The Neuroprotective Actions of a Calcium Channel Antagonist, Flunarizine, in the Infant Rat

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ABSTRACT. One postulated final common pathway leading to neuronal death after hypoxic-ischemic insults is an increase in intracellular calcium concentrations. We examined the effect of pretreatment with flunarizine, a calcium channel antagonist known to pass the blood brain barrier, on the behavioral and histologic changes after an hypoxic-ischemic insult in the infant rat. The 21-d-old rats were subjected to unilateral carotid ligation, then to 2 h of hypoxia. They were pretreated with either flunarizine (30 mg/kg, intraperitoneally) or with an equal volume of diluent. After 5 days of observation they were killed for histology. Acute behavioral abnormalities were observed in more controls than treatment animals, 52 vs 11% ($p < 0.002$). Cerebral injury was almost entirely confined to the ligated side and was significantly worse in the control rats. Full thickness cortical infarction was noted in 56% of controls ($n = 27$) vs 4% of flunarizine-treated rats ($n = 24$), ($p < 0.001$). Mean and maximum damage scores for all areas assessed including cortex, corpus striatum, thalamus, amygdala, and hippocampus were improved markedly in treatment rats ($p < 0.005$). These observations confirm that flunarizine, when given prophylactically, has a neuroprotective effect against hypoxic-ischemic injury in the developing brain. (*Pediatr Res* 25:573-576).

MATERIALS AND METHODS

Experimental preparation. The 21-d-old Wistar rats were anaesthetised with a 2% halothane/O₂ mixture. In each rat one carotid artery was exposed and ligated with 6.0 silk sutures. The rats were removed from anaesthesia and then received, by intraperitoneal injection, either flunarizine (Janssen Pharmaceutica, Beerse, Belgium), 30 mg/kg or an equal volume of the diluent, 10% ethanol, 1.5 ml/kg, given in equally divided doses. The first dose was given immediately after surgery, the second immediately before the hypoxia, 2 h later.

Each rat was next subjected to an hypoxic environment of 8% O₂ and 2% CO₂ [to prevent hypocapnia induced vasospasm (24)], maintained at 37° C, for 120 min. The pups were then returned to their cages, with their litter mates. Daily after the hypoxia each rat was behaviorally tested by a blind, independent observer (LB), who scored each for the presence of five possible abnormalities: fits, absence of startle response, abnormal turning, impaired righting, and impaired response to tail pinch (23, 25). On the day of death a further scoring for residual abnormalities was made. The presence of definite unilateral weakness was scored as 2, whereas other behavioral problems such as excessive or reduced activity or persistent piloerection were scored as 1. Death was by the injection of pentobarbital. These experiments were approved by the Animal Ethical Committee of the University of Auckland.

Pilot study. This study included the two main groups. 1) Flunarizine pretreated, with ligation and hypoxia, the treatment group ($n = 5$). 2) Ethanol pretreated, with ligation and hypoxia, the control group ($n = 9$). Four groups of sham animals were also studied. 1) Flunarizine, with hypoxia, but sham ligation ($n = 5$); 2) ethanol, with hypoxia, but sham ligation ($n = 9$); 3) ethanol, no hypoxia, with ligation ($n = 4$); 4) ethanol, no hypoxia, sham ligation ($n = 5$).

Behavioral testing was performed for 10 days. The brains were snap frozen after death on dry ice. Coronal sections were cut at 40 μ M and every fourth section stained with cresyl violet. Hemispheric diameter was compared between the ligated and the unligated side at the level of the anterior commissure, and the larger of the two sides noted. Neuronal density in a randomly selected area of the corpus striatum (1.638×10^{-8} M²) in 5 to 10 sections per brain was calculated. Each neuron was counted as undamaged only if the cell structure was entirely intact.

Main study. Flunarizine pretreated ($n = 32$) and 10% ethanol pretreated ($n = 40$) groups of infant rats, subject to the Levine procedure as above, were compared.

Each rat was behaviorally tested daily for 5 days after the hypoxia for the five possible abnormalities. At death the brains were perfused through the left cardiac ventricle with normal saline followed by 10% Formaldehyde then 10% Formaldehyde/10% sucrose. The brains were embedded in paraffin, coronally

Perinatal hypoxic-ischemic encephalopathy is associated with a significant mortality and increased risk of cerebral palsy (1). The majority of cases appear to be due to antepartum or intrapartum insults (2). Recently attention has focused on the role of intracellular calcium accumulation as one common mechanism by which asphyxia leads to neuronal death (3, 4). Agents that inhibit calcium flux may therefore be neuroprotective. Although many studies have examined this hypothesis in adult species (5-19), only two previous studies have examined the protective effect of calcium antagonists in immature animals (20, 21). We studied the behavioral and histologic outcome in infant rats using a model of unilateral carotid ligation followed by hypoxia, originally developed by Levine (22) with some later modifications (23), and investigated the protective effects of a calcium channel antagonist, flunarizine. After a pilot study a larger study was performed using more sensitive histopathologic procedures.

