Physiol. Res. 52: 497-502, 2003

Electroencephalogram is Activated by Addition of Pentobarbital in the Isolated Perfused Head of the Rat

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Received November 15, 2001
Accepted August 7, 2002

Summary

To evaluate the direct effects of a barbiturate on cerebral functions without its influence on brain perfusion pressure, circulatory hormones and metabolites, the electroencephalogram (EEG) was studied in the isolated rat head. Male Wistar rats were anesthetized, and EEG electrodes were inserted into the cranium. A Krebs-Ringer bicarbonate buffer solution containing heparinized rat whole blood, 20 mmol/l glucose, 200 mmol/l mannitol and 0.1 mg/ml dexamethasone was used for the perfusate. The bilateral common carotid arteries were cannulated, pumped at a rate of 6 ml/min and the head was isolated. The venous effluent was reoxygenated and recirculated into the brain. Twenty-five min after isolation of the heads pentobarbital was added to the perfusate at concentrations of 0.1, 0.5 and 2.5 mg/ml. EEG was recorded before and during perfusion. EEG activity could be recorded for more than 25 min after the beginning of perfusion. EEG activity gradually declined from 42±5 μV before perfusion (*in vivo*) to 4±1 μV at 25 min after the beginning of perfusion. Then, 3 min after the addition of pentobarbital, the EEG activity became significantly higher in the high dose groups; 12±3 μV in the 0.5 mg/ml group ($p<0.05$) and 12±1 μV in 2.5 mg/ml group ($p<0.05$) compared with the group without pentobarbital (2±2 μV). The present study suggests that a barbiturate has mitigating effects on the brain damage induced by the *in vitro* brain perfusion.

Key words
In vitro • Isolated brain perfusion • Electroencephalogram • Pentobarbital

Introduction

The *in situ* brain perfusion technique is often used to study the transport kinetics of substances across the blood-brain barrier (Takasato *et al*. 1984, Zlokovic *et al*. 1986, Ennis *et al*. 1994, Murakami *et al*. 2000). Previous perfusion method was performed by unidirectional perfusion from the arteries to the brain, and perfusion is usually possible for a few minutes. As previously reported, EEG examination *in vivo* is useful for evaluating the alteration of electric cortical functions in rats (Tagawa *et al*. 2000). However, there is no report examining cerebral functions using EEG activity as a parameter in the perfused brain. In a study of *in vitro* brain perfusion, it may be important to assess whether the EEG activities respond favorably to brain metabolites or anesthetics. Barbiturates are known to produce characteristic changes of EEG pattern in relation to the depth of anesthesia (Kiersey *et al*. 1951, Shimazono *et al*. 1953). It is also recognized that barbiturates protect the

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brain against several types of hypoxic insults (Smith 1977). However, the relation between EEG alterations and the mechanism of brain protection is still unclear.

The anesthetics also have strong systemic effects on blood pressure and hormones such as catecholamines and energy sources such as glucose and ketone bodies. In the present study, to exclude the influence of systemic elements on brain function, the rat head was isolated and vascularly and recirculatory perfused to observe the dose-dependency and the time-course of the influence of pentobarbital on the EEG.

Methods

Animals. Twenty male Wistar rats weighing 200-250 g were used for the experiments. Whole blood was obtained from other fifteen rats weighing 250-300 g which was used in the perfusion medium. All procedures were approved by the institution's animal care committee.

Perfusate. Krebs-Ringer bicarbonate buffer (KRBB) of pH 7.4 consisted of 118 mmol/l NaCl, 2.5 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 25 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4, and 1.2 mmol/l $MgSO_4$. The perfusate consisted of KRBB and heparinized rat whole blood (KRBB: whole blood = 3:1), in addition to 20 mmol/l glucose, 200 mmol/l mannitol and 0.1 mg/ml dexamethasone and was equilibrated with 95 % O_2 + 5 % CO_2 gas.

