Embryonic Growth Inhibition Induced by Cocaine Is Associated with the Suppression of Ornithine Decarboxylase Activity (43565)

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Abstract. **Cocaine use during pregnancy results in significant increases in fetal morbidity and mortality. Multiple maternal and environmental variables influence the fetal response to cocaine, and growth suppression of the developing child is frequently associated with *in utero* cocaine exposure. Using intact chick embryos as well as cultured embryonic tissue as a model, we report that the growth suppression induced by cocaine exposure is correlated with molecular changes occurring directly in the embryonic cells and that these molecular changes appear to be distinct from other maternal, placental, or environmental effects of the drug, including anoxia. Specifically, embryonic cocaine exposure suppresses the normal developmental increase in ornithine decarboxylase (ODC) enzymatic activity. The loss of ODC activity during the early stages of development is dose dependent and is correlated with the degree of growth suppression. The cocaine-induced loss of decarboxylase activity is specific to ODC, but cocaine, per se, has no effect on ODC activity *in vitro*. Moreover, a single dose of exogenous putrescine given at 120 hr of incubation blocks the cocaine-induced growth suppression. In cultured embryonic tissue, cocaine exposure inhibits the ability of a known trophic factor (insulin) to induce growth and also blocks the associated increase in ODC activity. Preliminary data suggest that cocaine hinders the binding of insulin to embryonic cells. Because ODC is a focal enzyme for the regulation of growth, the data suggest that cocaine-induced changes in the mitogenic induction of embryonic/fetal ODC activity may be a part of the biochemical mechanism by which cocaine-induced growth inhibition occurs.**
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Consumption of cocaine has multiple adverse effects on reproductive functions and maternal cocaine use is associated with significant increases in premature deliveries (1), with increased abruptio placentae (1, 2) and with increased fetal and perinatal morbidity and mortality (see, for example, the paper by Chasnoff *et al.* [3]). Multiple factors influence the magnitude of the fetal response to cocaine, including the quantity and frequency of maternal drug use, the route of cocaine administration (2, 4), the concurrent use (abuse) of other drugs (5, 6), and such environmental factors as socioeconomic class, the extent of maternal prenatal care, and maternal nutrition (2).

Human and animal model studies suggest that cocaine exposure causes growth inhibition of the developing organism (1, 3, 6–10). These reports are of concern because decreased intrauterine growth is a manifestation of poor pregnancy outcome and is a strong predictor of neonatal problems. Cocaine-induced growth inhibition in human infants appears to result in an under growth of height, weight, and head circumference (6, 7), with microcephaly being the most persistent deficit (3, 6, 11). The developing central nervous system (CNS) is sensitive to the growth inhibitory effects of cocaine (6, 7, 12, 13) and maternal cocaine exposure in humans and in animal models is associated with changes in brain structure/function and in neurochemistry (14–20) as well as with behavioral changes (1, 6, 13, 21–25) among the offspring.

