Oral Administration of Sildenafil Restores Learning Ability in Rats With Hyperammonemia and With Portacaval Shunts

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Patients with liver disease with overt or minimal hepatic encephalopathy show impaired intellectual capacity. The underlying molecular mechanism remains unknown. Rats with portacaval anastomosis or with hyperammonemia without liver failure also show impaired learning ability and impaired function of the glutamate-nitric oxide-cyclic guanine monophosphate (glutamate-NO-cGMP) pathway in brain. We hypothesized that pharmacological manipulation of the pathway in order to increase cGMP content could restore learning ability. We show by *in vivo* brain microdialysis that chronic oral administration of sildenafil, an inhibitor of the phosphodiesterase that degrades cGMP, normalizes the function of the glutamate-NO-cGMP pathway and extracellular cGMP in brain *in vivo* in rats with portacaval anastomosis or with hyperammonemia. Moreover, sildenafil restored the ability of rats with hyperammonemia or with portacaval shunts to learn a conditional discrimination task. In conclusion, impairment of learning ability in rats with chronic liver failure or with hyperammonemia is the result of impairment of the glutamate-NO-cGMP pathway. Moreover, chronic treatment with sildenafil normalizes the function of the pathway and restores learning ability in rats with portacaval shunts or with hyperammonemia. Pharmacological manipulation of the pathway may be useful for the clinical treatment of patients with overt or minimal hepatic encephalopathy. (HEPATOLOGY **2005;41:299-306.**)

Hepatic encephalopathy is a neuropsychiatric syndrome covering a wide range of neuropsychiatric disturbances ranging from minimal changes in personality or altered sleep-and-waking cycle to deep coma and death. Patients with liver cirrhosis with normal neurological or mental status examination results may have minimal forms of hepatic encephalopathy showing intellectual function impairment as revealed by neuropsychological testing.[1,2]

Hyperammonemia is considered one of the main factors responsible for the neurological alterations in hepatic encephalopathy,[3] and the classical clinical treatments are directed toward reducing blood ammonia levels. However, the molecular mechanisms by which hyperammonemia and liver failure lead to neurological alterations and to impairment of intellectual function remain unclear.

The molecular mechanisms involved in different types of learning are not well known. *N*-methyl-D-aspartate (NMDA) receptors are involved in some types of learning. Activation of NMDA receptors increases calcium in postsynaptic neurons. Calcium binds to calmodulin and activates neuronal nitric oxide (NO) synthase, increasing NO, which activates guanylate cyclase, increasing cyclic guanine monophosphate (cGMP), part of which is released to the extracellular space. Activation of this glutamate-NO-cGMP pathway may be involved in some forms of learning. Some recent reports indicate that guanylate cyclase and cGMP are important in learning and memory. Administration of a membrane permeant analog of cGMP facilitated memory consolidation,[4] whereas bilateral intrahippocampal administration of an inhibitor

Abbreviations: NMDA, N-methyl-D-aspartate; NO, nitric oxide; cGMP, cyclic guanine monophosphate.

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of guanylate cyclase caused amnesia for inhibitory avoidance when given immediately after training.[5] Blocking NMDA receptors with dizocilpine or inhibiting NO synthase impaired spatial working memory in mice, suggesting that the reduction in NO and cGMP production in brain may be responsible for dizocilpine-induced learning impairment.[6] These results support the idea that the glutamate-NO-cGMP pathway and cGMP modulate some forms of learning and memory.

Both liver failure and chronic hyperammonemia impair the function of the glutamate-NO-cyclic GMP pathway in rat brain *in vivo*.[7,8] Chronic hyperammonemia also impairs the ability of rats to learn a conditional discrimination task.[9]

The step of the glutamate-NO-cGMP pathway altered in rat models of hyperammonemia and hepatic encephalopathy is the activation of soluble guanylate cyclase by NO,[8,10] which is also altered in brains of patients who died with hepatic encephalopathy.[10]

We hypothesized that the alterations in the function of the glutamate-NO-cGMP pathway in the brain in hyperammonemia and liver disease may be responsible for the impairment in learning ability and intellectual function and that pharmacological modulation of the pathway may restore learning ability in hyperammonemia and hepatic encephalopathy.

