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Brain enzyme activities after intracerebroventricular injection of streptozotocin in rats receiving acetyl-L-carnitine

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Abstract

Intracerebroventricular (i.c.v.) injection of streptozotocin has been introduced as a means to inhibit glucose utilization in the rat brain, and to induce changes in neurotransmitter systems and behavior which resemble those seen in Alzheimer's disease. In this study, enzyme activities previously investigated in Alzheimer's disease (peptidases, dehydrogenases and acetyltransferases) were measured in the septum and hippocampus of control and streptozotocin-treated rats. Streptozotocin-treated rats receiving acetyl-L-carnitine were also included in the experiments, to assess possible neuroprotective effects of this substance. All enzyme activities in the septum were affected by streptozotocin, with the exception of choline acetyltransferase activity. By contrast, choline acetyltransferase activity was the only enzyme activity affected in the hippocampus. The weight of the septum was reduced in streptozotocin-treated animals. These findings indicate that i.c.v. injection of streptozotocin causes septal damage and enzymatic changes that do not closely resemble those seen in Alzheimer's disease, which are more specific. Acetyl-L-carnitine partly prevented this damage, as reflected by an attenuation of the streptozotocin-induced decrease in hippocampal choline acetyltransferase activity. This finding indicates that streptozotocin-treated rats may be valuable to test possible neuroprotective effects of drugs.

Keywords: Streptozotocin; Acetyl-L-carnitine; Brain; Alzheimer's disease; Oxidative stress

1. Introduction

Recently, Nitsch and Hoyer (1991) introduced intracerebroventricular (i.c.v.) injection of streptozotocin as a means to reduce brain glucose utilization. Besides a reduced glucose utilization after i.c.v. injection of streptozotocin, cholinergic and monoaminergic deficits and behavioral impairments have been observed (Ding et al., 1992; Hellweg et al., 1992; Blokland and Jolles, 1993, 1994). These deficits have been suggested to resemble those of Alzheimer's disease and, therefore, steptozotocin treatment was assumed to be of relevance to investigate pathogenetic changes involved in Alzheimer's disease (Nitsch et al., 1989; Blokland and Jolles, 1993; Plaschke and Hoyer, 1993). The mechanism through which streptozotocin causes its effects on the brain has not been investigated as yet. Hoyer and collaborators speculated that its action on the central nervous system might be similar to its peripheral action, i.e. the destruction of insulin-secreting cells or interference with the insulin receptor system (Nitsch et al., 1989; Nitsch and Hoyer, 1991; Plaschke and Hoyer, 1993). Systemic administration of streptozotocin is used to induce diabetes mellitus in experimental animals (Rakieten et al., 1963). Pancreatic β-cells are selectively destroyed by streptozotocin (Rakieten et al., 1963), although the selective vulnerability of these cells to streptozotocin is not entirely understood. Several studies have demonstrated that inhibitors of poly-ADP ribosylation, which is stimulated by single-strand DNA breaks, decrease the effect of streptozotocin on pancreatic β-cells (e.g. Dulin and Wyse, 1969; Bolaffi et al., 1987). This suggests that streptozotocin causes DNA

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damage, which is in accordance with the finding that streptozotocin generates DNA-damaging free radicals in cultures of pancreatic islets (Takasu et al., 1991).

The present study addresses the question whether changes in enzyme activities as observed in Alzheimer's disease are also observed after i.c.v. injection of streptozotocin. Thus, the possible relevance of the streptozotocin-treated rat as a model for Alzheimer's disease was studied. Several enzyme activities previously studied in Alzheimer's disease (Davies, 1979; Kalaria and Harik, 1992; Mastrogiacomo et al., 1993; Terwel et al., 1992, 1994) were measured in the septum and hippocampus three weeks after i.c.v. injection of streptozotocin or saline. The enzyme activities measured in the studies were those of prolyl endopeptidase, α-ketoglutarate dehydrogenase, carnitine acetyltransferase and choline acetyltransferase, which are affected in Alzheimer's disease, and those of aminopeptidase and glutamate dehydrogenase, which are not affected in Alzheimer's disease. Choline acetyltransferase activity was used as a marker of cholinergic neurons, which die or shrink in Alzheimer's disease. The other enzyme activities are of a ubiquitous nature. The present study also addresses the question whether in the brain α-ketoglutarate dehydrogenase is selectively affected by streptozotocin as compared to glutamate dehydrogenase, which has been shown to be the case in pancreatic β-cells (Rasschaert et al., 1992).

