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Increased ATP production during long-term brain ischemia in rats in the presence of propentofylline

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Abstract

Forty adult rats were subjected to stepwise two- and four-brain vessel occlusion and propentofylline 25 mg/day per kilogram body weight was intraperitoneally administered for 1 week or 3 weeks. Adenosine 5′-triphosphate, creatine phosphate, adenosine 5′-diphosphate and adenosine were determined in rat parietotemporal cortex by high-pressure liquid chromatography; lactate and pyruvate were measured spectrophotometrically. Stepwise and permanent long-term brain vessel occlusion gradually reduced the concentration of energy-rich phosphates and induced a marked increase in the concentration of adenosine, a parameter of ischemia. Three weeks of propentofylline treatment resulted in a significant increase in cerebral adenosine 5′-triphosphate concentration from 2.16 ± 0.15 [(−)-propentofylline] to 2.70 ± 0.24 nmol/mg wet weight during four-vessel occlusion (+25%). This was associated with an enhancement of the adenosine 5′-triphosphate/adenosine 5′-diphosphate ratio (+33%), mainly because of the significant reduction in adenosine 5′-diphosphate concentration. Propentofylline did not prevent the increase in lactate concentration during permanent brain vessel occlusion, but significantly reduced the tissue concentration of adenosine. In summary, the results demonstrate that continuous propentofylline administration over 3 weeks induced a striking increase in rat cortical adenosine 5′-triphosphate concentration during long-term brain vessel occlusion. Thus, propentofylline may have possible neuroprotective effects and could be used in the treatment of patients with chronic cerebrovascular disorders.

Keywords: Propentofylline; Adenosine; Energy metabolism; Brain vessel occlusion; Cerebral cortex; (Rat)

1. Introduction

Acute brain ischemia leads to significant disturbances in the oxidative energy metabolism of nervous tissue. The impaired availability of energy, in particular adenosine 5′-triphosphate (ATP), first reduces cellular function and subsequently cellular integrity (Buttgereit and Brand, 1995). Thus, when the energy state of the brain becomes compromised, longlasting disturbances in brain function may develop. Furthermore, excessive accumulation of excitatory amino acids and tissue acidosis play an important role in the cascade of biochemical processes contributing to ischemic cell damage (Choi, 1985; Meyer, 1989; Benveniste, 1991). For instance, several studies in which microdialysis and the cortical cup technique were used showed a rapid and substantial increase in extracellular glutamate and adenosine during ischemia (Rudolphi et al., 1992a,b; Obrenovitch and Richards, 1995; Siesjö et al., 1995), adenosine being an important inhibitory neuromodulator in the central nervous system (Sweeny, 1997). Because adenosine can be rapidly incorporated into the ATP pool via the salvage pathway, several pharmacological approaches have been undertaken to increase the intracellular availability of adenosine. The xanthine derivative propentofylline (HWA 285) is a combined inhibitor of adenosine transporters and cyclic nucleotide phosphodiesterases, which favors adenosine A_2 receptor-mediated actions (Fredholm et al., 1994). During acute ischemia, for instance, propentofylline is known to increase the cerebral

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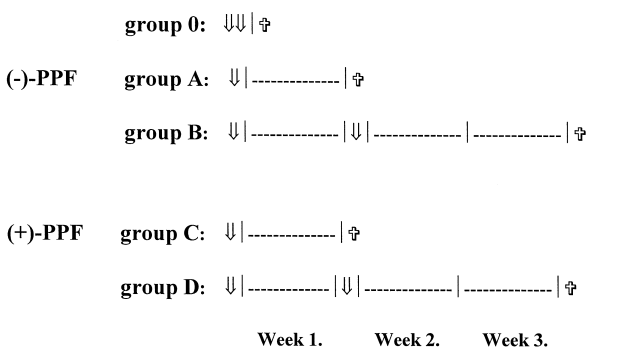
concentrations of adenosine (Andine et al., 1990) and γ-aminobutyric acid (GABA) (Vukadinovic et al., 1986), and to reduce the release of glutamate (Andine et al., 1990; Miyashita et al., 1992). Although the neuroprotective effect of propentofylline under conditions of acute brain ischemia has been demonstrated (De Leo et al., 1988; Park and Rudolphi, 1994), no studies have investigated the effect of propentofylline on cerebral energy metabolism during stepwise and permanent long-term cerebral vessel occlusion. Therefore, an animal model of stepwise reduction of cerebral blood supply was used to determine (i) the effect of permanent two- and four-vessel occlusion on cerebral energy metabolism and intracellular markers of ischemia, and (ii) the protective role of propentofylline on cerebral energy metabolism in the parietotemporal cortex of adult rats after permanent cerebral brain vessel occlusion. In addition, acute four-vessel occlusion was performed to compare the effects of acute and chronic four-vessel occlusion on rat cerebral energy metabolism.

