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Neuromodulatory effect of propentofylline on rat brain under acute and long-term hypoperfusion

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1 The effects of propentofylline (PPF, 25 mg kg^{-1} body weight per day) on rat cerebral energy state and cytokine expression as well as on behaviour and histopathology were studied after acute and long-term permanent bilateral common carotid artery occlusion (BCCAO).

2 In the absence of PPF, acute ischaemia led to a decrease in energy-rich phosphates in parietotemporal cortex and hippocampus which correlated with an increase in AMP and adenosine concentrations measured by high-performance liquid chromatography technique. The concentrations of cortical cytokines TNFα and IL1β were increased 12 and 19 fold, respectively.

3 PPF had a neuroprotective action after 20 min of BCCAO, reducing the deleterious effect of acute ischaemia on rat brain energy state and microglial reaction. Simultaneously, PPF treatment increased cyclic-AMP 3 fold.

4 Three weeks of permanent BCCAO did not significantly disturb brain energy metabolism, microglial reaction or histopathology. However, a significant reduction of 30–50% in rat memory capacities and a locomotor hyperactivity were obtained.

5 Continuous PPF-application, however, led to a marked increase in rat working memory and to reduced locomotor activity, which were returned nearly to control levels by 1 week after permanent BCCAO.

6 In summary, PPF showed a clear neuroprotective effect on cerebral energy state and pro-inflammatory cytokines under conditions of acute global ischaemia. Continuous administration of PPF led to memory improvement during permanent BCCAO. These results underscore the benefit of treatment with PPF in clinical practice, particularly during stroke, but also in cerebrovascular and neurodegenerative disorders.

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Abbreviations: ADO, adenosine; BCCAO, bilateral common carotid artery occlusion; Hb, haemoglobin; Hct, haematocrit; h.p.l.c., high-performance liquid chromatography; LA: locomotor activity; MAP, mean arterial blood pressure; PCr, phosphocreatine; PPF, propentofylline; WM: working memory

Introduction

The physiologic function of adenosine as a cell modulator is achieved by a mosaic of actions that influence intracellular metabolic signalling in neurons and in glial cells (Sweeney, 1997). Experimental data suggest a potential role of adenosine as an endogenous neuroprotective substance in acute brain focal and global ischaemia (Hagberg *et al*., 1987; DeLeo *et al*., 1988). For instance, the production of adenosine is closely related to ATP degradation and this nucleoside is a potent vasodilator of the vasculature to enhance the oxygen availability in brain tissue (Berne *et al*., 1974). Furthermore, adenosine inhibits the release of excitatory amino acids, e.g. glutamate (Phillis *et al*., 1994). Although the protective function of adenosine on microglial reaction is widely accepted (Rudolphi *et al*., 1992), the role of endogenous adenosine on animal behaviour is a controversial subject (Hooper *et al*., 1996; Ohno & Watanabe, 1996). Adenosine acts *via* a variety of cellular receptors, in particular adenosine A_1 and A_2 receptors, which are located on neurons and glial cells in the brain and are linked *via* G-proteins to different effector systems, such as adenylate cyclase. Thus, acting at specific receptors, endogenously formed adenosine can exert a number of effects counteracting the 'pathologic activation' of nerve and glial cells (for review see Sweeny, 1997).

In the past, pharmacological studies with adenosine receptor antagonists, adenosine analogues, or agents that inhibit the physiological mode of adenosine inactivation have been used to characterize the role of adenosine in brain ischaemia and/or to find methods of drug therapy for patients with acute ischaemia and chronic vascular insufficiency (Rudolphi *et al*., 1992). One of the most promising agents is the xanthine derivative propentofylline (PPF, HWA 285). Previous studies have clearly shown an 'energy-saving' effect of PPF on the cerebral energy state in rats subjected to

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stepwise cerebral vessel occlusion (Plaschke *et al*., 1998). Furthermore, treatment with PPF prevents the ischaemia-induced glial activation otherwise seen after transient brain ischaemia in such forms as early hyperplasia of astrocytes (DeLeo *et al*., 1988), suggesting that this drug may also interfere with the change in astrocyte function. Moreover, PPF can reduce cerebral concentrations of cytokines such as IL1β and TNFα and free radical formation of activated microglia (Banati *et al*., 1994), and PPF can enhance synthesis and release of nerve growth factor (NGF) by cultured mouse astroglial cells (Shinoda *et al*., 1990). In rats with amnesia, oral administration of PPF for 16 days restored the learning capacity which had been decreased by continuous infusion of anti NGF-monoclonal antibody (Nitta *et al*., 1993). In addition, PPF-dependent protection against ischaemia-induced secondary nerve cell damage was documented in various experimental studies with focal ischaemia (DeLeo *et al*., 1988; Dux *et al*., 1990; Park & Rudolphi, 1994).

Concerning the pathomechanism of PPF, it has been shown that (i) inhibition of cyclic AMP-phosphodiesterases (Fredholm & Duner-Engstrom, 1989; Parkinson *et al*., 1994), (ii) reinforcement of adenosine A_2 receptor-mediated effects in a synergistic manner (Fredholm *et al*., 1992), and (iii) inhibition of the adenosine transport (Meskini *et al*., 1994) are potent mechanisms responsible for the protective adenosine-mediated actions of PPF. Furthermore, there is evidence that PPF is a weak adenosine autoreceptor A_1 antagonist (Parkinson & Fredholm, 1991) which can additionally inhibit its re-uptake and the activity of the 5′-nucleotidase (Fredholm & Lindgren, 1983).

