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The effect of glucagon and cyclic adenosine monophosphate on acute liver damage induced by acetaminophen

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Summary. Recent investigations suggest that glucagon might have a potentially important hepatoprotective activity. We investigated the effect of glucagon in a model of acetaminophen-induced liver injury. CBA male mice were injected intraperitoneally with a lethal (300 mg/kg) or sublethal (150 mg/kg) dose of acetaminophen. The liver injury was assessed by observing the survival of mice, by liver histology and by measuring the concentration of alanine-aminotransferase (ALT). Inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF-κB) protein expressions were determined immunohistochemically. Hepatic levels of reduced glutathione (GSH) and cyclic adenosine monophosphate (cAMP) were also measured. Results show that glucagon, dose and time dependently, protects against acetaminophen-induced hepatotoxicity. This protection was achieved with a dose of 0.5 mg/kg of glucagon given intraperitoneally 15 min before or 1 h after acetaminophen. Treatment of animals with acetaminophen elevated ALT and nitrite/nitrate concentration in the plasma, enhanced iNOS and NF-κB expression and reduced GSH and cAMP concentration in the liver. Animals treated with glucagon had higher hepatic cAMP level, lower ALT and nitrite/nitrate concentration in plasma and lower expression of iNOS in liver cells than animals in control group, whereas there was no difference in the expression of NF-κB. Glucagon did not prevent the loss of GSH content caused by acetaminophen. Our investigation indicates that glucagon has a moderately protective effect against acetaminophen-induced liver injury, which is, at least partially, mediated through the downregulation of iNOS and through the increase in hepatic cAMP content, but it is not mediated through the modulation of NF-κB activity.

Key words: Glucagon, cAMP, Acetaminophen, Liver injury, iNOS

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Introduction

Glucagon is a 29 amino acid hormone synthesized and secreted from pancreatic α-cells in response to hypoglycemia. Primary action of glucagon is regulation of blood glucose via stimulation of hepatic gluconeogenesis and glycogenolysis. It also inhibits glycogen synthesis and glycolysis and is the primary counter-regulatory hormone to insulin. Glucagon exerts its effect by binding to the G protein-coupled receptor whose activation leads to an increased activity of adenylate cyclase with a consequent increase in intracellular level of cyclic adenosine-monophosphate (cAMP) (Jelinek et al., 1993; Jiang and Zhang, 2003). Several recent investigations suggested that glucagon might have an important hepatoprotective activity. For example, Harbrecht et al. reported a protective effect of glucagon against liver injury caused by lipopolysacharide (LPS) in rats (Harbrecht et al., 2004). Similarly, it was reported that administration of glucagon reduces apoptotical liver injury caused by Fas agonist in mice (Sinclair et al., 2008), prevents hepatocyte death after ischemia/reperfusion liver injury (Pediaditakis et al., 2010) and that livers for transplantation, during cold preservation, survive longer after the addition of glucagon in preserving solution (Li et al., 2005). Some data in these experiments indicated that its protective effect might be mediated, at least partially, by its

stimulatory effect on cAMP synthesis. This is supported by findings of the protective effect of exogenously added cAMP or its agonists in some models of liver injury (Arai et al., 1995; Taguchi et al., 1999; Akbar and Minor, 2001).

Acetaminophen (APAP) is the most widely used analgesic and antipyretic drug which is considered safe at therapeutic doses. However, overdose of APAP causes liver damage and for the last two decades is the leading cause of acute hepatic failure in the western world (Kaplowitz, 2005; Nourjah et al., 2006). Due to this, intoxication with a high dose of APAP is a widely used experimental model for the study of chemically-induced acute hepatotoxicity. The toxicity of APAP is initiated by formation of a reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) by liver microsomal enzymes (Mitchell et al., 1973a). It seems that CYP3A1 and CYP2E1 are the most important enzyme isoforms in mice. NAPQI first depletes glutathione (GSH), a major cellular antioxidant, and then covalently binds to cellular proteins (Mitchell et al., 1973b; Nelson, 1982; Zaher et al., 1998; Laine et al., 2009). Primary cellular targets of NAPQI toxicity have been postulated to be mitochondrial proteins, as well as proteins involved in cellular ion control (Nelson, 1990; James et al., 2003). Further events are a subject of numerous investigations and are still not fully understood.

It is believed that inflammatory mechanisms play a role in the later phases of APAP toxicity. Thus, several investigations indicated that oxidative stress, which is present after APAP poisoning, induces the expression of inducible nitric oxide synthase (iNOS) (Gardner et al., 1998, 2002) and leads to the activation of nuclear factor kappa B (NF-κB) (Bauer et al., 2000; Dambach et al., 2006; Cavar et al., 2010). Also, an increase in levels of pentraxin 3 and neopterin occurs, indicating a stimulation of the immune system (Demirbas et al., 2011; Yaman et al., 2011). It is suggested that these events might be important contributing factors during the development of APAP-induced hepatotoxicity.

Based on these data, the present study aimed to investigate the role of exogenously applied glucagon on APAP-induced liver injury, as well as to examine the effects of its administration on expression of NF-κB and iNOS in the liver tissue. The influence on hepatic GSH and cAMP synthesis in the liver was also monitored.

Materials and methods

Animals

Male CBA/H Zg mice aged 12-16 weeks and weighing 20-25 g were used in all experiments. They were raised in an animal facility unit at the Department of Physiology, School of Medicine, University of Zagreb. The cages were stored in room with a 12 h light period from 6 a.m. to 6 p.m., temperature of 21±2°C and relative humidity of 50±5%. The cages were sanitized twice weekly and mice were allowed free access to tap water and standard mouse chow diet (No. 4RF21, Diet Standard, Milan, Italy). All animal protocols were approved by the Ethics Committee of the University of Zagreb, School of Medicine (Zagreb, Croatia).