Table 1
Experimental procedure



Experimental design of two- and four-vessel occlusion in the absence (groups 0/A/B) and presence (groups C/D) of propentofylline.
Abbreviations: ⇓: vessel occlusion (ipsilateral A. carotis, contralateral A. vertebralis), PPF: propentofylline, ✞: final steady-state experiment.

2. Materials and methods

The experimental protocol was approved by the appropriate review committee of the Medical Faculty of the University of Heidelberg and complied with the guidelines of the responsible government agency.

2.1. Acute cerebral four-vessel occlusion

Ten adult male Wistar rats (breeder: Centre D'Elevage R. Janvier, France) underwent permanent four-vessel occlusion of both carotid and vertebral arteries. Animals were killed after 20 min of four-vessel occlusion under hemodynamic steady state conditions (group 0).

2.2. Chronic cerebral four-vessel occlusion

Forty adult male Wistar rats were subjected to stepwise and permanent vessel occlusion by performing cross-wise occlusions of the carotid and vertebral arteries before the final experiment was done under steady-state conditions. As shown in Table 1, two-vessel occlusions were investigated for 1 week in the absence (group A) or presence (group C) of propentofylline. In groups B and D, additional two-vessel occlusions were performed at the end of the first week after two-vessel occlusion. The four-vessel occlusion lasted for two more weeks. The vessel occlusions were carefully done under general anesthesia (0.5 vol % halothane, nitrous oxide/oxygen 70:30) by ligation of the common carotid arteries and electrical coagulation of the vertebral arteries. During vessel occlusion care was taken to avoid any damage to the surrounding tissue, in particular to the vagus nerve. Corresponding control groups of 10 adult rats underwent surgery without vessel occlusion or propentofylline application.

2.2.1. Propentofylline application

Propentofylline solution was intraperitoneally administered at a dosage of 25 mg/day per kilogram body weight (10 μl/h), using a subcutaneously implanted osmotic Alzet pump model 2ML1 (Alza, Palo Alto, USA). Propentofylline was administered for 1 week (group C) and 3 weeks (group D) after vessel occlusion until the final steady state experiment. Propentofylline (HWA 285) was kindly provided by Hoechst Marion Roussel (Wiesbaden, Germany). In sham-operated animals, distilled water was applied by a subcutaneously implanted tube.

2.2.2. Final steady-state experiment

During the final steady-state experiment all animals underwent general anesthesia with 0.5 vol % halothane and nitrous oxide/oxygen (70:30). After tracheal cannulation, rats were artificially ventilated and muscle relaxation was produced with pancuronium bromide (2 mg/kg body weight). A 20-min steady state of arterial normotension, normocapnia, normoxemia and normothermia was established (Hoyer and Krier, 1986). Thereafter, the brains were frozen in situ by means of liquid nitrogen poured into a skin funnel formed from the sagittally incised galea. The animals were decapitated, and the brains were chiseled out of the skull under liquid nitrogen and stored at −80°C. The cerebral parietotemporal cortex was prepared at −20°C.