The aim of this work was to reverse the impairment in learning ability of rats with portacaval anastomosis and of hyperammonemic rats without liver failure by pharmacological manipulation of the pathway in brain. We assessed whether the learning ability of rats with portacaval anastomosis or hyperammonemia may be restored by increasing cGMP by chronic oral administration of sildenafil, an inhibitor of cGMP-degrading phosphodiesterase. Tests of conditional discrimination learning were performed with control rats, rats with portacaval anastomosis, or hyperammonemic rats without liver failure treated or not with sildenafil.

Materials and Methods

Portacaval Anastomosis

Male Wistar rats were anesthetized with halothane and an end-to-side portacaval anastomosis was constructed as previously described.[11] At the moment of death, the liver was atrophic and the anastomosis was permeable. The rats were subjected to the Y-maze learning test 4 weeks after surgery.

Hyperammonemic Rats Without Liver Failure

Male Wistar rats (120-140 g) were made hyperammonemic by being fed an ammonium-containing diet, as previously described.[9] Animal models of hepatic failure (*e.g.*, portacaval shunt) show, in addition to hyperammonemia, other alterations (*e.g.*, loss of muscular mass and altered metabolism of other compounds in liver) that do not allow identification of the effects of hyperammonemia and of those resulting from other alterations. The model used in this work was developed to make available a rat model of pure hyperammonemia, reproducing the hyperammonemia present in patients with chronic liver disease (*e.g.*, liver cirrhosis) but without the other alterations. This model allows study of the contribution of hyperammonemia to the effects of liver failure and discernment of which effects are the result of hyperammonemia and which are the result of other factors. The model of hyperammonemia used was described in detail previously.[12] The rats were subjected to the Y-maze learning test after 4 weeks of treatment.

Administration of Sildenafil

Hyperammonemic Rats Without Liver Failure. Male Wistar rats were fed a normal diet (controls) or the diet containing ammonium acetate as indicated above for a period of 28 days before the initiation of the tests and were maintained on these diets during behavioral tests. Rats were divided in four groups (6 rats per group): two groups were fed the control diet and the other two groups were fed the ammonia-containing diet. One group of rats eating the control diet (control + sildenafil) and one group of hyperammonemic rats (ammonia + sildenafil) were treated daily with sildenafil (50 mg/L in the drinking water administered *ad libitum*). The treatment started 2 days before the learning test, and rats were allowed to drink always at the same time, 1 hour before the test. Rats were treated with sildenafil daily until death. For the other two groups (control group and ammonia group), the rats were treated in the same way with the tap water. Sildenafil was a gift from Pfizer, Inc. (New York, NY).

Rats With Portacaval Shunts. Four groups of 10 rats were used: the control group; the control + sildenafil group, the portacaval shunted rats group, and the portacaval shunted rats + sildenafil group. The treatment with sildenafil was initiated 28 days after surgery and was carried out as for hyperammonemic rats without liver failure.

The amount of sildenafil ingested by each group of rats is shown in Table 1.

Y-Maze Learning Test

Learning tests were initiated 2 days after the beginning of the treatment with sildenafil. Learning ability was tested as described previously[13] in a wooden Y-maze with three arms.[9] The whole area of the arms was covered by black or white inserts. In each trial, rats were rewarded for choosing the left arm when the inserts were black and the

Table 1. Ingestion of Sildenafil by the Rats of the Different Groups

	Ingestion (per 24 h)		
	Volume (mL)	Sildenafil (μg)	Sildenafil (μg/kg body weight)
Experiment with hyperammonemic rats without liver failure			
Control rats	19 ± 4	967 ± 208	4,509 ± 970
Hyperammonemic rats	19 ± 5	950 ± 233	5,138 ± 1,262
Experiment with rats with portacaval anastomosis			
Control rats (sham)	22 ± 5	1,082 ± 235	3,956 ± 781
Rats with portacaval anastomosis	20 ± 5	1,017 ± 255	4,658 ± 1,169