These data suggest that cocaine's multiple effects

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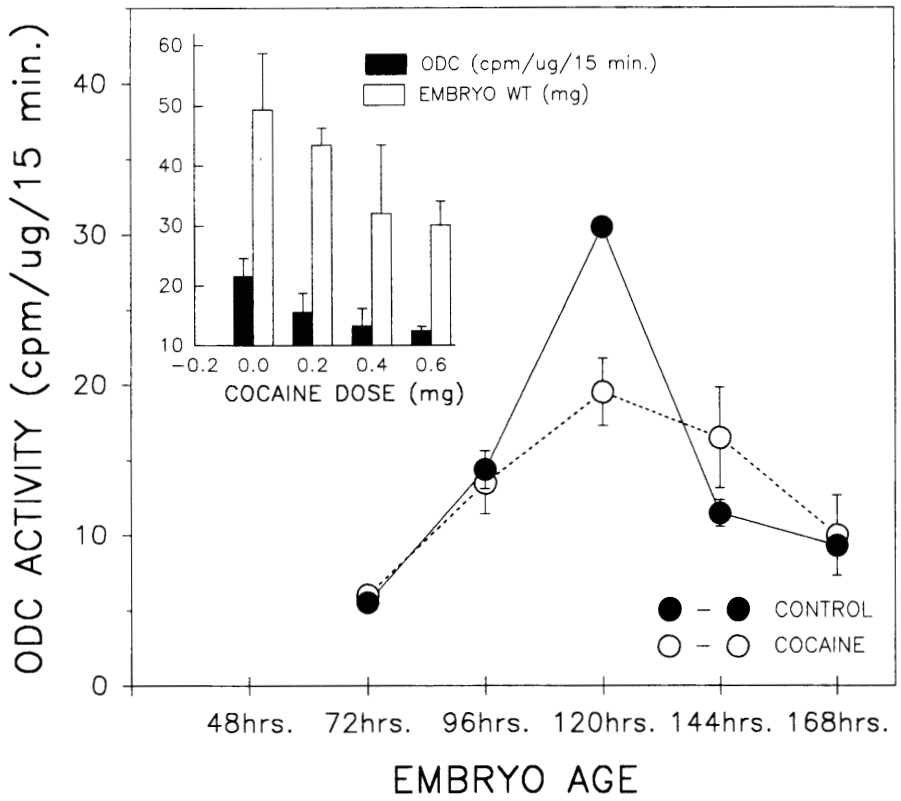

Figure 1. Suppression of ODC activity by a single dose of cocaine. Eggs were injected at the start of incubation with cocaine (0.15 mg/embryo) dissolved in chick Ringer solution. Vehicle-treated animals received chick Ringer solution only. The overall effect on ODC activity was significant ($f = 16.7$; $df = 9,30$; $P < 0.0001$). There was a significant hour × drug effect ($f = 5.04$; $df = 4$; $P = 0.0032$). The inset shows the effect of cocaine dose on ODC activity and weight of 96-hr embryos. There was no significant difference in ODC activity between sedentary and vehicle embryos at any stage of development (data not shown). For this and all subsequent figures, individual points in the figures represent group means, with the SE of the estimate indicated as error bars. *Note*: This figure was redrawn from Ref. 27 by the permission of the publisher.

on the mother, on the placenta, and on the embryo/fetus can all influence the embryonic/fetal response to cocaine and that growth inhibition could be mediated by multiple mechanisms. Studies in humans and in animal models (1, 26) indicate that cocaine-induced growth inhibition may involve the vasoconstrictive properties of the drug. However, animal model studies also provide compelling evidence that cocaine can also directly inhibit fetal growth via mechanisms that are distinct from its effects on the mother, the placenta, or on the fetal blood supply. For example, cocaine-induced growth inhibition occurs in placental models in which the embryos are dosed directly (9), in nonplacental models (27), in placental models dosed *in vitro* (8, 28), and in embryonic tissue grown in culture (29). Although there have been questions concerning the amount of cocaine used in some of these studies, the findings indicate that cocaine can inhibit growth by directly interacting with the embryo/fetus.

Studies using rodent models have provided insight into the biochemical alterations associated with cocaine-induced growth suppression. For example, in the perinatal rat CNS, acute and chronic (3 days) cocaine exposure markedly inhibited DNA synthesis (9). In addition, cocaine decreased the rate of CNS protein synthesis in the same model. There have been two recent reports of a loss of ornithine decarboxylase (ODC) activity in the perinatal rat CNS as the result of pre- and perinatal exposure to cocaine. Koegler *et al.* (26) reported that acute (1–4 hr) *ex utero* perinatal exposure of rat pups to cocaine inhibited ODC by a mechanism that appeared to be distinct from the drug's effect on central dopaminergic processes and from the anesthetic properties of cocaine. Acutely, perinatal cocaine appeared to inhibit ODC activity via a mechanism that involved the drug's vasoconstrictive action because a peripheral α-adrenergic blocker (phenoxybenzamine) reversed the ODC inhibition caused by cocaine. Bondy and co-workers (30) gave cocaine to pregnant rats on 3 consecutive days during mid- to late gestation and found a significant decrease in brain ODC at birth followed by a marked elevation in ODC activity by postnatal Day 11. Using a nonplacental chick model, our laboratory has recently reported a cocaine-induced alteration in ODC activity (27). These studies showed that the cocaine-induced growth suppression was asso-