L-Carnitine and its acetylated form, acetyl-L-carnitine, are endogenous substances in various organs, including the brain (Bieber, 1988). L-Carnitine has a role in the β-oxidation by mitochondria of organs such as liver and muscle. This role of L-carnitine is of minor importance in the brain, since the brain almost exclusively depends on the oxidation of glucose to meet its energy demands. However, L-carnitine and acetyl-L-carnitine may be involved in the regulation of metabolic pathways in the brain (and other organs) by having an influence on the regeneration of CoASH and modulation of the level of acetyl-CoASH in the mitochondria. In addition, acetyl-L-carnitine may have a role as a precursor of acetylcholine, by providing the acetyl moiety for acetylcholine synthesis (White and Scates, 1990).

Chronic treatment with acetyl-L-carnitine has been shown to prevent impaired neuronal functioning in aged rats, both at the neurochemical level (Napoleone et al., 1990; Patacchioli et al. 1989; Taglialatela et al. 1994) and at the behavioral level (Barnes et al., 1990; Caprioli et al, 1990; Ghirardi et al., 1988, 1992). Moreover, it has been found that chronic treatment with acetyl-L-carnitine increases cholinergic functioning after fimbria-fornix transection (Piovesan et al. 1994), and attenuates behavioral deficits induced by i.c.v. treatment with cyanide (Blokland et al., 1993) or streptozotocin (Prickaerts et al., 1995). However, no uniform explanation has been offered to explain these neuroprotective effects of acetyl-L-carnitine. As neuronal functioning may be affected after streptozotocin treatment, the effects of chronic treatment with acetyl-L-carnitine on the enzyme activities mentioned were also evaluated in the streptozotocin-treated rats.

2. Materials and methods

2.1. Animals

For the first experiment 23 male Lewis rats of 18 months of age were used. The rats were divided over three groups of seven to nine animals. One group of rats was given acetyl-L-carnitine (75 mg/kg per day) in the drinking fluid (0.1% saccharine). After 2 weeks on acetyl-L-carnitine the rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic frame. A skin incision was made to free the skull and two holes were drilled above the lateral ventricles. The animals were given an injection of 2 μl of streptozotocin solution in each lateral ventricle (total dose 1.5 mg/kg body weight). The stereotaxic coordinates were -0.8 mm anterior, 1.55 mm lateral and -3.8 mm ventral from bregma (Paxinos and Watson, 1988). Streptozotocin was dissolved in saline just prior to injection. Another group of rats was not given acetyl-L-carnitine in the drinking fluid, but received injections of streptozotocin only. A third group of animals received injections with saline. 3 weeks after injection the animals were decapitated and the brains were removed from the skull in a cold room for dissection of the septum and hippocampus. For the second experiment 16 rats were used, divided over two groups of eight animals. The rats were treated in the same manner as in the first experiment, except that none of the rats was treated with acetyl-L-carnitine.

2.2. Enzyme assays

Brain tissues were homogenized in 19 volumes of 50 mM sodium phosphate (pH 7.4). Part of the homogenates was centrifuged at high speed to obtain particle-free fractions. In both experiments prolyl endopeptidase, aminopeptidase and choline acetyltransferase activities were determined. In addition, carnitine acetyltransferase activity was determined in the first experiment and α-ketoglutarate dehydrogenase, and glutamate dehydrogenase activities in the second experiment. All enzyme activities were not determined in a single experiment because of insufficient material.

