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Rat Liver Catalase Protection from α-Amanitin in Extracorporeal Liver Perfusion

Ochrona katalazy hepatocytów szczura przed działaniem α-amanityny w modelu pozaustrojowej perfuzji wątroby

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Abstract

Background. Death in death cap toadstool (*Amanita phalloides*) poisoning is caused principally by acute failure of the liver, which takes up 57% of circulating amanitins. α-Amanitin – the main toxin of this mushroom – binds to RPB1 subunit of RNA polymerase II, thereby blocking synthesis of cellular enzymes, moreover α-amanitin inhibits catalase activity and increases free radicals generation in hepatocytes leading to cell death.
Objectives. The aim of this study was to investigate the potential protective effect of amanitin uptake inhibitors on catalase activity in α-amanitin exposed rat hepatocytes.
Material and Methods. The experiment was performed using extracorporeal liver perfusion on 80 Wistar rats divided into following groups: control group C0 (perfusion fluid without α-amanitin and without inhibitors), control group C, and 6 treatment groups (I–VI). In groups C and I–VI perfusion fluid was supplemented with α-amanitin at 25 ng/ml, while treatment groups were perfused with the fluid containing also potential inhibitors of α-amanitin uptake at the following concentrations: I. penicillin G 0.5 mM; II. rifamycin SV 10.0 μM; III. silibinin 20.0 μM; IV. acetylcysteine 1.0 mM; V. β-estradiol-17-β-D-glucuronide 320 μM; VI. taurocholate sodium 270 μM.
Results. Catalase activity in α-amanitin-exposed hepatocytes after 2-hours extracorporeal rat liver perfusion was significantly inhibited in comparison to the catalase activity in the unexposed hepatocytes. As catalase protectors from α-amanitin-induced inactivation act only these substances which exert inhibitory effect on α-amanitin uptake by the rat liver (groups II–VI).
Conclusion. The protection of catalase is probably not a result of direct influence on the enzyme, but it results only from inhibiting of α-amanitin influx into hepatocytes (**Adv Clin Exp Med 2008, 17, 3, 269–273**).

Key words: α-amanitin, catalase, rat, hepatocytes, extracorporeal liver perfusion.

Streszczenie

Wprowadzenie. Śmierć w zatruciu muchomorem sromotnikowym (*Amanita phalloides*) jest wynikiem ostrej niewydolności wątroby, która wychwytuje aż 57% krążących we krwi amanityn. α-Amanityna – główna toksyna tego grzyba – łączy się z podjednostką RPB1 polimerazy II RNA, przez co blokuje syntezę enzymów komórkowych, α-amanityna ponadto hamuje aktywność katalazy, zwiększając powstawanie wolnych rodników, co prowadzi do śmierci komórki.
Cel pracy. Określenie, czy substancje hamujące wątrobowy wychwyt α-amanityny mogą ochronić katalazę hepatocytów szczura przed inaktywacją.
Materiał i metody. Badania przeprowadzono z użyciem pozaustrojowej perfuzji wątroby na 80 szczurach szczepu Wistar podzielonych na następujące grupy: grupa kontrolna C0 (płyn perfuzyjny bez α-amanityny i bez inhibitorów jej wychwytu), grupa kontrolna C oraz 6 grup badanych (I–VI). W grupach C oraz I–VI do płynu perfuzyjnego dodawano α-amanitynę w stężeniu 25 ng/ml, w grupach badanych płyn perfuzyjny zawierał dodatkowo potencjalne inhibitory wątrobowego wychwytu α-amanityny w następujących stężeniach: I penicylina G 0.5 mM; II ryfamycyna SV 10.0 μM; III silibinina 20.0 μM; IV acetylocysteina 1.0 mM; V glukuronian 17-β-D-estradiolu 320 μM; VI taurocholan sodu 270 μM.
Wyniki. Aktywność katalazy w hepatocytach szczura eksponowanych na α-amanitynę podczas 2-godzinnej pozaustrojowej perfuzji wątroby była istotnie mniejsza niż aktywność katalazy w hepatocytach nieeksponowanych. Ochronne działanie na katalazę hepatocytów szczura eksponowanych na α-amanitynę wykazano tylko dla tych substancji, które we wcześniejszych badaniach okazały się inhibitorami wątrobowego wychwytu tej toksyny (grupy II–VI).