While the neuroprotective effects of PPF on cerebral pathology have been described in a focal ischaemia model (DeLeo *et al*., 1988), there is no evidence on the role of this xanthine derivative on cerebral metabolism and brain function under conditions using the bilateral common carotid artery occlusion (BCCAO) model. Therefore, the present study investigates the effect of PPF on cerebral energy metabolism, cytokine expression, brain histopathology, and rat behaviour during acute (20 min) and long-term (3 weeks) cerebral hypoperfusion.

Methods

Animal procedure

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.

In all, 122 adult (1-year-old) male Wistar rats weighing between 450 and 650 g were housed individually in cages in a temperature-controlled animal room ($21 \pm 0.5^{\circ}$C) with reversed day/night cycle (12 h : 12 h). Free access to food and water was allowed throughout the experimental period. Two rats died in the course of operation to achieve vessel occlusion.

In 80 animals, biochemical determinations were performed under conditions of acute (20 min) and permanent (3 weeks) BCCAO in both hemispheres. A further 40 animals were used for psychometric testing and pathomorphologic investigations 3 weeks after BCCAO.

Rats were investigated according to the following acute and permanent experimental groups with $n = 10$ each: (1) sham; (2) sham [+]PPF; (3) BCCAO; (4) BCCAO [+]PPF.

Bilateral common carotid artery occlusion

Rats underwent BCCAO by ligation. The vessel occlusions were carefully performed under general anaesthesia with 1.5 vol% halothane and nitrous oxide–oxygen (70 : 30). During vessel occlusion care was taken to avoid damage of the surrounding tissue, particularly near the vagus nerve. For bilateral occlusion, a ventral midline incision was made to expose both carotid arteries. The arteries were gently isolated from the carotid sheath and vagus nerve. Each carotid artery was ligated with 5-0 silk suture (ETHICON®) just below the carotid bifurcation (Plaschke *et al*., 2000).

Rats with 3 weeks of permanent BCCAO were used for permanent hypoperfusion studies. Sham animals underwent the same operation procedure without vessel ligation.

Administration of propentofylline

In acute cerebral hypoperfusion experiments, propentofylline (PPF, HWA 285) was i.v. injected over a time interval of 1 min immediately after BCCAO at a dosage of 25 mg kg^{-1} body weight. This particular dosage was chosen because of its significant effect on cerebral energy metabolism in a comparable rat model of global ischaemia (Plaschke *et al*., 1998). PPF dissolved in 1 ml of sterile saline solution was administered for exactly 20 min into the femoral vein.

For long-term experiments PPF solution was administered i.p. directly after BCCAO and for 3 weeks at a dosage of 25 mg per day kg^{-1} body weight (10 μl h^{-1}), using a s.c.-implanted osmotic Alzet pump model 2ML1 (ALZA Corporation, Palo Alto, CA, U.S.A.). PPF was kindly provided by Hoechst Marion Roussel AG (Wiesbaden, Germany). In sham-operated animals, identical volumes of physiological saline solution were administered i.v. or by a s.c.-implanted tube.

Psychometric studies

Animals were housed in a temperature-regulated room ($21 \pm 0.5^{\circ}$C) with a reversed 12 h : 12 h light/dark cycle (at 0600 to 1800 h). Rats ($n = 40$) underwent psychometric testing with handling three times within 5 days. After 2 weeks of adaptation to the apparatus psychometric experiments were conducted during the dark part of the cycle with respect to the enhanced rat activity during night. The experimental period for psychometric testing lasted from 0900 to 1300 h. There was free access to food and water throughout the experimental period. During habituation and training periods and for 2 days before a retest, food restriction (5 g day^{-1}) was imposed to enhance motivation for performing the holeboard tests.

To measure rat locomotor activity and memory capacity, a holeboard memory test system was used (Lannert & Hoyer, 1998) to perform activity and memory testing before BCCAO, and also 24 h, 7, 14, and 21 days after the surgical intervention. All psychometric tests were performed four times, i.e. four tests were carried out using the same animals at each of the time-points.

In detail, habituation, training and retest for memory measurements were performed in a holeboard box. This square closed-field area ($70 \times 70 \times 40$ cm) contains 16 holes on the flat in a 4×4 array. Each hole contains a metal cup (3.5 cm in diameter, 3 cm deep) which has a perforated bottom. On one side of the wall, a starting box is attached and separated from the testing area by means of a guillotine door which can be operated from a distance. For habituation, the rats were placed into the starting box and allowed to enter the testing area to explore the holeboard with all 16 holes baited with 50 mg of food pellet. A trial started when the door was opened and ended when the rat dipped into all 16 holes. Even if the rat could not find all food pellets, the trial ended after 10 min. After four times of habituation the rats were trained to search a fixed order of four out of 16 baited holes. The trial was terminated when the rat found all food pellets or 5 min had elapsed, whatever occurred first. Four trials were performed each day. After sham operation and vessel occlusion the rats were tested by the same procedure as during training, but different combinations of food holes were applied to avoid the possibility of habituation as experienced the order of baited set of foodholes during the training period.

The following psychometric parameters were calculated: (i) rat locomotor activity as the mean intervisit interval [(elapsed time) − (latency time to the first hole)] [(number of visits and revisits to all holes) $-1]^{-1}$, and (ii) working memory (WM) = (number of food-rewarded holes) (number of visits and revisits to the baited set of holes)$^{-1}$ (Plaschke *et al*., 1999).

