

Intrauterine Hypoxia-Ischemia Increases *N*-Methyl-*D*-Aspartate-Induced cGMP Formation and Glutamate Accumulation in Cultured Rat Cerebellar Granule Cells

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ABSTRACT

Effects of intrauterine hypoxia-ischemia (HI) on brain functional development in the term rat were examined in cerebellar granule cell cultures obtained from an *in utero* HI model. On gestation d 17, HI conditions were achieved by complete clamping of the uterine vasculature for designated durations followed by removal of the clamps to permit reperfusion. Sham operation (surgery without vasculature clamping) was performed as the control. After surgery, the uterine horns were returned to dam's abdomen to let the pups deliver naturally. Severe HI insult from the surgical manipulation was evident in that the lactate levels of fetal brain increased, and fetal blood pH decreased with the duration of vasculature clamping up to 1 h. The experimental HI insult up to 1 h did not affect fetal survival rate, but retarded growth of fetuses or newborns was observed in the 1 h HI group. *N*-Methyl-*D*-aspartate (NMDA)- and kainate (KA)-stimulated cGMP formation and glutamate accumulation were measured in cerebellar granule cell cultures from 8-d-old pups suffering from various durations of antenatal HI insult. NMDA (100 μ M)-induced elevation of cGMP was further augmented by a 10–35-min HI insult as compared with the control cells (62.4–78.2 *versus* 49 pmol/mg protein). In the presence of *L*-*N*^G-monomethyl-arginine (*L*-NMMA, 150 μ M), a nitric oxide syn-

thase inhibitor, NMDA-induced cGMP formation was blocked, and the blockade of cGMP formation by *L*-NMMA (10 μ M) could be reversed by simultaneous application of 1 mM arginine in both control and HI cells. Antenatal HI insult (20–35 min) also augmented NMDA-, but not KA-, stimulated accumulation of extracellular glutamate. The hypersensitive response in NMDA-induced glutamate accumulation could be suppressed by 150 μ M *L*-NMMA. The overall results suggest that antenatal HI occurring in the last half of gestation may result chronically in adverse effects on NMDA receptor-mediated neurotransmission and that nitric oxide is possibly involved in these effects. (*Pediatr Res* 38: 107–112, 1995)

Abbreviations

HI, hypoxia-ischemia
EAA, excitatory amino acid
NO, nitric oxide
L-NMMA, *L*-*N*^G-monomethyl-arginine
NMDA, *N*-methyl-*D*-aspartate
KA, kainate
ANOVA, analysis of variance
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

In utero HI is a major contributor to long-term neurologic abnormalities. The severe sequelae include learning disabilities, motor disturbances, behavioral alterations, and mental retardation (1, 2). The establishment of many perinatal HI animal models (3, 4), including several rat models (5–8), has greatly expanded our knowledge of the critical events and biochemical alterations occurring during or after the initial HI insult. However, in most of these experimental models the HI insult is given acutely near term or during the first a few days of postnatal life (in case of the rat model), which is thought to be a developmental stage equivalent to the third trimester in

humans, and brain injury is determined immediately after the insult. It is not fully understood whether and how HI occurring at a relatively early gestational stage results in chronic adverse effects on CNS development. Recent animal studies have provided evidence that intermittent antenatal hypoxia has long-term effects on rat brain ornithine decarboxylase activity and causes several behavioral changes, although with maturation those abnormal behaviors are not evident (9). Clinical evidence also indicates that neurologic damage in newborn infants due to antenatal insults is demonstrable (10–12) and that the observed neonatal encephalopathy, after the exclusion of infants with major malformations or infections, is not always due to birth asphyxia and is sometimes due to preexisting abnormalities (13). It is thus desirable to have an animal model for

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studying the chronic effects of *in utero* HI on postnatal brain development.