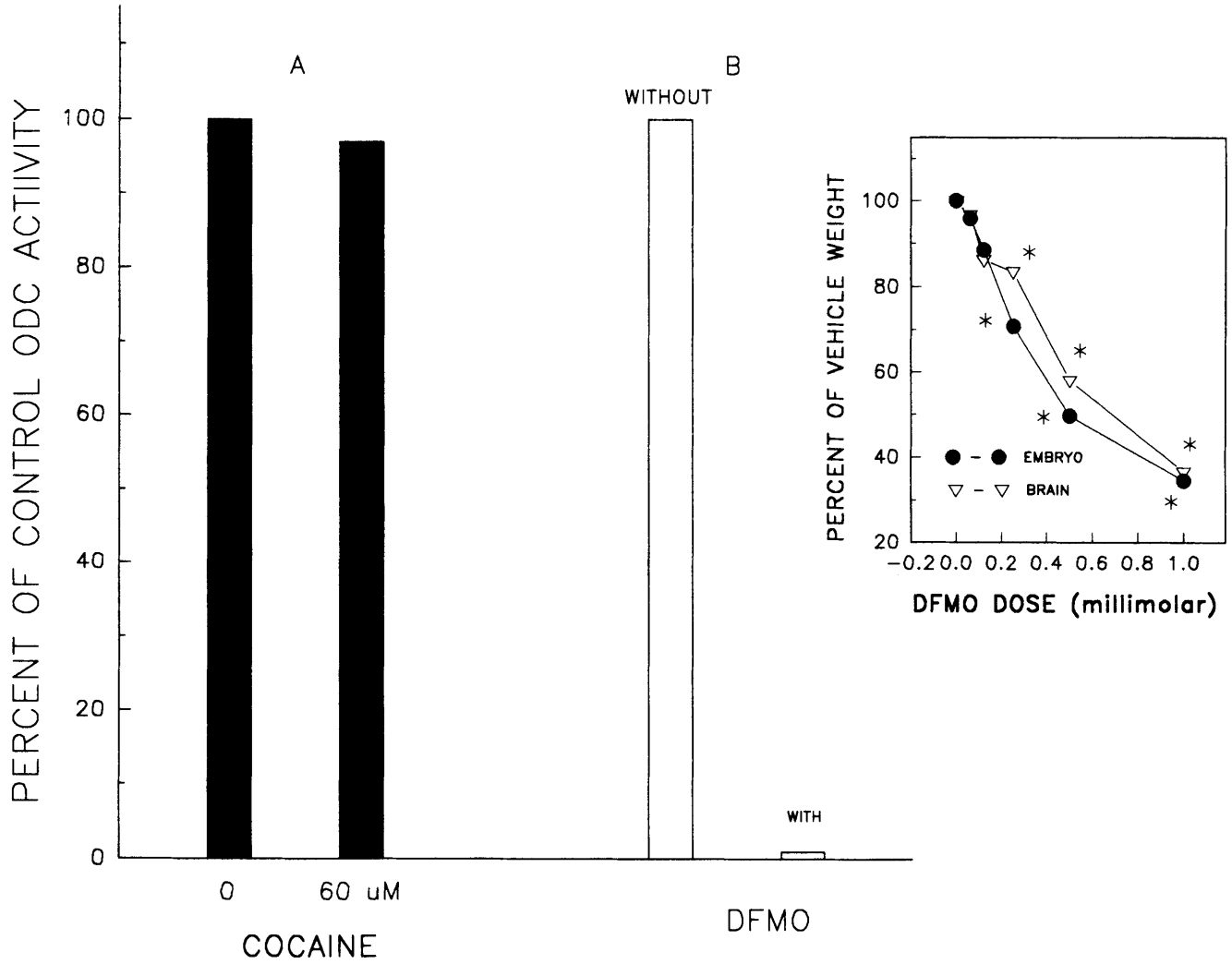

Figure 2. The effect of (A) *in vitro* cocaine or (B) DFMO treatment on ODC activity of a pooled tissue sample. Sedentary embryos were used to prepare a pooled tissue homogenate to which the indicated amount of drug was added *in vitro*. The inset of the figure shows the inhibition of embryo and brain growth by low, *in ovo* doses of DFMO. Eggs were dosed after 96 hr of incubation (the start of incubation = 0 hr) with the indicated concentration of DFMO dissolved in chick Ringer solution. The eggs were opened and the embryos were examined at 168 hr. For embryo weight, the treatment effect was significant ($f = 49.2$; $df = 5,45$; $P < 0.0001$), as it was for brain weight ($f = 15.4$; $df = 5,45$; $P < 0.0001$). The asterisk indicates values that are significantly different from the O (vehicle)-dosed values. Giving higher doses of DFMO or administering the dose early in the incubation period resulted in total arrest of development and embryo death.

ciated with a decrease in the normal developmental peak of ODC activity during early development. As pointed out in all three of these papers, the focal role of ODC activity in regulating growth suggests that cocaine-induced changes in the activity of this enzyme may represent a critical molecular defect. The present report describes studies of the biochemical changes underlying the cocaine-induced loss of embryonic ODC activity.

Materials and Methods

Embryo Incubation and Cocaine Dosing. Unincubated fertile chicken eggs ($n = 10$ for each treatment group; Arbor Acre or Hubbard strains obtained from Webber's Hatchery, Goldsboro, NC) were stored at 10°C for no more than 5 days before incubation. Eggs were incubated