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Chronic administration of selective adenosine A₁ receptor agonist or antagonist in cerebral ischemia

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Abstract

The effect of chronic administration of selective adenosine A₁ receptor agonists and antagonists on the outcome of cerebral ischemia is entirely unknown. Therefore, we have investigated the impact of such regimens on the hippocampal adenosine A₁ receptor density, and on the recovery from 10 min forebrain ischemia in gerbils. While acutely administered *N*⁶-cyclopentyladenosine (CPA) given at 0.02 mg/kg resulted only in a significant reduction of mortality, at 1 mg/kg it improved both survival and neuronal preservation in the hippocampal CA1 region. Acute treatment with 1,3-dipropyl-8-cyclopentylxanthine (CPX) significantly worsened the outcome and enhanced neuronal destruction. The effects of chronic administration of these drugs (15 days followed by 1 drug-free day) were opposite. Thus, although chronic CPA at 0.02 mg/kg did not have any effect at all, at 1 mg/kg both survival and neuronal preservation were significantly poorer than in controls, while chronic CPX resulted in a significant improvement of both measures. These results were not accompanied by adenosine A₁ receptor up- or downregulation. Our study indicates that highly selective adenosine analogues may have therapeutic potential in treatment of cerebral ischemia/stroke and possibly other neurodegenerative disorders as well.

Keywords

Adenosine; Ischemia; Adenosine receptor; Therapy; (Gerbil)

1. Introduction

Almost 10 years ago, Newby (1984) described endogenously released adenosine as a 'retaliatory metabolite'. Several studies confirmed this concept showing that suitable stimulation of adenosine receptors appears to counteract the effect of traumatic tissue/organ challenges (for reviews, see Spielman and Arend, 1991; Olsson and Pearson, 1990; Dragunow and Faull, 1988; Rudolphi et al., 1992a, b).

In the brain, apart from having a regulatory effect upon cerebral blood flow (reviewed by Van Wylen et al., 1991), endogenously released adenosine has a direct influence upon

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neuronal functions. It has been thus shown that stimulation of neuronal adenosine A₁ receptors leads to reduction of calcium influx (Dolphin, 1983), neurotransmitter release (Coradetti et al., 1984; Dolphin and Archer, 1983; Harms et al., 1978), depression of neuronal excitability and firing rate (Phillis et al., 1974; Kostopoulos and Phillis, 1977), and hence, to lowering of the neuronal metabolic rate (Phillis and Wu, 1983).

Cerebral ischemia is associated with excessive release of excitatory amino acids (Globus et al., 1990) and overstimulation of NMDA receptors leading to calcium overload of the neuronal cytoplasm (Choi, 1992). The 'excitotoxic' impact of glutamate and other excitatory amino acids released during ischemia (Olney, 1978) is believed to be one of the chief perpetrators of the subsequent neuronal death (Rothman and Olney, 1987; Choi, 1992). Moreover, recent evidence indicates that activation of metabotropic glutamate receptors results in potentiation of responses to endogenous adenosine (Winder and Conn, 1993), while stimulation of both metabotropic glutamate and NMDA receptors results in enhancement of adenosine release (Hoehn and White, 1990). Thus, the functional relation of the excitatory amino acid receptors and adenosine supports a possibility that the system of adenosine release, receptors, and uptake may constitute an endogenous self-defense apparatus activated in stroke and other brain disorders involving excitotoxicity (Von Lubitz and Marangos, 1992).

Several independent groups demonstrated that acute treatment with selective adenosine A₁ receptor agonists prior to or after ischemia, or inhibition of the uptake of endogenously released adenosine led to a significant neuroprotection (for reviews see Dragunow and Faull, 1988; Rudolphi et al., 1992a, b; Miller and Hsu, 1992). Acutely administered antagonists have, on the other hand, a converse effect and aggravate the outcome (reviewed by Rudolphi et al., 1992a, b). Despite encouraging results of acute administration of selective adenosine A₁ receptor agonists, the extent of eventual neuroprotection following chronic exposure to these agents is virtually unknown. Importantly, however, it has been shown that chronic exposure to caffeine, a nonselective adenosine antagonist, results in a significant reduction of postischemic damage in gerbils (Rudolphi et al., 1989). Furthermore, we have recently shown (Von Lubitz et al., 1993b, in press) that, in contrast to the acute treatment with adenosine A₁ receptor agonists, chronic treatment of mice with N⁶-cyclopentyladenosine (CPA) is procognitive. Also in opposition to the acute administration of CPA, chronic exposure to this drug increases susceptibility to NMDA induced seizures (Von Lubitz et al., 1994). Chronic administration of 1,3-dipropyl-8-cyclopentylxanthine (CPX) produces opposite results. Thus, chronic treatment with highly selective agents acting at adenosine A₁ receptor appears to have a converse effect to that seen following their acute administration. We have explored this possibility further by investigating the effect of CPA and CPX administered either acutely or chronically prior to forebrain ischemia in gerbils.