Experiment. Rats were anesthetized with pentobarbital (50 mg/kg) by intraperitoneal injection. Three silver-chloride EEG electrodes, one as reference electrode in the mid-anterior region and the other two as recording electrodes in the bilateral temporal region. They were implanted in the cranium by using a drill. Then, EEG activity during the pre-perfusion period was recorded. The neck and chest were then opened. According to the method previously performed (Mokuda *et al.* 2001), both the right and left common carotid arteries were cannulated through an incision made in the aorta, the cannulae were fixed in the vessel with a silk thread, and 40 ml of perfusate was infused at a rate of 3 ml/min into each cannula, i.e. with 6 ml/min into both cannulae. After stabilization of the perfusion for 5 min, the head, the common carotid arteries and opened veins were isolated from the body. The venous effluent was collected, reoxygenated by the method previously reported by Mokuda *et al.* (1999) through an oxygenator of a monilated glass cylinder filled with 95 % O_2 + 5 % CO_2 gas and recirculated through the brain. The perfusion system was maintained at 37 ^oC.

EEG was recorded every minute during the perfusion. Then, EEG activity was analyzed for 20 s at each one-minute period. In the present study, rats were divided into three groups due to the dose of added pentobarbital as follows: control group (group I: perfusion was performed with the above-mentioned perfusate); low-dose group (group II: 0.1 mg/ml of pentobarbital was added into the perfusate 25 min after head isolation); two high-dose groups (group III: 0.5 mg/ml of pentobarbital was added into the perfusate 25 min after head isolation, group IV: 2.5 mg/ml of pentobarbital was added into the perfusate 25 min after head isolation). Five rats were examined in each group.

Analysis. Amplitude of EEG activity was determined by measuring the distance between the peak of each wave and the baseline. All values of the EEG amplitude were given as mean ± S.E.M. Student's *t* test was used for statistical analysis.

Results

During the perfusion, partial O_2 and CO_2 tension in influent perfusate were maintained at 250-350 mm Hg and 30-35 mm Hg, respectively, and the perfusion pressure was in the range of 80-120 mm Hg.

Table 1. Alteration of EEG amplitude (μV)

	Group I (n=5)	**Group II (n=5)**	**Group III (n=5)**	**Group IV (n=5)**
Pre-perfusion	42±5	40±4	36±5	39±4
Perfusion 3 min	15±3	13±2	13±6	14±5
10 min	7±2	8±4	8±6	9±4
25 min	4±1	3±2	4±1	3±3
●*28 min*	2±2	2±1	12±3*	12±1*

*All values are mean ± S.E.M., *p<0.05 from group I, ●28 min: 3 min after addition of pentobarbital into the perfusate in group II-IV.*

In Table 1, the EEG amplitude before and during perfusion are shown. All the groups were perfused under the same conditions until 25 min after the beginning of perfusion. No difference in the EEG amplitude was observed among the four groups for EEG until 25 min of perfusion. Frequency of EEG activity was

8~12 Hz in all rats before the isolation of the head and it became 3~5 Hz 1 min after the beginning of perfusion.

EEG amplitude 28 min after head isolation, namely 3 min after the addition of pentobarbital, was significantly higher in the two high-dose groups (group III 12±3 μV, $p<0.05$; group IV 12±1 μV, $p<0.05$) than in the control group. In this period, frequency in group III and IV was about 20-30 Hz. There was no significant difference in the EEG amplitude 28 min after head isolation between the control group (2±2 μV) and the low-dose group (2±1 μV).

Table 2. Time (s) at which EEG activity appeared in each period

Perfusion	**Group I (n=5)**	**Group II (n=5)**	**Group III (n=5)**	**Group IV (n=5)**
25min	1.0±0.2	1.1±0.4	1.1±0.3	1.0±0.3
●*28 min*	0.5±0.2	0.3±0.2	19.2±0.2*	19.4±0.3*

*All values are mean ± S.E.M., *p<0.001 from group I.*
●28min: 3 min after addition of pentobarbital into the perfusate in group II~IV.

The total time in which EEG activities could be recorded during a 20 s record is shown in Table 2. The time at which EEG activity appeared at the period of 25 min after head isolation was decreased compared to the beginning of the perfusion, but it was not significantly different among the four groups. On the other hand, the time at which EEG activity appeared at the period of 28 min after head isolation, namely 3 min after addition of pentobarbital into the perfusate, was significantly longer in group III and IV (19±0.2 s, $p<0.001$; 19.4±0.3 s, $p<0.001$, respectively) than in the control group. There was no significant difference in the time when EEG activity appeared at the period of 28 min after head isolation between the low-dose group (0.3±0.2 s) and the control group (0.5±0.2 s).