2.2.3. Biochemical analysis

Adenosine 5′-triphosphate, adenosine 5′-diphosphate, creatine phosphate and adenosine were determined by high-pressure liquid chromatography after disruption of cell membranes with an ultraturrax in a chloroform/acetic acid mixture (1:2) at −20°C. Samples (0.1 ml) were automatically injected onto a Partisil SAX column (4.6 ID,

25 cm, 10 μm pore size) for determination of energy-rich phosphates. The linear gradient started at 100% 0.01 M H_3PO_4, and the 0.75 M KH_2PO_4 buffer was increased from 0% to 100% within 20 min, the flow rate being 2.0 ml/min. Absorbance of the column eluate was continuously monitored (Shimadzu UV Detector SPD 6A) at 210 nm. For adenosine analysis, samples (0.2 ml) were automatically injected onto a C18 column (Nova-Pak C18, 3.9 mm × 150 mm, Waters). The linear gradient started at 100% KH_2PO_4 (0.001 M, pH 4.0) and increased to 60% of 60/40 methanol/water (vol/vol) in 15 min, the flow rate being 1.0 ml/min. Then the gradient was reversed to restore the initial conditions over the next 3 min. Absorbance of the column eluate was continuously monitored at 254 nm, using photodiode array detection. Purine compounds were quantitated by using a computer-assisted program. Concentrations of pyruvate and lactate in the parietotemporal cerebral cortex were measured spectrophotometrically at 340 nm according to Bergmeyer (1974) and Folbergrova et al. (1974).

2.2.4. Calculations and statistics

The following functional parameters were calculated: '*P*' representing the total load of available energy (0.18 × adenosine 5′-triphosphate + 0.15 × creatine phosphate (Siesjö, 1978)), the lactate/pyruvate ratio as an indicator of the cytoplasmic redox state, and the ATP/ADP ratio.

Data are given as means ± standard deviation (S.D.). Statistically significant differences were calculated by one-way analysis of variance (ANOVA) with post-hoc Tukey-test and were assumed to be significant at $P < 0.05$.

3. Results

3.1. Hemodynamic changes

In the absence and presence of propentofylline no significant changes in hemodynamic parameters and arterial blood gases were obtained between the groups. In addition, hemoglobin and hematocrit values and central body temperature did not differ between the groups (Table 2).

3.2. Effect of long-term brain vessel occlusion

Table 3 summarizes the tissue concentrations of purine compounds and the parameters of the intracellular redox potential (pyruvate, lactate) (i) during sham operation (Table 3 (I)), (ii) during two- and four-vessel occlusion in the absence of propentofylline (Table 3 (II)), and (iii) during two- and four-vessel occlusion in the presence of propentofylline (Table 3 (III)).

In sham-operated rats [Table 3 (I)] the concentrations of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and creatine phosphate amounted to 2.38 ± 0.39, 0.48 ± 0.09, and 6.52 ± 0.90 nmol/mg wet weight, respectively, resulting in an ATP/ADP ratio of 5.60 ± 1.65. Under control conditions the energy load '*P*' in the parietotemporal cortex was calculated to be 1.40 ± 0.20 nmol/mg wet weight. In controls the tissue concentration of adenosine was 36.72 ± 4.97 pmol/mg wet weight. Pyruvate and lactate concentrations (0.12 ± 0.05 and 1.11 ± 0.30 nmol/mg wet weight, respectively) were maintained within the physiological range and the lactate/pyruvate ratio was calculated to be 10.54 ± 4.43.

During graded and permanent brain vessel occlusion (Table 3 (II)) four-vessel occlusion induced a marked decrease in ADP concentration. There was a progressive decrease in the energy load '*P*' after two- and four-vessel occlusion which was mainly caused by the significant decrease in creatine phosphate concentration from 6.52 ± 0.90 under control conditions to 5.46 ± 0.37 and 5.00 ± 0.65 nmol/mg wet weight during two- and four-vessel occlusion, respectively. The cortical concentration of adenosine 5′-triphosphate and the ratio of ATP/ADP were not changed significantly. After two-vessel occlusion, adenosine was significantly increased, but no further change in its concentration was obtained after four-vessel occlusion. Thus increasing the number of vessels occluded did not affect the redox state.