at 37.5°C in 90% humidity in a forced air incubator and turned automatically every 4 hr. A single dose of cocaine (dose levels indicated in the Figure Legends) was injected into the air space of the eggs at the start of incubation (0 hr). Sterile chick Ringer solution was used as the vehicle. Vehicle-treated embryos (0 dose) received chick Ringer solution only and a total volume of 200 μl was used for both the vehicle and drug injections. Untreated (sedentary) eggs were incubated to control for handling and seasonal variations in fertility and viability.

Embryo Tissue Isolation. At 72, 96, 120, 144, and 168 hr of incubation, individual eggs ($n = 10$ for each treatment group at each time point) were opened at the blunt end and the embryos were removed and freed of the associated membranes. The embryos were blotted

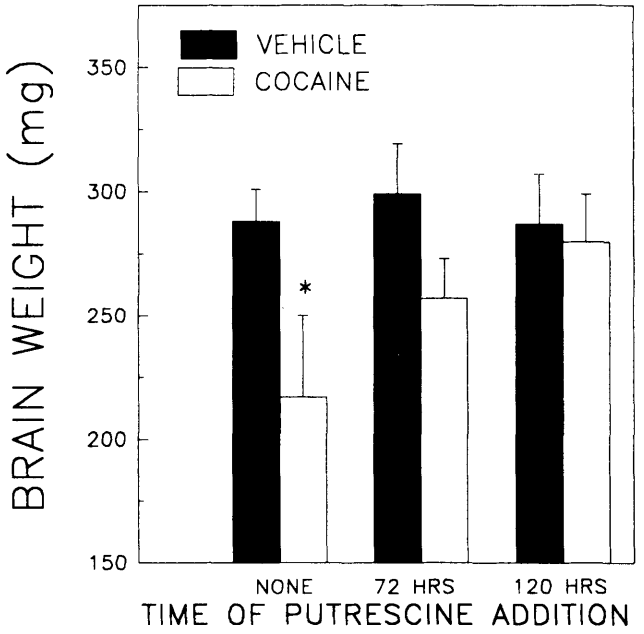

Figure 3. The effect of exogenous putrescine on the growth of embryos treated previously with cocaine. Fertile eggs were given a single dose of cocaine (0.15 mg/embryo) at 0 hr of incubation, and a single injection of putrescine (1 mg) was subsequently given at either 72 or 120 hr of incubation. The eggs were opened at 168 hr of incubation and total embryo weight and brain weight were determined. The cocaine-treated brains that received no putrescine (none) were significantly different from all vehicle-treated brains ($P < 0.05$). No other differences were significant. Total embryo weight gave an analogous result (data not shown).

and weighed to the nearest milligram. For embryos older than 72 hr, the cranial tissue was also isolated and weighed to the nearest milligram. The tissue was collected on wet ice and immediately assayed for ODC activity.