The most typical EEG alterations in all four groups are depicted in Figure 1. It is evident that 3 min after the addition of pentobarbital there were fast waves in the EEG recording. Moreover, these fast waves containing slow waves were recorded for additional 10 min. Because EEG activity in the control and low dose pentobarbital groups had completely disappeared until 28 min of perfusion, we discontinued the examination in all rats.

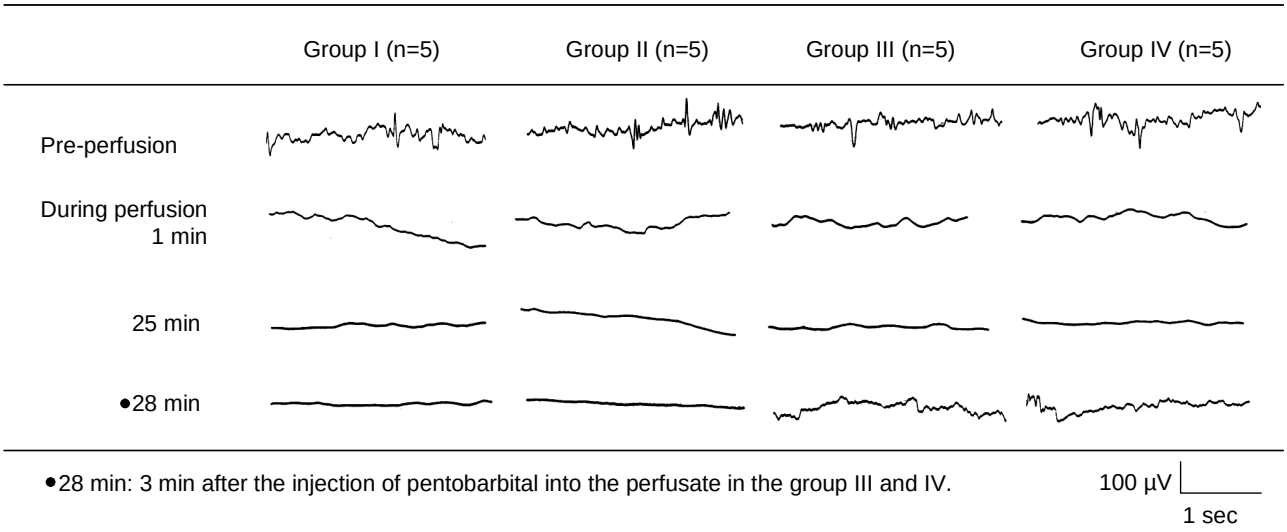

Fig. 1. *Most typical EEG alterations in particular groups before and during perfusion. The amplitude was markedly reduced 25 min after the beginning of perfusion. Fast waves appeared in the 28 min record, i.e. 3 min after the injection of pentobarbital in Groups III and IV.*

Discussion

The present study demonstrated that the isolated rat head perfusion experiment is useful for the examination of the direct effects of anesthetics on the EEG independently of their effects on circulatory hormones and metabolites. The *in situ* brain perfusion was previously studied for evaluating the permeability

and transport system of the substrates through the blood brain barrier (Takasato *et al.* 1984, Zlokovic *et al.* 1986, Ennis *et al.* 1994). The perfusion systems in these previous studies were designed to examine unidirectional flux from blood to the brain, so that the possible perfusion time was usually limited to a maximum of 1-5 min (Takasato *et al.* 1984). It was shown, however, that by using the perfusates containing red blood cells, the viability of the brain perfused *in situ* was maintained for 20-30 min (Zlokovic *et al.* 1986, Ennis *et al.* 1994). In the present study, the perfusate was reoxygenated through an oxygenating device and recirculated to the brain.

Using this method, the EEG could be recorded for at least 25 min after isolation of the head. EEG activity gradually declined and became less than 10 μV 25 min after the beginning of perfusion in all rats. In our preliminary study, EEG activity of the rat brain declined and became flat immediately after cardiac arrest. On the other hand, ^{14}C-glucose oxidation in the isolated rat brain was maintained for at least 5 min. It is therefore suggested that glucose oxidation in the brain tissue is preserved during the EEG activity. However, 25-30 min of *in vitro* brain perfusion seems to be a maximum in the because of the disturbance of cerebral circulation due to brain edema and increased intracranial pressure. In a further study, it will be necessary to observe the glucose oxidation in the isolated perfused rat brain.

