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Acute hypoxia increases ornithine decarboxylase activity and polyamine concentrations in fetal rat brain

(development/ornithine decarboxylase mRNA/hippocampus/cerebrum)

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ABSTRACT **The cellular responses to hypoxia are poorly understood. To test the hypothesis that ornithine decarboxylase (ODC; L-ornithine carboxy-lyase; EC 4.1.1.17) activity and polyamine concentrations change in response to acute hypoxia, we performed the following studies. Pregnant Sprague–Dawley rats inspired various O_2 concentrations (9–21%) for various time periods (0.5–48 h) from days 15 to 21 of gestation. In fetal brains we measured the activity of ODC, ODC mRNA, and polyamines. In response to 4-h acute mild hypoxia, ODC activity in fetal rat brain (cerebrum, cerebellum, and hippocampus) increased to 330–450% from control values ($P < 0.001$), after which it declined to control levels in 6–8 h. The 4-h ODC response varied inversely with inspired O_2 concentration and was not mimicked by β_2 agonist or blocked by β_2-antagonist administration. The ODC response was associated with an increase in fetal brain putrescine concentration to 190% above control at 4–6 h ($P < 0.01$) and an increase in the polyamines spermidine and spermine to about 115% above control at 6–8 h. We also observed that ODC mRNA increased significantly after 2–4 h of hypoxia. ODC activity and polyamine concentrations appear to be useful enzymatic markers for fetal brain hypoxia. The magnitude and time course of the acute hypoxic ODC increase were similar to responses to extracellular signals that result in differentiation or cell growth. Thus, the well-defined and regulated ODC activity response may represent a protective mechanism in brain to hypoxia.**

The brain is known to be particularly vulnerable to oxygen deprivation. Recently, considerable interest has centered on the role of ischemia (lack of blood flow) on brain neurochemistry (1, 2), but relatively little recent work has focused on the neurochemical effects of hypoxia (relative O_2 lack) *per se* (3, 4). In addition, there are few well-documented biochemical or cellular markers of hypoxic effects in brain or other tissues.

In this report we present the hypothesis that ornithine decarboxylase (ODC; L-ornithine carboxy-lyase; EC 4.1.1.17) activity could be an enzymatic marker responsive to hypoxia in fetal rat brain. ODC activity is tightly regulated in growing tissue (5) and is sensitive to extracellular signals (6). ODC is the rate-limiting enzyme in polyamine biosynthesis, is highly inducible, has a short half-life (about 20 min), and is absolutely required for cell growth and proliferation (7–9). Thus, ODC activity is an excellent marker for changes in polyamine metabolism, which plays key roles in DNA, RNA, and protein synthesis. Our rationale was that if mild hypoxia affected any of these processes, then ODC activity and polyamine concentrations could be sensitive markers for these changes. Furthermore, because ODC activity is relatively high in growing fetal tissue (10, 11) we expected to be able to detect altered ODC activity in response to hypoxia.

We specifically chose maternal hypoxic exposures (9–16% inspired O_2), which might be experienced during fetal development, and not ischemic exposures, which would cause extensive tissue and cellular damage. Thus, our purpose was to develop a sensitive biochemical marker for hypoxia rather than to measure the consequences of cellular damage due to ischemia or anoxia.

This report details the rather surprising finding that acute maternal hypoxic exposures (e.g., 4 h or less) in rats caused an inspired O_2 concentration-dependent and time-dependent increase of ODC activity in fetal brain. Furthermore, hypoxia also raised ODC mRNA and polyamine levels (putrescine, spermidine, and spermine).

MATERIALS AND METHODS

We obtained pregnant female Sprague–Dawley rats (≈120 days of age, 250 g) at ≈13 days of gestation (E13) (Charles River Breeding Laboratories) that had previously reared one litter. Dams were housed one per "shoebox" cage and maintained in a normal vivarium environment, as described (12). For acute hypoxia, dams inspired various O_2 concentrations (9–16%) for 0.5–48 h on E15 to E21 (term = E22). In seven chronically catheterized pregnant rats, we obtained maternal arterial blood gas values.

