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ORIGINAL ARTICLE

Effect of L-arginine on metabolism of polyamines in rat's brain with extrahepatic cholestasis

Dušan Sokolovic · Gordana Bjelakovic · Jelenka Nikolic · Boris Djindjic · Dusica Pavlovic · Gordana Kocic · Ivana Stojanovic · Voja Pavlovic

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Abstract Cholestatic encephalopathy results from accumulation of unconjugated bilirubin and hydrophobic bile acids in the brain. The aim of this study was to determine disturbances of polyamine metabolism in the brains of rats with experimental extrahepatic cholestasis and the effects of L-arginine administration. Wister rats were divided into groups: I: sham-operated, II: rats treated with L-arginine, III: animals with bile-duct ligation (BDL), and IV: cholestatic-BDL rats treated with L-arginine. Increased plasma γ-glutamyltransferase and alkaline phosphatase activity and increased bile-acids and bilirubin levels in BDL rats were reduced by administration of L-arginine ($P < 0.001$). Cholestasis increased the brain's putrescine ($P < 0.001$) and decreased spermidine and spermine concentration ($P < 0.05$). The activity of polyamine oxidase was increased ($P < 0.001$) and diamine oxidase was decreased ($P < 0.001$) in the brains of BDL rats. Cholestasis increased the activity of arginase ($P < 0.05$) and decreased the level of citrulline ($P < 0.001$). Administration of L-arginine in BDL rats prevents metabolic disorders of polyamines and establishs a neuroprotective role in the brain during cholestasis.

Keywords Polyamines · Cholestasis · L-Arginine · Brain

D. Sokolovic (✉) · G. Bjelakovic · J. Nikolic · D. Pavlovic · G. Kocic · I. Stojanovic
Institute of Biochemistry, Medical Faculty in Nis, Bul. dr Zorana Djindjica 81, 18000 Nis, Serbia
e-mail: soko@medfak.ni.ac.yu

B. Djindjic
Institute of Pathophysiology, Medical Faculty in Nis, Nis, Serbia

V. Pavlovic
Institute of Physiology, Medical Faculty in Nis, Nis, Serbia

Introduction

Chronic bile-duct ligation (BDL) in the rat is a method very frequently used to produce an experimental model of liver and brain with cholestasis. Cholestasis represents stagnation of bile due to impairment of bile flow along its outflow tract leading to accumulation of bile components (bilirubin and bile acids) in the blood (Kullak-Ublick and Maier 2000).

The effect of bile salts at concentrations of 1.5 mM and above is largely due to their lytic action as strong detergents on endothelial cell membranes, but at lower concentrations a more subtle modification of the blood-brain barrier (BBB) occurs (Greenwood et al. 1991). The accumulation of hydrophobic bile acids plays a role in the induction of apoptosis and necrosis and increases oxidative stress (Yerushalmi et al. 2001).

The data confirm the deleterious effects of bilirubin on neuronal viability, protein synthesis, and metabolic rates (Vert et al. 2001). The pathogenesis of bilirubin encephalopathy is multifactorial, involving the accelerated transport of unconjugated bilirubin (UCB) across the deteriorated BBB and delivering bilirubin to target neurons (Wennberg 2000). UCB induces neuronal apoptosis involving caspase activation (Grojean et al. 2000), diminished mitochondrial transmembrane potential and increased mitochondrial-associated Bax protein levels (Rodrigues et al. 2002).

Polyamines (spermine, spermidine, and putrescine) are aliphatic cations widely distributed in prokaryotic and eukaryotic cells (Moinard et al. 2005). Polyamines play an important role in vital processes of growth, division, and differentiation, as well as in regulation of permeability and stability of the cell membrane (Schuber 1989). They inhibit the process of lipid peroxidation and prevent apoptosis (Thomas and Thomas 2001). They are synaptic transmission modulators in the central nervous system (CNS) with both

neuroprotective and neurotoxic effects (Halonen et al. 1993). As intracellular messengers, they contribute to intracellular Ca^{2+} increase and modify NMDA receptor activity, increasing their affinity for the ligands (Camon et al. 2001).

