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Original scientific paper

The Effect of Cyclic Adenosine Monophosphate (cAMP) on Acute Liver Toxicity in Mice Induced by D-galactosamine and Lipopolysaccharide

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A B S T R A C T

The aim of this study was to examine the effect of cyclic adenosine monophosphate (cAMP) and its possible interference/synergism with calcium channel blocker in mice with acute liver injury induced with D-galactosamine (D-GalN) and lipopolysaccharide (LPS). C57Bl/6 mice were given i.p. simultaneously 300 mg/kg D-GalN and LPS 0.01 mg/kg. This treatment induced severe hepatitis, as evidenced by high mortality (80–90%) of control mice and large increase in concentration of aminotransferases in plasma (AST, ALT). Injection of stabile analogue of cAMP (dibutyryl-cAMP, db--cAMP) one hour before hepatotoxic agents increased survival of mice in dose-dependent manner and in medium dose significantly decreased plasma ALT level. Similar (protective) effect had also verapamil, calcium channel blocker, when given in non toxic doses and at the same time schedule as db-cAMP. Combination of db-cAMP and verapamil had not synergistic effect in protection from D-GalN+LPS hepatotoxicity; the survival of mice was similar to that seen in protection caused by each agent alone.

Key words: *cyclic adenosine monophosphate, cAMP, verapamil, D-galactosamine, LPS, acute liver toxicity*

Introduction

Liver failure (damage) is frequently final result of liver infections and severe complication of highly mortality diseases, like sepsis[1]. Various experimental models are used to study the mechanisms of liver damage in infection diseases involving liver, which generally include the agent that is hepatotoxic *per se* and/or blocks the process of transcription in endogenous protein synthesis, specifically in liver (D-galactosamine – D-GalN) or in general (actinomycine D – Act D), and the agent which stimulates the synthesis or accumulation of inflammatory mediators in liver[2,3]. Among the latter agents, most frequently used are: the active component of endotoxin from gram-negative bacteria – lipopolysaccharide (LPS), T-cell mitogenes – Concanavalin A (Con A) or phytohemagglutinin (PHA), bacteries Corynebacterium parvum (CP), exogenously administered mediators in D-GalN sensitized mice, etc.

Various inflammatory mediators, depending on its nature, may have either noxious (pathogenic) or protective effect on liver damage in these models. Thus, tumor necrosis factor-alpha (TNF-α) is well proven to be pathogenic in mice given LPS+D-GalN, since administration of monoclonal antibodies to this cytokine greatly reduced liver damage (as assessed by reduction of animal mortality, serum aminotransferase level, and histopathological damage)[4–6]. Similar effects were obtained in transgenic mice with nonfunctional gene for TNF-α or its type 2 receptor[7]. Also interferon-γ (IFN-γ) appears to mediate liver damage in this model as well as in hepatotoxicity induced with Con A+D-GalN or with D-GalN alone[8]. The data also show that IL-1 and IL-6 can mediate liver toxicity[9]. The results obtained with stimulation of synthesis or inhibiting of action of nitric oxide (NO) are controversial[10–12]. Prostaglandin of series E (PGE1 or PGE2) were shown to be protective in different »infection like« models of hepatic damage[13,14], as well as in hepatotoxicity induced by acetaminophen[15]. On the contrary, it was shown in one experiment that thromboxane

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A2 might mediate liver damage in mice intoxicated with TNF-α + D-GalN[16]. As one would expect, generally suppressive cytokine, IL-10, alleviated liver damage in several models of hepatotoxicity when it was administered exogenously or is produced in increase quantity when the synthesis of TNF-α or its actions were blocked[9].

Agents that raise the level of cyclic adenosine monophosphate (cAMP), which is known for its general anti-inflammatory or immunosuppressive actions, have generally beneficial (protective) effect in described models of hepatic damage. By analyzing the mortality of mice, serum aminotransferase concentrations, level of histopathological damage by classical means or by the level of apoptosis in liver and/or liver-production of TNF-α, it was shown that inhibitors of cAMP breakdown (especially inhibitors of type IV phosphodiesterase)[17,18] and stimulators of adenylyl cyclase[18] have protective effect on hepatitis induced by LPS, Con A or TNF-α in D-GalN sensitized mice. Similar protective effect was obtained by use of stabile, cell permeable analogue of cAMP (dibutyryl-cAMP, db-cAMP)[19]. In model of acute hepatotoxicity induced with acetaminophen, we and others have shown that PGE_2 as well as IL-1 and IL-6 have hepatoprotective effect[20–22]. Similar effect was shown with PGI_2[23]. Since it is known that PGE_2, IL-6, and PGI_2 increase the synthesis of cAMP in many cells, the increase of cAMP in liver cells might be common pathway for protective effect of these prostaglandins and inflammatory cytokines against APAP toxicity.