The adult rats were sacrificed by cervical dislocation. As rapidly as possible, we exposed the uterine horns through a midline abdominal incision, and the fetuses were removed and weighed. Pups were decapitated, and the brain was removed, placed in ice-cold phosphate-buffered saline, and dissected into component parts (cerebrum, cerebellum, and both hippocampi) on cold, wet filter paper covering a Petri dish containing dry ice and ethanol. In some experiments we also examined the ODC response in brain stem, hypothalamus, and striatum.

We measured ODC activity by the method of Russell and Snyder (8) and soluble protein by the method of Bradford (13). We quantified the polyamines putrescine, spermidine, and spermine by high-performance liquid chromotography (HPLC) employing precolumn derivatization with dansyl chloride (14). We extracted total RNA from brain tissue with 4 M guanidine (RNAzol, Tel-Test, Friendswood, TX). Thirty micrograms of total RNA per lane was electrophoresed in a 1.2% agarose/0.66 M formaldehyde denaturing gel. Separated RNA was capillary blotted to nylon membranes (Hybond, Amersham) and UV crosslinked. Prehybridization and hybridization (18 h) were performed in 50% formamide at 42°C (15). For hybridization of the ODC probe, a 1-kDa

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Abbreviations: ODC, ornithine decarboxylase; E, embryonic day; HSP, heat shock protein.

*Hin*dIII fragment (16) was labeled with [α-^{32}P]dCTP (Primagene, Promega). Final wash was in 75 mM sodium chloride/5 mM sodium phosphate/0.5 mM EDTA, pH 7.4, at 65°C for 15 min.

Pups of three or four pregnant rats in each group were used for each datum point. We measured tissue ODC activity in five pups per litter and calculated the mean value. Thus, each litter constituted an n of one. In general, the coefficient of variation of the five measurements per litter was <20%. All data are presented as mean ± SEM. Significant differences between different brain regions, times, and treatments were evaluated by analysis of variance (ANOVA) and Duncan's multiple-range test. Differences were considered to be statistically significant at a level of $P < 0.05$.

RESULTS

ODC Activity. In response to acute, continuous, maternal hypoxia, fetal brain ODC activity increased 3- to 5-fold in the three brain regions. For instance, Fig. 1 shows that in the hippocampus at E20, after 1, 2, or 3 h of hypoxia ODC activity rose to 168%, 260%, and 370% of control values, respectively. By 4 h ODC activity reached a maximum of 446% of the control value. Thereafter ODC activity decreased, so that by 8 h of continuous exposure it was only 122% of control levels (see also Table 1, group A). After 24 and 48 h of continuous hypoxia ODC activity continued to decrease to about 90% and 60% (not significant), respectively, of control values (Fig. 1). ODC activity also increased 3- to 4-fold in the fetal cerebellum (Fig. 1), as it did in the cerebrum, brainstem, hypothalamus, and striatum (data not shown). By 4 h ODC activity also increased about 2-fold in fetal liver (Table 1, group A). In the chronically catheterized dams (n = 7), which inspired 10.5% O_2, arterial O_2 partial pressure averaged 37.6 ± 1.4 torr (1 torr = 133 Pa) for hypoxemic dams as compared to 87.4 ± 3.4 torr for normoxic controls.

To ascertain the ODC activity response to transient versus continuous hypoxia, we exposed pregnant rats (three litters per time point) to 10.5% O_2 for 4 h and measured ODC activity at various times following the end of hypoxia (Fig. 1 *Inset*). Subsequently, and following return to room air, hippocampal ODC activity decreased in a manner similar to that seen when the hypoxic exposure was continued (Fig. 1 *Inset*).