Wniosek. Ochrona katalazy hepatocytów szczura przed inaktywacją wynika najprawdopodobniej nie z bezpośredniego oddziaływania użytych substancji na enzym, lecz jedynie z blokowania napływu α-amanityny do komórek (**Adv Clin Exp Med 2008, 17, 3, 269–273**).

Słowa kluczowe: α-amanityna, katalaza, szczur, hepatocyty, pozaustrojowa perfuzja wątroby.

Mushroom poisoning is still a real problem in clinical toxicology. Toadstool death cap (*Amanita phalloides*) and its subspecies (*Amanita vernalis*, *Amanita virosa)* are responsible for the highest number of all fatal cases of mushroom intoxication. α-Amanitin (α-AMA) – main toxin of these toadstools is one of the most dangerous natural toxins causing hepatic damage. The mortality rate in death cap poisoning is still high and is estimated to range 30–40% [1, 2].

Death by the toadstool death cap poisoning is caused principally by acute failure of the liver, which takes up 57% of the circulating amanitins. The mechanism of amanitin toxicity has not been fully elucidated, what makes the discovery of an efficient antidote difficult. The amanitin uptake by hepatocytes is mediated by both organic anion-transporting polypeptide (OATP–human, Oatp–rat) and Na^+–taurocholate cotransporter polypeptide (NTCP–human, Ntcp–rat) located in plasma membrane [3–5]. Thus, α-AMA hepatic uptake is mediated by sodium-dependent (OATP/Oatp) and sodium-independent (NTCP/Ntcp) transporters. Subsequently, α-AMA binds to the RPB1 subunit of RNA polymerase II, thereby inhibiting synthesis of cellular enzymes and leading to cell death [1, 6, 7]. Moreover α-amanitin *in vitro* and *in vivo* inhibits catalase (CAT) activity and increases free radicals generation which can contribute to severe liver damage [8–10]. It was found that penicillin G, acetylcysteine, silibinin, rifamycin SV, β-estradiol-17-(β-D-glucuronide), and taurocholate sodium inhibit α-amanitin hepatic uptake [3, 11], therefore it could be presumed that inhibitors of the α-AMA hepatic uptake may protect CAT in hepatocytes from amanitin and may diminish amanitin toxicity.

The aim of this study was to investigate the potential protective effect of amanitin uptake inhibitors on CAT activity in α-AMA exposed rat hepatocytes.

Material and Methods

Animals

The experiment was performed on 80 Wistar rats of both sexes weighing 240.0 ± 16.4 g. Before experiments animals were kept under standard conditions with water and pellet feed (LSM, Agropol, Motycz) available *ad libitum*.

Chemical and Drugs

Perfusion fluid: modified Krebs – Henseleit buffer (NaCl – 118 mM, $NaHCO_3$ – 25 mM, KCl – 4.8 mM, $CaCl_2$ – 1.5 mM, $MgSO_4$ x $7H_2O$ – 1.2 mM, KH_2PO_4 – 1.2 mM, glucose 4.9955 mM); α-Amanitin (Sigma, Cat. No. A2263); Penicillin G potassium (Sigma, Cat. No. P4687); Rifamycin SV sodium (Fluka, Cat. No. 83909); Silibinin (Sigma, Cat. No. S0417); Acetylcysteine sodium (Hexal AG/Lek Polska, amp. 0.3 g/3 ml); β-Estradiol-17-(β-D-glucuronide) sodium (Sigma, Cat. No. E1127); Taurocholic acid sodium salt hydrate (Sigma, Cat. No. T4009); Thiopental (Sandoz/Lek Polska, amp. 0.5 g).