Chemicals

Pure APAP substance was purchased from Belupo pharmaceutical company (Koprivnica, Croatia). Phenobarbitone-sodium and trichloroacetic acid (TCA) were obtained from Kemika (Zagreb, Croatia). L-Glutathione reduced, bovine serum albumine (BSA) and 5,5-dithiobis 2-nitro-benzoic acid (DTNB) were purchased from Sigma-Aldrich (St. Louis, U.S.A) and glucagon was obtained from Novo Nordisk (Copenhagen, Denmark).

Treatments of animals

In all experiments mice were given phenobarbitone in drinking water for 7 days (0.3 g/L) in order to induce hepatic cytochromes P450 (CYPs). 12-14 h before the experiment the food was removed. The next morning mice were randomly divided in different groups and received appropriate treatment. Food was returned to animals 4 hours after the APAP administration (Guarner et al., 1988; Cavar et al., 2010). The doses and manner of drug application *in vivo* were chosen from scarce data in the literature (Harbrecht et al., 2004; Sinclair et al., 2008) and according to the toxicity data in our preliminary experiments, in which the effects of the drugs on survival of mice and gross macroscopic changes of liver and other visceral organs were observed. APAP was dissolved in warm saline, under light magnetic stirring and administered to mice intraperitoneally (i.p.) in a volume of approximately 0.5 mL in all experiments. To observe the survival of the mice, APAP was administered to animals in a dose of 300 mg/kg which in our previous experiments induced 43 to 72% mortality of untreated animals. The survival of mice was monitored for 48 h, since almost all mice either died within this period or fully recovered thereafter. In all other experiments mice were treated with a high sublethal dose of APAP (150 mg/kg), and in most of them glucagon was given 15 minutes before APAP.

In the first experiment, different doses of glucagon (0.05. 0.5, and 1.5 mg/kg, i.p.) or saline were given 15 minutes before APAP (150 mg/kg, i.p.). Twenty four hours after administration of APAP animals were first bled by venipuncture of the medial eye angle for determination of ALT in plasma, after which they were sacrificed and livers were taken for pathohistological analysis.

In the second experiment, the time effect of glucagon administration was investigated by monitoring the survival of animals. Glucagon (0.5 mg/kg) or saline were injected 15 min before or 1 h after the APAP (300 mg/kg) and survival was monitored for 48 h.

In the third experiment, the time effect of glucagon administration was investigated by determining the ALT levels. Glucagon (0.5 mg/kg) or saline were injected 1 or 2 h after the APAP (150 mg/kg), mice were sacrificed after 24 h and livers (for patohystological analysis) and plasma (for ALT determination) were taken.

In the fourth experiment, the kinetics of changes in ALT concentration, nitrite/nitrate levels and hepatic GSH and cAMP content was investigated. Mice were injected with saline or glucagon (0.5 mg/kg) i.p. 15 min before APAP (150 mg/kg), or only with saline or glucagon (i.e. without APAP). Animals were sacrificed at various time points (2.5, 6 or 24 hours) after APAP and livers (for immunohistochemical analysis and cAMP and GSH content determination), and plasma (for ALT activity and nitrite/nitrate levels measurements) were taken.

Plasma ALT activity

After collection, blood was centrifuged and plasma was separated and stored at -80°C for 24 h before ALT determination. ALT levels were determined by standard laboratory techniques in a clinical diagnostic laboratory, using an Olympus AU400 analyser.

Liver histology

Liver lobes of each animal (9-10 animals per group) were processed and stained with hematoxyllin and eosin as described previously (Cavar et al., 2010). Microscopically, liver damage was classified using an arbitrary scale from 0 to 5 as follows: degree 0-no damage; degree 1-minimal lesions involving single to few necrotic cells; degree 2-mild lesions, 10-25% necrotic cells or mild diffuse degenerative changes; degree 3-moderate lesions, 25-40% necrotic or degenerative changes (i.e. loss of normal tissue structure without evident necrosis); degree 4-marked lesions, 40-50% necrotic or degenerative cells; degree 5-severe lesions, more than 50% necrotic or degenerative cells. Sections with scores higher than 2 were considered to exhibit significant liver injury (Fig. 1) (Silva et al., 2001; Cavar et al., 2010).

Immunohistochemistry

Samples of liver were taken 6 hours after intoxication with APAP. Processing and staining of liver tissue for immunochemistry was performed exactly as described previously (Cavar et al., 2010). Briefly, sections were first incubated with diluted (1:100) polyclonal rabbit anti-NF-κB (p65 subunit) or anti-iNOS antibodies (No. sc-109 and sc-651, respectively, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and then with a biotinylated secondary antibody (rabbit UniTect ABC Kit, Oncogene, Boston, Mass., USA) for 30 min at room temperature. Afterwards, sections were incubated with avidin biotinylated horseradish peroxidase complex (ABC) and stained with diaminobenzidine (DAB). Finally, sections were counterstained with hematoxyllin. Cells positive to NF-κB had brown-stained cytoplasm or nuclei (depending of NF-κB activation) and cells positive to iNOS had brown-stained cytoplasm. Positive internal controls for NF-κB and iNOS staining were smooth muscle cells in vascular walls (Cavar et al., 2010). No staining was observed when sections were incubated with secondary antibodies alone or when only primary antibodies were used. Images were captured with digital camera (SPOT Insight, Diagnostic Instruments, USA) mounted on an Olympus BX51 microscope using the SPOT software.