Dose–Response Relations. To assess the relation of increased ODC activity to inspired O_2 concentration, we exposed four pregnant dams each (E20) to 16, 13.2, 10.5, or 9% O_2 for 4 h. Fetal brain ODC activity immediately after exposure increased progressively with lowered inspired O_2 concentrations (Table 1, group B). For instance, hippocampal ODC activity was increased above control values as follows: 172% at 16% O_2, 193% at 13.2%, 337% at 10.5%, and 404% at 9% O_2. A similar increase in ODC activity occurred in cerebellum. The response in the liver, though statistically significant, was less striking.

Acute Intermittent Hypoxia. To test whether the ODC response became either attenuated or augmented with repeated hypoxic exposure, we exposed pregnant dams to 10.5% O_2 for 4 h/day from E15 to E19 or E20 and measured ODC activity on E20 (four litters per time point). On E20, immediately following the sixth daily 4-h hypoxic exposure, ODC activities in the several brain regions were similar to those seen following a single 4-h exposure on that day (Table 1, group C). In addition, on E20 24 h following five such daily 4-h hypoxic exposures (E15 to E19) ODC activities in the several brain regions were not significantly different from control values (Table 1, group C).

Effect of β-Adrenergic Stimulation and Stress on ODC Activity. To examine the possibility that this hypoxic-induced ODC response was secondary to β-adrenergic stimulation, we administered to the dam at E20 either the β_2 agonist terbutaline (2 mg/kg i.p.) or the β-antagonist propranolol (1 mg/kg i.p.) (four litters per group). Terbutaline administration was followed by a slight increase in fetal hippocampal and cerebellar ODC activity at 4 h; however, this did not compare to the 340–430% increase seen with hypoxia alone (Table 1, group D). Propranolol failed to block or otherwise affect significantly the fetal ODC response to hypoxia. To rule out the possibility that the ODC increase was a generalized "stress" response, we placed pregnant rats (three litters per group) in a plastic restrainer for 15 min every hour for 4 h. Additional dams swam in cold water at 22°C for 15 min or for 15 min every hour for 4 h. At the end of these 4-h periods, fetal brain ODC activities were not significantly

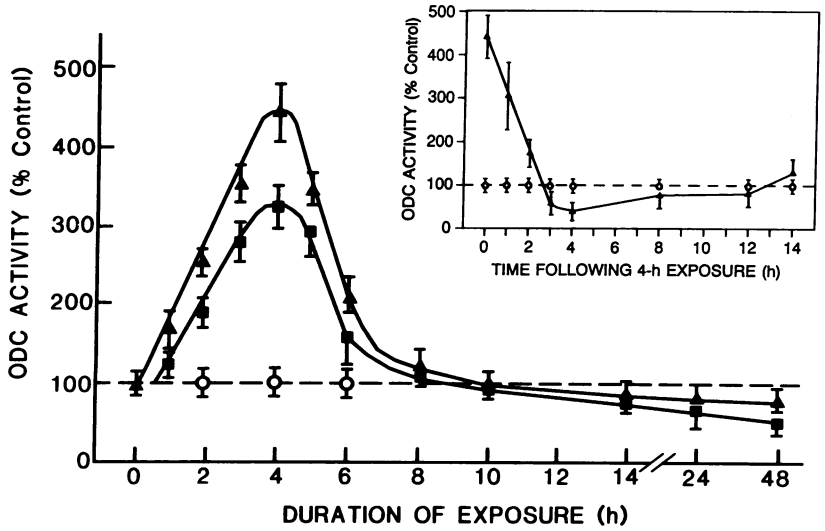

FIG. 1. Changes in ODC activity in fetal rat brain (E20) in response to continuous hypoxia (10.5% inspired O_2) for various periods of time. ODC activity was measured in hippocampus (▲) and cerebellum (■) immediately following hypoxic exposure for the indicated period. Each time point represents the mean ± SEM (shown by bars) of at least 12 litters from four separate experiments. All values are significantly different from control values ($P < 0.05$) except those at 15 min and 8, 10, 14, and 24 h. (*Inset*) Change in ODC activity in hippocampus (▲) following 4 h of hypoxia (10.5% inspired O_2) with recovery in room air for the indicated times. Each time point represents the mean value of 4 litters (five fetuses per litter) ± SEM.

