at term or on neonatal viability. As found earlier, the postnatal treatment regimens had no significant effects on body, heart, or liver weights 24 hr or 6 days after the end of CPF treatment (Auman et al. 2000; Song et al. 1997).

In adulthood, animals receiving prenatal or postnatal CPF regimens did not display any consistent differences in body weights (data not shown), in agreement with earlier reports (Dam et al. 1999; Qiao et al. 2003); the group exposed to CPF on GD9–12 showed a small (< 10%) reduction in body weight at 1 mg/kg ($p < 0.006$) but not at 5 mg/kg. Heart weights were similarly unaffected by all treatments except the GD9–12 regimen, which displayed the same inconsistent effect, a 7% reduction at 1 mg/kg ($p < 0.006$) without any significant difference at 5 mg/kg. Comparisons of liver weights were not done because only one lobe was sampled on PN60.

CPF exposure on GD9–12. In the heart, animals treated with CPF on GD9–12 showed immediate (GD21) and long-term (PN60)

Table 1. Development of βAR binding and AC activities in controls.

	GD21 (n = 16)	PN60 Male (n = 28)	PN60 Female (n = 28)
Heart			
βAR binding (fmol/mg protein)	19.4 ± 0.8	10.4 ± 0.3*	10.4 ± 0.2*
Basal AC	12.2 ± 0.4	9.1 ± 0.3*	9.6 ± 0.3*
Isoproterenol-stimulated AC	16.2 ± 0.5	25 ± 1*	29 ± 1*,**
Glucagon-stimulated AC	12.6 ± 0.4	23.8 ± 0.9*	26 ± 1*
NaF-stimulated AC	42 ± 2	64 ± 2*	69 ± 3*
Forskolin-stimulated AC	235 ± 12	304 ± 13*	300 ± 9*
Mn^{2+}-stimulated AC	135 ± 7	100 ± 5*	103 ± 4*
Liver			
βAR binding (fmol/mg protein)	27.7 ± 0.7	4.9 ± 0.1*	6.5 ± 0.2*,**
Basal AC	15.1 ± 0.4	3.52 ± 0.07*	3.35 ± 0.06*
Isoproterenol-stimulated AC	27 ± 1	4.7 ± 0.1*	4.91 ± 0.09*
Glucagon-stimulated AC	30 ± 2	27.1 ± 0.4*	26.2 ± 0.5*
NaF-stimulated AC	46 ± 1	28.0 ± 0.6*	27.5 ± 0.5*
Forskolin-stimulated AC	41 ± 3	49 ± 2*	45 ± 1*
Mn^{2+}-stimulated AC	63 ± 2	50 ± 1*	49 ± 1*

Values shown are in picomoles per minute per milligram of protein except where noted. Values were combined across multiple cohorts (controls used for CPF administration on GD9–12, GD17–20, PN1–4, and PN11–14); however, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.
*Significantly different from GD21. **Significantly different from male.

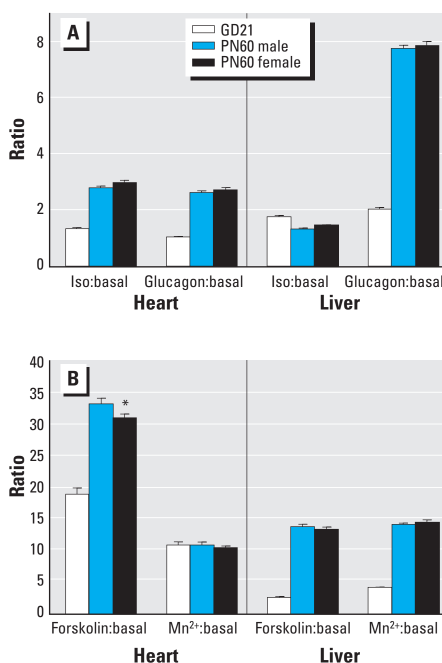

Figure 2. Development of responses to AC stimulants in control animals. (*A*) Responses to receptor-mediated stimulation by isoproterenol (Iso) or glucagon. (*B*) Responses to direct AC stimulants forskolin and Mn^{2+}. Data represent means and SEs of the ratios of each stimulant to basal AC activity, obtained from 16–28 animals in each group. With the exception of cardiac Mn^{2+} responses, ANOVA indicates a significant change with age ($p < 0.0001$).
*The only response for which a sex difference was seen on PN60.