Experimental Procedure

Rats were assigned to 2 control groups (C0 and C) and 6 treatment groups (I–VI). Each group comprised 10 animals (5 males and 5 females).

In all groups, two-hour extracorporeal liver perfusion was carried out using an universal apparatus for extracorporeal organ perfusion (Universal Perfusion System, Harvard Apparatus, Uniper UP-100, Type 834).

Once the rats were under deep barbiturate anesthesia (thiopental 100 mg/kg *i.p.*), their peritoneal cavity was opened by the middle section and cannulae were inserted into the portal vein, inferior jejunal vein and bile duct. Then the liver was dissected, removed from rat's body and placed in a perfusion chamber while cannulae were attached to the perfusion system. Perfusion fluid was delivered through the cannula in the portal vein, then it passed through the network of intrahepatic vessels perfusing the hepatic parenchyma and left the liver through a cannula inserted into the inferior caval vein. Perfusion fluid flow was controlled by a peristaltic pump at a rate 20–25 ml/min, maintaining the pressure in the portal vein between 0.04–0.08 mm Hg (5.32–10.64 Pa). Perfusion fluid was gassed with carbogen (94.93% O_2 and 5.07% CO_2) and its temperature was kept at 37.8°C (310.9 K). The liver was perfused with Krebs buffer pH 7.35–7.4, with our modification, consisting in higher glucose concentration (4.9955 mM). The perfusion fluid contained no blood.

In group C0 the perfusion fluid without α-AMA and without examined substances was used. In groups C and I–VI, perfusion fluid was

supplemented with α-amanitin at 25 ng/ml (0.027 μM). The perfusion fluid for treatment groups (I–VI) contained different potential antidotes at the following concentrations:

Group I. Penicillin G potassium: 0.5 mM (186.24 mg/l);
Group II. Rifamycin SV sodium: 10.0 μM (7.19 mg/l);
Group III. Silibinin: 20.0 μM (9.64 mg/l);
Group IV. Acetylcysteine sodium: 1.0 mM (184.96 mg/l);
Group V. β-Estradiol-17-(β-D-glucuronide) sodium: 320 μM (150.56 mg/l);
Group VI. Taurocholic acid sodium salt hydrate: 270 μM (145.16 mg/l).

In our experiment the same α-AMA concentration was chosen in which α-AMA significantly inhibited CAT activity *in vitro* [10]. The concentration of penicillin G in group I was equal to concentrations of this substance in other experiments in which penicillin was examined as a potential α-AMA hepatic uptake inhibitor in rats [11, 12]. Concentration of this agent was also similar to concentrations of penicillin, which were observed after administration of therapeutic doses of penicillin G in the treatment of toadstool death cap poisoning in humans [13]. The other examined substances (groups II–VI) were used at concentration in which they presented an inhibitory effect on α-AMA hepatic uptake during 2-hour extracorporeal liver perfusion in rats [11].

After two-hour extracorporeal liver perfusion, 500 mg of livers were homogenized carefully o ice in PBS buffer containing 1mmol EDTA and centrifuged at 4°C (277 K) for 10 min at 14.000 rpm. Supernatants were collected and assessed for CAT activity and protein concentration [9]. CAT activity was determined following decrease in the initial H_2O_2 concentration (30 mM used as a substrate) at 240 nm at 25°C (298 K) in 60 s according to Johanson and Borg [14]. Briefly, 100 μl of supernatants isolated from rat liver homogenates were placed in a cuvette in PBS buffer (50 mM) to a final volume 2 ml. After adding of 1 ml H_2O_2 the decrease of the absorption at 240 nm for 60 s was followed. One unit of CAT was defined as the amount of enzyme that degraded 1 μl H_2O_2 per min [9].