NOTE. The volume of water containing sildenafil and the total amount of sildenafil ingested by each rat were measured every day for 30 days starting 2 days before the beginning of learning tests. The value given is the mean of the 30 days. Body weight of each rat was determined every 3 to 4 days. Eight measurements were obtained during the 30 days. To calculate the ingestion of sildenafil per kilogram of body weight, we divided the total amount of sildenafil ingested in 24 hours by the mean of the eight determinations of body weight. In all cases, values are the mean ± SD of six rats per group. No significant difference was found in the ingestion of sildenafil between the different groups of rats.

right arm when they were white. The reward consisted of four food pellets placed in a cup at the end of the correct arm. Rats performed 10 trials per day, with an intertrial interval of 5 minutes in their home cage, until the completion of a required 10 correct responses in 10 consecutive trials.

Analysis of the Function of the Glutamate-NO-cGMP Pathway in Rat Brain by In Vivo *Brain Microdialysis*

Analysis of the function of the glutamate-NO-cGMP pathway in rat brain by *in vivo* brain microdialysis was carried out as described previously.[8] Animals were allowed to recover for 24 to 48 hours in the microdialysis bowl of the system for freely moving animals (BAS Bee-Keeper; Bioanalytical System, Lafayette, IN), with free access to food and water.

The day of the experiment, a microdialysis probe (CMA/12; 3 mm length, 500 μm outer diameter) was implanted carefully in the freely moving animal. The probes were perfused continually with artificial cerebrospinal fluid at a flow rate of 3 μL/min using a BAS Baby Bee microperfusion pump. The composition of artificial cerebrospinal fluid was (in mM): NaCl, 145; KCl, 3.0; $CaCl_2$, 2.26; buffered at pH 7.4 with 2 mM phosphate buffer, as described elsewhere[14]; however, we replaced $MgCl_2$ with $CaCl_2$ to avoid the blocking effect of Mg^{2+} on NMDA receptors. The artificial cerebrospinal fluid was filtered through 0.45-μm pore size Millipore filters (Millipore, Billerica, MA). The probes were dialyzed continuously for 2 to 3 hours. After this stabilization period, consecutive samples were collected every 30 minutes. NMDA was perfused through the microdialysis probe at the intervals indicated in the figure legends to activate the glutamate-NO-cGMP pathway. Samples were 4 mM in ethylenediaminetetraacetic acid and were stored at −80°C until determination of cGMP.

Determination of cGMP

cGMP was measured by using the BIOTRAK cGMP enzyme immunoassay kit from Amersham (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Determination of Ammonia

Ammonia concentration in microdialysis samples was measured as described previously.[15]

Statistical Analysis

Statistical analyses were performed by one-way ANOVA followed by the Newman-Keuls post test. A confidence level of 95% was considered to be significant.

All experiments were approved by the Fundación Valenciana de Investigaciones Biomédicas, Valencia, Spain, and were performed in compliance of the rules of the European Union concerning the use of laboratory animals.

Results

Treatment With Sildenafil Restores Learning Ability in Rats With Portacaval Anastomosis

Rats with a portacaval shunt showed a reduced learning ability (Fig. 1) and needed 96 ± 19 trials to learn a conditional discrimination task, which was significantly higher ($P < .001$; $F = 17.49$) than the number of trials needed by control rats (67 ± 8). Treatment with sildenafil restored the ability of rats with portacaval anastomosis to learn, reducing the number of trials required to 57 ± 11, which was significantly lower ($P < .001$; $F = 17.49$) than the number of trials for rats with portacaval anastomosis and was not different from the number of trials required by control rats. The number of trials for control rats treated with sildenafil (76 ± 10) was not significantly different from control rats drinking tap water.

Treatment With Sildenafil Normalizes the Function of the Glutamate-NO-cGMP Pathway and Extracellular cGMP in Rats With Portacaval Anastomosis

The effects of portacaval anastomosis and of treatment with sildenafil are shown in Fig. 2. The experiments were performed after 28 days of treatment with sildenafil. A microdialysis probe was inserted in the cerebellum, and