In the two high-dose groups to which pentobarbital was added into the perfusate, fast wave EEG activity continuously appeared 3 min after the injection, i.e. 28 min after the beginning of perfusion. Despite the fast oscillations that can be generated by the intrinsic properties of single neuronal cells, complex neuronal circuits are required for the synchronization of EEG activity (Steriade 1999). There are two factors which contribute to synchronization of the EEG: synchronizing effect on each unit of activity in neighboring parallel fibers, thus of the intracortical circuits and corticothalamic projections (Steriade 1999). However, the reason why the fast waves appeared after injection of pentobarbital in this study is not clear.

It is known that barbiturates produce characteristic changes of EEG activity dependent upon the depth of anesthesia and their time course (Kiersey *et al.* 1951, Shimazono *et al.* 1953). After intravenous injection of the low dose (1 mg/kg) pentobarbital, 10-35 Hz rapid waves of high amplitude appear. Early change in EEG activity after the induction of barbiturate anesthesia is characterized by high-amplitude and spike activity with fast waves of near 20 Hz (Shimazono *et al.* 1953). Although the present study was performed under *in vitro* conditions, the fast waves after pentobarbital addition may indicate the first pattern of barbiturate anesthesia.

Under a continuous infusion of pentobarbital, the early pattern of fast wave gradually changes into the predominance of slower wave forms of very irregular contour and random occurrence, progressive suppression of cortical activity and reduction in the amplitude of all components (Kiersey *et al.* 1951). These EEG changes after addition of pentobarbital could not be detected in the present *in vitro* examination. It is also known that EEG patterns after the intravenous injection of barbiturates change in a dose-dependent manner. In the present study, EEG was examined after the administration of various doses of pentobarbital. No effect was observed on EEG activity in the low dose group (0.1 mg/ml), whereas the EEG was obviously activated in the two high-dose groups (0.5 mg/ml and 2.5 mg/ml).

Barbiturates exert several distinct effects on excitatory and inhibitory synaptic transmission. They also have an effect in protecting the brain from variable insults due to injury, hypoxia and ischemia (Smith 1977, Warner *et al.* 1991). Cerebral protection by barbiturates chiefly results from the reduction of intracranial hypertension (Millson *et al.* 1982) and lowering of cerebral oxygen consumption due to a decrease of cerebral blood flow (Nilsson and Siesjö 1975) and blood-brain glucose transfer (Gjedde and Rasmussen 1980). In the present study, the perfusion pressure was not influenced by the addition of pentobarbital. There was a possibility that increased intracranial pressure resulted from isolation of the heads and perfusion was reduced after infusion of pentobarbital. In general, the basis of EEG is due to postsynaptic potentials. It is not obvious, however, whether the EEG waves which appeared after the addition of pentobarbital in this study were due to the activation of postsynaptic potentials or due to a change in intracranial conditions.

A simple sign of the nervous tissue other than EEG activity will represent an alternative possibility. In general, if the reaction of the brain tissue to a flash or sound is improved due to the injection of pentobarbital, the brain protection by pentobarbital will be elucidated. In a further study, it will be necessary to observe whether the isolated perfused rat brain will react to a stimulus or not. In addition, biochemical research of measuring ATP concentrations in brain samples will be necessary to confirm the protective mechanism.

In the present study, 200 mmol/l mannitol and dexamethasone were added to the perfusate to prevent intracranial hypertension. Hyperosmolar drugs such as mannitol cause reversible opening and disruption of the blood brain barrier (Cosolo *et al.* 1989). It is reported that large doses of pentobarbital attenuated the BBB disruption caused by hyperosmolar mannitol in the *in vivo* animal study (Chi *et al.* 1998). In further studies, it may be necessary to observe directly the intracranial pressure and to examine the interaction between barbiturate and mannitol in the *in vitro* brain perfusion.

In summary, the study of EEG activity in the isolated perfused rat head would be useful for examining the direct influences of non-physiological factors and anesthetic agents on cerebral function. In a further study, it will be necessary to assess the brain damage following the perfusion procedure by other tools than the EEG record, e.g. the microscopic study.

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