The diamine putrescine may be formed directly from L-ornithine by ornithine decarboxylase (ODC), the initial and limiting reaction in the biosynthesis of polyamines, or indirectly from L-arginine by arginine decarboxylase out of which agmatine is formed. Putrescine is the base for spermidine and spermine synthesis generated by the activity of decarboxylated *S*-adenosylmethionine (Wallace et al. 2003). The brain cells are extremely sensitive to reductions in polyamine level. They are capable of both recognizing the reduction in their concentration and starting up the regulatory mechanisms for maintaining the normal level of these compounds (Slotkin et al. 1984).

Spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO) regulate the interconversion of spermine/spermidine back to spermidine/putrescine (Casero and Pegg 1993). In normal brain tissue, it has been estimated that ODC accounts for 30% of putrescine, whereas the rest is formed through the SSAT/PAO pathway (Seiler 1995). Diamine oxidase (DAO) primarily catalyzes the conversion of putrescine to ammonia, H_2O_2, and pyrroline.

In the CNS, where the concentration of spermine and spermidine can reach the high micromolar range, polyamines are important for the regulation of cell proliferation, can exert a protective function (Gilad and Gilad 1992), and can be toxic (Segal et al. 1999).

L-Arginine is common substrate for the synthesis of polyamines, citrulline, nitric oxide (NO), agmatine, creatine, proline, and glutamate. L-Ornithine, as a product of arginase activity, is necessary for the production of polyamines (Nikolic et al. 2007). Nitric oxide (NO) and citrulline are metabolites of L-arginine in a reaction catalyzed by nitric oxide synthase activity. Nitric oxide is involved in intracellular signaling, and it is a mediator in glutamate-induced neurotoxicity (Moller et al. 1995).

The focus of this research was to determine disturbances in polyamine metabolism in the brains of rats with experimental extrahepatic cholestasis and the effects of L-arginine administration.

Materials and methods

Chemicals

The reagents were purchased from Sigma (St. Louis, MO, USA) and were of the highest commercial grade available. All used chemicals were of analytical grade. All drug solutions were prepared on the day of the experiment. Animals used for the procedure were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

Animal

Male adult Wistar rats, body mass 250–300 g, were used for the experiment. The animals were housed in an air-conditioned room at a temperature of 23 ± 2°C with 55 ± 10% humidity and with lights on 12 h/day (0700–1900 h). The animals were given a commercial rat diet and tap water ad libitum.

Surgical procedure—biliary obstruction

Laparotomy was performed under general anesthesia induced by intraperitoneal (i.p.) injection of ketamine HCl (50 mg/kg). In BDL animals, the bile duct was isolated, doubly ligated, and resected between the ligatures.

In sham-operated animals, laparotomy was performed and the abdominal cavity exposed without any surgical procedure, in order to eliminate the effects of the operation on the examined parameters. All the animals were sacrificed 9 days after surgery under ketamine anesthesia.

All animal experiments were approved by the Animal Ethics Board of the Medical Faculty in Nis and were performed according to these guidelines.

Experimental procedure

The animals were allocated into four experimental groups (each consisted of ten animals): (I) sham-operated (control) group: animals with simulated surgery, treated by saline, intraperitoneally (i.p.) applicated; (II) L-arginine group: rats treated with L-arginine monochloride (Sigma) (150 mg/kg b.w. i.p); (III) BDL group: animals with common BDL; and (IV) BDL + L-arginine group: cholestatic rats treated with L-arginine (150 mg/kg b.w. i.p).

The animals were killed after 9 days under ketamine anesthesia, after 15 h of starvation. The blood was collected by heart puncture after medial laparatomy, and the brain was rapidly removed. The brain tissue was cut in small pieces and homogenized in ice-cold water, by using a homogenizer (IKA Works de Brasil, Taquara, RJ). The homogenates (10% w/v) were centrifuged at 1,500×*g* for 10 min at 4°C.