Considerable experimental evidence indicates that alteration in glutamate neurotransmission is one of the important factors in the HI-induced brain injury (1, 14–18). As a major EAA neurotransmission system, the glutamate receptors can be categorized into two subgroups, metabotropic and ionotropic receptors, which include three subtypes based on selective activation with the agonists: NMDA, KA, and (\pm)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate. During HI insult, an excessive amount of glutamate is released (19, 20) and results in massive influx of ions and water and eventual neuronal cell death (15). In addition to the direct neuronotoxic effects of glutamate, activation of EAA receptor subtypes has been shown to trigger the Ca^{2+} -dependent synthesis of the novel intercellular messenger, NO, from L-arginine (21, 22). Inhibition of NO synthesis from L-arginine has been reported to reduce brain injury associated with HI in mice and immature rats (23, 24), suggesting that formation of NO is possibly involved in the brain injury during and after HI insults. Because a major action of NO so formed is to stimulate the enzyme guanylate cyclase and thus trigger cGMP synthesis, cGMP levels could be taken as an indirect measurement of NO formation (21). The objective of the present study is to examine the chronic effects of *in utero* HI occurring at a relatively early gestational stage on the functional changes in the glutamate neurotransmission system.

METHODS

Chemicals. Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). [^3H]cGMP assay kit was obtained from Amersham Corp. (Arlington Heights, IL).

Animal surgery. Pregnant Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) at 17 d of gestation were used for the *in utero* HI surgery. Under anesthesia induced by isoflurane (4% induction, 1% maintenance), an abdominal midline incision was performed, and the uterine horns were exposed. The HI conditions were achieved by complete clamping of the uterine vasculature for designated time periods followed by removal of the clamps to permit reperfusion. Sham operation (surgery without vasculature clamping) was conducted in the control groups. After surgery, the uterine horns were returned to the dam's abdomen to let the pups deliver naturally. One day after the pups were delivered, the litter size was culled to nine (in some batches, 10). Cerebella from the pups at 8 d of age were used for neuronal cell cultures.

For determination of fetal brain lactate levels during HI, only blood vessels leading to one uterine horn were clamped in some surgeries, and the fetuses in the other uterine horn served as the sham-operated group. Immediately after the designated time periods of HI insult, fetal brains were collected within 10 s after decapitation and kept on dry ice until the brain lactate levels were measured the same day.

Due to the small size of the fetuses at 17 d of gestation, it was difficult to collect the fetal blood samples. We conducted the surgery on some rats at 20 d of gestation. Fetuses were

delivered immediately after the HI insult, and blood samples were collected directly from the fetal heart. Fetal blood pH was measured by a pH/blood gas analyzer (Instrumentation Laboratory, Lexington, MA).

Cell culture. Cerebellar granule cells were cultured as described by McCaslin and Ho (25) with minor modifications. Six milliliters of dissociating medium per rat pup cerebellum were used. Cells were grown in Dulbecco's modified Eagle's medium containing 12% defined/supplemented bovine calf serum (Hyclone, Logan, Utah), 4 mM glutamine, 25 mM glucose in the absence of antibiotics on glass slides coated with poly-L-lysine at 37°C under 10% CO_2 /90% room air. Fresh medium containing cytosine arabinoside (5 μM) and KCl (25 mM) was added 24 h later. Cells grown 8 d *in vitro* were used for the cGMP measurement and 11–15 d *in vitro* cells for the glutamate cumulation measurement.

Lactate determination. Fetal brain lactate levels were determined following the methods described by Magal *et al.* (7) with minor modification. Briefly, brain tissues were homogenized in two volumes of 20 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM DTT, and two volumes of cold perchloric acid (8%) were added to one volume of the homogenate. The mixtures were centrifuged at $1500 \times g$ for 10 min, and aliquots of 0.1 mL of the supernatant were analyzed by the lactic dehydrogenase enzymatic method using a kit from Sigma Chemical Co. in the presence of excessive NAD.

Determination of cGMP accumulation. The culture medium was removed from cells by three washes with a physiologic balanced salt buffer containing the following mM concentrations: 154 NaCl, 5.6 KCl, 2.5 CaCl_2 , 10 glucose, 40 bicarbonate, and 5 HEPES (pH 7.4; PSH buffer). The cells were placed in 4 mL of PSH buffer and preincubated at 37°C for 20 min before the addition of agonists. Three minutes after addition of stimulants, the reaction was terminated by aspirating the incubation buffer, and cells were harvested in 0.35 mL of hot 1 M perchloric acid (100°C). The cell suspension was sonicated and centrifuged at $4500 \times g$ for 2 min. The supernatant was neutralized with potassium carbonate (2 M) and assayed for cGMP by using the [^3H]cGMP assay kit from Amersham. The resulting pellet was dissolved in 0.4 N NaOH for measuring cellular protein by the method of Bradford (26).