Immunohistochemical quantification

The number of cells was evaluated quantitatively by two independent investigators and classified as negative (not stained) cells, nuclear positive for NF-κB counting and cytoplasm positive for iNOS counting. Counts were made along the length of the liver, as described previously (Cavar et al., 2010). The examination was performed on an Olympus BX51 microscope equipped with a DP11 digital camera. Images were analyzed using DP-SOFT version 3.1 software.

Measurement of nitrite and nitrate in plasma

Samples of plasma were taken 2.5, 6 or 24 hours after intoxication with APAP. For quantification of total NO production plasma nitrite and nitrate, the end products of NO metabolism were assessed. Nitrate (NO3-) was first reduced into nitrite (NO2-) using copper-coated cadmium granules (NITRALYZER-II, Nitrate to Nitrite reduction kit, World Precision Instruments, Sarasota, FL, USA). Concentration of total nitrite was measured by Griess reaction according to the manufacturer's instructions. Finally, absorbance readings were performed with the use of an automated microplate reader (Dynatech MR 5000, Dynatech Technology, Inc., Horsham, PA, USA) at 540 nm.

Hepatic GSH determination

Samples of liver were taken 2.5, 6 or 24 hours after intoxication with APAP. Reduced glutathione (GSH) in liver tissues was determined according to the Ellman method (Ellman, 1959). A portion (approximately 100 mg) of liver was homogenized in Tris-HCl buffer (25 mmol/L, pH 7.4) and 0.1 mL of the homogenate was mixed with 0.2 mL 5% TCA, followed by centrifugation at 860 g for 10 minutes. Supernatant was mixed with DTNB. The absorbance was determined at 412 nm. The GSH level was compared with a standard curve prepared with six different GSH concentrations. From the rest of the homogenate, the protein concentrations were determined using the Bradford method with BSA as the standard (Bradford, 1976) and GSH content was calculated in nmoles/mg proteins.

Hepatic cAMP determination

Samples of liver were taken 2.5, 6 or 24 hours after intoxication with APAP. A piece of liver (approximately 200 mg) was snap frozen in liquid nitrogen. Later, it was weighed and homogenized in TCA acid 1:5. After centrifugation (10 minutes at 4°C and 860 g), the supernatant was separated and trichloroacetic acid was extracted with water-saturated ether. Concentration of cAMP was determined using a commercial kit, cAMP Complete Enzyme Immunometric Assay (EIA) kit (No. 900-163, Assay-Designs, USA) according to the manufacturer's instructions.

Statistical analysis

Results are expressed as mean ± S.E. Differences in survival between groups of mice were compared by chi-square test using Yates's correction. Statistical comparisons between two groups were made using a Student's t-test. Comparisons between multiple groups were carried out using one-way analysis of variance (ANOVA) with a post hoc test of significance between individual groups. Statistical significance level was established at $p<0.05$.

Results

Effect of different doses of glucagon on ALT plasma levels and liver histology

ALT levels

Mice received a saline or three different doses of glucagon (0.05, 0.5 and 1.5 mg/kg) 15 min before APAP. Treatment of mice with APAP highly increased plasma ALT levels and pre-treatment of mice with glucagon showed a beneficial effect which was dose dependant (Fig. 2). The group of mice which received 0.5 mg/kg of glucagon before APAP had 3 fold lower ALT levels than the control group treated with saline before APAP ($p<0.05$). The dose of 1.5 mg/kg of glucagon also markedly reduced ALT levels although the difference was not statistically significant, whereas the dose of 0.05 mg/kg had no influence. Glucagon per se had no influence on ALT levels at any dose: ALT levels of mice treated only with glucagon were similar to that of normal (untreated mice) ≈30 U/L (data not shown).

Liver histology

Macroscopically, the whole liver surface of APAP treated animals had a mottled appearance with dark-red hemorrhagic-necrotic spots scattered on the yellowish background. Microscopically, liver damage was graduated using the arbitrary scale from 0 to 5 (Fig. 1), as described in Materials and methods. Although the severity of necrosis was quite variable both between animals and also within different parts of the same liver, it was found that animals pre-treated with glucagon (0.5 mg/kg) had a significantly lower number and size of necrotic foci in the liver (Table 1).

Effect of varying the time of glucagon treatment in relation to APAP administration

Survival of animals

In this experiment the effective dose of glucagon (0.5 mg/kg) was applied either15 min before APAP (300 mg/kg), or in an early therapy manner, i.e. 1 h after APAP. Both groups which received glucagon had significantly better survival than the control group (Fig. 3A).

Table 1. Effects of glucagon on APAP-induced liver injury.

	Histopathological scores[c]						
Treatment of mice[a]	0	1	2	3	4	5	%>2[d]
Saline + APAP	0	1	1	5	3	0	80
Glucagon (0.5 mg/kg) + APAP	0	4	5	2	0	0	16*
	Histopathological scores[c]						
Treatment[b]	0	1	2	3	4	5	%>2[d]
Saline + APAP	0	0	3	0	1	3	57
Glucagon (+ 1h) + APAP	0	4	4	0	0	0	0*
Glucagon (+ 2h) + APAP	0	2	2	3	0	1	50

Mice received APAP (150 mg/kg), and were sacrificed 24 h later and livers were collected for analysis. [a]: Glucagon (0.5 mg/kg, i.p.) or saline were given 15 min before APAP administration. [b]: Glucagon (0.5 mg/kg, i.p.) or saline were given at indicated times after APAP administration. [c]: Histopathological scores were determined and graded by intensity of hepatocellular necrosis from 0 to 5 as described in Materials and Methods. [d]: Scores greater than 2 were considered as significant necrosis. N=8-10 mice per group. *: Statistically significant in comparison to group treated with saline and APAP ($p<0.05$).