It is known that many hepatotoxicants cause the rise of concentration of intracellular calcium, which interferes with many important cellular reactions, leading finally to cell death[24,25]. Indeed, administration of calcium channel blocker (verapamil) alleviated the hepatotoxicity in mice given lethal dose of TNF-α or LPS[26,27]. However, it appears that the rise of concentration of intracellular calcium mediates apoptotic but not necrotic death of hepatocytes[28]. The possible effect of increased intracellular level of calcium on cAMP – activity is largely unknown.

In these experiments we investigated the effect of db-cAMP and verapamil on liver injury in mice induced by simultaneous application of D-GalN and LPS, when they were administered separately or combined (in order to see if they might have synergistic or antagonistic effect).

Materials and Methods

Animals

C57Bl/6 mice were raised in an animal colony unit at the Department of Physiology, School of Medicine, Zagreb. Mice of both sexes aged 12–16 weeks and weighing 20–25 g were used in all experiments. In particular experiment, only mice of the same sex were used. The cages were stored in rooms with a 12h light period from 6 a.m. to 6 p.m., and the temperature and relative humidity in the animal room were 21±2°C and 50±5%, respectively. The cages were sanitized twice weekly. All mice were given free access to tap water and standard mouse chow diet (Diete Standard, Milano, Italy).

Chemicals

Db-cAMP – Cat. No. A9501, LPS from *Escherichia coli* (strain 0111 : B4) – Cat. No. L2630, and D-galactosamine – Cat. No. G0500, were purchased from Sigma-Aldrich (St Louis, USA). Verapamil (calcium channel blocker, ampoules for human use, 2 mL/5 mg) was obtained from Belupo (Koprivnica, Croatia).

Induction of hepatitis with D-GalN and LPS

In our previous experiments[20] mice were given i.p. route 650 mg/kg of D-GalN and 0.01 mg/kg (0.2 μg/mouse) of LPS. This caused 100% mortality of control mice and almost all animals died within 10 hours. Therefore, in order to reduce or delay the morality of mice we tested lower dose of D-GalN. In these experiments we injected to mice 300 mg/kg of D-GalN and the same dose of LPS (0.01 mg/kg, 0.2 μg/mouse). Both agents were given simultaneously, i.p., in volume of 0.2 to 0.25 mL. Most animals died within 8 to 16 hours; all animals which survived 24 hours recovered completely. For bleeding and determination of aminotransferases, mice were given the same dose of LPS but the lower dose of D-GalN (150 mg/kg).

Treatment

Db-cAMP was dissolved and verapamil diluted to appropriate concentrations in saline and injected to mice one hour before D-GalN and LPS i.p. in volume of 0.2 to 0.25 mL. Experimental and control groups of mice contaited 10–12 animals (for determining survival of animals) or 6–8 animals (for determining ALT and AST activity). Control animals received appropriate vehicle.

Plasma ALT and AST activity

ALT and AST levels were measured 20–22h after D-Galn+LPS administration. Plasma samples were obtained by a procedure in which hemolysis was undetectable. Mice were given 250 U heparin i.p. 15 min before bleeding. Blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes. Plasma was stored at –70°C for 24h before ALT and AST determination. ALT and AST concentrations were measured by standard diagnostic laboratory techniques[20].

Statistical analysis

Results are expressed as mean±SEM. Parametric variables were compared by Student's t-test. Differences in survival between groups of mice were compared by chi-square test, using Yates's correction of the test when indicated.

Results

The effects of db-cAMP on survival of mice given LPS sensitized with D-GalN

In two separate experiments mice were given different doses of db-cAMP (5 mg/kg, 25 mg/kg and 125 mg/kg) one hour before LPS+D-GalN. Control mice obtained saline instead cAMP. There were 22 to 23 animals *per* group. Figure 1 shows the summary survival of mice obtained in two experiments. We can see that cAMP increased the survival of animals in dose-dependent manner. The survival of mice at the dose 125 mg/kg cAMP was significant in comparison to control group given saline ($p<0.05$), while the survival at the dose 25 mg/kg cAMP was on the verge of significance ($p=0.047$). Since at the dose 125 mg/kg cAMP three animals died immediately after its administration, we considered this dose as toxic one, and the dose 25 mg/kg cAMP was used in further experiments.