Accumulation of extracellular glutamate. In determination of glutamate accumulation, cells were washed and preincubated in a similar manner as those used for cGMP determination except that 100 mM sucrose was added to the buffer to prevent EAA agonist-induced cell death. In this way, only the agonist-induced amino acid accumulation, not the release as the result of cell death, was measured (27). After addition of receptor agonists, cells were incubated at 37°C for 1 h. Culture buffer was then collected for the determination of amino acids secreted into the buffer, and cells were harvested with 0.35 mL perchloric acid for protein measurement. Amino acid content was separated and quantified by an HPLC method as described by Ellison *et al.* (28).

Statistics. Data of measured variables from various HI groups stimulated with the same receptor agonist were subjected to conventional one-way ANOVA. Duncan's multiple comparison was used to determine the significance of differ-

ences between groups when one-way ANOVA indicated a significant F value. The level of statistical significance was set at $p < 0.05$.

RESULTS

Clamping of uterine vasculature in the present study resulted in a severe HI insult as indicated by the increase of brain lactate levels correlating with the duration of blood flow restriction (Fig. 1a). Although sham operation up to 1 h also caused a slight increase of fetal brain lactate levels (0.92–1.51 mg/g of brain), the lactate levels in HI fetal brains were significantly higher than the corresponding sham-operated group 5 min after blood flow restriction (1.32–2.74 mg/g of brain). Consistent with the lactate level data, fetal blood pH in the HI groups was significantly lower than in the sham-operated groups (Fig 1b).

The *in utero* HI insult up to 1 h did not affect fetal survival rate significantly, although the number of stillbirths in the 1 h HI group was slightly more than in the other groups. As shown

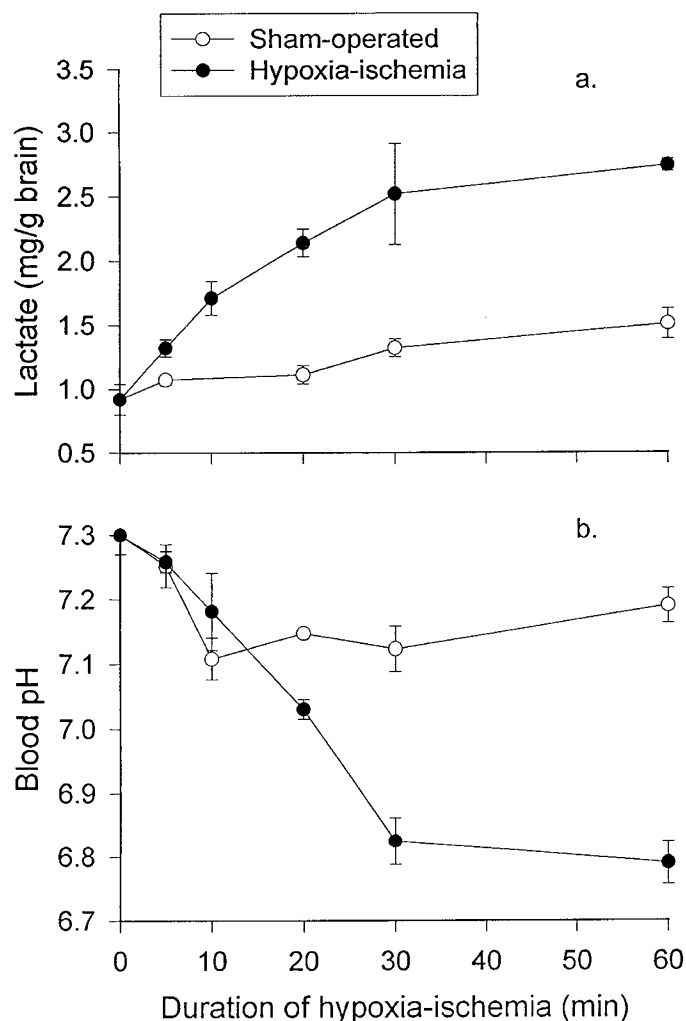


Figure 1. Fetal brain lactate levels (a) and blood pH (b) during intrauterine HI for various duration. The HI conditions were achieved as described in the footnote of Table 1 and in the text. Only one uterine horn vasculature was clamped for designated time and the other horn served as the sham operation. Each point represents the mean \pm SEM of at least three samples. Data were analyzed by an ANOVA followed by the Duncan's multiple range test.

in Table 1, the mean body weight of the pups at 8 d of age slightly decreased with the increase of HI duration, but the differences in body weight among the sham-operated group and the various HI groups (up to 35 min) were not statistically significant. Significantly retarded growth of fetuses or newborns was only observed in the 1 h HI group (13.9 versus 16.1 g).