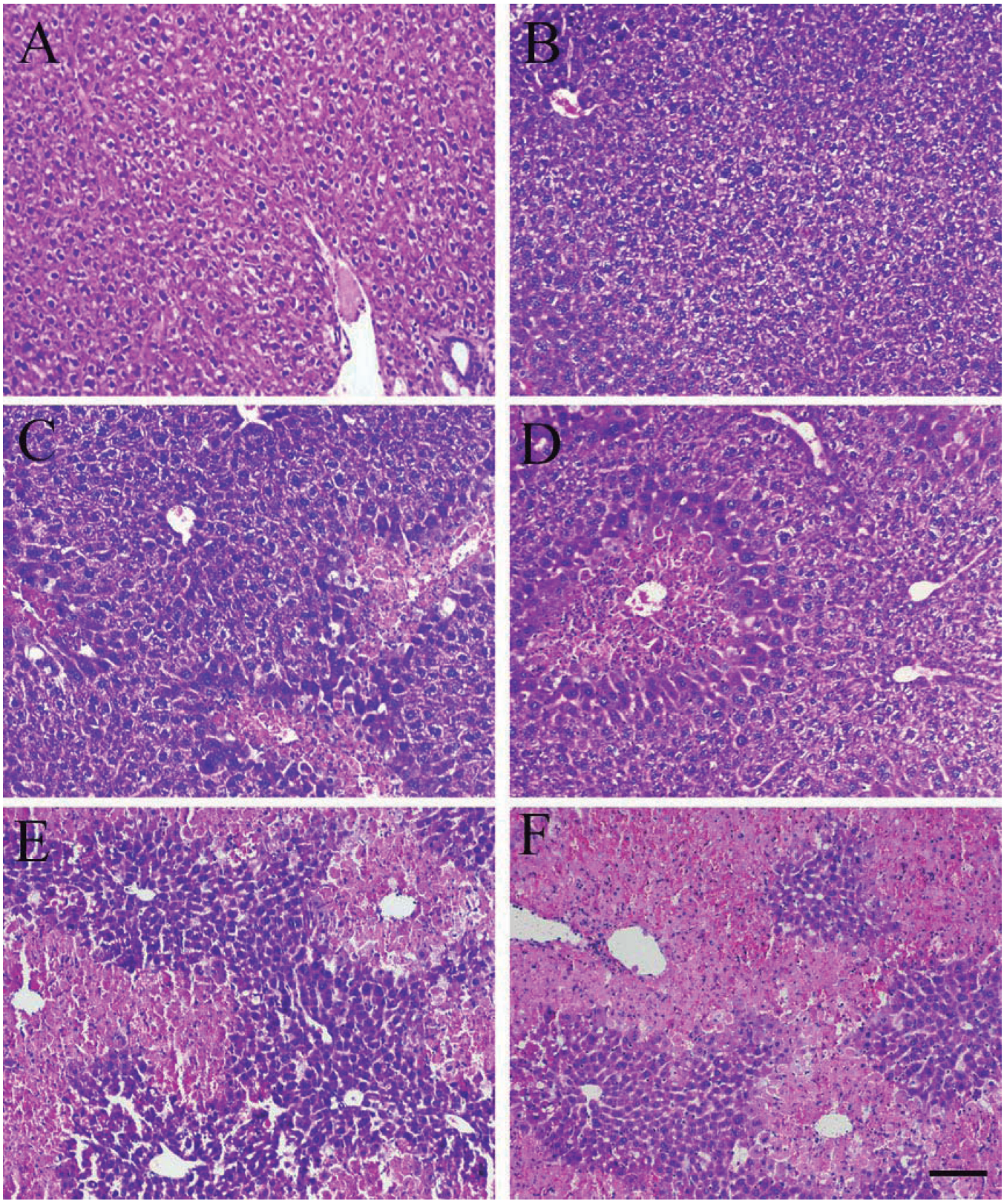

Fig. 1. Histopathological changes in livers from normal and APAP-intoxicated mice. Livers were collected 24 h after APAP administration (150mg/kg). Graduation of liver damage was determined according to the arbitrary scale as follows: degree 0 **(A),** degree 1 **(B)**, degree 2 **(C)**, degree 3 **(D)**, degree 4 **(E)** and degree 5 **(F)**. Descriptions of each degree are explained in Materials and Methods. Sections were stained with hematoxylin and eosin, x 100; Scale bar: 10 μm.

ALT levels and liver histology

We further investigated the time-effect of glucagon application by monitoring ALT levels in plasma and liver histopathological changes. Glucagon (0.5 mg/kg) was applied 1 or 2 h after APAP poisoning. In accordance with the results of previous experiments glucagon effectively reduced ALT levels (Fig. 3B) and ameliorated the histopathological signs of liver injury when applied 1 h after APAP, but had no influence when given as a late therapy, i.e. 2 h after APAP administration (Fig. 3B, Table 1). We also found no effect when glucagon was given 90 min before APAP (data not shown).

Kinetics of changes in ALT concentration, nitrite/nitrate levels and in hepatic GSH and cAMP content

In this experiment mice were treated with glucagon (0.5 mg/kg) or saline 15 min before the APAP and sacrificed at various time points (2.5, 6 or 24 h) after APAP treatment. Some animals received only glucagon or saline (without APAP).

ALT levels

In agreement with our previous results, mice which received glucagon before APAP had lower ALT levels than mice in control group. The difference was visible at 6 and at 24 h after APAP (Fig. 4A).