The effects of db-cAMP on concentration of aminotransferases in plasma of mice given D-GalN+LPS

Saline or 25 mg/kg db-cAMP were given to mice which one hour latter received 150 mg/kg D-GalN and 0.2 μg/mouse LPS. Concentrations of AST and ATL were determined 21 h thereafter (Table 1). As seen, in comparison to normal (non-treated) mice, administration of D-GalN+LPS greatly increased plasma concentrations of both AST and ALT. Treatment with db-cAMP decreased partially this rise of aminotransferase level, in the case of ALT significantly ($p<0.05$).

The effects of calcium channel blocker–verapamil on concentration of ALT in plasma of mice given D-GalN+LPS

Verapamil at three different doses (5 mg/kg, 20 mg/kg or 80 mg/kg) was injected to mice one hour before they received D-GalN+LPS (150 mg/kg D-GalN and 0.2 μg/mouse LPS). Concentration of ALT was determined 20–22 hours after that. Summary results of two different experiments are shown in Figure 2. As seen, when injected in two lower doses (5 mg/kg and 20 mg/kg), verapamil decreased significantly the concentration of ALT in comparison to mice given saline before hepatotoxic agents. On the other hand, high dose of the drug (80 mg/kg) appears to be toxic, since almost half animals died in half to one hour after treatment and level of ALT in survived animals was higher than in controls.

TABLE 1
THE EFFECT OF DB-cAMP ON PLASMA AMINORNSFERASE LEVEL IN MICE WITH HEPATITIS INDUCED WITH D-GalN+LPS

Treatment[a]	AST (U/L)[b]	ALT (U/L)[b]
Saline	1207.5±201.1	993.3±128.4
db-cAMP (25 mg/kg)	585.3±84.7	516.0±61.75*
Normal (untreated) mice	75.2±4.0	23.1±1.2

[a] db-cAMP (or saline) were given i.p. one hour before administration of D-GalN+LPS (300 mg/kg + 0.2 μg/mouse)
[b] Mean±SE; 8–9 mice *per* group
* Significantly different to group of mice given saline ($p<0.05$)

The effects of verapamil on survival of mice given D-GalN+LPS

In this experiment only one dose of verapamil was tested – 10 mg/kg. As in previous experiments, verapamil was given one hour before D-GalN (300 mg/kg) + LPS. Figure 3 shows that verapamil increased the survival of intoxicated animals, but (probably due to low number of animals in groups, 10 mice in each group) the difference was not significant ($p=0.06$).

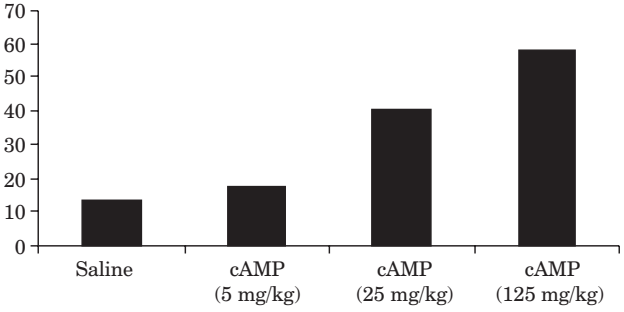

Fig. 1. The survival of mice given different doses of db-cAMP one hour before simultaneous injection of D-GalN and LPS. The survival was recorded 24 hours after administration of D-GalN and LPS. Number of mice: 22–23 per group, except in group treated with 125 mg/kg cAMP (19 mice). Asterisk indicates significance to mice given saline before D-GalN and LPS at $p<0.05$ level.

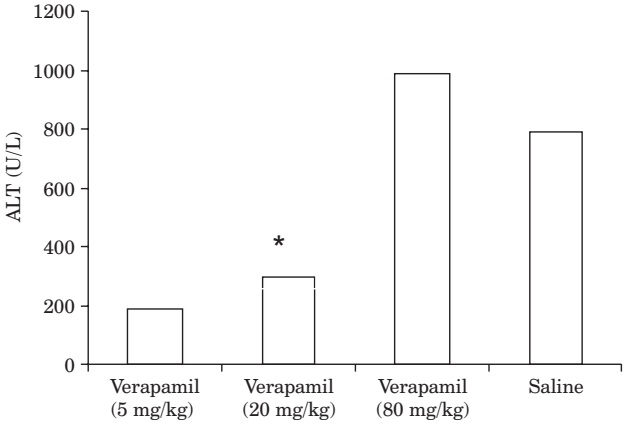

Fig. 2. Effects of verapamil on concentration of ALT in plasma of mice given D-GalN+LPS. The concentration of ALT was determined 20–22 hours after administration of D-GalN and LPS. Shown are the summary results of two different experiments. Number of mice: 10–12 per group, except in group treated with 80 mg/kg verapamil (6 mice). Asterisk indicates significance to mice given saline before D-GalN and LPS at $p<0.05$ level.