alterations in βAR binding and AC responses. On GD21, there was significant up-regulation of cardiac βARs at the two lowest CPF doses, but the effect disappeared when the dose was raised to 5 mg/kg, above the threshold for systemic toxicity (Figure 3A). Consistent with this pattern, AC activities also showed augmentation at subtoxic CPF exposures and loss of the effect at 5 mg/kg. This hormetic effect was statistically significant across all AC measures ($p < 0.02$ for CPF 5 mg/kg vs. 1 mg/kg; $p < 0.01$ for CPF 5 mg/kg vs. CPF 2 mg/kg). For AC activities, the most profound effect was on the response to Mn^{2+}, which showed elevations as high as 60% above control values, effects that far exceeded the smaller induction of the other AC measures. In particular, there was a statistically significant ($p < 0.005$) preferential effect on the Mn^{2+} response compared with the forskolin response, suggestive of an AC isoform shift. A smaller but significant ($p < 0.03$) shift was seen in the ratio of NaF to basal activity, indicative of a defect in G-protein signal transduction: control, 3.27 ± 0.21; CPF 1 mg/kg, 2.70 ± 0.09 ($p < 0.005$); CPF 2 mg/kg, 2.76 ± 0.05 ($p < 0.02$); CPF 5 mg/kg, 2.93 ± 0.11.

In contrast to the effects seen in the heart, the immediate (GD21) alterations in hepatic βARs and AC were much less notable, and neither set of variables achieved overall statistical significance (Figure 3B). The lack of significant effect in the liver was statistically distinguishable from the changes seen in the heart, as evidenced by interactions of treatment × tissue ($p < 0.0001$) and treatment × tissue × measure ($p < 0.0001$).

At adulthood (PN60), animals exposed to CPF on GD9–12 showed major alterations in βARs and AC signaling profiles. In the heart (Figure 4A), animals that received the low dose of CPF (1 mg/kg) displayed overall increases in AC measures that were sex selective, with significant elevations in males but not in females. There were no differential effects detected among the various AC measures, implying that activity was enhanced globally (i.e., induction of total AC activity), rather than involving specific changes in receptor-mediated responses, G-protein coupling, or AC isoform shifts. To evaluate whether these effects were secondary to systemic toxicity of CPF, we also evaluated a higher dose (5 mg/kg) that was at the threshold for impairment of maternal weight gain during the treatment period; presumably, effects due to toxicity would show an enhancement at the higher dose. Instead, however, we found the same pattern but with smaller effects: AC elevations in males were no longer statistically significant, and values in females tended to be subnormal.

Across both the heart and liver on PN60, the main effect of CPF was statistically significant ($p < 0.04$), but when hepatic values were

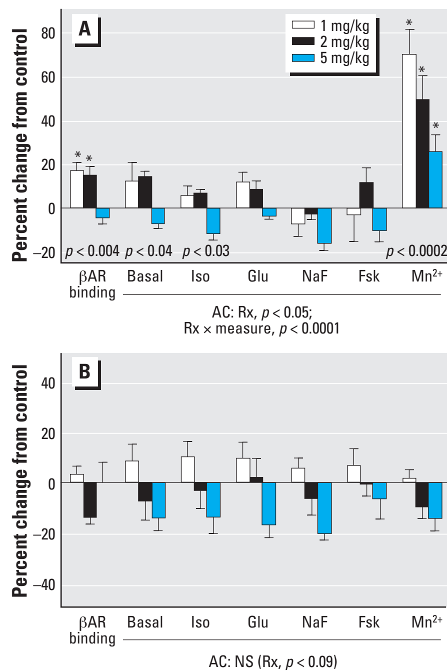

Figure 3. Effects of GD9–12 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on GD21. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from six animals in each treatment group, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant treatment effects that differed between the two tissues, justifying separate comparisons for heart and liver: βAR binding, $p < 0.004$ for treatment × tissue; AC activities, $p < 0.0001$ for treatment × tissue, $p < 0.0001$ for treatment × tissue × measure. Lower-order ANOVAs for each tissue are shown within the figure.

*Individual groups differ significantly from the control (calculated only for variables showing a significant overall effect by ANOVA).