Hepatic GSH concentration

The APAP treatment caused depletion of hepatic GSH after 2.5 h and glucagon did not prevent this depletion. However, animals which were pre-treated with glucagon had a somewhat faster recovery of GSH content, which was significant at 6 h after APAP. Glucagon per se slightly reduced GSH content at 2.5 h (Fig. 4B). Interestingly, an increase in GSH content at 24 h was present in all four groups. This can probably be explained by a negative effect of fasting on GSH content (Siegers et al., 1989) (as mentioned previously in Materials and Methods, after overnight fasting food was returned to animals 4 h after the APAP treatment).

Nitrite/nitrate levels

Nitrite/nitrate concentration in serum markedly increased at 2.5 h after the APAP treatment and after 6 h a further increase was present. After 24 h, the concentration of nitrite/nitrate returned to the basal level. Glucagon pre-treatment suppressed this increase, whereas glucagon per se had no influence on nitrite/nitrate concentration (Fig. 4C).

Hepatic cAMP concentration

The level of cAMP in liver 2.5 h after intoxication

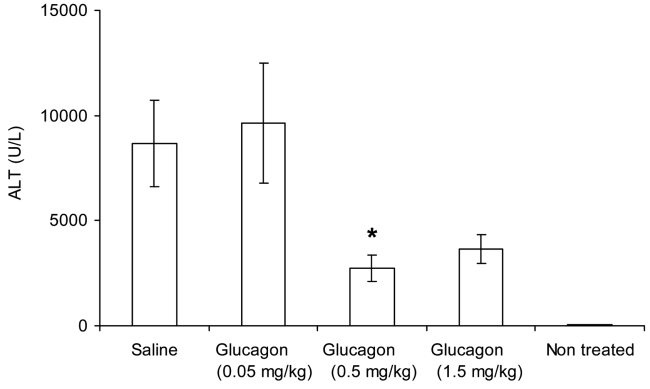

Fig. 2. Dose-effect of glucagon on ALT levels in mice with APAP-induced liver injury. Mice were treated with glucagon (at indicated doses, i.p.) or saline prior to APAP (150 mg/kg, i.p.). ALT levels were determined 24 hours after APAP. Each value represents the mean ± S.E. of 8 to 11 mice. *: Significantly different from mice treated with saline before APAP (ANOVA followed by Dunnetts' multiple comparison test, p<0.05).

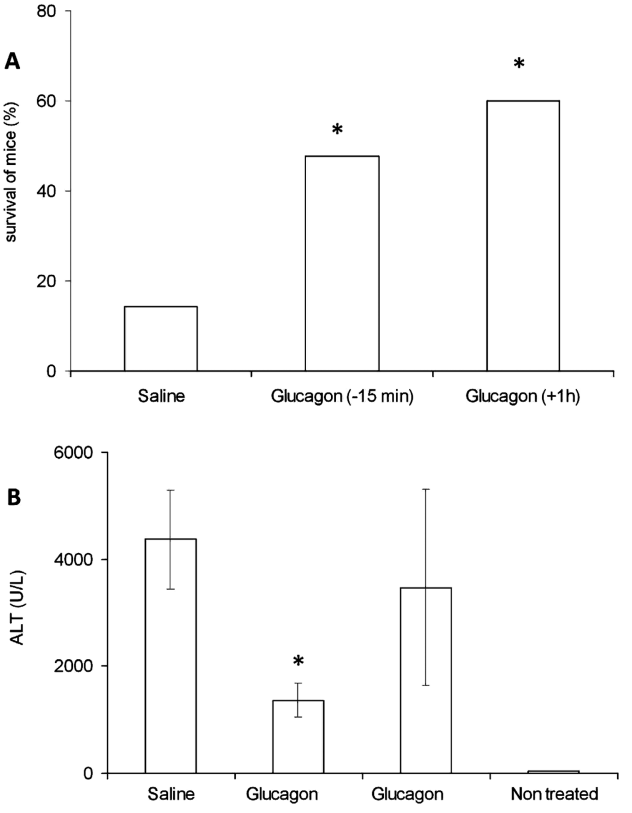

Fig. 3. Time-effect of administration of glucagon. Mice were treated with saline or glucagon (0.5 mg/kg, i.p.) at indicated times before, or after APAP (300 mg/kg, i.p. in **A**, and 150 mg/kg, i.p. in **B**. **A** Survival was monitored for 48 h (n=20 animals per group). **B.** ALT levels were determined 24 hours after APAP. Each value represents the mean ± S.E. of 7 - 8 mice. *: Significantly different from mice treated with saline before APAP (**A:** chi-squared test, p<0.05, **B:** ANOVA followed by Dunnetts' multiple comparison test, p<0.05).

with APAP significantly declined; however it returned to normal values already after 6 h. Treatment with glucagon before APAP reduced the decrease of cAMP at 2.5 h after administration of APAP, whereas at two remaining time points there were no significant differences (Fig. 4D).

Expression of iNOS after treatment with glucagon - immunohistochemistry analysis

Saline-treated (normal) mice

In the group of normal mice, iNOS positivity was low. Diffuse granular staining pattern was present in the cytoplasm of small groups of hepatocytes and in some cells on the inner surface of sinusoid capillaries (endothelial or Kupffer cells). There were 3.9% of cells with cytoplasmic positivity to iNOS (Fig. 5A, Table 2).