Tissue Culture Media. The basal medium for tissue culture consisted of a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 media and was used for all experiments to maintain a fixed nutrient level in view of ODC's sensitivity to the depletion of basic nutrients, e.g., amino acids or glucose. For most experiments, the basal Dulbecco's modified Eagle's medium/Ham's F12 medium (D/F) was supplemented with insulin, transferrin, and selenium. In certain experiments, as indicated below, a "mitogen-rich" medium was prepared by adding fetal calf serum (FCS; 5%) to the basal D/F medium.

Culture of Chick Embryo Tissue. Untreated embryos (120 hr of development) were isolated under sterile conditions. A sufficient number of embryos were pooled to yield 6×10^7 cells and their tissue was minced and incubated in sterile trypsin solution (0.25%, at 37°C for 10 min), followed by washing in basal D/F medium containing 5% FCS. The tissue was removed from the FCS-containing medium, washed in basal D/F medium, and dissociated by repeatedly drawing the tissue into a fire-polished Pasture pipette. The cells were centrifuged, resuspended, counted, and viability estimated using trypan blue exclusion. The cells were then diluted in D/F medium plus insulin (1.6×10^{-6} *M*) and plated at 1×10^6 cells/well in 24-well plates.

Ornithine Decarboxylase Assay. ODC was assayed by one of two radiochemical methods. Embryonic tissue was rinsed in phosphate-buffered saline and homogenized (using a Polytron) in a buffer containing 50 m*M* Tris (pH 7.4), 5 m*M* NaF, 2 m*M* dithiothreitol, 0.1 m*M* pyridoxal 5-phosphate, and 0.08 m*M* EDTA. The protein content of the homogenate was determined by the bicinchoninic acid procedure using a kit purchased from Pierce, Rockford, IL. Three 50-μl samples of each homogenate were assayed. ODC activity was measured at 37°C as the release of $^{14}CO_2$ from added [1-^{14}C]ornithine (0.1 μCi plus 0.03 m*M* cold ornithine). Using an air-tight reaction container, the released $^{14}CO_2$ was trapped on a filter paper saturated with 2*N* NaOH and placed above the incubation mixture in a plastic well. At the end of the 15-min reaction period, 20 μl of 50% trichloroacetic acid were injected into each tube and the tubes were incubated an additional 15 min at room temperature. The NaOH-saturated filter paper was then removed and the associated radioactivity was determined by liquid scintillation counting. For tissue in culture, the cells were rinsed in phosphate-buffered saline (twice) and a substrate solution was added (0.2 ml/well) that contained 50 m*M* Tris (pH 7.4), 5 m*M* NaF, 2 m*M* dithiothreitol, 0.1 m*M* pyridoxal 5-phosphate, and 0.08 m*M* EDTA plus [1-^{14}C]ornithine (0.1 μCi, 58 mCi/m*M*). The plates were incubated for 60 min at 37°C after being covered by a single sheet of filter paper saturated with 2*N* NaOH. Fifty microliters of 50% trichloroacetic acid were then added to each well and the plate was incubated at room temperature for an additional 30 min. The filter paper was removed and the positions of the various wells were lightly marked with a lead pencil. The radioactivity associated with the filter paper was quantitated by the use of a Raytest model 68000 TLC scanner. For some assays, the ODC specific inhibitor difluoromethylornithine (DFMO) was added to the assay mixture (0.1–1.0 m*M*).

Exogenous Putrescine Administration. Exogenous putrescine (1 mg) was administered *in ovo* to individual embryos previously dosed with cocaine (0.15 mg/embryo). The cocaine was given at the start of incubation (0 hr) and the putrescine then administered in 0.1 ml of chick Ringer's at 72 or 120 hr of incubation. Control embryos received cocaine at 0 hr and received only chick Ringer's solution at 72 or 120 hr. All embryos were extracted from the eggs after 168 hr of development and whole embryo and cranial weight were determined as described above.