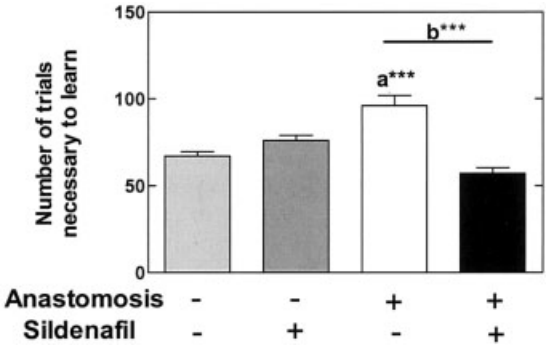

Fig. 1. Chronic administration of sildenafil restores learning ability in rats with portacaval anastomosis. Control rats and rats with portacaval anastomosis were subjected to the conditional discrimination learning test in the Y-maze as described in Materials and Methods. The tests were initiated after 2 days of treatment with sildenafil. Values are the mean ± SD of 10 rats per group and are given as the number of trials needed to learn. Values that are significantly different from controls are indicated by **a**. Values that are significantly different from rats with portacaval anastomosis are indicated by **b**. The statistical significance is indicated by asterisks: ***$P < .001$, $F = 17.49$ (one-way ANOVA after Newman-Keuls multiple comparison test). +, treatment with sildenafil, rats with portacaval anastomosis; −, control rats.

samples of the extracellular fluid were taken to measure cGMP.

The five initial fractions were taken before the rats were allowed to drink sildenafil or water. The concentration of

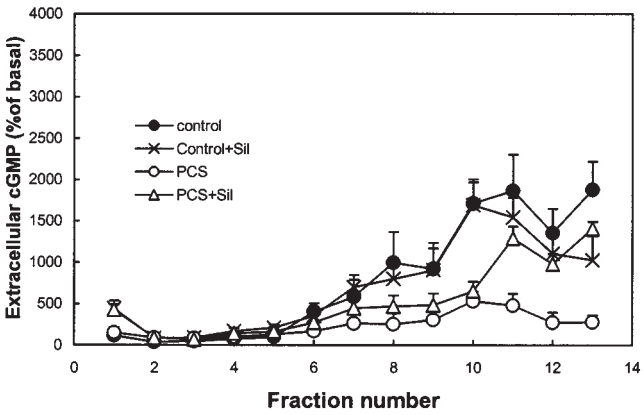

Fig. 2. Chronic administration of sildenafil restores the function of the glutamate- nitric oxide-cyclic guanine monophosphate (cGMP) pathway in rats with portacaval anastomosis. The function of the pathway was assessed by *in vivo* brain microdialysis in ccontrol rats and rats with portacaval anastomosis (PCS), as described in Materials and Methods. Perfusion was carried out at 3 μL/min, samples were collected every 30 minutes, and cGMP in the extracellular fluid was determined. Fractions 1 to 5 were taken before allowing the rats to drink. Drinking (water or sildenafil [Sil]) was allowed from the beginning of fraction 6. *N*-methyl-D-aspartate (0.3 mM) was administered in the perfusion stream for 20 minutes to activate the pathway at the beginning of fraction 9 (indicated by line). Data are presented as percentage of basal concentration of cGMP (mean of fractions 1–4). Values are the mean ± SE of six rats per group. ●, control rats; ×, control rats + sildenafil; ○, rats with portacaval anastomosis; △, rats with portacaval anastomosis + sildenafil.