2. Materials and methods

Female gerbils (60–70 g, Tumblebrook Farms, MA) were screened for susceptibility to spontaneous seizures using the 'triple handling' technique of Lee et al. (1984). Only animals in which seizures could not be elicited participated in subsequent experiments.

2.1. Drugs

Drugs were purchased from Research Biochemicals International (Natick, MA). Both N^6 -cyclopentyladenosine (CPA) and 8-cyclopentyl-1,3-dipropylxanthine were dissolved in a 20:80 mixture of Alkamuls EL-620 (Rhone-Poulenc, Cranbury, NJ) and saline. Drug dosing was based upon our previous studies (Von Lubitz et al, 1993b, c, 1994). CPA was therefore injected in a volume of 0.15 ml at either 0.02 mg/kg or 1.0 mg/kg, while CPX was given at 1.0 mg/kg. Drug solutions or the vehicle (0.15 ml) were injected i.p. using a 25 gauge needle according to treatment schedules shown in Table 1.

2.2. Adenosine A₁ receptor analysis

Adenosine A₁ receptor analysis was performed only in the non-ischemic animals. Three separate groups of gerbils were injected i.p. each day for 15 days with either vehicle (0.15 ml), CPA (1.0 mg/kg) or CPX (1.0 mg/kg). After a 24 h washout period, animals were decapitated, their brains were rapidly removed and placed on an ice-cold glass plate. Cortices, hippocampi and striata were freed of excessive myelin and frozen on dry ice. Cortices and striata were stored at -70°C for future use, while the hippocampi were homogenized in 10 volumes (v/w) of 0.32% sucrose solution. The homogenate was centrifuged at $1000 \times g$ for 10 min, and the supernatant was removed and recentrifuged at $32000 \times g$ for 40 min. The resulting pellet was resuspended in water, recentrifuged, and the pellet resuspended in Tris-HCl buffer (pH 7.4) at a concentration of 1.5–2 mg protein/ml. All the above procedures were carried out at 4°C . Protein content was determined using the BCA protein assay reagents (Pierce Chemical Co., Rockford, IL). [^3H]CPX (DuPont NEN, Boston, MA) saturation studies were carried out as described previously (Bruns et al., 1987) at 25°C , using the radioligand in the range of 0.04–3 nM. Each incubation tube contained ca. 30–50 μg protein in a total volume of 500 μl Tris-HCl (pH 7.4), with adenosine deaminase (3 IU/ml) present. Scatchard analysis was used to determine B_{max} and K_D .

2.3. Ischemia

Ischemia was induced 24 h (washout period) after the last injection of either drugs or vehicle. Animals were anesthetized with 2% halothane in O_2 and N_2O (1 : 2) followed by ligation of both carotid arteries with monofilament nylon sutures (Ethicon) for 10 min (Von Lubitz et al., 1989, 1992a). Previous experiments (Von Lubitz et al., 1992b, and unpublished data) indicated that this procedure results in a 95–99% reduction of the cortical blood flow rate as measured with a Perimed PF3 laser-Doppler flowmeter.

2.4. Monitoring of physiological parameters

Rectal temperature was monitored either with an electronic Harvard or YSI rectal thermometer, and epicranial and tympanic temperatures with Exergen Infrared Veterinarian Temperature Monitors. Measurements of rectal, epicranial, and tympanic temperatures were made immediately prior to the injection of all drugs. During the period between injection and surgery, rectal and epicranial temperatures were measured every 3 min, while during both surgery and ischemia temperature was monitored continuously. Rectal temperature was maintained by means of a heating blanket (Harvard). Epicranial temperature was maintained by means of an independent heating spotlight illuminating the head. During ischemia,

tympanic temperature was measured every 5 min, assuring that both hypothalamic and epicranial temperatures were within 0.5°C of each other. During recovery, rectal and epicranial temperatures were measured every 15 min until the return of spontaneous thermoregulation (Table 2).