Table 2
Effect of stepwise long-term occlusion of brain vessels and propentofylline treatment on hemodynamic parameters and arterial blood gases

Treatment	(−)-Propentofylline			(+)-Propentofylline		
Groups	Sham group	Two-vessel occlusion	Four-vessel occlusion	Sham group	Two-vessel occlusion	Four-vessel occlusion
n	10	10	10	10	10	10
MABP (mmHg)	99 ± 5	106 ± 2	99 ± 6	99 ± 5	103 ± 3	99 ± 5
pO_2 (mmHg)	122.5 ± 7.0	123.9 ± 6.4	115.7 ± 9.0	122.5 ± 7.0	119.9 ± 4.4	116.5 ± 8.0
pCO_2 (mmHg)	38.2 ± 2.7	37.7 ± 2.9	39.2 ± 2.4	38.2 ± 2.7	38.7 ± 2.9	39.0 ± 2.2
pH	7.47 ± 0.10	7.47 ± 0.10	7.43 ± 0.10	7.47 ± 0.10	7.47 ± 0.10	7.44 ± 0.11
Hb (g/100 ml)	13.3 ± 1.3	13.4 ± 1.3	13.0 ± 0.4	13.3 ± 1.3	13.3 ± 1.1	13.2 ± 0.5
Hct (%)	37.0 ± 0.5	37.7 ± 1.0	37.2 ± 1.3	37.0 ± 0.5	37.0 ± 0.9	37.3 ± 1.2
T (°C)	37.0 ± 0.4	37.1 ± 0.1	37.0 ± 0.1	37.0 ± 0.4	37.1 ± 0.2	37.0 ± 0.2

Mean ± S.D., *n*: number of animals/group; MABP: mean arterial blood pressure; Hb: hemoglobin; Hct: hematocrit; *T*: body temperature.

Table 3
Effect of stepwise permanent brain vessel occlusion and propentofylline treatment on cerebral energy state in adult rat brain

	ATP (nmol/mg ww)	ADP (nmol/mg ww)	Creatine phosphate (nmol/mg ww)	ATP/ADP (nmol/mg ww)	Energy load '*P*' (nmol/mg ww)	Pyruvate (nmol/mg ww)	Lactate (nmol/mg ww)	Lactate/Pyruvate (nmol/mg ww)	Adenosine (pmol/mg ww)
I sham									
	2.38 ± 0.39	0.48 ± 0.09	6.52 ± 0.90	5.60 ± 1.65	1.40 ± 0.20	0.12 ± 0.05	1.11 ± 0.30	10.54 ± 4.43	36.72 ± 4.97
II (−)-propentofylline									
two-vessel occlusion	2.28 ± 0.11	0.49 ± 0.05	5.46[a] ± 0.37	4.72 ± 0.61	1.23[a] ± 0.07	0.12 ± 0.02	1.60 ± 0.33	14.23 ± 3.91	56.15[a] ± 14.98
four-vessel occlusion	2.16 ± 0.15	0.33[b,c] ± 0.08	5.00[c] ± 0.65	7.06 ± 2.61	1.14[c] ± 0.10	0.11 ± 0.04	1.20 ± 0.42	12.97 ± 8.57	51.33[c] ± 16.19
III (+)-propentofylline									
two-vessel occlusion	2.41 ± 0.32	0.42 ± 0.08	4.94 ± 0.48	6.05 ± 1.95	1.17 ± 0.12	0.09 ± 0.02	1.81 ± 0.62	19.24 ± 4.95	41.82[d] ± 1.93
four-vessel occlusion	2.70[e] ± 0.24	0.30[f] ± 0.07	5.38 ± 0.31	9.39[f] ± 2.21	1.29 ± 0.08	0.11 ± 0.03	1.75[e] ± 0.24	17.66 ± 5.52	40.68 ± 6.23
ANOVA *P*	0.007	< 0.0001	< 0.0001	< 0.0001	0.0002	0.2870	0.0004	0.0096	0.0007