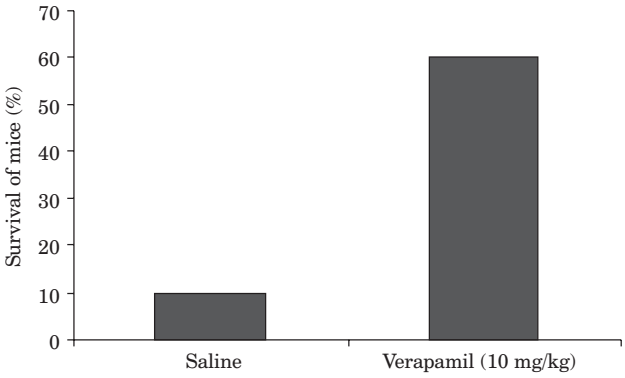

Fig. 3. Effects of verapamil (10 mg/kg) on survival of mice given D-GalN+LPS. The survival was recorded 24 hours after administration of D-GalN and LPS. Number of mice: 10 per group. The difference to control group given saline was not significant (p=0.06).

The effects of combined treatment with cAMP and verapamil on survival of mice given D-GalN+LPS

In the next experiment we checked whether there is a synergy of cAMP and calcium channel blocker in their protective effect on liver injury caused by heptotoxicants. Before intoxication with D-GalN (250 mg/kg) and LPS (0.2 μg/mouse), mice received one hour before 25 mg/kg db-cAMP and half hour before verapamil (10 mg/kg). As seen in Figure 4, it appears that combination of db-cAMP and verapamil has not synergistic effect in protection from hepatotoxicity – the survival of mice was similar to that seen earlier in protection of each agent alone. However, this effect should be investigated on higher number of animals and by using different schedules of application.

Discussion

Cyclic adenosine monophosphate (cAMP, cyclic AMP) is a second mesenger that is present in almost all cells in organism and is important in many biological processes. It is synthesized by enzyme adenylyl cyclase and decomposed by enzymes called phosphodiesterases (PDE), of which type IV of PDE appears to be the most important[29]. Of variety actions of cAMP, the most important for purpose of this study is its generally inhibitory action on synthesis and action of proinflammatory cytokines. This is the main reason that the cAMP or its derivatives are used clinically for treatment of obstructive and degenerative diseases in lungs[30]. In variety experimental models it was shown that cAMP inhibits production of proinflammatory cytokines, like TNF-α, IL-1, IFN-γ, IL--18, etc. Thus, raising its level in cells, by stimulation of adenylyl cyclase or by inhibition of type IV PDE (especially by drug rolipram) and less frequently by exogenous administration of its stabile cell permeable analogue – db-cAMP, has beneficial effect in many models of liver toxicity[17–19]. On the other hand, there are evidences that it stimulates the production of immunosuppressive cytokines (IL-10) and PGE_2, which have similar (suppressive) effect on production of inflammatory cytokines[31].

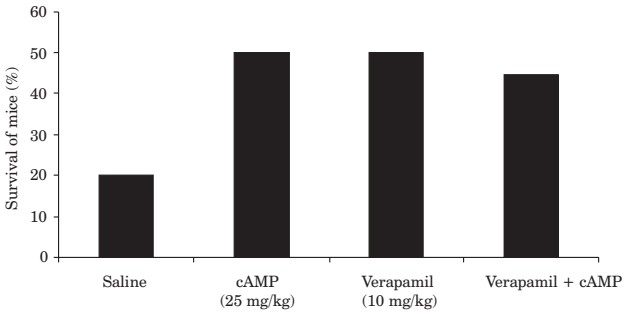

Fig. 4. Effects of db-cAMP and verapamil on survival of mice given D-GalN+LPS. db-cAMP (25 mg/kg) was injected i.p. one hour and verapamil (10 mg/kg) half hours before D-GalN+LPS. The survival was recorded 24 hours after administration of D-GalN and LPS. Number of mice: 10 per group, except in group given db-cAMP and verapamil-9 mice per *group.*