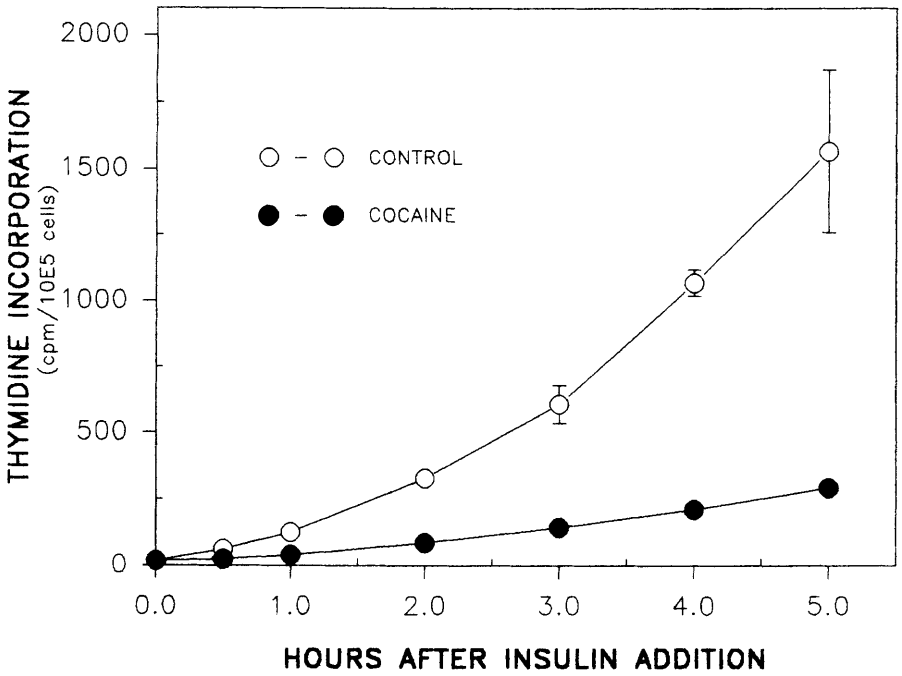

Figure 4. Inhibition of insulin-induced incorporation of [^{3}H]thymidine into cultured chick embryo cells by cocaine exposure (0.5 μM/ml). Tissue from untreated, 120-hr embryos was grown in culture and DNA synthesis was assayed as described in Ref. 32. DNA synthesis activity was induced in synchronous cells by exposure to insulin (1.6×10^{-6} M) for the indicated time periods. Values represent the group means ± SE (n = 3 cultures for each treatment group at each time point). The differences between group means are significant at all time points beyond the 1-hr time period.

Mitogenic Induction of ODC Activity and Thymidine Uptake. Embryonic tissue cultured as described above was used to test the effect of cocaine on the ability of insulin (a potent mitogen in the chick embryo [31]), to induce ODC activity. After 24 hr in the basal D/F medium plus insulin, cultures (n = 3 for each treatment) were washed (twice) in phosphate-buffered saline and synchronized by placing them in D/F medium only for the next 24 hr. The cultures were then refed D/F plus insulin or 5% FCS and the ODC activity was determined at 0, 2, 3, and 4 hr after the initiation of the refeeding. Additional cultures (n = 3 for each treatment) were assayed for thymidine uptake under identical conditions as described in Ref. 32. As indicated in the Legends for Figures 4 and 5, cocaine was present (0.5 μM/ml) during the mitogen refeeding and/or cell synchronization time periods in both experiments.

Statistical Analyses of Data. Group means and standard errors as well as post-hoc testing of significant differences between means for the various treatments were calculated using the general linear model procedure of the SAS/PC statistical program. Statistically significant differences between group means for vehicle and cocaine-treated preparations were determined by a one-way analysis of variance with $P < 0.05$ accepted as significant. Significant differences between individual groups were determined by the use of Duncan's post-hoc test.