Mean ± S.D., $n = 10$ per group.
PPF: propentofylline; vo: vessel occlusion, vs.: versus, ww: wet weight.
Significance at $P < 0.05$, one-way analysis of variance (ANOVA), Tukey test: (a) 2 vo (−)-PPF vs. sham, (b) 4 vo (−)-PPF vs. 2 vo (−)-PPF, (c) 4 vo (−)-PPF vs. sham, (d) 2 vo (+)-PPF vs. 2 vo (−)-PPF, (e) 4 vo (+)-PPF vs. 4 vo (−)-PPF, (f) 4 vo (+)-PPF vs. 2 vo (+)-PPF.

3.3. Effect of propentofylline on cortical energy state

In the presence of 3 weeks of propentofylline (Table 3 (III)), the cerebral concentration of ATP was significantly increased after four-vessel occlusion in comparison to the respective control values [four-vessel occlusion, (−)-propentofylline].

No significant changes in tissue concentration of creatine phosphate, energy load, pyruvate and the lactate/pyruvate ratio were determined after 1 week and 3 weeks of propentofylline. The cerebral concentration of ADP and the ATP/ADP ratio showed time-dependent significant changes after 3 weeks of propentofylline as compared to those after 1 week of drug administration.

Interestingly, propentofylline significantly reduced the cortical concentrations of adenosine when applied for 1 week, but had no further effect on tissue adenosine concentrations in the four-vessel occlusion condition. Tissue lactate concentration increased by 46% in the four-vessel occlusion condition.

3.4. Effect of acute brain vessel occlusion

After acute ischemia (20 min of cerebral four-vessel occlusion), the cortical concentrations of ATP, ADP, creatine phosphate, the ATP/ADP ratio, and the energy load were determined to be 1.70 ± 0.55, 0.313 ± 0.166, 1.995 ± 1.453 nmol/mg wet weight, as well as 5.44 ± 5.52, and 0.605 ± 0.35, respectively. The significant differences between the effects of acute and chronic ischemia on the concentrations of energy-rich phosphates in comparison to those of controls are shown in Fig. 1.

4. Discussion

Long-term propentofylline application does not influence the hemodynamic parameters and the blood gases when administered by means of Alzet pumps. This result differs from that of studies in which propentofylline was applied intravenously after acute brain injury by bolus injection. Under these conditions, the drug induced marked dose-dependent vasodilation and decreased blood pressure in rabbits (Hudlicka et al., 1981). Obviously, compensatory mechanisms are activated to maintain stable hemodynamic conditions when propentofylline is continuously applied.

In brain tissue, ischemia is the most powerful stimulus for enhanced degradation of intracellular adenosine 5′-triphosphate (ATP) (Berne et al., 1974; Hagberg et al., 1987). As a consequence of ATP depletion and the subsequent failure of ATP-dependent ion pumps, in particular the Na^+/K^+-ATPase, there is a rapid derangement of ion homeostasis, leading to sustained and marked membrane depolarization and a subsequent Ca^{2+} influx, which presynaptically triggers the release of neurotransmitters, including the excitatory amino acids glutamate and aspartate (Meyer, 1989; Benveniste, 1991; Schubert et al., 1994).

Adenosine production is closely related to ATP degradation. This nucleoside has been shown to play a physiological role as an important modulator of different cell functions. For instance, adenosine is a sensitive indicator of impaired tissue oxygenation during acute cerebral ischemia (Berne et al., 1974; Winn et al., 1981) and is a potent vasodilator of the vasculature to enhance the oxygen availability in brain tissue. Adenosine can act via