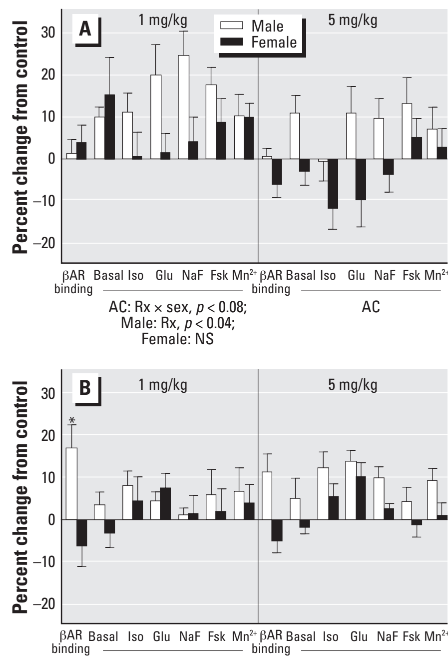

Figure 4. Effects of GD9–12 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from eight animals in each treatment group for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant sex-dependent effects on βAR binding ($p < 0.04$ for treatment × sex). For AC activities, the overall ANOVA showed treatment effects that differed between the two tissues and among the different AC measures ($p < 0.04$ for treatment × tissue × measure), necessitating separate comparisons for heart and liver. ANOVAs across both CPF doses are as follows: for (*A*), Rx × measure, $p < 0.1$; Rx × sex × measure, $p < 0.02$; for (*B*), NS ($p < 0.09$). ANOVAs for each dose are shown within the figure. Significance testing for individual AC values was not done because of the absence of treatment × measure interactions after subdivision of the data into the separate tissues and doses.

*Individual group differs significantly from control.

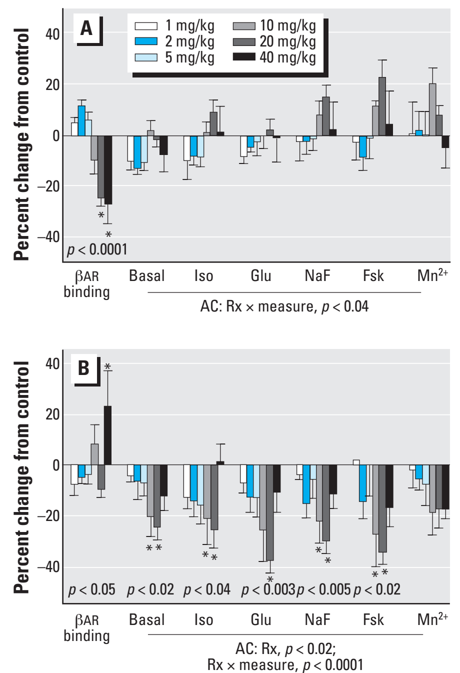

Figure 5. Effects of GD17–20 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on GD21. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; Rx, treatment. Data represent means and SEs obtained from six animals in each treatment group, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant treatment effects that differed between the two tissues, justifying separate comparisons for heart and liver: βAR binding, $p < 0.0001$ for treatment × tissue; AC activities, $p < 0.004$ for treatment × tissue, $p < 0.02$ for treatment × tissue × measure. Lower-order ANOVAs for each tissue are shown within the figure.

*Individual groups differ significantly (calculated only for variables showing a significant overall effect by ANOVA).

examined separately, the tendency toward elevated AC activities was only at the margin of significance (Figure 4B). Hepatic βAR binding was significantly elevated only in males.

CPF exposure on GD17–20. Animals treated with CPF during late gestation (GD17–20) presented a different pattern of immediate and delayed-onset alterations in βAR binding and AC activity from those receiving treatment earlier in gestation. For the immediate effects on GD21, the response patterns for animals receiving CPF on GD17–20 were statistically distinguishable from those treated on GD9–12 ($p < 0.05$ for treatment × regimen). In the heart (Figure 5A), GD17–20 exposure at CPF doses below (1 or 2 mg/kg) and up to the threshold (5 mg/kg) for systemic toxicity failed to cause significant alterations in βAR binding or any of the AC measures on GD21. Raising the dose further elicited significant decrements in cardiac βAR binding but still failed to cause any consistent changes in AC. In the liver, CPF exposures of 1, 2, or 5 mg/kg similarly did not alter any of the parameters, but higher doses suppressed virtually all AC measures in a parallel manner (Figure 5B).

For examination of effects in adulthood (PN60), we again chose two dose groups, one (1 mg/kg) well below the threshold for fetal cholinesterase inhibition or impaired maternal weight gain (Qiao et al. 2002) and one (5 mg/kg) at or above the threshold. Animals exposed to 1 mg/kg CPF on GD17–20 displayed sex-dependent effects on cardiac βARs and AC activities in adulthood (Figure 6A). Males showed elevated βAR binding, although this effect did not correspond to an elevation in the cardiac AC response to isoproterenol. Instead, most of the AC measures found to be increased were in females, with the notable exception of glucagon-stimulated AC, which was unchanged. Because both βARs and glucagon receptors operate through G_s to stimulate AC, we compared the effect on the isoproterenol response with that on the glucagon response and found a significant reduction ($p < 0.006$) in the relative response to glucagon (glucagon:isoproterenol response ratio, 0.97 ± 0.04 in control females, 0.82 ± 0.03 in females exposed to CPF 1 mg/kg). Again, raising the CPF exposure above the threshold for systemic effects did not produce significant enhancement of the long-term effects on cardiac βARs or AC activity and instead diminished the βAR effect in males; in addition, the higher dose elicited significant reductions in cardiac AC measures that were not seen at the lower exposure.