Final steady state experiment

The final steady state experiment was performed during anaesthesia to reach well controlled and stable haemodynamic parameters in all animals thereby preventing deleterious effects of the procedure of sacrifying on rat cerebral energy state. Therefore, anaesthesia was started with 3.0 vol% halothane. After tracheotomy, the trachea was intubated with a 2-mm endotracheal tube. Anaesthesia was then continued with 1.5 vol% halothane and nitrous oxide/oxygen 70 : 30. The animals were immobilized with pancuronium bromide (2 mg kg^{-1} body weight) and artificially ventilated to maintain end-tidal CO_2 between 35 and 40 mmHg with animal respirator 4600 (RHEMA Labortechnik). Femoral arteries and veins were exposed and catheterized (polyethylene PE 90) to measure mean arterial blood pressure (MAP) by means of a blood pressure transducer and a Hellige™ polygraph. In addition, arterial blood samples were taken for gas analysis and for measurements of haemoglobin (Hb), and haematocrit (Hct) with Chiron Diagnostics gas analyzer. Anaesthesia was continued with 0.5 vol% halothane and nitrous oxide/oxygen 70 : 30. After a 'steady state' of arterial normoxaemia, normocapnia, and normothermia was reached the brains were frozen *in situ* by means of liquid nitrogen. The animals were decapitated, and the whole brains were chiselled out of the skull under liquid nitrogen for morphologic determinations and stored at −80°C. For biochemical measurements, the right and left cerebral parietotemporal cortex and the hippocampus were prepared at −20°C.

Brain energy metabolism

Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), phosphocreatine (PCr), and adenosine (ADO) levels were determined in cerebral cortex and hippocampus of 80 rats by high-performance liquid chromatography (h.p.l.c.) analysis (Waters 2690 Alliance) after disruption of cell membranes with an ultraturrax (Brandel) in a chloroform–acetic acid mixture (1 : 2) with subsequent neutralization (3 M KOH) at −20°C.

Energy-rich phosphates Samples (0.1 ml) for determination of the energy-rich phosphates were automatically injected onto a Partisil SAX column (4.6 ID, 250 mm, 10 μm pore size, Waters). Two eluents were used: (i) 0.01 M H_3PO_4 (pH = 2.85); and (ii) 0.75 M KH_2PO_4 (pH = 4.4) for a total running-time of 60 min with a constant flow rate of 1.0 ml min^{-1}. The linear gradient started with 100% H_3PO_4 and 0% KH_2PO_4. After 10 min, the gradient reached 98% H_3PO_4 and 2% KH_2PO_4, changing within 45 min to 0% H_3PO_4 and 100% KH_2PO_4. Thereafter, eluent composition was reversed to reach 100% H_3PO_4 and 0% KH_2PO_4, allowing the column to equilibrate. Absorbency of the column eluate was continuously monitored at 210 and 254 nm using a photodiodearray-detector (Waters 966).

Adenosine analysis For adenosine analysis, samples (0.1 ml) were automatically injected onto a C-18 column (Nova-Pak C18, 3.9×150 mm, Waters). The linear gradient started with 100% KH_2PO_4 (0.001 M, pH 4.0) and changed to 60% of 60 : 40 methanol–water (vol : vol) in 15 min, the flow rate being 1.0 ml min^{-1}. This was followed by a reversal of the gradient to restore the initial conditions over the next 3 min. Absorbency of the column eluate was continuously monitored using photodiodearray detection at 254 nm.

Purine compounds and energy rich phosphates were quantified using a computer-assisted program (Millennium^{32}, Waters).

Cyclic AMP analysis For determination of cyclic AMP, a nonisotopic enzyme immunoassay test kit was used from Amersham™ (RPN 225). The assay is based on the competition between unlabelled cyclic AMP and a fixed quantity of peroxidase-labelled cyclic AMP for a limited number of binding sites on a cyclic AMP specific antibody. Rat samples were incubated with 100 μl rabbit anti-cyclic AMP serum in 0.05 M acetate buffer containing 0.02% (w v^{-1}) bovine serum albumin (BSA). The plate was covered with a lid and was incubated at 3–5°C for exactly 2 h. Thereafter, 50 μl cyclic AMP-horseradish peroxidase conjugate was transferred into all samples except the blank. The duration of the second incubation time at 3–5°C was exactly 60 min. After washing, 150 μl enzyme substrate [tetramethylbenzidine (TMB)/hydrogen peroxidase] was added. The reaction was stopped by addition of an acid solution, and the resultant colour was determined at 450 nm in the Bio-Rad ELISA reader.

Histopathology

Histological assessment was performed 21 days after permanent BCCAO. To this end, the animals were sacrificed

under steady state conditions and decapitated, and the brains were removed under liquid nitrogen for histopathological investigations. Thereafter, 8-μm-thick cryoslices were cut with LEICA cryostat through the rostral–caudal extent of the brain and stained with haematoxylin and eosin (HE). The sections were examined with a light microscope (LEICA), and ischaemic neuronal damage was graded on a scale of 0–3, where 0 = normal cells, 1 = few neurons damaged, 2 = many neurons damaged, and 3 = majority of neurons damaged. Each hemisphere was evaluated independently by an examiner blinded to the experimental conditions.