The cultural cell densities were not significantly different among intact, sham-operated and up to 35 min HI groups (0.5×10^6 /slide). Cell cultures from the 1 h HI group had lower densities (0.3×10^6 /slide), probably due to the small body size. Therefore, only the cultures with similar cell densities were used for cGMP and extracellular glutamate measurements.

The basal level of cGMP in cells cultured from various groups was 7.4 pmol/mg protein and it was not affected by *in utero* HI (Fig. 2). Upon the application of NMDA or KA (100 μ M, each), the cGMP level in cells from the intact group was increased by 7- and 4-fold, respectively. The NMDA-induced elevation of cGMP was further augmented in HI groups. The NMDA-induced cGMP formation in the 10–35-min HI groups was significantly higher than in the sham-operated group. Antenatal HI for 10–20 min, but not 25–35 min, also augmented the KA-stimulated elevation of cGMP levels.

To further ascertain whether NO formation is involved in the NMDA receptor-mediated cGMP production and the augmentation of cGMP formation by HI, we measured NMDA-induced cGMP in the presence of L-NMMA, a NO synthase inhibitor, in a separate experiment. As shown in Fig. 3, NMDA-induced cGMP formation was inhibited by increasing concentrations of L-NMMA. In the presence of 150 μ M L-NMMA, NMDA-induced cGMP increase was completely blocked in both control and 25-min HI groups. The blockade of cGMP increase by 10 μ M L-NMMA was reversed by simultaneous application of 1 mM L-arginine in both groups. In the presence of L-NMMA, the augmentation of cGMP formation by intrauterine HI disappeared.

The baseline level of extracellular glutamate was low and stable in control and various HI groups as presented in Table 2,

Table 1. Effects of *in utero* HI on pup's body weight (g) at 8 d of age

Duration of antenatal HI (min)	Litter size	
	9	10
Intact	17.3 \pm 0.3 (18)	15.5 \pm 0.6 (20)
Sham-operated	16.1 \pm 0.3 (18)	14.6 \pm 0.2 (30)
5	16.9 \pm 0.2 (18)	14.5 \pm 1.2 (10)
10	—	14.3 \pm 0.5 (20)
20	15.0 \pm 0.5 (18)†	14.4 \pm 0.5 (20)
25	—	14.3 \pm 0.3 (20)
35	14.9 \pm 0.4 (18)†	13.7 \pm 0.3 (30)†
60	13.9 \pm 0.5 (27)*	—

On gestation d 17, intrauterine HI conditions were achieved by complete clamping of the uterine vasculature for designated time periods followed by removal of the clamps to permit reperfusion. Sham operation (surgery without vasculature clamping) was conducted as the control. The uterine horns were returned to dam's abdomen and pups were born naturally. One day after birth, litter size was culled to nine or 10. Values are the mean \pm SEM of (n) pups.

* $p < 0.05$ from the value for the sham-operated group.

† $p < 0.05$ from the value for the intact group.