2.2.1. Prolyl endopeptidase

Prolyl endopeptidase activity was determined spectrophotometrically with *N*-succinyl-Gly-Pro-Leu-Gly-Pro-7-amido-4-methylcoumarin as substrate. The sub-

strate (0.1 mM) was incubated with 10 μl cytosol in 200 μl of a buffer consisting of 50 mM Tris (pH 7.4), 1 mM DTT and 1 mM EDTA, at 37°C. After 1 h of incubation the reaction was stopped by the addition of 0.3 ml 1 M acetic acid. Extinction was measured at a wavelength of 373 nm.

2.2.2. Aminopeptidase

Aminopeptidase activity was measured with alanine *p*-nitroanilide as substrate. The substrate (0.5 mM) was incubated with 10 μl cytosol in 200 μl 50 mM Tris (pH 7.4) at 37°C. After 30 min of incubation the reaction was stopped by the addition of 0.3 ml ethanol. Extinction was measured at a wavelength of 410 nm.

2.2.3. α-Ketoglutarate dehydrogenase

α-Ketoglutarate dehydrogenase activity was determined in the homogenates according to the procedure of Lai and Cooper (1986).

2.2.4. Glutamate dehydrogenase

Glutamate dehydrogenase activity was determined spectrophotometrically. The reaction mixture consisted of 50 mM Tris (pH 8.0), 2.5 mM EDTA, 100 mM ammonium acetate, 0.2 mM NADH, 1 mM ADP, 7 mM α-ketoglutarate, and 20 μl 2% homogenate in a final volume of 1 ml. The reaction, at ambient temperature, was started by the addition of α-ketoglutarate. The disappearance of NADH was monitored at a wavelength of 340 nm. To calculate enzyme activity a correction was made for the change in extinction in the absence of α-ketoglutarate.

2.2.5. Choline acetyltransferase and carnitine acetyltransferase

Choline acetyltransferase activity was measured in the homogenates according to the method of Fonnum (1975). The method for the determination of carnitine acetyltransferase activity was identical to the method for the determination of choline acetyltransferase activity, except that L-carnitine hydrochloride was used as substrate instead of choline chloride and the reaction was stopped with 10 mM phosphate (pH 3.0) instead of 14% trichloroacetic acid.

2.3. Statistics

Data of the first experiment were subjected to a one-way analysis of variance with treatment as the dependent variable. Treatment effects between the three experimental groups were evaluated in more detail by using Student-Newman-Keuls test. Treatment effects in the second experiment were assessed by using Student's *t*-test. Relationships between measures were assessed by calculating Pearson's correlation coefficient (R).

3. Results

3.1. Experiment 1

Septum weights ± S.E.M. (mg) were 17.0 ± 1.0, 9.2 ± 1.3, and 11.9 ± 1.9 in the control, the streptozotocin-injected and the acetyl-L-carnitine-treated, streptozotocin-injected rats, respectively. The weight of the septum was reduced after injection of streptozotocin in both the animals treated with acetyl-L-carnitine and the untreated animals ($P < 0.01$). The weight of the hippocampus was unchanged (data not shown).

The enzyme activities in the septum of the saline-injected, the streptozotocin-injected and the acetyl-L-carnitine-treated, streptozotocin-injected rats are given in Table 1. The three experimental groups differed in septal prolyl endopeptidase activity ($P < 0.05$), aminopeptidase activity ($P < 0.05$) and carnitine acetyltransferase activity ($P < 0.01$). Septal choline acetyltransferase activity only tended to be different between the groups ($0.05 < P < 0.1$). Post hoc analysis revealed that prolyl endopeptidase, aminopeptidase and carnitine acetyltransferase activities were reduced in the septum of the streptozotocin-injected animals, whereas choline acetyltransferase activity was not changed. Treatment with acetyl-L-carnitine did not prevent prolyl endopeptidase and carnitine acetyltransferase activities from being reduced. Aminopeptidase activity was spared by treatment with acetyl-L-carnitine. However, septal choline acetyltransferase activity of the acetyl-L-carnitine-treated, streptozotocin-injected rats was even

Table 1
Enzyme activities in the septum of saline-injected, streptozotocin-injected and acetyl-L-carnitine-treated, streptozotocin-injected rats of experiment 1