Results

In an effort to circumvent the maternal and placental variables associated with placental models, the chick embryo (*Gallus domesticas*) dosed *in ovo* has been utilized as a vertebrate model for the study of the growth inhibitory effects of drugs. Recent studies (27) using the chick model have shown that a single dose of cocaine administered *in ovo* at the start of incubation results in significant growth inhibition in the embryo (inset of Fig. 1). Whole embryo weight was lowered by cocaine and the growth inhibition was correlated with the cocaine dose. As also shown in Figure 1, cocaine-induced growth suppression was associated with a failure of the embryos to express the normal developmental increase in ODC enzymatic activity. Peak levels of ODC activity occurred at 120 hr of development in both the control and cocaine-treated embryos, but the cocaine-treated animals had approximately 30% less ODC activity. The decrease in decarboxylase activity was particular to ODC because the ODC-specific inhibitor DFMO added *in vitro* ablated all of the decarboxylase activity, including that of the cocaine- and vehicle-treated embryos (Fig. 2B), indicating that only ODC activity was being measured in both groups. Cocaine added *in vitro* to cell-free preparations had no effect on ODC activity (Fig. 2A), indicating that cocaine is not a direct inhibitor of ODC. Furthermore, a depletion of the decarboxylase cofactor does not appear to be in-

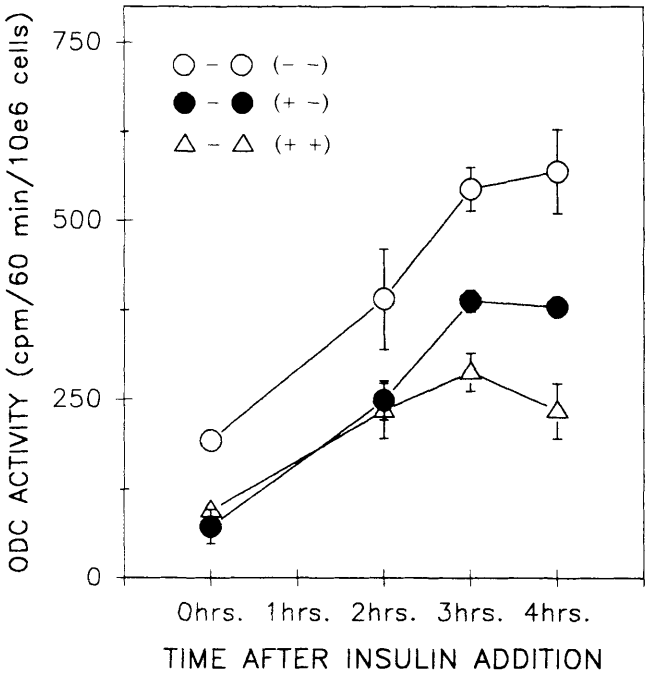

Figure 5. Inhibition by cocaine (0.5 μM/ml) of the insulin-dependent expression of ODC activity in cultured embryonic tissue. Tissue from untreated, 120-hr embryos was grown in culture and ODC assayed as described in the text. ODC activity was induced in synchronous cells by exposure to insulin (1.6×10^{-6} M) for the indicated time periods. Values represent the group means ± SE ($n = 3$ cultures for each treatment group at each time point). The treatment groups were as follows: − −, control cultures with no cocaine ever present; − +, cultures that had cocaine present during the 24-hr synchronization period; and + +, cultures that had cocaine present during both the synchronization and refeeding periods. Cultures refed media containing 5% FCS were not different from the control cultures refed insulin only (data not shown). Both cocaine-treated cultures were significantly different from the control culture at all time points. Furthermore, at 3 and 4 hr, all treatment groups were significantly different from each other.

volved because in all cases, the assay buffer contained excess pyridoxal 5-phosphate.

As seen in the inset of Figure 2, the inhibition of ODC activity by low, *in ovo* doses of DFMO resulted in a dose-dependent decrease in both whole embryo and brain weights. A similar result has been reported by others (33–35). These data suggest that embryonic putrescine levels are a critical factor for development and that a cocaine-induced decrease in putrescine synthesis would inhibit growth. To indirectly test this assumption, cocaine-dosed embryos were supplemented with exogenous putrescine *in ovo* to circumvent the loss of ODC activity and thus prevent the cocaine-induced growth suppression. The results are shown in Figure 3. Compared with cocaine-treatment without added putrescine (none), a single injection of exogenous putrescine (1.0 mg) given at 120 hr of development was sufficient to prevent brain growth suppression by cocaine (dose = 150 μg/embryo). A similar effect was observed for whole embryo weight (data not shown). Given at 72 hr of development, exogenous putrescine gave a comparable result, but the lower mean increase in brain weight suggested that the timing of the putrescine addition could be important, relative to the cellular events that occur in concert with the developmental increase in ODC activity.