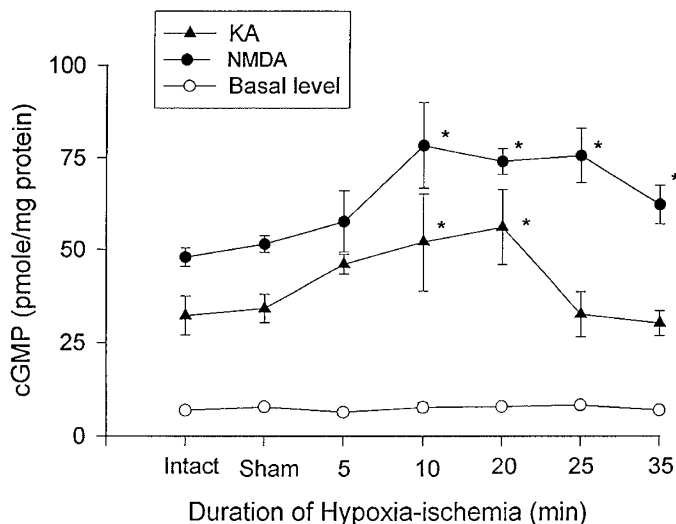


Figure 2. cGMP induced by NMDA or KA ($100 \mu\text{M}$, each) in rat cerebellar granule cell cultures from pups suffered from various durations of intrauterine HI insults. Cells, 8 d *in vitro*, were stimulated with NMDA or KA in a physiologically balanced HEPES buffer for 3 min. Each point represents the mean \pm SEM of at least five samples. Data were analyzed by a one-way ANOVA followed by the Duncan's multiple range test. * $p < 0.05$ from the value for the sham-operated group stimulated with the same agonist.

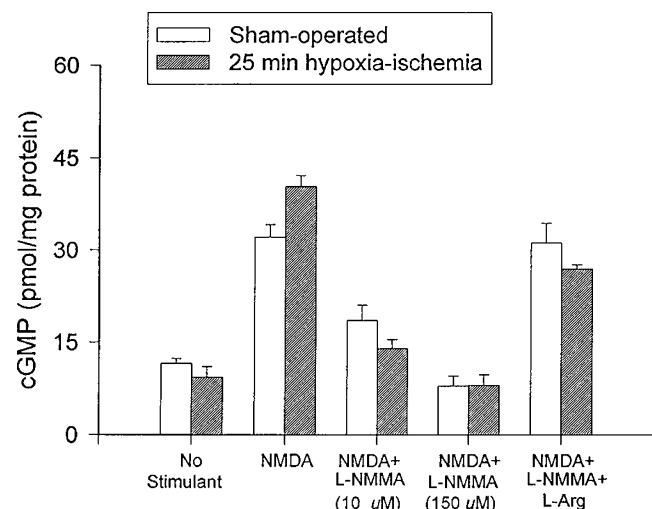


Figure 3. cGMP formation induced by NMDA ($100 \mu\text{M}$) in the presence or absence of L-NMMA and L-arginine (L-Arg, 1 mM) in rat cerebellar granule cells cultured from the pups experiencing 25 min intrauterine HI or sham operation. Each bar represents the mean \pm SEM of at least five measurements.

apparently through a reuptake mechanism. Stimulation with NMDA or KA ($100 \mu\text{M}$, each) selectively increased glutamate (but not other amino acids) accumulation by 2–3-fold in the sham-operated group. The NMDA-stimulated, but not the KA-stimulated, increase in glutamate accumulation was further augmented in the 20 and 35 min HI groups by 50–60%. As shown in Fig. 4, simultaneous application of $150 \mu\text{M}$ L-NMMA reduced NMDA-induced glutamate accumulation in the 35 min HI group. However, addition of L-NMMA did not affect the KA-induced elevation of glutamate accumulation.

DISCUSSION

Our major observation in this study is that an *in utero* HI insult occurring in the last half of gestation in rats results in an augmentation in NMDA-induced cGMP formation and glutamate accumulation in cultured cerebellar granule cells of the rat progeny. This observation implies that the HI occurring at an early gestational period may have long-lasting effects on EAA neurotransmission and brain development in perinatal life.

The experimental *in utero* HI rat model in the current study is modified from previous models used for studying intrauterine growth retardation (29, 30). The current method avoids a long period of severe anoxic conditions for all the fetuses owing to the placental blood vessel ligation as adopted in the previous models and provides a possibility of investigating long-term effects of intrauterine HI during postnatal brain development. The tissue lactic acid level is one of the most widely used parameters to indicate the magnitude of hypoxic-ischemic insults (31). The time course of brain lactate accumulation in the present study (Fig. 1) is comparable to that reported by Magal *et al.* (7) in a near term fetal rat ischemia model. It is noticeable that the brain lactate levels in the sham-operated group were also increased with the duration of surgical operation. The increase in lactate levels in sham-operated groups appeared to be due to maternal hypoventilation resulting from anesthesia. Inasmuch as no significant differences in receptor-stimulated cGMP elevation and glutamate accumulation were observed between intact and sham-operated groups, hypoxia resulting from anesthesia contributed little, if any, to the augmentation of NMDA-induced cGMP and glutamate accumulation in HI groups.