Enzyme activity (nmol · h^{-1} · mg^{-1} protein)	Control	Streptozotocin	Acetyl-L-carnitine, streptozotocin
Prolyl endopeptidase	72 ± 5 [a]	46 ± 5 [b]	56 ± 6 [a,b]
Aminopeptidase	568 ± 24 [a]	486 ± 17 [b]	564 ± 24 [a]
Carnitine acetyltransferase	1219 ± 31 [a]	858 ± 70 [b]	949 ± 71 [b]
Choline acetyltransferase	100 ± 9 [a]	110 ± 7 [a,b]	135 ± 13 [b]

Data represent means ± S.E.M. Means of enzyme activities with the same superscript are not different (Student-Newman-Keuls test, $P < 0.05$).

Table 2
Enzyme activities in the hippocampus of saline-injected, streptozotocin-injected and acetyl-L-carnitine-treated, streptozotocin-injected rats of experiment 1

Enzyme activity ($nmol \cdot h^{-1} \cdot mg^{-1}$ protein)	Control	Streptozotocin	Acetyl-L-carnitine, streptozotocin
Prolyl endopeptidase	125 ± 5 [a]	123 ± 7 [a]	127 ± 4 [a]
Aminopeptidase	569 ± 28 [a]	535 ± 25 [a]	621 ± 41 [a]
Carnitine acetyltransferase	854 ± 27 [a]	786 ± 35 [a]	857 ± 21 [a]
Choline acetyltransferase	117 ± 3 [a]	44 ± 13 [b]	84 ± 16 [a]

Data represent means ± S.E.M. Means of enzyme activities with the same superscript are not different (Student-Newman-Keuls test, $P < 0.05$).

Table 3
Pearson's correlation coefficients for data from experiment 1 on septum weight and enzyme activities

Enzyme activity	R	P
Prolyl endopeptidase	0.838	< 0.001
Aminopeptidase	0.594	< 0.01
Carnitine acetyltransferase	0.754	< 0.001
Choline acetyltransferase	−0.272	> 0.05

Table 4
Enzyme activities in the septum of saline-injected and streptozotocin-injected rats from experiment 2

Enzyme activity ($nmol \cdot h^{-1} \cdot mg^{-1}$ protein)	Control	Streptozotocin
Prolyl endopeptidase	66 ± 3	35 ± 6 **
Aminopeptidase	567 ± 12	418 ± 29 **
α-Ketoglutarate dehydrogenase	545 ± 33	306 ± 27 **
Glutamate dehydrogenase	5938 ± 218	2602 ± 149 **

Data represent means ± S.E.M. Asterisks indicate differences with respect to control (t-test, $P < 0.001$).

Table 5
Enzyme activities in the hippocampus of saline-injected and streptozotocin-injected rats from experiment 2

Enzyme activity ($nmol \cdot h^{-1} \cdot mg^{-1}$ protein)	Control	Streptozotocin
Prolyl endopeptidase	137 ± 10	128 ± 8
Aminopeptidase	682 ± 48	607 ± 26
α-Ketoglutarate dehydrogenase	615 ± 45	650 ± 49
Glutamate dehydrogenase	8106 ± 308	8065 ± 279
Choline acetyltransferase	92 ± 2	39 ± 13 *

Data represent means ± S.E.M. Asterisk indicates a difference with respect to control (t-test, $P < 0.01$).

Table 6
Pearson's correlation coefficients for data from experiment 2 on septum weight and enzyme activities

Enzyme activity	R	P
Prolyl endopeptidase	0.977	< 0.001
Aminopeptidase	0.901	< 0.001
α-Ketoglutarate dehydrogenase	0.691	< 0.01
Glutamate dehydrogenase	0.845	< 0.001

higher than that of the saline-injected rats. In the hippocampus of all experimental groups prolyl endopeptidase, aminopeptidase, and carnitine acetyltransferase activities were unaffected (see Table 2, P values > 0.05), whereas choline acetyltransferase activity was affected ($P < 0.01$). Post hoc analysis showed that treatment with acetyl-L-carnitine attenuated the decrease in choline acetyltransferase activity in the hippocampus elicited by streptozotocin.