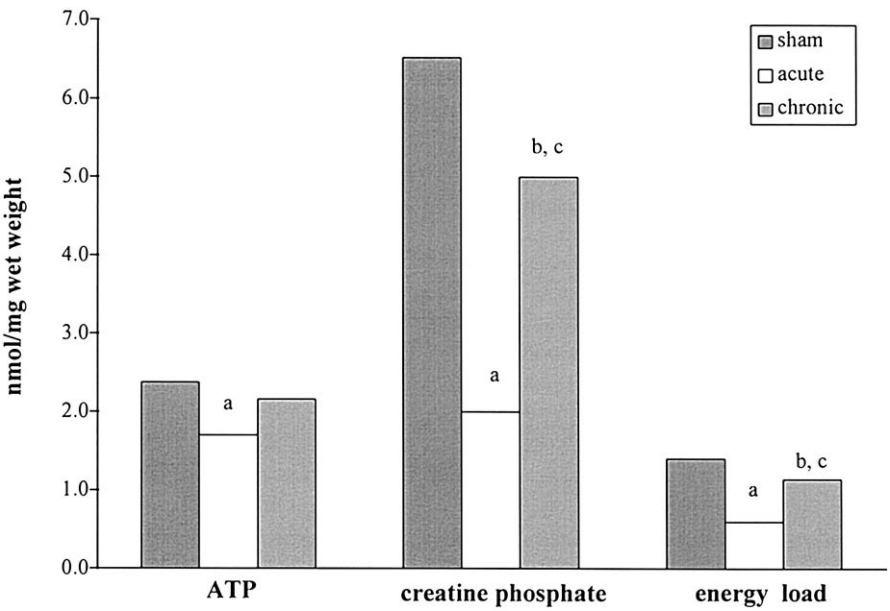


Fig. 1. Effect of acute and chronic cerebral four-vessel occlusion on cortical energy state in rat brain.

adenosine A_1 and A_2 receptors to limit further metabolic disturbances and hence to decrease its formation by means of a feedback system. One of the most important actions of adenosine in the brain is its inhibition of the release of excitatory amino acids and, in particular, the presynaptic inhibition of the excitatory actions of glutamate (Corradetti et al., 1984; Phillis et al., 1994). In this sense adenosine can be assumed to be an endogenously formed neuroprotective agent.

In the past, pharmacological studies with adenosine receptor antagonists, adenosine analogues, or agents that inhibit the physiological inactivation of adenosine have been used either to characterize the role of the nucleoside in brain ischemia and/or to find methods of drug therapy for patients with chronic vascular insufficiency (Rudolphi et al., 1992a,b). The most promising agent is the xanthine derivative propentofylline, which is a combined inhibitor of adenosine transporters (Fredholm et al., 1994; Parkinson et al., 1994) and enzymes such as 5′-nucleotidase (Fredholm and Lindgren, 1983). Several investigators have shown that propentofylline can protect against brain damage after global ischemia (Mrsulja et al., 1985; Dux et al., 1990) and can enhance ATP levels in anoxic rats (Stefanovich, 1985). Moreover, the neuroprotective effect of adenosine seems to be amplified in the presence of propentofylline, which can influence the balance between adenosine A_1 and A_2 receptor-mediated actions, depress free radical formation in activated microglia, and modulate astrocyte reactions (Schubert et al., 1994).

Animal models of acute cerebral ischemia have been used to demonstrate the relationship between disturbances in brain circulation and morphological and metabolic abnormalities (Ljunggren et al., 1974; Kirino, 1982; Hoyer and Krier, 1986; Petito and Pulsinelli, 1993). As can be seen from our data for acute brain ischemia, the concentrations of cortical energy-rich phosphates are significantly reduced after four-vessel occlusion, leading to a substantial decrease in the energy load of cerebral tissue. However, the increasing number of patients with chronic cerebrovascular disorders make it necessary to have an animal model in which cerebral perfusion and energy metabolism deteriorate chronically. Up to now, no adequate animal models of a stepwise reduction in cerebral blood supply have been available. Therefore, we established a potential clinically relevant animal model in which graded reductions in cerebral blood flow were induced by cross-wise occlusion of the common carotid and vertebral arteries (two- and four-vessel occlusion) in rats. This model was characterized by the lack of significant changes in hemodynamic and respiratory parameters after 1 week of two-vessel occlusion and after a further 2 weeks of four-vessel occlusion.