Although the role of the activation of glutamate receptors, especially the NMDA receptors, in HI associated neuron death and brain damage has been extensively studied as one of the important mechanisms (1, 14–18), information about the functional changes of the receptor itself after HI insults is sparse. In a recent study, an increased sensitivity of the NMDA receptor to activation owing to intrauterine hypoxia was reported in fetal guinea pig brain (32). The increased sensitivity of the NMDA receptor to activation was indicated by a greatly enhanced ligand binding affinity and basal activation of NMDA receptor, as well as its increased activation regulated by spermidine (32). As another example of the increased glutamate receptor sensitivity after hypoxic insults, perinatal HI has been found to enhance quisqualate-stimulated phosphoinositide turnover in rat brain slices (33). Consistent with these results, we found in the current study that intrauterine HI insults caused hypersensitive responses in NMDA-stimulated cGMP formation and glutamate accumulation during the postnatal period. This functional alteration may contribute to the HI brain dysfunction seen in perinatal life. The NMDA receptor has important roles in long-term potentiation and the learning and memory process. Recent studies have provided evidence that NO production inhibits the glutamate receptor, especially the NMDA receptor activation (34–36) by a negative feedback system (35). In the current study, the increased production of NO in the HI cells, as indicated by the increased formation of NMDA-induced cGMP (Fig. 2), may in turn cause suppression

Table 2. Extracellular amino acid concentrations (nmole/mg protein)

HI duration	Intact	Sham	5 min	20 min	35 min
Glutamate					
Basal	8.8 ± 0.8	8.0 ± 1.5	6.4 ± 0.9	6.5 ± 1.8	9.7 ± 0.6
NMDA-induced	19.4 ± 0.9	18.5 ± 0.7	18.2 ± 1.3	30.9 ± 2.8*	27.0 ± 1.7*
KA-induced	25.6 ± 0.9	25.8 ± 1.9	19.9 ± 0.7	26.4 ± 1.9	25.0 ± 3.0
Alanine					
Basal	51.4 ± 6.0	57.3 ± 3.4	51.5 ± 4.5	64.4 ± 8.3	54.6 ± 4.1
NMDA-induced	51.7 ± 4.4	57.5 ± 6.4	61.9 ± 4.9	66.0 ± 8.6	64.3 ± 7.8
KA-induced	57.3 ± 5.6	49.4 ± 2.3	47.6 ± 6.0	57.2 ± 3.7	54.6 ± 7.4
Glycine					
Basal	34.0 ± 2.2	40.2 ± 2.8	35.4 ± 3.0	42.0 ± 4.5	33.8 ± 2.1
NMDA-induced	39.2 ± 1.7	39.5 ± 2.4	40.1 ± 3.1	38.8 ± 5.0	44.6 ± 2.0
KA-induced	33.7 ± 1.8	39.1 ± 4.2	46.8 ± 5.1	42.6 ± 3.3	35.1 ± 4.2
Histidine					
Basal	7.9 ± 0.4	9.4 ± 0.5	8.2 ± 0.7	10.1 ± 1.5	8.0 ± 0.7
NMDA-induced	9.1 ± 0.6	9.1 ± 0.9	10.4 ± 0.9	11.5 ± 0.7	10.5 ± 0.6
KA-induced	7.3 ± 0.7	9.3 ± 0.9	9.2 ± 0.6	9.9 ± 0.6	8.8 ± 1.2

Extracellular amino acid concentrations were determined in cerebellar granule cells cultured from 8-d-old pups suffering from various durations of intrauterine HI insults. Cells, 11–15 d *in vitro*, were stimulated with NMDA or KA, 100 μ M each, in a physiologically balanced HEPES buffer added with 100 mM sucrose at 37°C for 1 h. Amino acid contents were separated and quantified by HPLC. Values are mean \pm SEM of at least five measurements. Data were analyzed by a one-way ANOVA followed by Duncan's test.

* $p < 0.05$ from the value for the sham-operated group stimulated with the same agonist.