Biochemical analysis

Total bilirubin concentrations were measured by standard biochemical analyses on the apparatus BTS-370 (BioSystems). The activity of the enzymes γ-glutamyltransferase (γ-GT) and alkaline phosphatase (ALP) in the plasma was determined by the test of Ellitech Company, on the

Table 1 Effects of L-arginine on plasma cholestatic marker [γ-GT and AP (U/L)] activity, as well as on bile-acids and bilirubin levels (μmol/l)

	Control	L-Arginine	BDL	BDL + L-arginine
γ-GT	2.5 ± 0.3	3.4 ± 0.2	19.9 ± 1.1**	5.3 ± 1.4##
ALP	194.2 ± 12.0	139.3 ± 18.3	340.5 ± 45.1**	258.8 ± 42.9##
Bile acids	3.0 ± 0.5	2.8 ± 1.9	22.8 ± 2.58**	13.6 ± 9.8##
Total bilirubin	2.01 ± 0.9	7.4 ± 1.9	112.7 ± 51.1**	58.3 ± 8.9##
Unconjugated bilirubin	1.2 ± 0.3	4.9 ± 0.6	43.6 ± 5.8**	28.3 ± 3.7##

**$P < 0.001$ (vs. control), ##$P < 0.001$ (vs. BDL)

apparatus BTS-370 (BioSystems). Total bile-acids level in the plasma was determined by the direct spectrophotometry method (Mashige et al. 1981).

Arginase activity was measured on the basis of formed ornithine by ninhydrin color reaction, with maximum absorption of 515 nm (Porembska and Kedra 1975).

L-Citrulline, a co-product of the NO biosynthetic reaction, was determined using a colorimetric assay (Boyde and Rahmatullah 1980).

Examination of polyamine concentrations was done with *n*-butanol extraction followed by ninhydrin identification of separated polyamines by the technique of electrophoresis (Russell et al. 1970).

Activity of PAO (spermine tetra hydrochloride was used as a substrate) and DAO (putrescine dihydrochloride was used as a substrate) was measured by modified spectrophotometric method of Bachrach and Reches (1966) based on the determination of the formed amount of amino aldehydes (Quash et al. 1976). Activity was expressed as units per mg (U/mg) protein.

Brain proteins were determined according to Lowry's method (Lowry et al. 1951), using bovine serum albumin as standard.

Statistical analysis

Data were analyzed using a commercially available statistics software package (SPSS for Windows, v. 9.0, Chicago, IL, USA). Results are presented as means ± SD. Statistical significance was determined at level of $P < 0.05$ using the Student's *t*-test.

Results

For the purpose of evaluating the effect of bile-duct ligation and cholestasis confirmation, the activity of markers of cholestatic impairment (γ-GT an ALP) and bile-acids and bilirubin (total and unconjugated) levels were determined. Increased activity of these enzymes and higher concentration of bile acids and bilirubin were registered in the plasma of cholestatic rats compared to sham-operated ones. Application of L-arginine prevented the increase in the enzyme activity and level of bile acids and bilirubin compared to BDL rats (Table 1).

In the brain of cholestatic rats, an increase in the arginase activity was recorded ($P < 0.05$) as well a considerable decrease in citrulline level ($P < 0.001$), when compared to sham-operated ones. The administration of L-arginine to the cholestatic rats led to an increase in citrulline level ($P < 0.05$) compared to the BDL rats (Table 2).

The levels of spermine and spermidine in the brain tissue of cholestatic rats decreased ($P < 0.05$), while the amount of putrescine doubled in comparison to the sham-operated animals ($P < 0.001$; Fig. 1). The application of L-arginine to the BDL rats led to a decrease in putrescine

Table 2 The arginase activity (μmol/mg protein) and citrulline level (μmol/mg protein) in the brains of cholestatic rats

	Control	L-Arginine	BDL	BDL + L-arginine
Arginase	0.27 ± 0.05	0.3 ± 0.05	0.34 ± 0.06*	0.29 ± 0.04
Citrulline	8.03 ± 0.4	7.7 ± 0.43	6.52 ± 0.36**	7.03 ± 0.3#

*$P < 0.05$ (vs. control), **$P < 0.001$ (vs. control), #$P < 0.05$ (vs. BDL)