2.5. Histology

Seven days postischemia, all surviving animals were perfused with buffered formaldehyde. Two naive gerbils were also perfused and served as overall controls. The brains were removed, infiltrated with 20% sucrose, and sectioned on a freezing microtome at 40 μm . The sections were then stained with Nissl (Lin et al., 1990). Five randomly selected sections from the region 0–2 mm behind the bregma were obtained from each brain. Neurons showing unchanged morphology were counted in the ischemia-vulnerable CA1 region. All counts were made by an investigator unaware of the treatment protocol. Further details of histopathological methods have been published previously (Von Lubitz et al., 1992a, b).

2.6. Statistical analysis

Analysis of variance followed by the Tukey test was used to determine statistical significance of the histological data. End-point survival data were analyzed using Bonferroni-corrected Fisher's exact test, while Student-Newman-Keuls test was used to analyze the receptor data. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1. Receptor analysis

Chronic treatment with CPA (1 mg/kg) or CPX (1 mg/kg) for 15 days followed by a single washout day did not result in changes of either receptor density (B_{max}) or dissociation constants (K_d) (Table 3).

3.2. Control animals

The pattern of mortality among control gerbils was similar to that described in our previous studies (Von Lubitz et al., 1989, 1992a, b). Most of animals (34%) died within the initial 48 h postischemia. No animals died after the 4th day, and the end-point survival was 60% (Table 1, Fig. 1). Severe damage of the hippocampal CA1 region was present in all survivors (Fig. 2), with only 34% of the neuronal population showing normal morphology.

3.3. Acute administration of CPA and CPX

When CPA was administered acutely at 0.02 mg/kg, there was a significant increase in the end-point survival (94%, $P < 0.01$, see Table 1, Fig. 1). There was no effect on neuronal survival at this dose (26% neurons surviving, $P > 0.05$). Acute administration of CPA at 1.0 mg/kg also significantly improved survival at the end-point (90%, $P < 0.01$, Fig. 1). Moreover, 72% of the neuronal population in the CA1 was still intact ($P < 0.01$).

Compared to controls, the acute treatment with CPX significantly increased the mortality rate (Table 1, Fig. 1), with 63% gerbils dying during the initial 48 h postischemia. End-point

mortality was also significantly worse ($P < 0.01$). Furthermore, the number of surviving neurons was significantly lower than in the controls (22%, $P < 0.01$, Fig. 2).

3.4. Chronic administration of CPA and CPX

Chronic treatment with CPA at 0.02 mg/kg did not result in significant changes of either end-point survival or hippocampal morphology (Table 1, Figs. 1 and 2). However, when CPA was given at 1.0 mg/kg, the mortality rate and end-point survival were both significantly worse than in the controls ($P < 0.01$). Moreover, the values of both parameters did not differ statistically from those seen following acute administration of CPX (Fig. 1). Neuronal preservation in the two animals that survived to the end-point was very poor, and amounted to less than 5% (Fig. 2).

The mortality rate and the end-point survival following chronic treatment with 1.0 mg/kg CPX were improved to the extent that they were statistically indistinguishable from the values seen with acute doses of CPA at either 0.02 mg/kg or 1 mg/kg (Table 1, Fig. 1). Furthermore, the degree of neuronal preservation in the hippocampus obtained by the chronic CPX regimen was statistically indistinguishable from that seen with CPA administered acutely at 1 mg/kg (Fig. 2).

4. Discussion

Our study confirms results of previous investigations showing that acutely administered selective adenosine A₁ receptor agonists such as phenylisopropyl adenosine (PIA), cyclohexyl adenosine (CHA) or CPA are neuroprotective in models of stroke (Roucher et al., 1991) and global brain ischemia (Block and Pulsinelli, 1987; Von Lubitz et al., 1989). However, although micromolar doses of PIA reduced quinolinic acid neurotoxicity in vitro (Connick and Stone, 1989), no amelioration of cortical damage has been observed following middle cerebral artery occlusion (Roussel et al., 1991). Similarly, despite significantly better survival among animals treated acutely with a micromolar dose of CPA, there was no reduction of the hippocampal damage (present study). Nonetheless, we cannot exclude a possibility of protection in other regions where ischemic damage also develops (e.g., cortex and striatum, see Siesjö, 1988). Discrepancies between the degree of neuronal sparing and survival rate have been described before in the same model of cerebral ischemia (Von Lubitz et al., 1993a) and stress desirability of more than one measure in determination of therapeutic outcome following a drug treatment.