In the liver on PN60 (Figure 6B), animals exposed to 1 mg/kg CPF on GD17–20 displayed significant suppression of glucagon- and forskolin-stimulated AC activity, without demonstrable sex selectivity. Raising the dose above the threshold for systemic toxicity (5 mg/kg) did not intensify the effects but rather reduced them.

CPF exposure on PN1–4. In an earlier study (Song et al. 1997), we found that exposure to 1 mg/kg CPF on PN1–4 evoked short-term (PN5, PN10) deficits in basal cardiac AC activity without significant effects on the response to AC stimulants; there were no effects on βAR binding. Evaluation of these animals in adulthood (PN60) indicated no significant overall effect (Figure 7A), although AC values tended to be decreased by the same magnitude (10%) as that obtained for the immediate posttreatment effects.

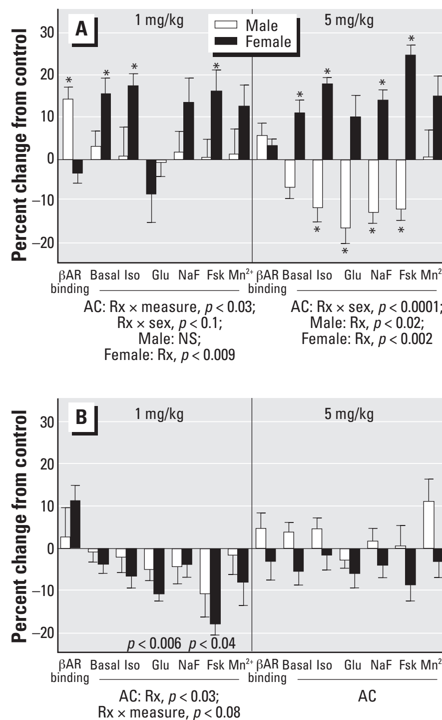

Figure 6. Effects of GD17–20 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from eight animals in each treatment group for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant sex- and tissue-dependent effects on βAR binding ($p < 0.05$ for treatment × sex × tissue). For AC activities, the overall ANOVA showed treatment effects that differed between the two tissues, between sexes, and among the different AC measures ($p < 0.02$ for treatment × tissue; $p < 0.007$ for treatment × measure; $p < 0.006$ for treatment × sex × measure), necessitating separate comparisons for heart and liver. ANOVAs across both CPF doses are as follows: for (*A*), Rx × sex, $p < 0.003$; Rx × measure, $p < 0.05$; male: NS; female: Rx, $p < 0.005$; for (*B*), Rx × measure, $p < 0.08$. ANOVAs for each dose are shown within the figure.
*Individual groups differ significantly from the control (calculated only for variables showing significant treatment × measure and treatment × sex interactions after separation by dose; otherwise, only the main effects are shown.

As reported previously (Auman et al. 2000), CPF treatment on PN1–4 produced transient elevations in hepatic AC responses to glucagon and Mn^{2+} that disappeared by PN10. In the present study, we also assessed short-term effects on hepatic βAR binding, none of which was statistically significant: PN5—control