Cytokine concentration

For the determination of cerebral cytokine concentrations, samples of rat cerebral cortex and hippocampus were homogenized in 0.02 M Tris HCl buffer containing 0.1 mM DTT, 0.1 mM EDTA, 0.25 M sucrose, pH 7.5 and 10 μl of 10% Triton X-100 by means of a Potter homogenizer at 0°C. Protein concentration was determined by the method of Bradford (1976) (Bio-Rad) using BSA as standard.

Biosource International Cytoscreen® ELISA test kits (KRC0012, KR3012) were used for determination of rat IL1β and TNFα concentrations. The assays were performed according to the manufacturer's instruction. All ELISA measurements were performed in duplicate, and the means of the two measurements for each brain sample were used in the statistical analysis.

RT–PCR for mRNA of IL1β and TNFα

Mouse primers for reverse transcriptase-polymerase chain reaction (RT–PCR) of IL1β (No. 302420) and TNFα (No. 302124) were received from Stratagene (Germany).

The mouse TNFα sense and antisense oligonucleotide sequences were as follows: 5′-ATGAGCACAGAAAGCATGATC-3′ and 5′-TACAGGCTTGTCACTCGAATT-3′, respectively. As a IL1β positive control, a 447-bp PCR product has been used. Primers for RT–PCR of human β-actin were commercially synthesized by using data from published primer sequences (DeWerra *et al*., 1997).

The cortical brain tissue RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Adema & Baas, 1992). The final RNA pellet was air dried and then resuspended in ribonuclease-free water. Thereafter, the RNA preparation and cDNA synthesis was performed as described by Oberhofer *et al*. (1999).

PCR procedure A 2 μl sample of the first-strand cDNA was used in 25 μl Hot-Start PCR. The final PCR composition was optimized in preliminary experiments and contained 1 × PCR buffer (10 mmol Tris-HCl, pH 9.0; 50 mmol KCl; 0.1% Triton X-100), 1.0 mmol $MgCl_2$, 0.2 mmol deoxyribonucleoside triphosphate, 0.4 μmol of the primer, 1.0 U Taq DNA polymerase (Promega Corp.), and the indicated amount of cDNA.

Samples underwent PCR amplification with DNA-Thermal-Cycler 9600 (Perkin-Elmer Corp.) with 5 min of initial denaturation at 94°C and 5 min of annealing at 60°C, followed by 30 cycles of 30 s at 72°C, 15 s at 94°C, 20 s at 60°C, and the final extension of 7 min at 72°C. Subsequently, an aliquot of 5 μl of each amplified product underwent electrophoresis through a 2% agarose gel (Sigma) stained with SYBR Green I (Molecular Probes) to separate PCR fragments. The gels were documented and analysed with a video image capture and analysis system (Easy, Herolab). Semiquantification was realized by integrating fluorescence intensity over a given band, and the relative amount of the final RT–PCR product for IL1β and TNFα mRNA in rat brain cortex was normalized to the intensity of the corresponding β-actin band (Oberhofer *et al*., 1999).

Statistics and calculations

Statistical significant differences were calculated by ANOVA followed by a *post hoc* Tukey test using an SPSS statistic programme. Data were expressed as means ± standard deviation (s.d.) or means ± standard error of means (s.e.m.) and significant differences were assumed at $P<0.05$.

Drug section

Propentofylline, PPF, HWA 285, Hoechst Marion Roussel AG, Wiesbaden, Germany, 3-Methyl-1-[5-oxohexyl]-7-propylxanthine, $C_{15}H_{22}N_4O_3$, FW 306.4, dosage: 25 mg per day kg^{-1} body weight according to Plaschke *et al*. (1998) was used in the present study.

Results

Because no significant differences in all haemodynamic, metabolic and functional parameters were obtained between the sham groups in the absence (1) and presence (2) of PPF the data were combined to one sham group only.

Haemodynamic parameters

After 20 min of acute BCCAO the mean arterial blood pressure (MAP) was significantly higher than in non-occluded and PPF-treated animals. During permanent BCCAO no significant differences were observed in rat haemodynamic parameters and arterial blood gases. PPF did not markedly alter PO_2, PCO_2, pH and haematocrit in rats (Table 1).

Energy metabolism

Twenty minutes of acute BCCAO led to significant decreases in cortical ATP and phosphocreatine to 65 and 52%, respectively (Table 2). Simultaneously, the cortical tissue concentrations of AMP, cyclic AMP and adenosine were significantly increased 3.7 fold, 1.7 fold and 15.4 fold, respectively. In contrast, treatment with PPF normalized metabolic disturbances in rat energy-rich phosphates during acute BCCAO. However, PPF administration was associated with a 3 fold and 2 fold increase in the intracellular concentrations of cyclic AMP and adenosine, respectively.