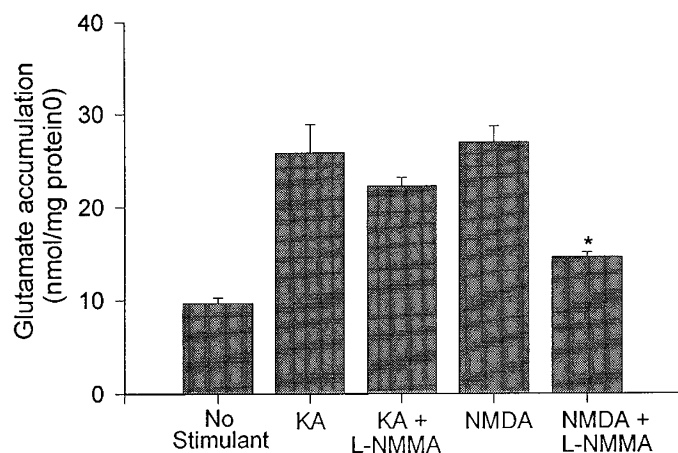


Figure 4. Extracellular glutamate accumulation induced by NMDA or KA (100 μ M each) in the presence or absence of L-NMMA (150 μ M) in rat cerebellar granule cells cultured from the pups suffering from 35 min of intrauterine HI insult. Experiments were performed as described in the footnote of Table 2 and in the text. Each bar represents the mean \pm SEM of at least five measurements. * $p < 0.05$ from the glutamate accumulation stimulated with NMDA alone by a *t* test.

of NMDA receptor-mediated neurotransmission and affect the learning and memory process. NO production has also been found to enhance NMDA receptor-mediated release of glutamate in guinea pig cerebral cortex (37). This finding is supportive to our observations that the NMDA-induced glutamate accumulation in HI cells was 50% higher than in sham-operated cells. The augmentation of NMDA-induced glutamate accumulation in HI cells can be attributed to the increase in NO production, as indicated by the cGMP formation, although blockade of uptake mechanism may also cause increase in extracellular glutamate accumulation, as done by KA (38). In the current study, NMDA- and KA-induced glutamate accumulation seems to occur by different mechanisms, because HI insults only augmented NMDA-induced glutamate accumulation and L-NMMA could block NMDA-induced but not KA-

induced glutamate increases. Nevertheless, because excessive glutamate is neurotoxic (14–16), the increased accumulation of NMDA-induced glutamate in HI groups may make the brain more vulnerable to glutamate neurotoxicity in EAA neurotransmission.

The mechanism underlying the hypersensitive responses in cGMP formation and glutamate accumulation upon NMDA receptor activation in antenatal hypoxic-ischemic cells is unclear at present time. Although reduced NMDA receptor number and [3 H]glutamate binding site measured immediately or 24 h after HI insults have been reported in fetal guinea pig brain (32) and neonatal rat brain (39), the availability of an increased number or higher affinity of NMDA receptors in the antenatal hypoxic-ischemic cells remains one of the possible mechanisms. The chronic changes after intrauterine HI insults may differ from the responses measured right after the insults. This possibility is currently under investigation in our laboratory by using the specific radioactive receptor binding ligand. Another question regarding our results is raised. It is known that in the rat cerebellum, neuronal synaptosomal clefts and EAA receptors develop postnatally (40). In the present study, the intrauterine HI insults occurred on gestation d 17, when the external germinal layer from which the granule cells derive just begins to develop (41). How do the antenatal insults affect postnatal brain receptor functional development? Perinatal HI brain injury has been found to stimulate glial fibrillary acidic protein mRNA and protein expression in neonatal rats (42). The investigators found that the increased glial fibrillary acidic protein mRNA was not detected in lesioned hippocampus and striatum until postnatal d 12, whereas the hypoxic-ischemic injury occurred on postnatal d 7. It is possible that the long-lasting effect of intrauterine HI on neurons placed in cultures are mediated by modifying the gene expression for NMDA receptor subunits that have been reported to be altered after forebrain ischemia in adult rats (43). To test this possibility, further investigations are obviously needed and are planned.

In summary, intrauterine HI enhances NMDA receptor-stimulated cGMP formation and glutamate accumulation in rat cerebellar granule cell cultures and NO production may be involved in the altered responses of NMDA receptor activation. This antenatal HI experimental model may be used for studying the chronic effects of intrauterine asphyxia on brain development.

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