Mice which received saline and APAP

iNOS expression was present in the cytoplasm of hepatocytes in a granular and vacuolar staining pattern and in cytoplasm of some endothelial or Kupffer cells (Fig. 5B). The number of liver cells with immuno-

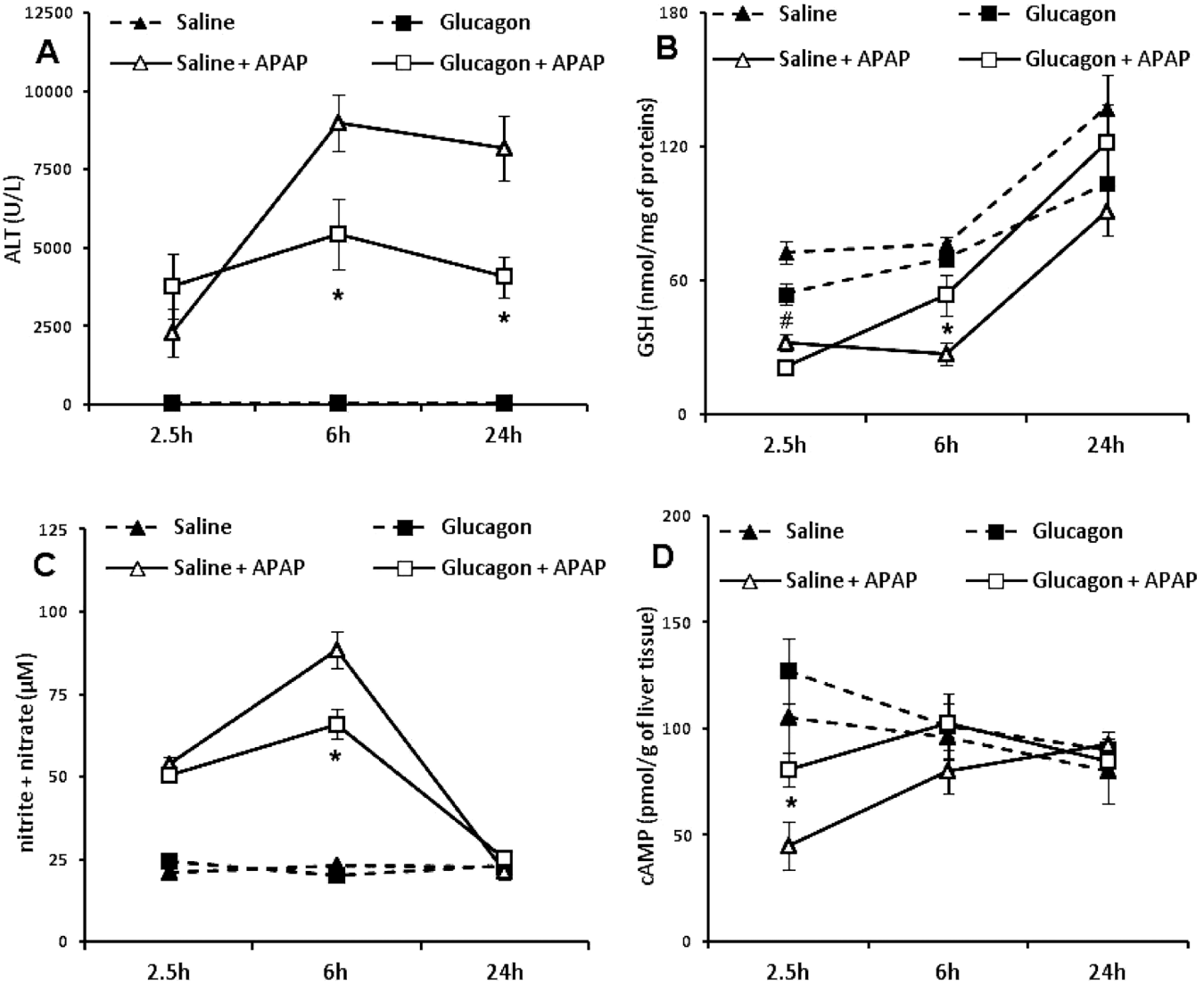

Fig. 4. Effect on ALT and nitrite/nitrate concentration in plasma and hepatic GSH and cAMP content. Mice were treated with glucagon (0.5 mg/kg, i.p.) or saline 15 min before APAP (150 mg/kg, i.p.) or with glucagon or saline alone. **A** ALT levels, **B** GSH content, **C** nitrite/nitrate levels and **D** cAMP were measured at indicated times. Each value represents the mean ± S.E. (n=8-16 for groups treated with APAP, n=4-8 for other two groups). *: Significantly different from mice treated with saline before APAP (Student's t-test, $p<0.05$). #: significantly different from mice treated with saline (Student's t-test, $p<0.05$).

reactivity to iNOS was significantly higher in comparison to normal mice (3.9 vs. 44.5%, Table 2, $p<0.05$, ANOVA test).

Mice which received glucagon and APAP

In the group of glucagon treated mice, a diffuse staining pattern of iNOS was seen in the cytoplasm of single or small groups of hepatocytes (Fig. 5C). The number of liver cells positive to iNOS was significantly lower when compared with the group treated with saline and APAP (23.5 vs 44.5%, Table 2, $p<0.05$ ANOVA test).

Expression of NF-κB after treatment with glucagon - immunohistochemistry analysis

Saline-treated (normal) mice

NF-κB was expressed in the form of numerous small granules which were diffusely scattered in the cytoplasm of liver cells situated around blood vessels in Kiernan spaces (afferent arterioles), and also in the cytoplasm of some cells in the lobules and around the central vein (efferent arterioles). Almost all NF-κB immunoreactivity was present in the cytoplasm and there were only 2.9% cells with nuclear positivity to NF-κB (Fig. 5D, Table 2).

Mice which received saline and APAP

The whole liver tissue was infiltrated with reactive cells. Layers of cells containing numerous vacuoles in nuclei and cytoplasm were situated around the blood

Table 2. Quantification of immunohistochemistry to NF-κB and iNOS in mice liver cells.