During 3 weeks of permanent BCCAO there was no marked alteration in the cerebral energy rich phosphates. Although the changes in cortical adenosine concentration did not reach statistical significant differences, however, a 7 fold increase in adenosine was obtained with permanent BCCAO. In addition, comparison of the results of treatment with PPF

Table 1 Rat physiological parameters

	MAP mmHg	PO_2 mmHg	PCO_2 mmHg	*pH*	*Hct* %
Acute					
Sham	111±7.7	116±9.8	38±3.5	7.45±0.05	36.9±1.4
BCCAO	136±8.6[a]	136±16.5	39±2.2	7.46±0.04	35.6±4.1
BCCAO[+]PPF	109±4.6[b]	122±12.7	40±1.5	7.51±0.05	33.4±4.9
Permanent					
Sham	106±3.4	115±8.0	40±2.6	7.46±0.03	35.2±0.7
BCCAO	102±2.9	138±15.5	38±3.1	7.50±0.03	34.4±4.9
BCCAO[+]PPF	101±1.9	132±12.6	38±2.5	7.50±0.05	34.4±1.9

The influence of PPF on rat haemodynamic parameters and arterial blood gases after acute and permanent BCCAO was investigated. Mean±s.d., MAP: mean arterial blood pressure, BCCAO: bilateral common carotid artery occlusion; hct: haematocrit, ANOVA, $P<0.05$, Tukey-test, [a]BCCAO *vs* sham, [b]BCCAO[+]PPF *vs* BCCAO.

Table 2 Cortical energy metabolism

Cortex (μmol g^{-1})	*ATP*	*ADP*	*AMP*	*PCr*	*ADO* (nmol g^{-1})	*cyclic AMP* (pmol g^{-1})
Acute						
Sham	2.614±0.072	0.176±0.038	0.016±0.004	3.872±0.529	2.302±1.197	68.1±16.55
BCCAO	1.703±0.553[a]	0.313±0.166[a]	0.059±0.039[a]	1.995±1.453[a]	35.37±5.670[a]	118.11±30.74[a]
BCCAO[+]PPF	2.605±0.073[b]	0.296±0.085	0.020±0.025[b]	4.029±0.729[b]	66.54±31.41[b,c]	363.83±109.69[b,c]
Permanent						
Sham	2.590±0.198	0.191±0.034	0.024±0.007	4.001±0.336	1.906±1.210	68.51±22.60
BCCAO	2.521±0.319	0.189±0.058	0.021±0.007	3.909±1.203	13.37±22.03	39.25±34.62
BCCAO[+]PPF	2.611±0.073	0.119±0.010	0.011±0.003	4.218±0.571	18.56±35.88	25.37±25.73

The influence of PPF under acute and permanent BCCAO on rat cortical energy metabolism was investigated. Mean±s.d., PCr: phosphocreatine, ADO: adenosine, PPF: propentofylline, BCCAO: bilateral common carotid artery occlusion, ANOVA, $P<0.05$, Tukey-test, [a]BCCAO *vs* sham, [b]BCCAO[+]PPF *vs* BCCAO, [c]BCCAO[+]PPF *vs* sham.

under sham conditions and during BCCAO suggested that there was no significant effect on energy metabolism during long-term cerebral vessel occlusion.

A close relationship between cortical adenosine and cyclic AMP concentrations in rat cortex was determined after acute BCCAO in the absence and presence of PPF. The linear correlation coefficient ($r=0.68$, $P<0.001$) was calculated according to the Pearson coefficient from 30 animals using a SPSS statistic program.

Changes comparable to those seen in cortical tissue were obtained in rat hippocampus (Figure 1). In detail, acute ischaemia led to significant disturbances in the hippocampal energy state, which were characterized by decreased concentrations of energy rich phosphates to about 60% of control levels and by an 8 fold increase in AMP and adenosine levels. PPF administration normalized the ischaemia-dependent changes in energy rich phosphates under acute ischaemic conditions. However, a 3 fold increase in hippocampal adenosine concentration was obtained from 37.07±11.85 after BCCAO to 107.2±87.8 nmol g^{-1} in the presence of PPF.

Neither in the absence nor in the presence of PPF any marked effects of long-term hypoperfusion on hippocampal energy state were determined.

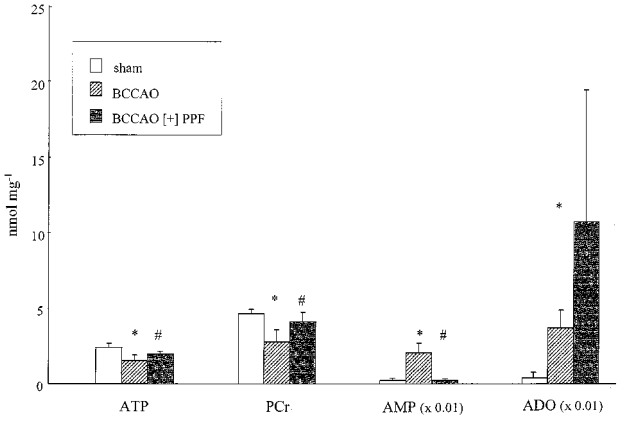

Figure 1 Hippocampal energy state. The effect of BCCAO on hippocampal energy metabolism was determined in rats after acute BCCAO in the absence and presence of PPF. Mean±s.d., BCCAO: bilateral common carotid artery occlusion, PCr: phosphocreatine, PPF: propentofylline, ADO: adenosine, ANOVA, $P<0.05$, * BCCAO *vs* sham; # BCCAO [+]PPF *vs* BCCAO.

Microglial reaction

In the absence of PPF, RT–PCR data showed a 4 fold and a 10 fold increase in IL1β and TNFα mRNA levels, respectively, after 20 min of acute vessel occlusion. PPF, however, substantially reduced the ischaemia-dependent increase in cortical cytokine levels (Figure 2).

Furthermore, the cortical concentrations of TNFα and IL1β were dramatically increased to 12 and 19 fold of the initial concentrations, respectively (Table 3). In contrast, in the presence of PPF no marked changes in TNFα concentrations were observed during acute ischaemia when they were compared with sham animals. In the case of IL1β,

however, PPF reduced this interleukin protein concentration to levels even lower than in sham group. Three weeks after permanent vessel occlusion, no changes were determined in TNFα and IL1β cerebral concentrations.