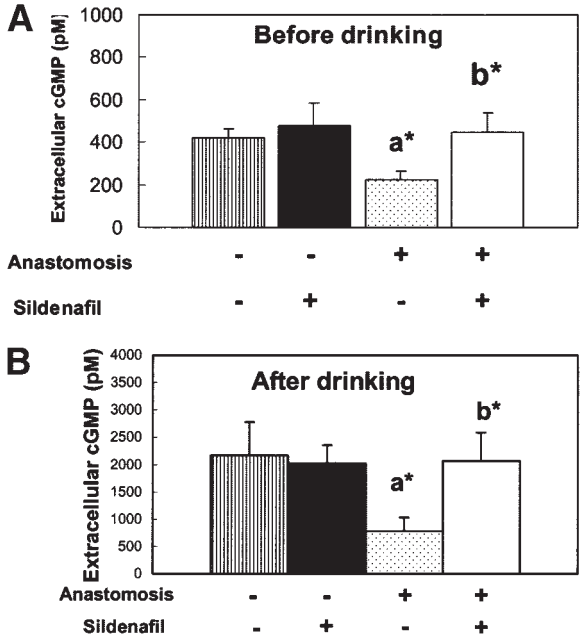

Fig. 3. Chronic treatment with sildenafil normalizes extracellular cyclic guanine monophosphate (cGMP) in rats with portacaval anastomosis. The experiments were the same as those shown in Fig. 2. (A) Basal concentration of extracellular cGMP before drinking was calculated as the mean ± SEM of the values for fractions 1 through 5. (B) Extracellular cGMP after drinking was calculated as the mean ± SEM of the values for fractions 6 through 9. Values are the mean ± SEM of six animals per group. Values that are significantly different from controls are indicated by **a**. Values that are significantly different from rats with portacaval anastomosis are indicated by **b**. The statistical significance is indicated by asterisks: *$P < .05$. +, treatment with sildenafil, rats with portacaval anastomosis; −, control rats.

cGMP in these samples is shown in Fig. 3A. The basal concentration of extracellular cGMP was significantly ($P < .05$; $F = 3.22$) reduced in the cerebellum of rats with portacaval anastomosis (226 ± 37 pM) compared with control rats (420 ± 43 pM). Treatment with sildenafil completely restored the extracellular level of cGMP in the cerebellum of the rats with portacaval anastomosis (446 ± 90 pM) and did not affect extracellular cGMP in the cerebellum of control rats (476 ± 105 pM).

After taking these five initial fractions, rats were allowed to drink sildenafil or water, starting at the beginning of fraction 6. As shown in Figs. 2 and 3B, drinking either water or sildenafil increased extracellular cGMP in all groups of rats, but the differences between the concentrations of cGMP in the cerebellum of the different groups of rats were maintained. For rats drinking tap water, the concentration of cGMP in rats with portacaval anastomosis (782 ± 257 pM) was significantly lower ($P < .05$; $F = 3.69$) than in control rats (2167 ± 617 pM). Treatment with sildenafil increased extracellular cGMP in rats with portacaval anastomosis, reaching a concentration similar to that of control rats drinking tap

water (2068 ± 519 pM). Treatment with sildenafil did not affect extracellular cGMP in control rats, remaining similar to rats drinking water (2026 ± 333 pM).

To assess the effect of treatment with sildenafil on the function of the glutamate-NO-cGMP pathway, NMDA receptors were activated by administering NMDA (0.3 mM) through the microdialysis probe for 20 minutes during fraction 10. As shown in Fig. 2, administration of NMDA activated the pathway and increased extracellular cGMP in the cerebellum in all groups of rats. The increase in extracellular cGMP was significantly lower in rats with portacaval anastomosis drinking water than in control rats. The increase in extracellular cGMP in the samples collected during the 90 minutes after application of NMDA reached 39-fold basal levels in control rats, but only 10-fold in rats with portacaval anastomosis, indicating a reduction of 74% in the function of the glutamate-NO-cGMP pathway.

Treatment with sildenafil significantly enhanced the function of the glutamate-NO-cGMP pathway in rats with portacaval anastomosis, reaching levels (29-fold increase) similar to that of control rats. The increase in extracellular cGMP in control rats treated with sildenafil (44-fold increase) was not significantly different from that of control rats drinking water (Fig. 2).

Treatment With Sildenafil Does Not Affect Ammonia Concentration in Extracellular Fluid

Ammonia was measured in the same microdialysis samples used to measure cGMP. The concentration of ammonia was significantly higher ($P < .01$; $F = 8.52$) in rats with portacaval anastomosis drinking water (218 ± 33 μM) or sildenafil (183 ± 13 μM) than in control rats drinking water (86 ± 15 μM) or sildenafil (108 ± 17 μM). Treatment with sildenafil did not affect ammonia concentration neither in control rats or portacaval shunted rats.

Treatment With Sildenafil Restores Learning Ability in Hyperammonemic Rats Without Liver Failure

Hyperammonemia is one of the main factors contributing to the neurological alterations in hepatic encephalopathy. To assess the role of hyperammonemia in the alterations in learning ability, extracellular cGMP, and the function of the glutamate-NO-cGMP pathway observed in rats with portacaval anastomosis, we carried experiments similar to those reported above using rats with chronic moderate hyperammonemia without liver failure.