Initial studies of cocaine's effect on mitogen-induced growth of embryonic cells in culture are shown in Figure 4. For embryonic tissue grown in serum-free media containing insulin as a mitogen, *in vitro* cocaine exposure resulted in significant growth suppression measured as the inhibition of [^{3}H] thymidine uptake. Furthermore, the insulin-induced expression of ODC activity in these same cells was significantly inhibited by *in vitro* exposure to cocaine. Dosing with cocaine for 24 hr significantly inhibited the ability of insulin to induce ODC activity after cell synchronization via mitogen starvation. Compared with control cultures not exposed to cocaine (− −), the presence of cocaine during the synchronization period (− +) caused a marked inhibition of the subsequent insulin-induced expression of ODC enzymatic activity. When cocaine was present during both the synchronization and the 4-hr mitogen refeeding periods (+ +), a further decline in ODC was seen.

Discussion

In utero growth suppression occurs in a substantial number of human pregnancies in which the mother uses cocaine. The molecular mechanism by which cocaine inhibits embryonic/fetal growth is unclear, but data suggest that the vasoconstrictive properties of cocaine are involved. The dopaminergic and anesthetic properties of cocaine do not appear to be critical components of this effect. However, the actual mechanism may involve multiple factors and is an area of current investigation.

In early embryonic tissue, ornithine decarboxylase activity is crucial to growth. ODC catalyzes the conversion of ornithine to putrescine, the rate-limiting step in the synthesis of the polyamines (putrescine, spermine, and spermidine) (36, 37). The polyamines are essential to growth and, during the cell cycle, increased ODC activity precedes DNA synthesis (36). In adults, ODC is a remarkably labile protein (37) whose level of activity is modulated by transcriptional (36–38) and translational mechanisms (37, 39, 40). ODC levels are regulated as well by proteases and antienzymes (41). In embryonic tissue, ODC appears to be a much more stable protein (33–35). Thus, during early development (e.g., before the midblastula transition in Xenopus embryos), ODC protein is stable and ODC enzymatic activity increases rapidly as the result of increased translation (42). In the Xenopus, embryonic ODC activity quickly peaks and then falls. Chick embryo ODC enzymatic activity also undergoes a transitory increase

during early periods of development (72–144 hr), a response analogous in form to that of Xenopus.

In the early chick embryo, the inhibition of ODC activity by low, *in ovo* doses of DFMO results in a dose-dependent decrease in both whole embryo and brain weights (Fig. 2 and reports by others [35]). Although neither the mechanism controlling the normal developmental increase in ODC activity nor the mechanism by which cocaine suppresses the increase in ODC are known, the present studies suggest that the loss of ODC activity is a critical factor because the administration of exogenous putrescine overcame the growth inhibitory effects of cocaine. Because ODC enzyme levels are responsive to a variety of mitogenic factors, the molecular mechanism by which cocaine prevents the normal developmental increase in ODC activity could involve changes in the embryonic response to mitogenic induction of ODC. Several studies have shown that exposure to drugs of abuse (43, 44) alters embryonic trophic factor (mitogen) levels and also lowers tissue responsiveness to these factors. While, several trophic factors modulate ODC activity (44–51), we choose to study the effects of cocaine on the response of chick cells to insulin, a potent tyrosine kinase-linked mitogenic factor. The data shown in Figure 4 indicate that in this model, cocaine exposure blocks the rapid increase in cell division induced by insulin exposure. Furthermore, Figure 5 shows that cocaine also blocks the ability of insulin to induce the expression of ODC in these same cells. The molecular mechanism of this response may involve changes in insulin's ability to interact with the cells, i.e., initial studies suggest that cocaine exposure blocks the receptor-mediated binding of insulin by these cells (data not shown). Additional studies are currently under way to determine the specificity of cocaine's effect on insulin-mediated mediated growth and the expression of ODC activity.

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