Acute hepatotoxicity induced with D-GalN and LPS is one of the first experimental models in which the pathogenic role of TNF-α was proven. Thus, the hepatotoxicity in mice can be almost completely prevented (and animals saved) if mice were given monoclonal antibodies to TNF--α[4–6] or if transgenic mice with nonfunctional TNF-α receptor (type I) were used[7]. It was shown that inhibitors of cAMP breakdown (especially rolipram, inhibitor of PDE IV)[17,18] and stimulators of adenylyl cyclase[18,19] inhibit production of TNF-α and/or alleviated hepatitis induced by LPS, Con A or TNF-α in D-GalN sensitized mice. Similar protective effect was obtained by use of stabile, cell permeable analogue of cAMP (dibutyryl-cAMP, db-cAMP)[19]. The latter effect was obtained in mice sensitized with D-GalN and pretreated with recombinant TNF-α – liver injury was assessed by serum aminotransferase levels and histopathological changes.

In our experiments, using mice in which liver injury was induced by D-GalN and LPS, we obtained the dose--dependent increase in survival of mice pretreated with db-cAMP (which was significant when the highest dose of the drug was used – 125 mg/kg) and significant decrease of serum ALT concentration (when medium dose of the drug – 25 mg/kg was used).

It is known that many hepatotoxicants, like acetaminophen[25,27] as well as TNF-α or LPS in D-GalN sensitized mice[16,26] increase concentration of intracellular calcium, which interferes with many important cellular reactions, leading finally to cell death. Administration of calcium channel blocker (verapamil) at non-toxic dose alleviated the liver injury, as assessed by reduction of plasma aminotransferase level and by increase of survival of mice (Figure 2 and 3). Essentially, our results are in line with those obtained by others[16,26].

In our preliminary investigations, we did not obtain the synergism in hepatoprotective action of db-cAMP and calcium channel blocker (verapamil) (Figure 4). The interrelationship of cAMP and intracellular calcium ions

is probably very complex, although in one *in vitro* experiment it was shown that synthesis of cAMP (and consecutive intracellular signaling) is possible when the cell influx of calcium was blocked[25,32]. However, the effect of modulation of intracellular calcium concentration on cell injury may be very dependent on the time of its modulation in respect to time of application of noxious liver agent.

In addition, it is necessary to repeat this experiment with higher number of animals, different time schedules of drug administration, and by analyzing different end points of liver injury (histopathology, serum aminotransferase level, etc.).

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ULOGA CIKLIČNOG ADENOZIN MONOFOSFATA U MODELU AKUTNOG TOKSIČNOG OŠTEĆENJA JETRE D-GALAKTOZAMINOM I LIPOPOLISAHARAIDOM U MIŠEVA

SAŽETAK

Svrha ovog istraživanja bila je ispitati učinak cikličnog adenozin monofosfata (cAMP) i njegovo moguće zajedničko djelovanje/sinergizam s blokatorom kalcijskih kanala u miševa u modelu akutnog toksičnog oštećenja jetre uzrokovanog primjenom D-galaktozamina (D-GalN) i lipopolisaharida (LPS). Pokusi su bili učinjeni na visokosrodnim miševima soja C57Bl/6 kojima su istodobno intraperitonealno (i.p.) uštrcani D-GalN (300 mg/kg) i LPS (0,01 mg/kg). Primjena ovih hepatotoksičnih agenasa dovela je do oštećenja jetre, što se očitovalo u visokoj smrtnosti životinja (80–90%) i velikom porastu jetrenih aminotransferaza u plazmi (ALT i AST) u kontrolnoj skupini miševa. Uštrcavanje stabilnog analoga cAMP (dibutiril-cAMP, db-cAMP) jedan sat prije D-GalN i LPS, dovelo je do značajnog povećanja preživljenja životinja (učinak ovisan o dozi) i smanjenja porasta koncentracije ALT u plazmi. Sličan (protektivni) učinak ostvario se primjenom blokatora kalcijskih kanala – verapamila, koji je bio uštrcan miševima u netoksičnim dozama (5 i 20 mg/kg) te na isti način kao i db-cAMP. Kombiniranim davanjem db-cAMP i verapamila u ovom modelu, nije se ostvario očekivani sinergistički hepatoprotektivni učinak: nije bilo razlike u preživljenju životinja u odnosu na pojedinačnu primjenu jednog ili drugog agensa.