Our data demonstrate that permanent brain vessel occlusion leads to a graded and significant decrease in creatine phosphate concentrations in the parietotemporal cortex of rats. Interestingly, the cortical ATP concentration was not significantly reduced after chronic vessel occlusion. Possibly, ATP production is favored at the cost of reduced tissue concentrations of ADP and degradation of creatine phosphate. It has been shown under conditions of acute ischemia (Allen et al., 1988; Tsuji et al., 1995) that an increase in creatine kinase activity causes the formation of ATP from creatine phosphate, the concentration of which is significantly diminished. In comparison to the situation during acute ischemia, compensatory mechanisms are likely to be activated during permanent brain ischemia (3 weeks) to restore high-energy phosphate concentrations in brain tissue (Fig. 1). Although adenosine concentrations generally reflect disturbances in cerebral tissue oxygenation during chronic brain vessel occlusion, the concentrations of this nucleoside did not distinguish between two- and four-vessel occlusions. Thus, data for chronic brain vessel occlusion differ from data for acute vessel occlusion (Berne et al., 1974), in which cerebral adenosine concentrations correlate with the extent of brain tissue ischemia.

When propentofylline was continuously applied for 1 week or 3 weeks, there was a significant increase in the cortical concentrations of ATP only after 3 weeks. The subsequent increase in the ATP/ADP ratio can be interpreted to mean that ATP metabolism is greatly stimulated in the presence of propentofylline, and more adenosine 5′-diphosphate is phosphorylated to ATP via the salvage pathway. Since adenosine may act as a homeostatic metabolite (Newby, 1984), i.e. a substance which decreases its own production, it is possible that the overall reduction in tissue adenosine concentration reflects a propentofylline-dependent improvement of brain energy metabolism. The data for long-term ischemia differ from those for in vitro experiments with short-term ischemic interventions of seconds or even minutes. Using microdialysis techniques, Andine et al. (1990) demonstrated in rats subjected to a 20-min period of bilateral forebrain ischemia that the transient increase in adenosine release was enhanced during reperfusion when propentofylline was present. Moreover, Fredholm et al. (1994) measured an increased concentration of adenosine in the superfusate, when isolated brain slices of rats were stimulated in the presence of propentofylline. Their data were interpreted to indicate that propentofylline significantly inhibits the reuptake of released adenosine, most likely via adenosine A_2 receptors. Obviously, other mechanisms may be responsible for the propentofylline-associated reduction in tissue adenosine concentrations in our animal model of chronic ischemia. It is well known that propentofylline can also inhibit the activity of the 5′-nucleotidase (Fredholm and Lindgren, 1983) which is responsible for the degradation of adenosine 5′-monophosphate to adenosine. Assuming that the activity of the 5′-nucleotidase is reduced during chronic propentofylline administration, one would expect the formation of adenosine to be lowered and hence more adenosine 5′-monophosphate may be available to significantly increase the concentration of ATP. Although the mechanism of action of propentofylline may differ during

acute and chronic ischemia, the common findings of in vitro and in vivo studies show that the neuroprotective effect of endogenously formed adenosine is strengthened in the presence of propentofylline. This action of propentofylline in the brain may also explain some of its pharmacological properties, such as learning and memory improvements, and its potential for the treatment of neurodegenerative diseases (Torigoe et al., 1994; Rother et al., 1996). The changes observed in the present study are in accordance with reports demonstrating that propentofylline effectively protects the brain against severe brain ischemia (De Leo et al., 1988).

5. Conclusion

In summary, the present study demonstrates for the first time that 3 weeks propentofylline-application effectively increases the concentration of ATP in the parietotemporal cortex of adult rats used as an animal model of permanent brain vessel occlusion. These findings could be the basis for chronic studies into the effect of propentofylline and can open up new indications for this drug in patients with chronic cerebrovascular disorders.

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