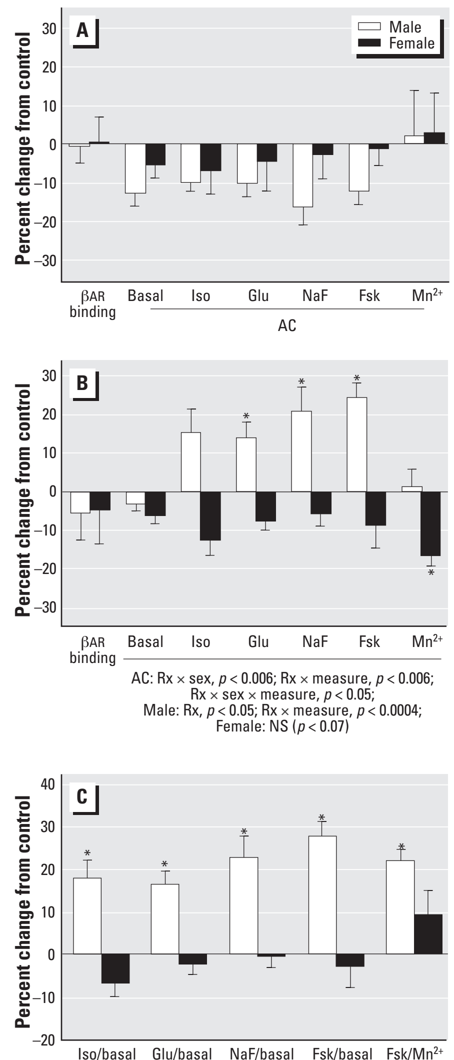

Figure 7. Effects of PN1–4 CPF exposure (1 mg/kg) on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60, and (*C*) activity ratios for liver values. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from six animals for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates no significant effects on βAR binding. For AC activities, the overall ANOVA showed treatment effects that differed between the two tissues, between sexes, and among the different AC measures ($p < 0.04$ for treatment × sex × tissue; $p < 0.04$ for treatment × tissue × measure), necessitating separate comparisons for heart and liver. Lower-order ANOVAs are shown within the figure.
*Individual groups differ significantly from the control (calculated only for variables showing significant treatment × measure and treatment × sex interactions).

male, 16.9 ± 0.5 fmol/mg protein (n = 5); control female, 14.5 ± 0.6 (n = 4); CPF male, 15.8 ± 1.1 (n = 4); CPF female, 14.7 ± 0.5 (5); PN10—14.5 ± 0.8 (n = 6), 12.5 ± 0.6 (n = 4), 13.2 ± 0.7 (n = 7), and 12.3 ± 0.8 (n = 7), respectively. When these animals reached adulthood, we still did not detect alterations in βAR binding, but there were major, sex-dependent effects on AC activities (Figure 7B). Basal AC activity was unaffected, but males showed elevations of AC responses to stimulants with the notable exception of Mn^{2+}. In females, AC responses to stimulants were not augmented and tended instead to be reduced. Because of the strong, differential effect on AC responses to specific stimulants, we characterized the pattern by calculation of response ratios (Figure 7C). CPF exposure on PN1–4 elicited specific activation of the responses to stimulants operating through G-proteins (isoproterenol, glucagon, NaF) and preferentially enhanced the response to forskolin as opposed to Mn^{2+}.

We also performed an additional experiment to identify whether the delayed-onset effects on hepatic AC signaling appeared earlier (Figure 8). By PN30, the elevations of AC stimulant responses had not yet appeared, and in fact, activities were significantly lower than in controls, again with a preferential effect toward males.

CPF exposure on PN11–14. As evaluated earlier (Song et al. 1997), animals exposed to 5 mg/kg CPF on PN11–14 showed reductions in cardiac βAR binding and AC responses to βAR stimulation on PN20. In the liver, the short-term effects consisted of transient elevations in AC responses to glucagon and Mn^{2+} that disappeared by PN20 (Auman et al. 2000). We did not find significant effects on hepatic βAR binding on PN15 or PN20: PN15—control male, 9.3 ± 0.5 fmol/mg protein (n = 6); control female, 9.0 ± 1.7 (n = 6); CPF male, 9.8 ± 0.6 (n = 6); CPF female, 6.9 ± 0.8 (n = 6); PN20—4.4 ± 0.5 (n = 6), 4.5 ± 0.3 (n = 6), 4.2 ± 0.4 (n = 6), and 4.0 ± 0.5 (n = 6), respectively.

In adulthood, we found the same magnitude and direction of effect on cardiac AC as was seen previously for short-term (PN20) evaluations (Song et al. 1997), although without achieving statistical significance in the present study (Figure 9A). A similar pattern was seen in the liver (Figure 9B), and ANOVA combined across the two tissues indicated a significant overall AC reduction (p < 0.05) despite a lack of significance for either tissue considered separately.

Discussion

Results of this study indicate that developmental CPF exposure elicits immediate and late-emerging alterations in AC-mediated cell signaling in the heart and liver, extending the adverse effects of CPF beyond neurotoxicity. Although CPF at high concentrations can interact directly with neurotransmitter receptors and/or AC (Huff and Abou-Donia 1995; Huff et al. 1994, 2001; Ward and Mundy 1996), our results indicate that such direct actions are unlikely to explain the net effects on the development of AC signaling after *in vivo* exposure: we found distinct sex-, tissue-, and age-selective actions that are incompatible with a unitary, stoichiometric interaction between CPF and the signaling proteins. Indeed, the fact that many of the effects emerged after a prolonged delay (i.e., between PN30 and PN60) indicates instead that CPF exposure alters the programming of the development of cell signaling. In light of the disparate effects of the different CPF treatment windows, it is worthwhile to examine the characteristics that are shared by, or that differentiate among, the various treatment paradigms before arriving at general conclusions.