Table 1. ODC response to hypoxia and other factors in fetal brain and liver

Group	Condition	Hippocampus		Cerebellum		Liver	
		ODC activity	% control	ODC activity	% control	ODC activity	% control
A	Control	0.27 ± 0.02	—	0.22 ± 0.01	—	0.031 ± 0.002	—
	Hypoxia (10.5% O_2)						
	1 h	0.45 ± 0.07	168*	0.35 ± 0.07	163*	0.036 ± 0.004	117
	2 h	0.70 ± 0.04	260†	0.52 ± 0.06	238†	0.041 ± 0.007	132*
	3 h	1.00 ± 0.08	370†	0.46 ± 0.08	208†	0.056 ± 0.010	181†
	4 h	1.20 ± 0.10	446†	0.77 ± 0.08	348†	0.061 ± 0.012	197†
	6 h	0.67 ± 0.09	249*	0.48 ± 0.05	218*	0.054 ± 0.009	173†
B	Control (21% O_2)	0.25 ± 0.01	—	0.18 ± 0.01	—	0.029 ± 0.001	—
	Hypoxia						
	16%	0.43 ± 0.01	172*	0.28 ± 0.03	154*	0.041 ± 0.005	142*
	13.25%	0.48 ± 0.02	193*	0.30 ± 0.08	165*	0.049 ± 0.004	170*
	10.5%	0.84 ± 0.11	337†	0.49 ± 0.06	270†	0.056 ± 0.006	192†
	9%	1.01 ± 0.12	404†	0.65 ± 0.10	353†	0.061 ± 0.003	210†
C	Control	0.28 ± 0.04	—	0.22 ± 0.03	—	—	—
	Single hypoxia (4 h)	0.83 ± 0.09	296*	0.69 ± 0.11	313*	—	—
	Immediately after 6th daily						
	4-h hypoxic exposure	0.79 ± 0.15	282*	0.67 ± 0.04	304*	—	—
	24 h after five daily						
	4-h hypoxic exposures	0.30 ± 0.06	107	0.25 ± 0.05	113	—	—
D	Control	0.27 ± 0.02	—	0.15 ± 0.01	—	0.023 ± 0.001	—
	Hypoxia alone (10.5% O_2)	1.19 ± 0.10	436†	0.51 ± 0.06	343†	0.033 ± 0.004	144
	Terbutaline	0.29 ± 0.03	107	0.19 ± 0.02	130	0.017 ± 0.001	73
	+ hypoxia	0.48 ± 0.03	176*	0.29 ± 0.02	193*	0.021 ± 0.001	92
	Propranolol	0.19 ± 0.02	70	0.13 ± 0.01	91	0.022 ± 0.002	96
	+ hypoxia	1.03 ± 0.07	379†	0.48 ± 0.03	326‡	0.034 ± 0.002	165†
E	Control	0.25 ± 0.03	—	0.19 ± 0.02	—	0.040 ± 0.004	—
	Maternal stress						
	Restraint	0.28 ± 0.02	112	0.18 ± 0.01	94	0.036 ± 0.002	91
	Swimming × 1	0.17 ± 0.02	69	0.15 ± 0.02	81	0.046 ± 0.006	115
	Swimming × 4	0.22 ± 0.03	87	0.15 ± 0.03	79	0.034 ± 0.004	85
F	Control	0.37 ± 0.04	—	0.25 ± 0.02	—	0.042 ± 0.008	—
	Hypoxia	1.13 ± 0.16	305†	0.57 ± 0.08	228*	0.104 ± 0.014	247*
	Ornithine	0.30 ± 0.04	81	0.15 ± 0.02	60	0.031 ± 0.003	74
	+ hypoxia	0.87 ± 0.07	290*	0.44 ± 0.06	293*	0.048 ± 0.006	114