Psychometric analysis

Permanent BCCAO significantly increased rat locomotor activity (LA) to about 140% at 24 h and 220% at 7 days after surgery in the absence of PPF (Figure 3). In contrast, with PPF treatment a marked decrease in LA to about 10% of that in ischaemic animals was obtained until 2 weeks of permanent BCCAO had elapsed. No significant differences in rat LA were determined more than 14 days after surgery.

Working memory (WM) was markedly disturbed (-50%) over the whole experimental period of 3 weeks in rats subjected to permanent BCCAO (Figure 4) compared with the respective sham animals. Continuous administration of PPF was followed by a tendency to an increase in rat WM, which was most marked at 7 days after surgery ($P<0.05$).

Histopathology

Histological examination of subserial sections from animals in all experimental groups revealed no morphological lesions. Thus, hippocampal CA1 pyramidal cells appeared to be unaffected in acute BCCAO animals as well as 3 weeks after permanent BCCAO. No evidence of neural destruction was found in rat cerebral cortex, hippocampus or paramedian area in any of the experimental groups.

Discussion

In the present study, propentofylline (PPF) reduced the ischaemia-related increase in MAP to control levels. This result is in agreement with previous studies in rats by Matsumoto *et al.* (1996), in which PPF exhibited a vasodilatory effect after acute brain injury. Obviously, PPF-dependent vasodilatation is effective under conditions of acute hypertension such as were induced in the present study after BCCAO.

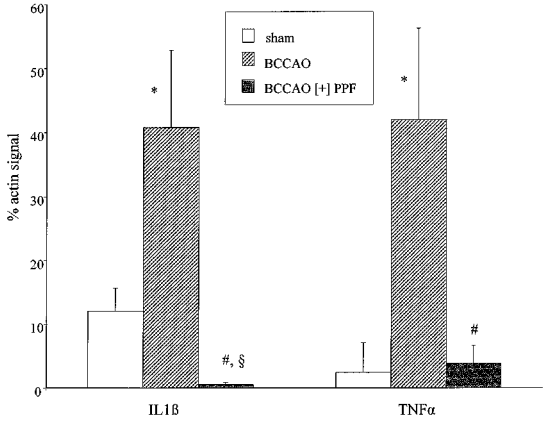

Figure 2 RT–PCR analysis of IL1β and TNFα. Mean$\pm$s.d., BCCAO: bilateral common carotid artery occlusion. The final RT–PCR product for rat cortical IL1β and TNFα mRNA after 20 min of BCCAO in the absence and presence of PPF was normalized to the intensity of the corresponding β-actin band. ANOVA, $P<0.05$, * BCCAO *vs* sham; # BCCAO [+]PPF *vs* BCCAO; § BCCAO [+] PPF *vs* sham.

After 20 min of BCCAO, there was a marked reduction in the concentrations of cortical and hippocampal ATP and phosphocreatine. Consequently, a corresponding increase in the ATP-degradation product adenosine occurred. While adenosine is a sensitive indicator of tissue ischaemia, this nucleoside can exert protective functions to limit metabolic disturbances by acting *via* adenosine receptors (Rudolphi *et al.*, 1992). One function of adenosine in the central nervous system is to amplify the cyclic AMP pathway directly *via* adenosine A_2 receptors (Lopes *et al.*, 1999). Recent data have indicated that the ischaemia-induced rise in cyclic AMP levels was obtained in the heart (Sandhu *et al.*, 1996) and brain after acute ischaemia (Prado *et al.*, 1992). These data are in agreement with results of the present study in which a significant increase in cerebral cyclic AMP concentration was obtained after acute BCCAO.

In the acute BCCAO model, PPF administration restored significantly the ischaemia reduced energy-rich phosphates ATP and phosphocreatine to near-normal values. As also shown by Parkinson *et al.* (1994) PPF induced a substantial increase in cortical intracellular adenosine concentration. According to its mechanism, PPF can inhibit the phosphodiesterase activity to reduce the breakdown of cyclic AMP to AMP (Fredholm *et al.*, 1994; Park & Rudolphi, 1994). This effect is demonstrated by a 3 fold increase in cyclic AMP in acute BCCAO with PPF as compared to BCCAO without PPF administration. Moreover, increased adenosine may be rephosphorylated particularly in the presence of PPF *via* the 'salvage pathway', to restore ATP. Consequently, more energy-rich phosphates are available to maintain cellular structure and cellular functions (Siesjö, 1978; Erecinska & Silver, 1989). With permanent BCCAO, however, no significant differences were determined between the groups. Possibly, under chronic conditions adaptive changes such as collateralization and pronounced vasodilatation (Coyle & Panzenbeck, 1990; Bronner *et al.*, 1998; Plaschke *et al.*, 2001, publication in preparation) might be activated to counteract the ischaemia-induced loss in energy-rich phosphates. Thus, with permanent BCCAO continuous PPF treatment did not influence the rat brain energy state.