Although the major effects of CPF emerged in adulthood, some acute effects were detected in the period immediately after exposure. With treatment on GD9–12, there was a shift in the cardiac AC isoform, evidenced by a preferential increase in the response to Mn^{2+} as opposed to forskolin (Zeiders et al. 1999b). For the GD17–20 treatment window, adverse effects on hepatic signaling profiles emerged at CPF doses above the threshold for systemic toxicity. Similarly, our earlier work detailed short-term effects of postnatal CPF treatments on AC (Auman et al. 2000; Song et al. 1997). However, none of these effects was sustained, nor are they likely to explain the long-term changes identified later. For example, there were no immediate effects for the GD17–20 regimen at the subtoxic doses that nevertheless produced robust effects on signaling in adulthood; similar discrepancies between acute and long-term effects have already been noted in the presentation of each set of results. In one case similar outcomes were obtained over both the immediate and the long-term time frames, namely, the cardiac effects of CPF exposure on PN11–14, but even in this situation the magnitude of impairment was small and nonsignificant. In general, then, the acute effects of CPF on AC signaling were poorly correlated with the subsequent emergence of major, sex-dependent, tissue-selective functional alterations,

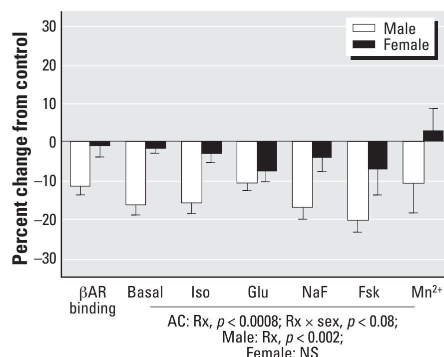

Figure 8. Effects of PN1–4 CPF exposure (1 mg/kg) on hepatic βAR binding and AC activity on PN30. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from six animals for each sex, shown as the percent change from corresponding control values for males and females, respectively: βAR binding, 5.7 ± 0.2 and 6.0 ± 0.3 fmol/mg protein; basal AC, 3.3 ± 0.1 and 2.8 ± 0.1 pmol/min/mg protein; isoproterenol-stimulated AC, 5.1 ± 0.2 and 4.7 ± 0.2 pmol/min/mg protein; glucagon-stimulated AC, 27 ± 1 and 25 ± 2 pmol/min/mg protein; NaF-stimulated AC, 33 ± 1 and 30 ± 1 pmol/min/mg protein; forskolin-stimulated AC, 65 ± 1 and 53 ± 2 pmol/min/mg protein; Mn^{2+}-stimulated AC, 51 ± 2 and 41 ± 1 pmol/min/mg protein. ANOVA indicates no significant CPF effects on βAR binding. For AC activities, the overall ANOVA (shown within the figure) displayed treatment effects that differed between the two sexes but not among the different AC measures, so lower-order tests were not done for the individual measures. Main treatment effects for each sex are also shown.

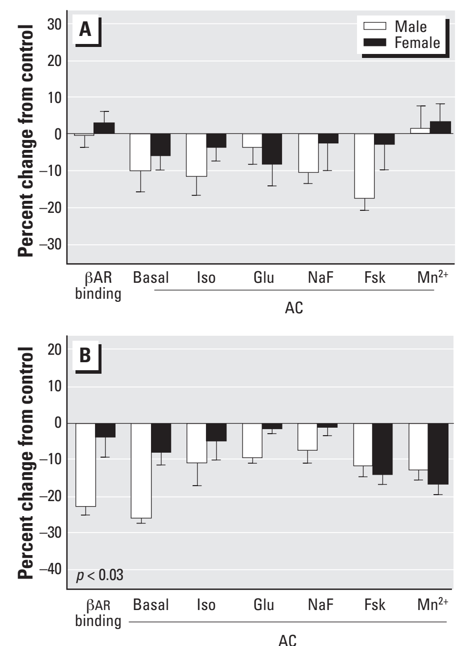

Figure 9. Effects of PN11–14 CPF exposure (5 mg/kg) on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; Rx, treatment. Data represent means and SEs obtained from six animals for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates effects on βAR binding that differed between tissues and sexes (p < 0.1 for treatment × sex; p < 0.03 for treatment × tissue), but the sex interaction was not maintained when the values were separated into the two tissues; accordingly, only the main effect is shown (*B*). For AC activities, the overall ANOVA showed only a main treatment effect (p < 0.05) without significant interactions of treatment with other variables, so separate tests or subtests were not done for the two tissues, although the values are shown separately.

implying that CPF affects the developmental program for cell signaling.