Chronic hyperammonemia significantly reduced the ability of rats to learn the conditional discrimination task, increasing the number of trials required to learn from 58 ± 3 in control rats to 83 ± 6 in hyperammonemic rats

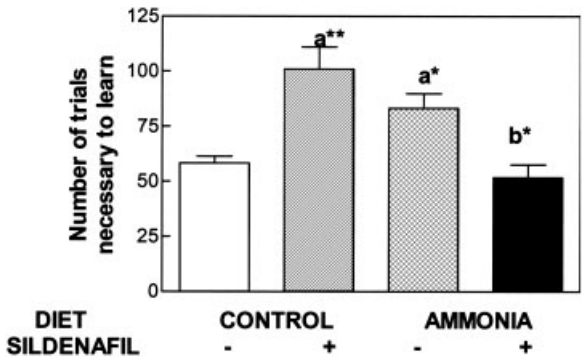

Fig. 4. Chronic treatment with sildenafil restores learning ability in hyperammonemic rats. Control and hyperammonemic rats without liver failure were subjected to the conditional discrimination learning test in the Y-maze as described in Materials and Methods. The tests were initiated after 2 days of treatment with sildenafil. Values are the mean ± SD of six rats per group and are given as the number of trials needed to learn. Values that are significantly different from controls are indicated by **a**. Values that are significantly different from hyperammonemic rats drinking water are indicated by **b**. The statistical significance is indicated by asterisks: *$P < .05$; **$P < .001$. +, treatment with sildenafil, rats with portacaval anastomosis; −, control rats.

(Fig. 4). Treatment with sildenafil restored the ability of hyperammonemic rats to learn the conditional discrimination test, decreasing the number of trials required to 52 ± 6, which was significantly lower ($P = .001$; $F = 10.89$) than for hyperammonemic rats and was not significantly different from that of control rats. The administration of sildenafil to control rats significantly increased the number of trials required to learn the test to 101 ± 10.

Treatment With Sildenafil Normalizes the Function of the Glutamate-NO-cGMP Pathway and Extracellular cGMP in Hyperammonemic Rats Without Liver Failure

The effects of hyperammonemia without liver failure and of treatment with sildenafil are shown in Fig. 5. The experiments were performed after 28 days of treatment with sildenafil. A microdialysis probe was inserted in the cerebellum, and samples of the extracellular fluid were taken to measure cGMP.

The five initial fractions were taken before the rats were allowed to drink sildenafil or water. The basal concentration of extracellular cGMP was significantly reduced ($P < .05$; $F = 9.84$) in the cerebellum of hyperammonemic rats (228 ± 30 pM) compared with that of control rats drinking water (396 ± 36 pM). Treatment with sildenafil increased basal extracellular cGMP in hyperammonemic rats (331 ± 32 pM), reaching levels that were not significantly different from those in control rats drinking water. Extracellular cGMP in control rats treated with sildenafil was 536 ± 72 pM, which was significantly higher than in control rats drinking water.