Experiments were performed on E20. ODC activity is given in nmol of CO_2 per mg of protein per h. Each value represents the mean of four litters ± SEM. *, $P < 0.05$; †, $P < 0.01$. Group A. These data differ from those shown in Fig. 1, in that they represent mean values of one experiment (four litters, five fetuses/litter). Group C. Dams were exposed to hypoxia for 4 h/day from E15 to E20. ODC activity was assayed either immediately after the end of the hypoxic exposure or 24 h thereafter, as indicated. Group D. On E20 dams were injected with terbutaline (2 mg/kg i.p.) or propranolol (1 mg/kg i.p.) followed with or without hypoxic exposure. After 4 h ODC activity was measured. Group E. On E20 dams were placed in a plastic tube (6.35 cm in diameter) for 15 min every hour for 4 h. Other dams swam in cold water at 22°C for 15 min or every 15 min for 4 h. Group F. Dams imbibed 5% L-ornithine in drinking water from E15 to the morning of E20, at which time half were subjected to 4 h hypoxia.

different from controls or were slightly depressed (Table 1, group E).

Maternal Ornithine Feeding. To test the hypothesis that elevated ornithine concentrations would alter ODC activity, we fed pregnant dams 5% L-ornithine in their drinking water from days E15 to E20. On E20 dams were subjected to 4 h of hypoxia. ODC activity was measured in four litters per time point (five pups/litter). Increased maternal ornithine intake resulted in lowered control fetal ODC activity. In absolute terms, the hypoxic-induced ODC response was lower; however, proportionally it was as great as in the non-ornithine-supplemented hypoxic rats (Table 1, group F).

Polyamines. In an effort to determine the relation of brain polyamines to ODC activity, we measured polyamines at 19 and 20 days of gestation in control animals and following various periods of hypoxia (three litters per time point). Fig. 2 shows that in response to 4 h of acute hypoxia, fetal hippocampal putrescine concentrations doubled. In contrast, hippocampal spermidine concentration rose and appeared to plateau at 6–8 h, remaining about 15% above the control value thereafter.

ODC mRNA. To obtain a preliminary understanding of the hypoxic-induced increase in ODC activity, we measured the time course of fetal brain ODC mRNA response. Fig. 3 shows a significant increase in fetal cerebral ODC mRNA at 1–4 h, peaking at 3 h. A similar increase in ODC mRNA at 2–4 h was observed in hippocampus and cerebellum. In contrast, there was no evidence of an increase in expression of the protooncogenes c-*fos* and c-*myc* or nerve growth factor 1β at 0.5, 1, or 2 h following the onset of hypoxia in fetal rat brain.

Lack of Increase in Heat Shock Protein (HSP). To examine whether the hypoxic-induced increase in ODC activity was, in fact, an increase in HSP, we quantified the inducible HSP-68 (a 68-kDa HSP) at 2, 4, 6, 8, 16, and 24 h of hypoxia and at 6 and 24 h following a 4-h hypoxic exposure. HSP-68 did not change significantly from control values at any of these times.

DISCUSSION

ODC Activity Response to Acute Hypoxia. The primary finding of the present study is that relatively mild maternal hypoxia resulted in an increase in fetal brain ODC mRNA,