The second common feature for all the exposure models is the fact that many of the emergent effects on signaling were heterologous; that is, they involved ubiquitous changes in the function of signaling proteins themselves rather than in the specific responses to receptor stimulation. Thus, the effects on AC tended to appear in "clusters," with increases or decreases shared by different receptor stimulants (isoproterenol, glucagon), by a G-protein activator (NaF), or by AC stimulants that bypass receptors (forskolin) or receptors and G-proteins (Mn^{2+}). These heterologous changes thus indicate that CPF alters the ability of the entire signaling cascade to respond to the multiple neuronal and hormonal inputs that elicit cellular responses through AC. Nevertheless, superimposed on these heterologous effects, we did detect instances where CPF exposure affected a specific input to the transduction pathway. As one example, the effects on cardiac AC signaling after exposure to 1 mg/kg CPF on GD17–20 included specific impairment of the response to glucagon in adulthood, superimposed on heterologous activation of other AC responses. We also found evidence for AC isoform shifts, such as the alteration in the preference ratio for forskolin versus Mn^{2+} in the liver after PN1–4 CPF exposure; because the various AC isoforms have distinct catalytic properties and differential responses to second messengers, these too may provide a basis for more selective functional alterations despite a common origin in heterologous AC effects.

An additional feature common to all the exposure models is the general dissociation of effects on βAR binding from the AC response to the βAR stimulant isoproterenol. Thus, on GD21, the two prenatal CPF exposure regimens affected cardiac βARs but not the corresponding AC response; in the liver, the GD17–20 treatment group displayed no change or an increase in βARs on GD21, but a decrease in the isoproterenol AC response. A similar lack of parallelism was seen for the other treatment paradigms and for the delayed-onset alterations determined in adulthood. These results are consistent with the view that the signaling proteins downstream from the receptors are the major determinants of functional responses (Gao et al. 1998, 1999; Navarro et al. 1991), so evaluations of receptor binding alone may be entirely misleading for the interpretation of physiologic consequences.

Despite the fact that all the CPF exposure windows shared major features of their effects on cell signaling, there were distinct differences that depended on the period of exposure. Focusing on the long-term effects of low-dose CPF exposures, cardiac AC showed augmentation with the prenatal treatment windows, with a different critical period for effects on males (GD9–12) versus females (GD17–20). Smaller, nonsignificant inhibitory effects were seen with postnatal exposures; therefore, the critical window of sensitivity for CPF-induced perturbation of cardiac AC signaling must begin to close soon after birth. The liver displayed a different pattern, with sensitivity first emerging late in gestation, characterized by long-term inhibitory effects after GD17–20 exposure. With later treatment on PN1–4, effects were stimulatory and restricted to males, whereas again, a shift to even later treatment (PN11–14) elicited only small, nonsignificant decrements. As a general observation, then, the effects of CPF on the programming of AC cell signaling occur during a distinct developmental window of vulnerability that closes in the second postnatal week.

An additional point of interest is the sex dependence of the effects of CPF on cardiac and hepatic cell signaling, which shifts radically according to the developmental window in which exposure occurs. In the present study, males were preferentially affected by most treatment paradigms, with the notable exception of the selective effects on females seen with the GD17–20 exposure. Although we have no information about the mechanisms underlying sex-selective actions, it is notable that similar shifts have been seen for effects on behavior from these particular treatment windows (Dam et al. 2000; Levin et al. 2001, 2002). The late gestational period is associated with sexual differentiation of the brain (Rodier 1988), and it is tempting to attribute the unique pattern seen with exposure on GD17–20 to effects on sexual dimorphism of neural pathways. CPF is only weakly estrogenic (Andersen et al. 2002; Vinggaard et al. 2000), but certainly, secondary endocrine effects are feasible (Guven et al. 1999). However, the fact that sex-selective effects were seen even earlier, with GD9–12 exposure, makes it unlikely that all the differences can be explained by a primary action on sexual differentiation of the brain. Obviously, this is an issue of considerable importance for future investigation.