The exact nature of mechanisms involved in amelioration of postischemic neuronal damage by acute administration of adenosine A₁ receptor agonists is not clear. Adenosine-mediated inhibition of neurotransmitter release is a well known phenomenon (Fredholm and Dunwiddie, 1988). Equally well described is inhibition of presynaptic calcium uptake (Wu et al., 1982; Proctor and Dunwiddie, 1983; Schubert et al., 1986). Postsynaptically, activation of adenosine A₁ receptors causes decrease in membrane resistance, increase in membrane potential, and enhancement of afterhyperpolarization (Haas and Greene, 1984; Gerber et al., 1989), all of which are obtained through activation of potassium (Haas and Greene, 1984; Greene and Haas, 1991) and chloride conductances (Schubert et al., 1991). As a further consequence of such stabilization of the membrane potential, adenosine is

capable of reducing input frequencies necessary to induce NMDA receptor mediated calcium influx (Schubert and Kreutzberg, 1990). It appears, therefore, that while several contributory processes are involved, the main source of neuroprotection obtained with acutely administered adenosine A₁ receptor agonists is maintenance of calcium homeostasis during the critical, immediately postischemic period (DeLeo et al., 1987; Dux et al., 1992; Andine, 1993). This conclusion is also supported by studies showing that acute treatment with adenosine A₁ receptor antagonists aggravates the damage both in vitro (Dux et al., 1992) and in vivo (Rudolphi et al., 1987).

Chronic treatment with adenosine A₁ receptor agonists decreases (Parsons and Stiles, 1987), while chronic treatment with caffeine (a nonselective antagonist) increases adenosine A₁ receptor density (Fredholm, 1982). Moreover, Rudolphi et al. (1989, 1990) showed that reduction of ischemic brain damage in gerbils may be the consequence of upregulation of adenosine A₁ receptors following chronic treatment with low doses of caffeine administered prior to ischemia. It is, therefore, surprising that during chronic administration of either CPA or CPX in both mice (Von Lubitz et al., 1994) and gerbils (present study) receptor up- or downregulation does not occur. Georgiev et al. (1993) reported similar results following chronic treatment of mice with very low doses of caffeine.

Although considerable progress has been made toward understanding of neuronal response to acute exposure to selective and non-selective agents acting at the adenosine A₁ receptor, virtually nothing is known about the consequences of a chronic treatment. The surprising absence of changes in the density of adenosine A₁ receptors indicates that the mechanism responsible for damage amelioration by chronic CPX, or its aggravation by chronic CPA, may involve processes independent of adenosine A₁ receptor shifts. However, it is possible that chronic treatment with a highly selective agonist or antagonist introduces perturbances of the inhibitory tone maintained by endogenous adenosine. As a consequence, release of several neurotransmitters could be affected, and result in subsequent density shifts of several non-adenosine receptor types. Many of these receptors may be involved in either generation of, or protection against ischemic damage. It is known that chronic treatment with caffeine upregulates not only adenosine A₁ receptors but also GABA_A (Wu and Coffin, 1984), and recently it has been demonstrated that, due to such treatment, the density of these receptors may increase by 65% (Shi et al., 1993). Whether chronic treatment with CPX also upregulates GABA_A receptors is unknown. Postischemic failure of GABA-mediated inhibition represents a significant component of selective ischemic damage (Sternau et al., 1989). One may speculate, therefore, that the chronic treatment with CPX caused preservation of the inhibitory modulation by GABA. Consequent decrease of the excitotoxic index (Globus et al., 1991) would then account for the significantly improved outcome of global ischemia after chronic CPX. If correct, this argument would also explain converse results obtained with chronic CPA. Interestingly, barbiturate therapies in which GABA_A receptors are also involved, appear ineffective in global ischemia, although their administration either during or soon after stroke results consistently in the reduction of the infarct (Martz and Hoff, 1990; Wauquier et al., 1987). Similar considerations may also apply to other receptor systems (e.g., serotonin, noradrenaline) which also participate in modulation of postischemic events (Koide et al., 1986; Spatz and Mrsulja, 1990). Studies have also shown that adenosine receptors may be functionally related to other receptor types