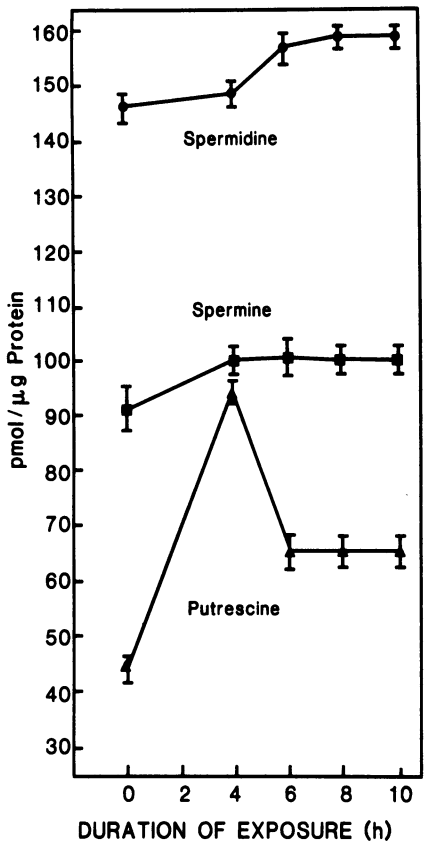

FIG. 2. Concentrations of putrescine (▲), spermidine (●), and spermine (■) in fetal hippocampus as a function of duration of hypoxia at E20. The concentration of putrescine was significantly increased from control values by 4 h ($P < 0.01$), whereas that for spermidine and spermine plateaued at 6 and 8 h. Each point represents the mean ± SEM for three litters (five fetuses per litter).

ODC activity, and polyamine concentrations. This increase in ODC activity was highly significant and reproducible in each of >30 separate experiments, the 4-h ODC activity being 300–500% above control values. This increase appears to be a general tissue response to acute hypoxia, as it was observed in all brain regions examined—cerebrum, cerebellum, hippocampus (as well as hypothalamus, striatum, and brainstem; data not shown). In addition to the fetus, the hypoxic-induced ODC activity increase occurred in these tissues in newborn and adult rats, albeit to a lesser extent. We have also observed a significant hypoxic-induced ODC increase in fetal and neonatal brain slice preparations (unpublished data). The fetal ODC activity increased in response to even mild hypoxia (16% inspired O_2, equivalent to ≈2100-m elevation); it was not mimicked by maternal stress, nor was it stimulated by adrenergic β_2 agonists or inhibited by a β blocker.

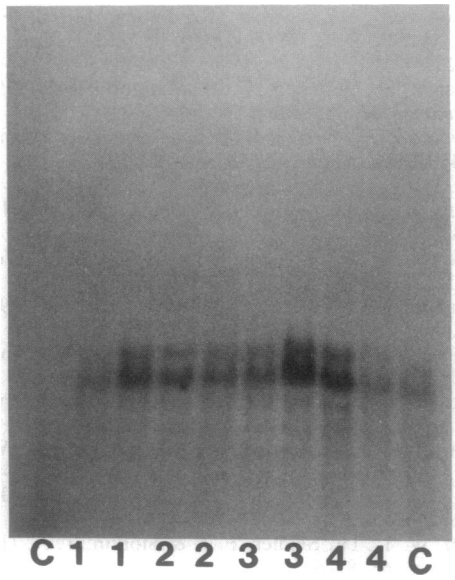

FIG. 3. Time course of fetal cerebral ODC mRNA response to acute hypoxia at 20 days of gestation as measured by Northern analysis and video densitometry. Lanes: C, control; 1–4, hours of hypoxia. The findings were similar in four separate experiments. Similar increases in ODC mRNA were seen in fetal hippocampus and cerebellum.

That the ODC response is well regulated, is suggested by its consistent temporal nature, increasing incrementally to peak at 4 h, and declining thereafter. This is also suggested by its dose–response characteristics. ODC activity can be induced by extracellular signals, and its induction is generally associated with the initiation of cell growth and differentiation. The temporal nature of this change in ODC activity was similar to that seen in response to extracellular signals such as growth factors and neurotransmitters (7, 17). Among many examples is the ODC response of PC-12 cells to nerve growth factor, which peaks at 4 h and leads to differentiation (6).

The effects of ornithine in the drinking water on fetal brain ODC activity (Table 1, group F) suggested that ODC activity was decreased because of increased concentration of the substrate ornithine. We concluded that less ODC activity was needed to maintain proper polyamine levels. Also, with ornithine in the drinking water ODC activity still rose proportionally in response to hypoxia. These data are also consistent with the idea that higher polyamine levels may be useful to the cell in response to hypoxia.