High correlations were found between septum weight and enzyme activities, except choline acetyltransferase activity (see Table 3). The correlation between choline acetyltransferase activity in the septum and that in the hippocampus was poor ($R = -0.093$, $P > 0.05$), but the correlation between septum weight and choline acetyltransferase activity in the hippocampus was high ($R = 0.832$, $P < 0.001$).

3.2. Experiment 2

Septum weights ± S.E.M. (mg) were 17.2 ± 0.5 and 7.9 ± 1.5 in the control and the streptozotocin-injected rats, respectively. The weight of the septum was reduced after injection of streptozotocin ($P < 0.001$). The weight of the hippocampus was unaffected (data not shown).

Prolyl endopeptidase, aminopeptidase, α-ketoglutarate dehydrogenase, and glutamate dehydrogenase activities in the septum were reduced after injection of streptozotocin (see Table 4), whereas in the hippocampus these enzyme activities were not affected by streptozotocin. However, choline acetyltransferase activity in the hippocampus of the streptozotocin-injected rats was reduced compared with that of the control animals (see Table 5).

High correlations were found between septum weight and enzyme activities (see Table 6). The correlation between septum weight and choline acetyltransferase activity in the hippocampus was high ($R = 0.917$, $P < 0.001$).

4. Discussion

In the present study the effects of streptozotocin on enzyme activities in the septum and hippocampus of

control rats and of rats treated with acetyl-L-carnitine were studied. Streptozotocin affected enzyme activities in the septum rather indiscriminately, except choline acetyltransferase activity, whereas it affected only choline acetyltransferase activity in the hippocampus. Acetyl-L-carnitine did not spare all the enzyme activities in the septum, but seemed to preserve the cholinergic function in the hippocampus to some extent. The general effect of streptozotocin on enzyme activities in the septum contrasts with its reportedly selective effects on enzyme activities in pancreatic β-cells (Raschaert et al., 1992). This difference might be explained by the possibility that streptozotocin may have caused cell death in the septum, whereas enzyme activities in pancreatic β-cells have been measured in cells surviving the insult by streptozotocin.

The above findings suggest that cholinergic neurons in the septum were relatively spared from the degenerative effects of streptozotocin. This does not necessarily mean that cholinergic neurons are less vulnerable to streptozotocin than other cells, since many of the non-cholinergic cells in the septum are closer to the injection site than the cholinergic neurons. The relative sparing of the cholinergic neurons may have been enhanced by acetyl-L-carnitine. Histological and immunocytochemical studies are in progress to assess which brain structures are affected by streptozotocin.

Specific choline acetyltransferase activity in the septum was unaffected by streptozotocin, whereas specific choline acetyltransferase activity in the hippocampus was reduced. This seems surprising, since the hippocampus receives a cholinergic input from the septum. However, the septum weight was reduced, whereas the hippocampus weight was unaltered, so that in both structures total choline acetyltransferase activity was reduced. In addition, total choline acetyltransferase activities in these two structures were strongly correlated ($R = 0.751$, $P < 0.001$). Therefore, the reduced choline acetyltransferase activity in the hippocampus can be explained by an effect of streptozotocin at the level of the septum.

Interestingly, choline acetyltransferase activity correlated highly with carnitine acetyltransferase activity in the hippocampus ($R = 0.712$, $P < 0.001$), although this latter activity was not significantly reduced after injection of streptozotocin. In addition, hippocampal carnitine acetyltransferase activity correlated both with septal carnitine acetyltransferase activity ($R = 0.499$, $P < 0.05$) and with septum weight ($R = 0.607$, $P < 0.01$). These correlations suggest that carnitine acetyltransferase may be linked to the septohippocampal pathway. This may have to do with the possibility that the enzyme is preferentially located in cholinergic nerve endings. This possibility is in accordance with some previous findings. First, lesion of the habenulointerpeduncular tract has been shown to result in reduced activity of both carnitine acetyltransferase and choline acetyltransferase in the interpeduncular nucleus (Sterri and Fonnum, 1980). Second, it has been reported that nerve growth factor (NGF) increases both carnitine acetyltransferase and choline acetyltransferase activities in PC12 cells (White and Scates, 1991). Moreover, these results suggest that preferential localization of carnitine acetyltransferase in cholinergic terminals is a general phenomenon.