Treatment	% of liver cells[a] positive to: NF-κB	iNOS
Saline	2.9±1.4	3.9±0.5
Saline + APAP	22.2±2.1[b]	44.5±2.1[b]
Glucagon + APAP	23±1.6[b]	23.5±3.2[b,c]

[a]: The percentage of positive cells was calculated and expressed as mean ± S.E. [b]: Significantly different from group treated with saline. [c]: Significantly different from group treated with saline and APAP. Differences were significant at $p<0.001$ level (ANOVA test followed by Bonferroni multiple comparison test).

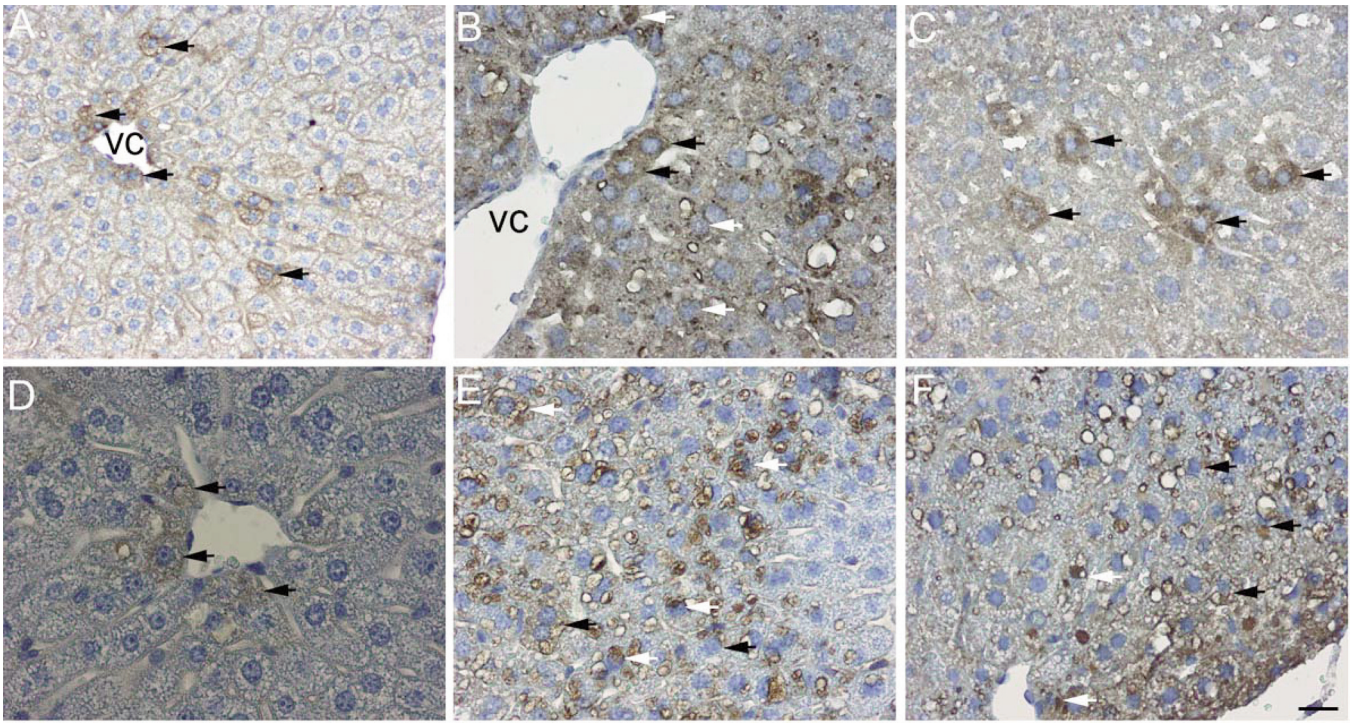

Fig. 5. A-C. Immunohistochemical expression of iNOS in liver cells of mice. **D-F.** Immunohistochemical expression of NF-κB in liver cells of mice. **A.** In a group of normal mice, iNOS positivity showed a diffuse granular staining pattern in the cytoplasm of small groups of hepatocytes (black arrows), x 20. **B.** In a group of control mice, iNOS positivity showed mostly diffuse granular (black arrows) and vacuolar (white arrows) staining pattern in the cytoplasm of hepatocytes, x 40. **C.** In the group of glucagon treated mice, a diffuse staining pattern of iNOS was seen in the cytoplasm of single or small groups of hepatocytes (black arrows), x 40. VC-cental vein. Immunohistochemical staining to iNOS, scale bar 25 μm. **D.** In the group of normal mice, NF-κB positivity showed mostly diffuse staining pattern within cytoplasm of hepatocytes (black arrows), x 100, scale bar 10μm. **E**. In control mice, NF-κB staining showed a perimembranous pattern, with vacuoles both in the nuclei (white arrows) and cytoplasm (black arrows), x 40. **F**. In glucagon treated mice, a similar expression pattern of NF-κB positive cells (white and black arrows) was observed as in group of control mice (see E), x 40. VC-cental vein. Immunohistochemical staining to NF-κB. Scale bar 25 μm.

vessels. NF-κB staining showed a perimembranous pattern with vacuoles both in the nuclei and cytoplasm (Fig. 5E). Immunoreactivity in nuclei was significantly higher than in the group of normal mice (22.2% vs 2.9, Table 2, $p<0.05$, ANOVA test).

Mice which received glucagon and APAP

NF-κB staining showed a similar pattern to that of the group treated with saline and APAP (Fig. 5F). There was no statistically important difference in nuclear immunoreactivity between these two groups (23 vs 22.2%, Table 2, $p>0.05$, ANOVA test).

Discussion

Our results further elucidate the hepatoprotective effect of glucagon which was reported by several recent investigations. We found that administration of glucagon protects against APAP-induced liver injury. However, the time of its administration is of the most importance; it had a hepatoprotective effect when it was administered to mice 15 min before or 1h after the APAP, but had no influence when given in a later (+2 h) or an earlier (-1.5 h) treatment (Figs. 2, 3). In previous investigations time-effect of glucagon administration was not investigated and glucagon was given either 30 min before or simultaneously with a noxious agent (Harbrecht et al., 2004; Sinclair et al., 2008).