Acute brain ischaemia which impaired cortical energy metabolism was associated with additional activation of rat microglia with the effect of 12 and 19 fold increases in the pro-inflammatory cytokines TNFα and IL1β, respectively. Circulating TNFα and other cytokines can be transported into the brain and severely impair brain function (Watkins *et al.*, 1995). In addition, cytokines can be synthesized in the brain mainly by microglia, but also by astrocytes and by neuronal and endothelial cells (Sawada *et al.*, 1989; Brenner *et al.*, 1993). Several investigators have demonstrated in the past that transient global and focal brain ischaemia induced pathological activation of microglia with resultant changes in tissue concentrations of cytokines and impaired expression (Morioka *et al.*, 1991; Gehrmann *et al.*, 1992; Hagberg *et al.*, 1996; Ohtsuki *et al.*, 1996). These findings have been confirmed in the present study by stimulated levels of mRNA for both TNFα and IL1β in rats undergoing acute vessel occlusion. Because pathologic cytokine expression of glia cells can lead to severe cell destruction, several attempts have been

Table 3 Cytokine concentrations

pg mg^{-1} protein	*Cortex*		*Hippocampus*	
	TNFα	*IL1β*	*TNFα*	*IL1β*
Acute				
Sham	26.35±7.46	1.82±0.71	81.8±12.46	11.90±1.53
BCCAO	311.06±192.14[a]	35.14±15.71[a]	204.37±114.25[a]	39.43±13.54[a]
BCCAO [+]PPF	28.92±20.33[b]	0.3±0.57[b,c]	62.04±47.57[b]	2.4±3.05[b,c]
Permanent				
Sham	26.96±6.79	1.75±0.77	66.47±14.72	10.37±2.44
BCCAO	25.43±5.62	1.38±0.45	53.24±11.00	11.30±3.54
BCCAO[+]PPF	24.94±7.61	1.87±1.51	42.49±12.68	9.20±5.05

The effect of PPF treatment on rat cortical and hippocampal cytokine concentrations after acute and permanent BCCAO was investigated. Mean±s.d., PPF: propentofylline, BCCAO: bilateral common carotid artery occlusion, ANOVA, $P<0.05$, Tukey-test, [a]BCCAO *vs* sham, [b]BCCAO[+]PPF *vs* BCCAO, [c]BCCAO[+]PPF *vs* sham.

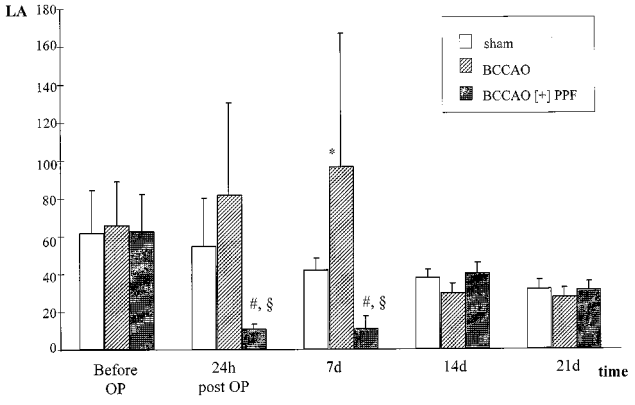

Figure 3 Rat locomotor activity. The effect of BCCAO on rat locomotor activity was determined in the absence and presence of PPF administration. Mean±s.e.mean, LA: locomotor activity in s, BCCAO: bilateral common carotid artery occlusion, PPF: propentofylline, ANOVA, Tukey-test, $P<0.05$, * BCCAO *vs* sham, # BCCAO [+]PPF *vs* BCCAO, § BCCAO [+] PPF *vs* sham.

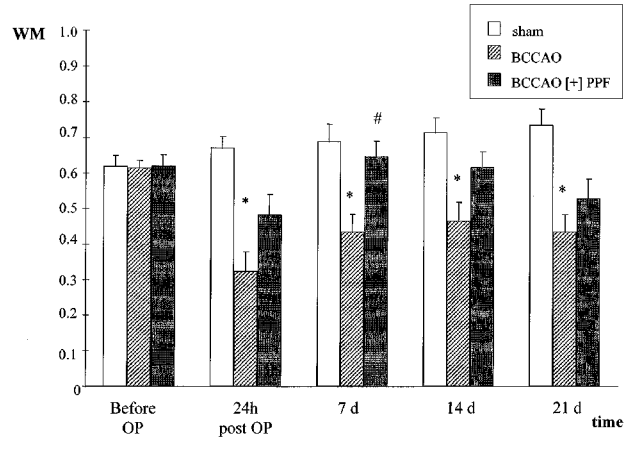

Figure 4 Working memory. The effect of PPF was determined on rat working memory (WM) under permanent BCCAO. Mean±s.d, OP: BCCAO, bilateral common carotid artery occlusion, d: day, ANOVA, Tukey-test, $P<0.05$, * BCCAO *vs* sham, # BCCAO [+]PPF *vs* BCCAO, WM=(number of food-rewarded holes) (number of visits and revisits to the baited set of holes)$^{-1}$.