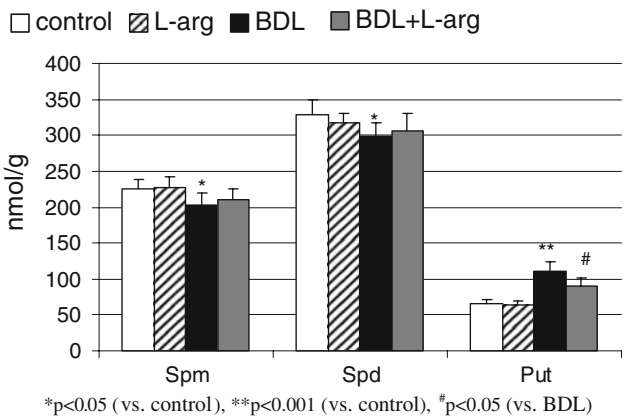

Fig. 1 Effects of L-arginine on polyamine levels (nmol/g wet weight) in the brains of rats with extrahepatic cholestasis. *Spm* Spermine, *spd* spermidine, *put* putrescine, *l-arg* rats treated with L-arginine, *BDL* rats treated with bile-duct ligation, *BDL + l-arg* rats treated with L-arginine and bile-duct ligation

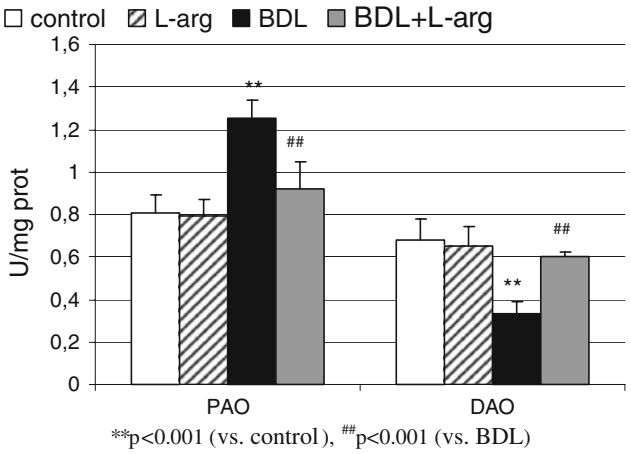

Fig. 2 The activities of polyamine oxidase (PAO) and diamine oxidase (DAO) (U/mg protein) in the brains of cholestatic rats. *L-arg* Rats treated with L-arginine, *BDL* rats treated with bile-duct ligation, *BDL + l-arg* rats treated with L-arginine and bile-duct ligation

level ($P < 0.05$) in the brain tissue in comparison to the cholestatic animals (Fig. 1).

The activity of PAO in the brain tissue of cholestatic rats was significantly larger in relation to the sham-operated animals ($P < 0.001$). The activity of DAO in the same tissue showed the opposite tendency, and the values in the brain of cholestatic rats were noticeably smaller than in sham-operated ones ($P < 0.001$; Fig. 2). In the group of cholestatic rats in which L-arginine was administrated intraperitoneally, a considerable decrease in PAO activity was observed ($P < 0.001$), as well as an increased DAO activity ($P < 0.001$) in relation to the BDL rats (Fig. 2).

Discussion

It is generally accepted that deposition of UCB in the CNS of extrahepatic cholestasis leads to the yellow staining and the neurological dysfunction characterizing UCB encephalopathy. UCB inhibits DNA and protein synthesis; modulation of neurotransmitter synthesis, release and uptake; and protein phosphorylation (Silva et al. 2001). UCB-induced toxicity is typically characterized by cell swelling, vacuole formation, disruption of plasma membrane integrity, and release of intracellular constituents following pigment accumulation. As a consequence, cell death has been characterized as loss of neuronal function associated with necrosis. UCB induces apoptosis in vitro in primary cultures of both rat astrocytes and neurons.

In cholestasis, the intracellular accumulation of toxic hydrophobic bile acids within hepatocytes and CNS promotes cellular injury and the subsequent development of hepatic cirrhosis and encephalopathy. Bile acid-mediated apoptosis is, in part, death receptor-dependent. For example, toxic bile acids trigger oligomerization of Fas in a ligand-independent manner by promoting trafficking of intracellular Fas to the plasma membrane (Faubion et al. 1999).