The question arises as to the mechanism of signal detection of the hypoxic-induced increase in ODC and polyamines. This could be either direct or indirect. Indirect mechanisms of signal detection could involve adrenergic stimulation or a generalized stress response. Our results suggested that the β_2-adrenergic receptor does not appear to be involved in the observed effect (Table 1, group D). In addition, it does not appear to be related to a general stress response, because neither restraint nor strenuous swimming in cold water resulted in increased ODC activity (Table 1, group E). The mechanism of detection of decreased O_2 levels may not involve membrane receptor activation, based on the lack of diminished responsiveness after repeated 4-h hypoxic exposures (Table 1, group C), usually associated with receptor down-regulation.

A distinct possibility is that the hypoxic signal is directly perceived by the cell. To our knowledge the only known molecular mechanism of detection of oxygen levels is direct oxidation of an OxyR protein in *Escherichia coli*, which activates transcription of oxidative stress-inducible genes that protect against oxidative damage (18). It remains to be determined if eukaryotic cells detect oxidative stress by a similar mechanism or whether this degree of hypoxia would stimulate cytochrome oxidase to cause an increase in ODC mRNA, ODC activity, and the polyamines.

Biologic Significance. Another question concerns the biologic meaning of these hypoxic-induced changes. The increases in putrescine, spermidine, and spermine concentrations may serve in cell defense mechanisms to hypoxia. In addition to the functions enumerated above, polyamines are known to stabilize cell membranes (19). Because hypoxia can generate superoxide radicals that damage cell membranes and subcellular organelles (20, 21), oxidize proteins (22), and damage DNA (23, 24), the polyamines, with their high charge-to-mass ratio, may be a defense against this or other such injury. The ODC activity response to mild hypoxia (16%

O_2), and the fact that it was observed in various tissues and ages, supports the idea of a general defense mechanism against hypoxia. That polyamines may play such a protective role is suggested by increased neuronal survival in hippocampal CA1 and striatal neurons in association with daily polyamine injections following cerebral ischemia in adult gerbils (1). In addition, pretreatment of rats with the polyamine precursor *S*-adenosyl-L-methionine prior to cerebral ischemia ameliorates brain lactate accumulation (25), while preventing an adverse increase in calcium concentrations and decreased glucose metabolism (26).

Alternatively, the possibility exists that the hypoxic-induced increase in ODC activity and polyamines may be a consequence of cell injury. For instance, polyamine increases have been related to cellular damage in studies of cerebral ischemia (2). Also, increased activity of amine oxidase (polyamine degrading enzyme) is associated with programed cell death (27). In addition, the role of polyamines in regulating *N*-methyl-D-aspartate (NMDA) receptor activation may be important in this regard (28), since NMDA receptors possess a specific polyamine recognition site (29). Increasing evidence indicates that NMDA receptor excitotoxicity and Ca^{2+} influx are involved in neuronal injury and death (30–33). Thus, further studies will be required to elucidate the biologic consequences of these changes.

Recent evidence suggests that in some instances HSP(s) also increases in response to acute hypoxia (34), although other researchers have reported that HSP-72 (a 72-kDa HSP) is only induced in response to hypoxia-ischemia, rather than hypoxia itself (35). The relation of increased ODC activity to the HSP(s) induction in hypoxia is not known; however, in this study there was no evidence of such a relation.

To our knowledge, no previous report has presented evidence of ODC activity or polyamine concentration increases of this magnitude in response to acute hypoxia in the fetus, neonate, or adult. This hypoxic-induced increase in ODC activity is in contrast to other examples of decreased ODC activity following neonatal acute hypoxia (10, 36, 37). However, small increases in ODC activity follow acute hypoxia in 8-day-old neonates (36), an effect increased by prenatal dexamethosone treatment (38), and ODC activity has been shown to increase slightly in response to treatment with nicotine (39).

Hypoxia represents a potentially important threat to the developing fetus, especially its brain. As shown in the present study, mild hypoxia resulted in sequential increases in ODC mRNA, ODC activity, and polyamine concentrations. This suggests some functional significance. The signal inciting these changes and the molecular mechanism(s) whereby these changes are mediated remain intriguing problems for future research.

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