undertaken to activate those homeostatic mechanisms that keep glial activation in a physiologic range (Schubert *et al*., 1998). One major regulator of glial cell state is the endogenous nucleoside adenosine, which may act *via* the cyclic AMP-signalling pathway. For instance, on the basis of *in vitro* studies it has been discussed that adenosine is able to block the production of TNFα possibly *via* the cyclic AMP-signalling pathway in human polymorphonuclear leukocytes (Thiel & Bardenheuer, 1994). Concerning the possible mechanism by which the production of TNFα might be inhibited, it is worthwhile to note that binding of adenosine to adenosine A_2 receptors is linked to the activation of adenylate cyclase resulting in increased production of intracellular cyclic AMP. Although a direct relationship between adenosine, cyclic AMP, and inhibition of TNFα production has not been studied in *in vitro* experiments there is abundant evidence that cyclic AMP is a potent regulator of the synthesis of TNFα in monocytes (Thiel & Chouker, 1995). On the other hand, Ramamoorthy *et al*. (1995) provided experimental results showing that the physiologic regulation of cerebral cytokine expression was rather independent from the cyclic AMP signalling pathway. The precise mechanism, however, by which adenosine modulates glial cell reaction still remains unknown, in particular under *in vivo* conditions. Therefore, PPF was used in the present study to elucidate the interrelation between adenosine, cyclic AMP and cytokine production in an *in vivo* model of rat BCCAO. PPF stimulated the formation of cerebral cyclic AMP and adenosine in a close linear relationship. However, the cytokine production and the related cytokine mRNA expression were significantly reduced compared with cytokine expression after BCCAO without PPF. Thus, PPF action on cytokine activation seems not to be controlled by cyclic AMP- or adenosine-dependent mechanisms alone. For instance, cyclic GMP-related actions and the 5′-nucleotidase mechanism of PPF action (Fredholm & Lindgren, 1983) might take place in PPF-mediated reduction of cerebral pro-inflammatory cytokine production.

Permanent BCCAO was associated with almost complete recovery in rat brain cytokine concentrations. This is in contrast to the results with acute BCCAO and also to the data of Block *et al*. (2000) demonstrating long-lasting microglial activation under transient cerebral ischaemia. We hypothesized that pronounced arterial collateralization indicated by recent angiography data on BCCAO rat model

(Plaschke *et al*., 2001, unpublished data) may improve cerebral blood flow and reduce microglial reaction.

Twenty-four hours after permanent BCCAO, rat working memory (WM) significantly decreased to about 50% which was consistently worse than in control animals for up to 3 weeks' occlusion time. While transient four-vessel occlusion induced changes in the structure of CA1 cells (Schmidt-Kastner & Freund, 1991) there were no histopathologic changes during permanent BCCAO in previous studies (Ni *et al*., 1995; Tanaka *et al*., 1996) and also in the present study. Ligation of the carotids alone seems to be ineffective in many animals in inducing substantial nerve cell damage, probably because of an ample collateral blood supply in chronic studies (Eklöf & Siesjö, 1972). This discrepancy between functional parameters and histopathologic changes has also been shown by other investigators. For instance, persistent cerebrovascular insufficiency causes visuo-spatial memory changes without structural hippocampal neuron damage (Olsen *et al*., 1994; Whishaw *et al*., 1994; Pappas *et al*., 1996). A direct correlation between locomotor changes and final degree of neuronal damage is a controversial subject (Poignet *et al*., 1989; Kuroiwa *et al*., 1991; Block & Schwarz, 1997; Plaschke *et al*., 1999). Concerning these results discussion is in progress as to whether at least part of the functional deficit during permanent brain vessel occlusions is more probably a result of 'transmission failure' (i.e. glutamate, Brown *et al*., 1974) rather than of 'energy failure' (Salford *et al*., 1973; Ljunggren *et al*., 1974). Confirming the findings of Ni *et al*. (1995) rats with mild hypoperfusion showed dysfunction of new information learning (WM).

The role of adenosine in the process of animal behaviour is a controversial subject. Thus, Hooper *et al*. (1996) put forward the hypothesis that endogenous adenosine does not normally participate in WM process in mice. On the other hand, Ohno & Watanabe (1996) suggested that activation of rat hippocampal adenosine A_1 receptors interferes with the neuronal processes involved in WM failure. Furthermore, adenosine A_2 but not A_1 receptor agonists reduced rat locomotor activity (LA) in a dose-dependent manner (Durcan & Morgan, 1989; Marston *et al*., 1998). The results of Von Lubitz *et al*. (1994) indicated that changes in mice's behaviour and histopathology were significantly different when agents acting *via* adenosine A_1 receptors were acutely rather than chronically administered. Thus, after chronic treatment with adenosine A_1 antagonist CPX (1,3-dipropyl-8-cyclopentylxanthine), animals showed no significant performance changes, and failed to develop spatial preference. However, acute application of the adenosine A_1 agonist CPA (N6-cyclopentyladenosine) led to significant behavioural reduction in rat spontaneous LA (Marston *et al*., 1998) and in mice memory as indicated by retention of one-trial inhibitory avoidance test (Normile & Barraco, 1991). In the present study, the administration of PPF did not influence rat behaviour under sham condition. However, PPF clearly caused a significant decrease of ischaemia-induced rat locomotor hyperactivity at 24 h and 7 days after permanent BCCAO. Two weeks of permanent BCCAO led to normalization of rat LA, indicating the transient character of these behavioural changes. Further studies with specific adenosine agonists and antagonists may result in new insights in the role of adenosine on rat behaviour during permanent BCCAO.

Summarizing the *in vivo* effects of PPF, we demonstrated the neuroprotective role of PPF in rat model of acute BCCAO on (i) rat cerebral energy state, and (ii) microglial reaction. In addition, continuous administration of PPF led to (iii) memory improvement during permanent cerebral vessel occlusion.

In conclusion, the results of the present study provide experimental evidence of the benefit of PPF treatment in clinical practice, particularly during stroke, but also in cerebrovascular and neurodegenerative disorders.

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