Finally, there are two elements of the delayed-onset emergence of abnormalities in AC signaling that need to be addressed. First, the alterations in receptor binding and AC biomarkers ranged up to about 20%; this is approximately the magnitude of the changes evoked by CPF in the developing brain (Aldridge et al. 2004; Meyer et al. 2003; Song et al. 1997), by other defined neurotoxicants such as nicotine (Slotkin et al. 1992), or by the loss of function associated with aging (Fraeyman et al. 2000; Kilts et al. 2002). Relatively small changes have important functional consequences because the AC pathway amplifies receptor signals by orders of magnitude: the activation of a single AC molecule leads to the production of numerous cAMP molecules and consequent activation of protein kinase A, which in turn elicits a plethora of downstream phosphorylation events (Freissmuth et al. 1989; Gilman 1989; Stiles 1989; Weiss et al. 1988). During development, these types of changes produce massive changes in cellular functions linked to cAMP despite modest changes in elements of the signaling cascade itself (Schuh et al. 2002; Song et al. 1997). Second, the dose–response relationships seen here are often complex. For example, although many of the acute effects of CPF show a typical, monotonic relationship to dose, we noted several instances where the effects did not progress beyond those seen at lower doses, and even cases where effects displayed hormesis, with larger alterations at low doses (e.g., Figures 3A, 4A). Indeed, a number of other studies indicate nonmonotonic effects of CPF at the biochemical and behavioral levels (Levin et al. 2001, 2002; Meyer et al. 2003; Qiao et al. 2002, 2003), and we recently found that this extends to AC signaling in the brain (Aldridge et al. 2003, 2004). One important distinction is whether a given dose lies above the threshold for cholinesterase inhibition, because, during fetal development, cholinergic input can lead to lasting improvements in neural performance (Meck and Williams 1997, 1999; Montoya et al. 2000). The doses used here bracket the threshold for fetal cholinesterase inhibition (Qiao et al. 2002). Given the clear involvement of neural input in the cardiac and hepatic effector mechanisms studied here, similar factors may then produce hormetic responses to AC in these peripheral tissues.

The results obtained here indicate that apparently subtoxic developmental exposures to CPF, even at levels that do not produce significant inhibition of cholinesterase (Qiao et al. 2002), alter cardiac and hepatic cellular function in adulthood. There are four important characteristics of these effects. First, the alterations involve heterologous changes in signaling components (G-proteins, AC itself) that are shared by multiple neuronal and hormonal stimulants. Accordingly, there will be global effects on the responses to diverse inputs. Second, these effects are sex selective, so future work with animal models or human populations will need to take sex differences into account. Third, the effects emerge late in development, thus requiring longitudinal evaluations. Finally, superimposed on the heterologous changes in AC signaling, we found specific alterations in the responses to glucagon and βAR stimulation, both of which play critical roles in cardiovascular and metabolic homeostasis, and with regard to the latter, specifically glucose homeostasis. The secretion of insulin, the counterbalancing hormone for glucagon, proceeds through activation of AC

(Gao et al. 2002), so the heterologous augmentation of AC signaling caused by CPF exposure is likely to amplify the physiologic effect of a superimposed deficiency in the glucagon response. It is now recognized that diseases that occupy a distinct cluster—hypertension, obesity, and diabetes—may have significant dependence on prenatal stress and/or toxicant exposures (Dodic et al. 1999, 2001; Nyirenda and Seckl 1998; Power and Jefferis 2002; Slikker and Schwetz 2003; Toschke et al. 2002). The present results point to the possibility that otherwise subtoxic, nonsymptomatic developmental exposure may provide predisposition to these types of diseases, with a specific component of delayed-onset effects. As such, the adverse adult outcomes predicted by the Barker Hypothesis may actually extend to exposures below the threshold for fetal growth impairment. Indeed, a number of the effects on signaling cascades seen here for CPF exposure have been examined in transgenic mice overexpressing the corresponding receptors or downstream transduction proteins, and clearly indicate the emergence of cardiac myopathies as well as hepatic dysfunction and damage (Andre et al. 1999; Asai et al. 1999; Du et al. 2000; Singh et al. 2001; Vatner et al. 1999). Nevertheless, given the multifactorial nature of hypertension and diabetes, it seems unlikely that the magnitude of the effects of developmental CPF exposure on the AC cascade alone would trigger these diseases, but instead might provide a risk factor that acts in concert with other comorbidities. Studies of CPF effects in animal models of hypertension, obesity, and diabetes are likely to resolve this issue, in concert with examination of human cohorts with significant fetal or childhood CPF exposures.

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