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subserially sectioned to 10 μ M, and then stained with thionin/acid fuchsin (26). Each section was examined by light microscopy. The presence or absence of full thickness cerebral infarction was noted at low power (25 $\times$), then each region was assessed under a magnification of 400 $\times$. Neurons with ischemic cell change as described by Brown and Brierley (27), with acidophilic (red) cytoplasm and contracted nuclei or with just a thin rim of red cytoplasm with pyknotic nuclei were assessed as dead whereas all others were considered viable (26, 27). Each region was scored for the proportion of dead neurones. The average value from four sections was used in all cases except for the lateral frontoparietal cortex where the average of four areas per section and four sections, spaced from anterior to posterior, were used. The damage scores were as follows: 0 = no dead neurones, 1 = >0–25%, 2 = >25–50%, 3 = >50–75%, 4 = >75–100% of neurones were dead.

Data analysis. Damage and behavioral scores were analyzed by two-way analysis of variance for litter and treatment group. *t* tests were performed to test differences between subgroups. Survival, the incidence of behavioral problems and of cerebral infarction were examined by χ^2 test, except when the numbers were low, where Fishers exact test was used. All values are expressed as mean $\pm$ SD.

RESULTS

Pilot Study. Behavioral testing. No sham controls ($n = 23$) or flunarizine rats ($n = 5$) had acute behavioral changes whereas eight of nine true controls (subject to hypoxia and ligation) showed significant abnormalities ($p < 0.01$). A consistent pattern was seen of acute disturbances that were worse in the 24 h after the insult and gradually resolving over 2 to 3 days with only mild unilateral weakness still apparent by 10 days.

Histology. Hemispheric size was symmetrical in all flunarizine-treated ($n = 4$) and sham rats ($n = 20$) but in six of eight control rats the hemisphere on the ligated side was smaller ($p < 0.05$). The density of intact neurons in the corpus striatum was markedly reduced on the ligated side vs the nonligated side (1.34 ± 0.76 vs 2.29 ± 0.91 , $\times 10^8$ neurons/ M^2) in the controls ($n = 8$, $p < 0.005$) but not in the flunarizine treated animals ($n = 4$, NS), (1.92 ± 0.61 vs 2.04 ± 0.70 , $\times 10^8$ neurons/ M^2) or in eight sham animals.

Main Study. A total of 77.5% of controls ($n = 40$) and 84% of flunarizine ($n = 32$) pretreated rats survived (NS). The majority of those who died did so during the hypoxia, and there were no deaths more than 12 h after cessation of the hypoxia.

Behavioral testing. The same pattern of acute and residual abnormalities was seen as in the pilot study. Acute problems included both generalized fits and focal motor convulsions with secondary generalization. The righting response was frequently absent or greatly slowed, although many circled repeatedly in one direction, usually away from the side of ligation although a few had an increased tendency to turn in that direction. Only one (control) animal showed an abnormal startle response and all responded to a tail pinch. Acute motor problems were less frequent ($p < 0.002$) in flunarizine pretreated animals (11%, $n = 27$) than in control animals (51.6%, $n = 31$). These resolved progressively and persisted at 5 days in only one control rat. As is shown in Figure 1 the mean duration of each of the three motor problems: fitting, abnormal circling, and impaired righting, was reduced in the flunarizine rats ($p < 0.005$).

Once the acute disturbance had resolved, residual deficits were more frequent in controls than flunarizine-treated animals. A total of 42% of controls vs 4% of flunarizine rats showed a unilateral palsy contralateral to the ligation ($p < 0.001$); other problems were infrequent. The acute and residual scores were highly intercorrelated ($r^2 = 0.69$, $p < 0.00001$).

Histology. Cerebral injury ranged from no damage, to laminar loss in layer 3 of the lateral cortex or selective loss in the CA1/2 region of the hippocampus, through to gross infarction. Only 4%

($n = 27$) controls vs 29% ($n = 24$) flunarizine animals had absolutely no dead neurons in any section examined ($p < 0.02$). When rats with less than 5% neuronal death in at most two regions were considered with the normals as having trivial damage the difference is striking: 11% of controls vs 58% of flunarizine rats ($p < 0.001$) had trivial damage.

Full thickness infarction of the cerebral cortex was much more frequent in controls (55.5%) than in flunarizine pretreated rats (4%, $p < 0.001$). The mean damage scores were significantly less in the flunarizine-pretreated pups in every region examined (Fig. 2), with the reduction in damage varying by >80% in the cortex ($p < 0.0001$) and by >50% in the CA1/2 region of the hippocampus ($p < 0.006$). The lesser degree of improvement in hippocampal scores compared with cortex or striatum was significant ($p < 0.01$). Although ischemic damage was documented

Fig. 1. Number of days each motor abnormality was observed (mean $\pm$ SD) in controls ($n = 40$, black bars) and flunarizine pretreated rats ($n = 32$, open bars). * $p < 0.05$, ** $p < 0.005$.