The fact that enzyme activities were affected rather indiscriminately in the septum together with a reduced weight of the septum suggests that streptozotocin acts as a non-selective neurotoxin near the site of injection. Hoyer and collaborators, who used the same dose of streptozotocin as used in this study, found rather widespread effects of streptozotocin on energy metabolism and neurotransmitter systems and ascribed these effects to a possible interference of streptozotocin with the action of insulin in the brain. However, our finding of septal degeneration is not in line with such a specific effect of streptozotocin on the action of insulin. The destruction of septal-hippocampal and septal-cortical connections may have contributed to the effects of streptozotocin in locations distant from the septum. Therefore, with respect to the mechanism of action of streptozotocin an alternative hypothesis may be formulated. Since it has been shown that streptozotocin causes its effects on pancreatic β-cells by the generation of hydrogen peroxide (Takasu et al., 1991), it can be hypothesized that streptozotocin does so in the central nervous system as well.

There is evidence that oxidative stress is important in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases (reviewed by Olanow, 1993). This evidence is for the most part indirect, consisting of alterations in the accumulation of peroxidation products in tissues (Andorn et al., 1990; Palmer and Burns, 1994) and changes in the activity of enzymes sensitive to changes in redox status (Terwel et al., 1992; Morrison et al., 1993). Interestingly, it has recently been shown that β-amyloid peptide increases the production of oxygen free radicals by stimulating a NADPH oxidase-like activity or by an auto-oxidative process (Behl et al., 1994; Goodman et al., 1994). However, i.c.v. injection of streptozotocin caused a general decline in the enzyme activities determined, whereas in Alzheimer's disease some of these enzyme activities are reduced and others are not (Terwel et al., 1992; Mastrogiacomo et al., 1993). The effect of streptozotocin on the septum may have been too strong to mimic what is going on in Alzheimer's disease. In addition, the septum may not be the most appropriate area to induce Alzheimer's disease-like features, since brain areas other than the septum are affected by

Alzheimer's disease. Future studies should investigate the effects of low doses of streptozotocin in brain areas that are affected in Alzheimer's disease.

Two effects of acetyl-L-carnitine reported in the literature may be of relevance to explain the positive effects of acetyl-L-carnitine on the septohippocampal cholinergic system. Firstly, acetyl-L-carnitine increases the binding of NGF to its receptor in aged rat central nervous system (Angelucci et al., 1988). Secondly, in PC12 cells acetyl-L-carnitine increases the synthesis of the NGF receptor (Taglialatela et al., 1992) and the subsequent internalization of NGF (Taglialatela et al., 1991), which is important in the action of NGF. Correspondingly, it has been found that acetyl-L-carnitine increases NGF levels and choline acetyltransferase activity in the central nervous system of fimbria-fornix transectioned rats (Piovesan et al., 1994) and aged rats (Taglialatela et al., 1994). Finally, the fact that NGF plays a role in oxidant homeostasis (Pan and Perez-Polo, 1993) strengthens the possibility that NGF is involved in the neuroprotective effect of acetyl-L-carnitine against the damaging effects of streptozotocin.

In conclusion, i.c.v. injection of streptozotocin causes structural damage in the septum. This damage causes enzymatic changes that do not closely resemble the more specific changes in enzyme activities as observed in Alzheimer's disease. Acetyl-L-carnitine partly prevents this damage, as reflected by an attenuation of the streptozotocin-induced decrease in hippocampal choline acetyltransferase activity. This finding indicates that streptozotocin-treated rats may be valuable to test neuroprotective effects of substances. Furthermore, it demonstrates that acetyl-L-carnitine has a neuroprotective action on cholinergic neurons. However, little is known about the neuroprotective effect of acetyl-L-carnitine on non-cholinergic neurons. Injection of low doses of streptozotocin in brain sites other than the septum in combination with assessment of markers of specific neuronal populations may be a way to study the neuroprotective effects of acetyl-L-carnitine on non-cholinergic neurotransmitter systems.

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