CAT activity was expressed as enzyme units per mg of total protein (U/mg protein) [9]. Total protein concentrations in liver homogenates were determined by the Total Protein Kit (Sigma, Cat. No. TP0300).

Statistical analysis

The results are presented as means ± SD. Differences between values were analyzed by individual comparison with the one-way ANOVA. The experiment was performed after approval by the Local Ethics Commission for Experiments on Animals in Wroclaw (license no. 19/2006).

Results

CAT activity in group C (32.32 ± 2.7) was significantly inhibited in comparison to the CAT activity in the group C0 (mean 52.25 ± 3.96, $p < 0.0001$). CAT activity in group I (mean 32.64 ± 4.83) was similar to the CAT activity in group C (32.32 ± 2.7, statistical insignificant, $p > 0.05$). CAT activity in group II (mean 43.0 ± 2.23), in group V (mean 41.99 ± 4.53), and in group VI (mean 41.31 ± 2.57) was significantly lower than in the control group C0 (mean 52.25 ± 3.96, $p < 0.005$), however CAT activity in these groups was significantly higher than in the control group C (mean 32.32 ± 2.7, $p < 0.005$). CAT activity in group III (mean 48.18 ± 4.42) and in group IV (mean 48.90 ± 5.04) was similar to the CAT activity in group C0 (52.25 ± 3.96, statistical insignificant, $p > 0.05$).

In the present study no statistically significant differences in the CAT activity between male and female rats were observed.

Discussion

In humans α-AMA is readily absorbed through the intestinal mucosa and carried to the liver via portal vein. In rats α-AMA is poorly absorbed from gastrointestinal tract, but in our experiment perfusion fluid with α-AMA was administered directly to the liver via portal vein. In *Amanita phalloides* poisoning clinical and laboratory symptoms of acute liver damage occur usually on 2–3 day after death cap ingestion [1]. However, in the present study we have demonstrated that the inhibition of CAT activity by α-AMA in the liver can be extremely rapid; after 2-hours extracorporeal rat liver perfusion CAT activity in α-AMA-exposed hepatocytes was significantly inhibited in comparison to the CAT activity in the unexposed hepatocytes. These results were in accordance with recently reported by Zheleva *et al.* inhibiting effect of α-AMA on CAT activity *in vitro* and *in vivo* [8–10]. A possible explanation concerning inhibitory effect of α-AMA on CAT activity may be the blockade of substrate (H_2O_2) access to CAT Fe-heme pocket [15]. A formed complex between CAT and the toxin probably hampers the access of the substrate to the amino acid moieties (histidine^{74}, asparagine^{147}) in the CAT molecule [16]. Hydrogen