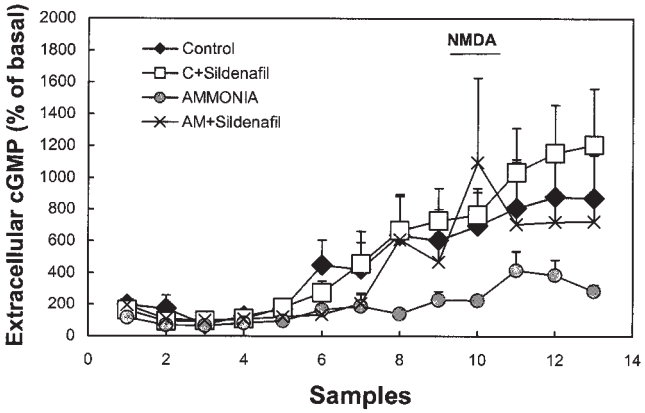

Fig. 5. Chronic administration of sildenafil restores the function of the glutamate- nitric oxide-cyclic guanine monophosphate (cGMP) pathway in hyperammonemic rats. The function of the pathway was assessed by *in vivo* brain microdialysis in ccontrol and hyperammonemic rats without liver failure as described in Materials and Methods. Perfusion was carried out at 3 μL/min, samples were collected every 30 minutes, and cGMP in the extracellular fluid was determined. Fractions 1 through 5 were taken before allowing the rats to drink. Drinking (water or sildenafil) was allowed from the beginning of fraction 6. *N*-methyl-D-aspartate (NMDA; 0.3 mM) was administered in the perfusion stream for 20 minutes to activate the pathway at the beginning of fraction 9 (indicated by line). Data are presented as percentage of basal concentration of cGMP (mean of fractions 1–4). Values are the mean ± SE of six rats per group. ◆, control rats; □, control rats + sildenafil; ●, hyperammonemic rats; ×, hyperammonemic rats + sildenafil.

After taking these five initial fractions, rats were allowed to drink sildenafil or water, starting at the beginning of fraction 6. As shown in Fig. 5, drinking either water or sildenafil increased extracellular cGMP in all groups of rats, but the differences between the concentrations of cGMP in the cerebellum of the different groups of rats were maintained. For rats drinking water, the concentration of cGMP in control rats was 624 ± 134 pM and was significantly lower in hyperammonemic rats (323 ± 46 pM). Treatment with sildenafil increased extracellular cGMP in hyperammonemic rats to levels similar to those of control rats drinking water (631 ± 110 pM). Treatment with sildenafil also increased extracellular cGMP in control rats (953 ± 186 pM).

To assess the effect of treatment with sildenafil on the function of the glutamate-NO-cGMP pathway, NMDA receptors were activated by administering NMDA (0.3 mM) through the microdialysis probe for 20 minutes during fraction 10. As shown in Fig. 5, administration of NMDA activated the pathway and increased extracellular cGMP in the cerebellum in all groups of rats. The increase in extracellular cGMP was significantly lower in rats with hyperammonemia drinking water than in control rats. The increase in extracellular cGMP in the samples collected during the 90 minutes after application of NMDA were 22-fold the basal levels in control rats and were significantly reduced to only sixfold in rats with hyperammonemia, indicating a reduction of 72% in the function of the pathway. The function of the pathway was significantly increased in hyperammonemic rats after treatment with sildenafil: cGMP increased 20-fold, which was not significantly different from control rats drinking water. The increase in cGMP was 29-fold in control rats treated with sildenafil (Fig. 5).

Treatment With Sildenafil Does Not Affect Ammonia Concentration in Extracellular Fluid

Ammonia was measured in the same microdialysis samples used to measure cGMP. The concentration of ammonia was significantly higher ($P < .001$; $F = 14.78$) in rats with hyperammonemia that drank water (220 ± 12 μM) or sildenafil (210 ± 17 μM) than in control rats that drank water (147 ± 27 μM) or sildenafil (127 ± 18 μM). Treatment with sildenafil did not affect ammonia concentration neither in control rats or hyperammonemic rats.

Discussion

Our results show that portacaval anastomosis reduces the ability of rats to learn a conditional discrimination task as well as the concentration of cGMP in the extracellular fluid and the function of the glutamate-NO-cGMP pathway in brain *in vivo*. Chronic treatment with sildenafil normalizes the function of the pathway, the extracellular concentration of cGMP, and the ability of rats to learn the task. These results indicate that the impairment in learning ability in rats with portacaval anastomosis is a consequence of the reduced cGMP formation and that learning ability may be restored by pharmacological manipulation of the glutamate-NO-cGMP pathway to increase cGMP.

The glutamate-NO-cGMP pathway shows similar patterns of activation in the hippocampus, striatum, and cerebellum.[16,17] Also in an acute paradigm, ammonia activates the pathway similarly in the cerebellum and striatum.[15,18] This study was performed in cerebellum because the expression of the pathway is high in this area, allowing more accurate quantitative determinations, which are more difficult to obtain in hippocampus (considered the main memory structure of the brain) because it has lower basal levels of extracellular cGMP. The changes in the function of the pathway and in extracellular cGMP in cerebellum may be considered a marker of the effects of sildenafil in other brain areas, including the hippocampus and striatum. Moreover, it should be noted that recent studies show that cerebellum also plays a role in spatial learning that is not the result of motor components but of cognitive components of spatial learning.[19] A

direct contribution of the glutamate-NO-cGMP pathway in cerebellum to modulation of learning therefore cannot be excluded.