and share second messengers via common G-proteins (Ramkumar and Stiles, 1988; Worley et al., 1987). Moreover, chronic ingestion of caffeine (Ramkumar et al., 1988) and theophylline (Fastbom and Fredholm, 1990) affects coupling of G-proteins and receptors. Since G-protein changes affect sensitivity to NMDA (Durcan and Morgan, 1991), the effects of chronically administered adenosine A₁ receptor agonists and antagonists may involve pre- and postsynaptic, as well as postreceptor phenomena (Georgiev et al., 1993). Whether similar events take place during chronic treatment with CPA, CPX and other highly selective adenosine A₁ receptor agents is entirely unknown.

Despite uncertainties concerning mechanisms involved in neuroprotective effects of both acutely and chronically administered adenosine analogues, there is convincing evidence for their therapeutic effects in several disorders of the central nervous system (Von Lubitz and Marangos, 1992). However, eventual clinical implementation of adenosine-based therapies will require that the selected treatment regimen (chronic/acute), and the selected form of the drug (agonist/antagonist) are carefully adopted to the type of the targeted disorder (i.e., chronic/acute).

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Fig. 1.

The effect of either vehicle, or acute or chronic administration of CPA or CPX on survival of 10 min forebrain ischemia. Symbols: black circles – controls; open squares – acute CPA, 0.02 mg/kg; black squares – chronic CPA, 0.02 mg/kg; open triangles – acute CPA, 1.0 mg/kg; black triangles – chronic CPA, 1.0 mg/kg; open diamonds – acute CPX, 1.0 mg/kg; black diamonds – chronic CPX, 1.0 mg/kg.

Fig. 2.

Neuronal survival in the CA1 sector of the hippocampus at 7 days after 10 min forebrain ischemia. Prior to ischemia, animals were treated either acutely or chronically either with the vehicle, CPA (0.02 or 1.0 mg/kg), or CPX (1.0 mg/kg). Details of treatment regimens are described in Table 1. Statistical significance: A – treatment to controls, B – between groups. $P < 0.05$ in both cases, Bonferroni corrected Fisher's exact test.

Table 1

Dose regimens and their outcome (end-point survival) in the treatment of 10 min cerebral ischemia in gerbils

Treatment	Survival (%)	<i>P</i> value ^c
Vehicle	60	
CPA (1.0 mg/kg)		
Acute ^a	90	< 0.01
Chronic ^b	14	< 0.01
CPA (0.02 mg/kg)		
Acute ^a	94	< 0.01
Chronic ^b	74	n.s.
CPX (1.0 mg/kg)		
Acute ^a	20	< 0.01
Chronic ^b	93	< 0.01

^a Acute treatment: drugs administered 15 min prior to the occlusion.^b Chronic treatment: drugs administered for 15 days followed by one drug-free day between the last injection and ischemia.^c Compared to controls. Bonferroni-corrected Fisher's exact test.

Table 2

Tympanic temperatures ($^{\circ}\text{C} \pm \text{S.E.M.}$) measured immediately before acute administration of either vehicle, CPA, or CPX (15 min prior to 10 min ischemia, doses given in mg/kg) and 30 min after reperfusion

	Controls	CPA 0.02	CPA 1.0	CPX 1.0
Before	37.3 ± 0.15	37.4 ± 0.16	37.4 ± 0.1	37.4 ± 0.14
After	37.2 ± 0.15	37.4 ± 0.5	37.3 ± 0.1	37.2 ± 0.6

Table 3

Saturation binding experiments in hippocampal membranes prepared from gerbil brain following chronic administration of an A₁ selective adenosine receptor agonist (CPA) or an A₁ selective adenosine receptor antagonist (CPX)^a

Group	<i>B</i> _{max} (pmol/mg protein)	<i>K</i> _d (nM)	<i>n</i>
Control	1.25 ± 0.09	0.40 ± 0.02	7
CPA-treated	1.45 ± 0.08	0.37 ± 0.05	7
CPX-treated	1.57 ± 0.20	0.40 ± 0.04	4

^aSaturation of binding of [³H]CPX (see Materials and methods), values expressed as mean ± S.E.M. No statistical differences were observed (Student-Newman-Keuls test). Both drugs given at 1 mg/kg for 15 days followed by one drug-free day.