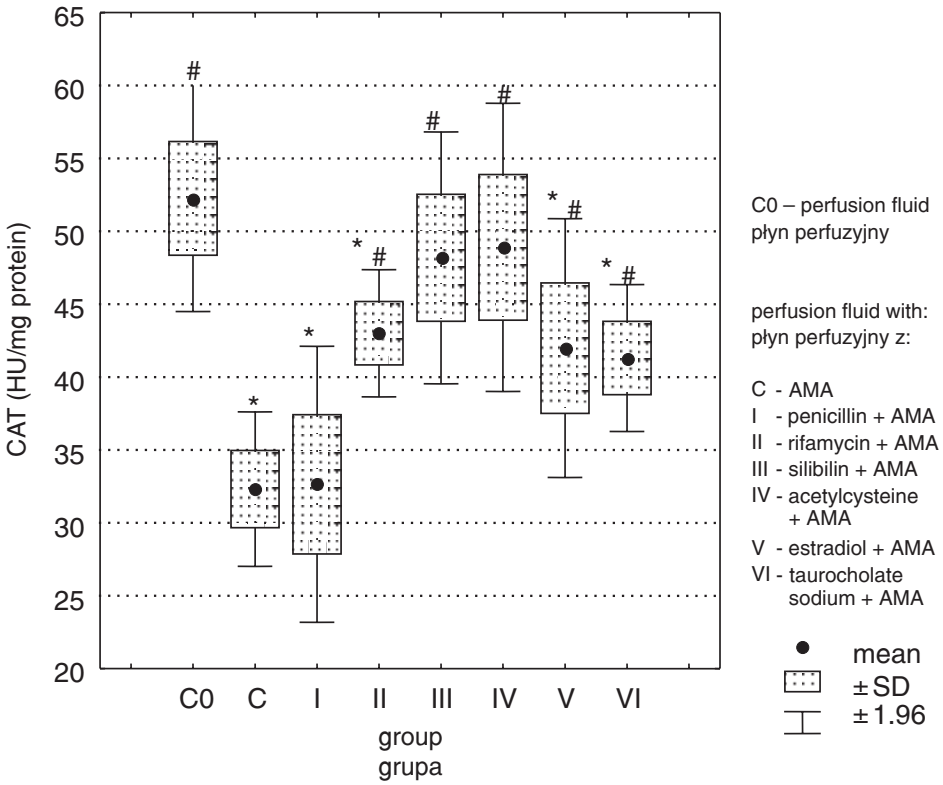

Fig. 1. Effect of the inhibitors of α-amanitin hepatic uptake on catalase activity in α-amanitin-exposed rat hepatocytes. * Statistical significant comparing to the group C0. # Statistical significant comparing to the group C

Ryc. 1. Wpływ inhibitorów wątrobowego wychwytu α-amanityny na aktywność katalazy w hepatocytach szczura eksponowanych na α-amanitynę. * Różnica znamienna statystycznie w porównaniu do grupy C0. # Różnica znamienna statystycznie w porównaniu do grupy C

bonds between CAT and α-AMA may be formed during *in vitro* incubation of the toxin plus enzyme or *in vivo* accumulation of the toxin in the liver [10].

Penicillin G is still the most commonly used antidote in death cap toadstool poisoning in humans [2]. In our study a protective action of penicillin G on liver CAT in α-AMA-exposed rat hepatocytes was not observed. However, the lack of this protective effect in rats does not exclude a protective action of penicillin G from α-AMA toxicity in humans because it was showed that penicillin G inhibits α-AMA hepatic uptake in humans [3], although that action was not observed in rats [11, 12]. Therefore, the extrapolation of results in experimental animals to humans always requires caution.

In our study as CAT protectors from α-AMA-induced inactivation act only these substances (groups II–VI) which exert inhibitory effect on α-AMA uptake by the rat liver in previous studies [11, 12]. Thus, CAT protection is probably not a result of direct influence on the enzyme, but it results only from inhibiting of α-AMA influx into hepatocytes.

Administration of silibinin (group III) or acetylcysteine (group IV) most forcefully protects CAT in rat hepatocytes from α-AMA-induced inactivation. Moreover, in these groups CAT activity was similar to the activity in α-AMA-unexposed hepatocytes (group C0).

The protective effect of silibinine and acetylcysteine from the prooxidative action of α-AMA may be probably very strong because both agents can inhibit α-AMA hepatic uptake [3, 11] what protects CAT in hepatocytes from inactivation. Moreover, these substances exert also a multiway antioxidative action; both substances exert free radical scavanger reactivity and inhibit lipid peroxydation, stabilize membrane structure and protect enzymes under conditions of oxidative stress [17, 18]. Enjalbert et al. using statistical comparison of survivors and death in 20-years retrospective analysis of death cap poisoning have reported that silibinin and acetylcysteine, administered either as mono-chemotherapy or in drug combination were the most effective antidotes [2]. We presume that acetylcysteine and silibinin are more effective than penicillin G in the treatment of *Amanita phalloides* poisoning in humans probably due to their strong antioxidant activity.

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