As is the case for patients with chronic liver disease, rats with portacaval anastomosis present a series of alterations that include hyperammonemia, loss of muscular mass, liver atrophy, and other alterations in liver-dependent, ammonia-independent parameters such as metabolization of compounds reaching the liver from gut, and so forth. Hyperammonemia is considered the main factor contributing to the neurological alterations in hepatic encephalopathy.[3] To assess whether hyperammonemia is responsible for the impairment of learning ability in rats with portacaval shunts, we also used an animal model of pure hyperammonemia without liver failure that presents hyperammonemia but not the other alterations associated with chronic liver failure.

The results reported confirm previous reports showing that, as is the case for rats with portacaval anastomosis, rats with hyperammonemia without liver failure also show reductions in learning ability,[9] extracellular cGMP, and function of the glutamate-NO-cGMP pathway.[8] Moreover, we show herein that chronic treatment of these rats with sildenafil also restores the function of the pathway, extracellular cGMP, and learning ability.

The fact that rats with portacaval anastomosis or with hyperammonemia without liver failure show the same alterations in the function of the pathway, extracellular cGMP, and learning ability indicates that hyperammonemia, which is the only common alteration in both models, is responsible for the alteration in the function of the pathway and, subsequently, of the impairment of learning ability. The effect of hyperammonemia on the function of the pathway may be a direct effect of ammonia or an indirect effect mediated by other ammonia-induced alterations. For example, Jones et al. showed that activation of $GABA_A$ receptors reduces the function of the glutamate-NO-cGMP pathway in cerebellum.[20] Ammonia may lead to increased GABAergic tone,[21] and this could mediate the effects of ammonia on the pathway.

In the experiments with hyperammonemic rats, sildenafil increased extracellular cGMP by 51% in control rats and reduced its ability to learn (Fig. 4), suggesting that an excessive increase in cGMP may impair learning and that cGMP must be kept high but below a certain threshold to reach maximum learning ability.

The fact that increasing extracellular cGMP by pharmacological manipulation is able to restore learning ability in hyperammonemic rats and in rats with portacaval anastomosis may have important clinical implications for the treatment of the impairment of intellectual function present in patients with evident hepatic encephalopathy but also in patients with minimal (subclinical) hepatic encephalopathy with reduced performance in psychometric tests.

The step of the glutamate-NO-cGMP pathway altered in brain *in vivo* in rats with portacaval anastomosis or with hyperammonemia without liver failure is the modulation of soluble guanylate cyclase by NO.[7,8] This modulation is altered in patients who have died of hepatic encephalopathy, as it is in rats with portacaval anastomosis.[10] Therefore, the function of the glutamate-NO-cGMP pathway also should be altered in the brains of these patients as in rats with portacaval anastomosis and also should be responsible for the impairment of some intellectual functions.

The results also indicate that concerning learning ability, the relevant alteration in hyperammonemia and hepatic failure is the reduced content of cGMP. Independently of other steps of the glutamate-NO-cGMP pathway, it is enough to normalize cerebral cGMP levels to restore learning ability by pharmacological manipulation using sildenafil or other inhibitors of the phosphodiesterases (*e.g.*, vardenafil) or by other means. Prickaerts et al.[22] showed that both sildenafil and vardenafil improve object recognition memory in rats and attributed the effect to increased levels of cGMP in brain. This indicates that cGMP may modulate different types of learning and memory processes.

Patients with liver cirrhosis or hepatitis C show cognitive impairment.[23-25] They also show reduced memory function, mainly attributed to deficits in attention and visual perception.[26] Although caution must be taken considering the possible deleterious increase in the existing vasodilatation in liver disease by sildenafil,[27] pharmacological manipulation of cGMP in brain by safe procedures may be a useful treatment to restore cognitive and intellectual functions in patients with overt or minimal hepatic encephalopathy.

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