In this research, the experimental cholestasis led to a considerable growth in γ-GT and ALP activities in the blood plasma. Hydrophobic bile salts with their detergent effect on the cell membrane (Schmucker et al. 1990) lead to leaking of γ-GT from hepatocytes into circulation. In addition, regurgitation of bile (the activity of γ-GT in the bile is ten times greater than in serum) increases enzyme activity in circulation. In all pathological processes that contribute to bile-flow disorder, the emphasis is on the solubilization effect of the bile salts, which causes the release of ALP on the peripheral side of the cell membrane (Alvaro et al. 2000).

There are contradictory views regarding the effects of polyamines in brain tissue. The possible neuroprotective effects of polyamines are ascribed to anti-apoptotic and anti-oxidant effects (Ha et al. 1998), with altered neuronal excitability by putrescine (Lukkarinen et al. 1998) and stabilization of chromatin (Brune et al. 1991). The eventual mechanisms of neuronal impairment injury on polyamines are (1) the activation of Ca^{2+} fluxes and neurotransmitter release in areas with an overproduction of putrescine (Koenig et al. 1983), (2) the increased stimulation of the NMDA receptor complex caused by a release of polyamines into the extracellular space during or after CNS trauma, and (3) production of hydrogen peroxide, 3-acetamidopropanal, and 3-aminopropanal through the interconversion of spermine and spermidine into putrescine via SSAT/PAO (Rao et al. 1998).

The cerebral ODC/polyamine system is sensitive to physiological and pathological stimuli in the brain tissue (including the impairment of CNS under cholestasis conditions). The main modifications are significant increases in ODC activity and putrescine concentration, with minor or no variations in spermidine and spermine levels (Paschen 1992). It has been confirmed that the changes in the metabolism of polyamines are connected to the degree of impairment of CNS in cholestasis conditions, and they have an important role in degeneration of neurons (Henley et al. 1997).

In the brain of cholestatic rats in this experimental model, a considerable growth in putrescine level was recorded. There are two contradictory views about the importance of high concentration levels of putrescine in the brain. The first point of view states that the rise of putrescine level in the brain (because of the increased ODC activity) represents the main cause of CNS injury. The role of putrescine in neuronal necrosis can be deduced only indirectly from the close relationship between putrescine accumulation and the extent of necrosis, the known

activities of putrescine, and the observation that pharmacological prevention of ischemia-induced neuronal necrosis also reduces the postischemic overproduction of putrescine (Paschen 1992).

The other point of view is that these biochemical factors (putrescine and ODC) do not cause neuronal damage even if they precede it (Kauppinen and Alhonen 1995), or that the increase in putrescine content associated with neuronal damage is a neuroprotective measure rather than a cause of the damage (Lukkarinen et al. 1998). Studies using transgenic animals overexpressing ODC have indicated that elevated ODC activity and putrescine accumulation exhibit either no effect or neuroprotection in transient focal cerebral ischemia (Lukkarinen et al. 1998; Kauppinen and Alhonen 1995). In line with this view are recent findings indicating that life-long overexpression of ODC in transgenic mice did not lead to neuronal degeneration and that polyamines promote regeneration of injured axons of cultured rat hippocampal neurons (Alhonen et al. 1995). Spermine also stabilizes chromatin, thus preventing endonuclease-mediated DNA fragmentation and apoptosis (Brune et al. 1991).

In this experiment it has been observed that in the brains of cholestatic rats, apart from the increase in the level of putrescine, there is a significant decrease in the levels of spermine and spermidine in relation to the sham-operated animals. It can be presumed that this impossibility of an increase in spermine/spermidine levels in these animals shows that putrescine, probably through sequestration in vacuoles or by means of some other unknown mechanism, becomes unattainable for further conversion into spermidine and spermine (Kauppinen and Alhonen 1995).