Fig. 2. Mean damage scores (mean $\pm$ SD) for different cerebral regions comparing controls ($n = 31$, black bars), and flunarizine pretreated 21-d-old rats ($n = 27$, open bars). * $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$. FR-PA, fronto-parietal cortex; CA1-4 are regions of the hippocampus.

primarily ipsilateral to the ligation, eight rats had minor (grade 1 or 2) damage on the unligated side: 26% of controls vs 4% flunarizine rats ($p < 0.05$). Of these, the majority (6/8) had CA1/2 lesions.

DISCUSSION

Flunarizine, a difluorinated piperazine derivative, is a calcium channel antagonist binding to the dihydropyridine binding site (28). It crosses the blood brain barrier and is relatively selective for the cerebral circulation, having little effect on the heart or systemic vasculature (29). In the present study pretreatment with flunarizine was associated with a dramatic reduction in histologic damage. The acute and chronic behavioral abnormalities were significantly improved.

Two other studies have examined effects of calcium antagonists in immature animals. In 7-d-old rats, subject to the Levine model, 30 mg/kg of flunarizine limited morphologic injury (20). Our study confirms and extends this result with behavioral measures and more detailed histologic assessment. Improved short term (2 h) improvement in evoked potentials was found after 10–15 min of cardiac arrest in newborn sheep using 3 mg lidoflazine (21). Unfortunately that study is confounded by the use of multiple other agents including free radical antagonists, short follow-up, and the absence of histologic data.

A range of studies in mature animals suggests that flunarizine is as or more effective than other calcium antagonists in preventing asphyxial damage. Flunarizine but not verapamil is effective in preventing death due to cyanide induced cellular hypoxia in rats (7). Recovery was improved in mice and rats by its administration prehypoxia/anoxia (8) and pre- (9) but not post- (10) cardiac arrest in the dog. In the adult Levine rat, pretreatment with 10–40 mg/kg of flunarizine reduced parietal neuronal damage (11), whereas only the maximum dose of nicardipine was minimally protective and diltiazem was not (12). In a rat model of partial ischemia, both pre- and postinsult treatment markedly improved histologic outcome in the hippocampus (14), striatum, and cortex (15). In the dog after cardiac arrest, 0.1 mg/kg intravenously reduced neuronal death only in the hippocampus suggesting that higher doses may be needed for cortical protection (16).

The most direct explanation for the overall neuroprotective action of flunarizine is inhibition of calcium uptake by neurones. Both enhanced excitatory neurotransmitter activity and depolarization due to energy failure favor excessive calcium entry, which in turn may activate a series of damaging events such as protein and phospholipid degeneration and release of eicosanoids and kinins, leading to delayed neuronal death (3, 4). Calcium ionophores, by promoting calcium entry produce neuronal death (30), whereas calcium free media are protective (4). Flunarizine inhibits depolarization induced uptake of Ca^{++} into rat synaptosomes *in vitro* (31) and delays the fall in extracellular Ca^{++} in the hypoxic rat brain (32).

The overall histopathologic pattern of damage in the controls corresponds with the territory of the middle cerebral artery (33): damage appears to be localized primarily by a preferential fall in middle cerebral artery perfusion on the ligated side during hypoxia (24, 34). In the infant Levine rat blood flow is dramatically reduced on the ligated side 4 h after ligation but not 24 h later, suggesting rapid development of physiologic shunting (34). Flunarizine that enhances and prolongs reactive hyperemia during hypoxia (35) might accelerate this process, maintaining blood flow during the insult. As well it has a protective effect on endothelium (36), and prevented secondary expansion of cerebral thrombosis in rats (13).

Other possible mechanisms have been suggested. As with other calcium antagonists, flunarizine is an anticonvulsant (29, 38) and it has been reported to increase levels of the inhibitory purine adenosine by affecting re-uptake (38). Adenosine reduces neuronal hyperexcitability, and may be an endogenous anticonvul-

sant (39). Seizures may aggravate cerebral damage induced by asphyxia (40, 41). The effective anticonvulsant dose in rats is similar to that used in this study where the flunarizine-treated rats had significantly less posthypoxic fitting than the controls.

The behavioral changes seen here of transient abnormal turning, impaired righting, fits and residual hemiparesis, are similar to those documented in adult rats (25, 27). In contrast in 7-d-old rats subjected to the Levine model, only trivial behavioral abnormalities can be identified (23, 42), most likely due to the rapid resolution of acute behavioral disturbances and the delay before the acquisition of motor skills.

The perinatal period is one situation where it is possible to predict likely episodes of asphyxia. Thus antepartum prophylaxis using calcium channel antagonists may be a potential therapeutic approach (43). However, considerable experimental investigation of the safety of such an approach will be required before therapeutic trials are considered.

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