Spermidine and spermine bind to a polyamine recognition site on the NMDA ionophore receptor complex and increase glutamate binding with anti-apoptotic properties (Rock and Macdonald 1995). Other authors reported a pro-apoptotic role of spermine in cerebellar granule neurons, which was independent of its actions at NMDA receptors (Segal and Skolnick 2000).

Spermine was reported to act as a potent anti-oxidant either by scavenging oxygen radicals (Ha et al. 1998) or through chelation of Fe^{2+} that catalyzes $OH^{\cdot}$ generation via the Fenton reaction (Lovaas 1997). The reaction of spermine with $OH^{\cdot}$ results in a spermine aldehyde derivative; this product is the same as that formed by serum amine oxidases and undergoes non-enzymatic, pH-dependent β-elimination to form acrolein and spermidine (Lee and Sayre 1998). This reaction could account for the loss of spermine but is expected to increase spermidine levels, which was not shown in cholestatic brain.

In our experimental model, it was evident that there was a decrease in spermine and spermidine levels in the brain tissue of cholestatic rats. It can be presumed that the overall anti-oxidant and anti-apoptotic capacity of the brain tissue has been decreased, and therefore the toxic brain impairment enlarges and progresses. Thus, while the polyamine response is now recognized to be a critical reaction of neurons to injury, whether this process is neurotoxic or neuroprotective is currently an issue of debate.

The increase in arginase activity and the decrease in citrulline level have been recorded in the brain of cholestatic rats. It is likely that in cholestatic conditions, the catabolism of L-arginine progresses towards the synthesis of L-ornithine and polyamines but not towards the synthesis of citrulline and nitric oxide. This is supported by the fact that in cholestatic rats the content of putrescine is increased.

The considerable increase in PAO in the brain of cholestatic rats can be clarified by intensifying the process of interconversion of spermine/spermidine back to spermidine/putescine. This process enables the regulation of polyamine levels and their disposition. The characteristic feature of the catabolism of spermine/spermidine into putrescine, with the help of the enzyme system SSAT/PAO, is the separation of toxic 3-aminopropanal and hydrogen peroxide (H_2O_2). The cytotoxicity of 3-aminopropanal is believed to be mediated by cleavage to acrolein (Ivanova et al. 1998; Calingasan et al. 1999). PAO generates potentially toxic reactive oxygen species (ROS), such as hydrogen peroxide, that can damage proteins, DNA, and lipids. Several reports have suggested that polyamine toxicity is a direct result of ROS produced by polyamine catabolism (Ivanova et al. 1998). This is a direct marker of brain injury, which presents as an increase in putrescine and a decrease in spermidine/spermine.

In the brain of cholestatic rats, the activity of DAO is considerably decreased in relation to the sham-operated animals. The change in DAO activity is connected to many pathological conditions, such as the collapse of DAO activity in brain tumor conditions (Berdinskikh and Lialiushko 1987). The decrease in DAO in cholestatic brain tissue probably decreases the level of putrescine catabolism, which leads to the increased concentration of this polyamine in the brain. With the increase in putrescine level in the brain tissue, its neuroprotective effects are intensified.

In this experiment, the application of L-arginine led to a decrease in putrescine level and to an increase in citrulline (and also of NO) in the brain tissue in relation to the cholestatic rats without supplementation. NO inhibits cell proliferation by inhibition of ODC, the rate-limiting enzyme in polyamine synthesis in the reaction of NO with Cys360—nitrosylation reaction (Bauer et al. 2001), and therefore the concentration of putrescine is decreased.

The fall in putrescine levels in the brain of cholestatic rats suggests that L-arginine can also be a consequence of the increased DAO activity in the brain. Moreover, the fall in

PAO activity in the brain of cholestatic rats with L-arginine can lead to a decrease in putrescine level in the brain of cholestatic rats and a reduction in oxydative stress injury.

On the basis of our results, it can be concluded that disorders of polyamine metabolism in the brains of cholestatic rats play an important pathogenetic role in brain injury. Application of L-arginine to cholestatic animals exerts modulatory effects and regulates the disorders of biosynthesis and catabolism of polyamines in the brain tissue. L-Arginine can be recommended as a neuroprotector in clinical practice for the treatment of patients with cholestasis.

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