Glucagon did not prevent depletion of GSH caused by APAP (Fig. 4B), which indicates that the protective effect was not mediated through blockade of APAP bioactivation. Glucagon per se had a slightly negative effect on GSH content and, after a detailed search of the literature we found one investigation indicating a similar effect of glucagon (Lu et al., 1991). GSH is an important antioxidant with a protective role in this and other models of liver injury (Sun et al., 2006; Cazanave et al., 2007). We assume that this negative effect of glucagon is exceeded by its other (protective) effects, one of which could be iNOS suppression. However, the GSH content should be studied in more detail in future research on the hepatoprotective effects of glucagon.

Basal levels of NO, mostly produced by endothelial and neuronal isoforms of nitric oxide synthase (NOS), are generally accepted to be necessary for protection of inner organs from ischemic injury. On the other hand, NO is a highly reactive oxidant which can be overproduced by iNOS in response to TNF-α, IFN-γ and other stimuli. Although its role is not yet firmly established, overproduction of NO in the liver has been implicated as a possibly important event in this as well as in other models of hepatic inflammation and injury. Overproduction of NO has been reported to suppress liver protein and DNA synthesis and to induce apoptosis; it also inhibits catalase activity, suggesting that it may alter the detoxification of cytotoxic oxygen radicals. NO reacts with superoxide anion, forming peroxynitrite, an even more potent oxidizing agent. Peroxynitrite can react directly with sulfhydryl residues in cell membranes leading to lipid peroxidation and cytotoxicity (Beckman et al., 1990; Grisham et al., 1999). For example, Gardner et al. showed that iNOS knockout mice are less sensible to APAP hepatotoxicity (Gardner et al., 2002) and Harbrecht et al. reported that the protective effect of glucagon in LPS-induced liver injury is probably mediated through iNOS suppression (Harbrecht et al., 2004). These results are not in agreement with the results of Liu et al. which showed a protective role of NO donor-VPYRRO/NO in APAP model of liver injury (Liu et al., 2003). However, it was later indicated that the vehicle used to dissolve VPYRRO/NO (absolute ethanol) could be the reason for this discrepancy (Ito et al., 2004). The use of organic solvents in vehicles is the leading cause of bias in this model of hepatotoxicity (Kelava et al., 2010). In our experiments, glucagon pre-treatment reduced iNOS expression and NO production in mice with APAP induced liver injury (Fig. 4C, Fig. 5A-C, Table 2) indicating that this could be, at least partially, the mechanism of protection of glucagon.

Our results further indicate that the protective effect of glucagon is not mediated by a modulation of NF-κB activity, which we found to be increased 6 h after APAP poisoning in both control and glucagon pre-treated group. In contrast to this (Fig. 5D-F, Table 2), Harbrecht et al. reported that glucagon decreases NF-κB activity in LPS-induced liver injury (Harbrecht et al., 2004). The difference could be caused by differences in experimental models. A change in NF-κB activity after APAP intoxication was first reported by Blazka et al., and after that the reports about its role are highly opposed (Blazka et al., 1995; Bauer et al., 2000; Dambach et al., 2006; Ghosh and Sil, 2009). A probable reason for this confusion could be the multitude of genes controlled by NF-κB. The activation of some of these genes (iNOS, TNFα) could contribute to hepatotoxicity, whereas the activation of others could contribute to hepatoprotection (cycloxigenase-2, hemoxigenase-1) (Ishida et al., 2006; Cavar et al., 2010; Wang et al., 2011). All this makes an exact role of increased NF-κB activity hard to predict and understand.

Administration of APAP caused a decrease in hepatic cAMP concentration, while the glucagon treatment opposed this (Fig. 4D). An important finding, from practical point of view, is that both glucagon and cAMP were shown to prevent hepatocyte death after ischemia/reperfusion injury (Pediaditakis et al., 2010) and that livers for transplantation, during cold preservation, survive longer, when glucagon was added to preservation solution (Li et al., 2005).). The mechanism(s) by which cAMP protects liver cells are not fully understood. It is generally known that cAMP conveys its intracellular signals mainly through activation of PKA, but also by activation of Akt (or protein kinase B) and phosphoinositide 3-kinase (PI3), which are generally connected with cell survival and proliferation. It was shown, in other models of hepatotoxicity, that cAMP either stimulates generation of

some cell surviving factors (Akbar and Minor, 2001) and/or inhibits the generation of pathogenic pathway of toxicity, like TNF-α (Arai et al., 1995), other inflammatory cytokines (Odashima et al 2006; Ji et al., 2012) and iNOS expression (Harbrecht et al., 2004). It also prevents changes in mitochondrial membrane potential (Pediaditakis et al., 2010). Since some agents, which increase hepatic cAMP production (PGE2, IL-1, rolipram), were shown to be hepatoprotective in other models of liver injury (Arai et al., 1995; Taguchi et al., 1999; Cavar et al., 2010; Erceg et al., 2010), the increase in intracellular cAMP could be a common way of protection for these various substances.

Sinclair et al. showed that glucagon protects liver from apoptotic damage caused by administration of Fas agonist (Sinclair et al., 2008). We did not investigate this possibility of protection, because in APAP model of liver injury most of the hepatocytes die in a necrotic and not in an apoptotic manner (Gujral et al., 2002).

In conclusion, our investigations indicate that glucagon has a moderate protective effect against APAP-induced liver injury. That protection could be partially mediated through the down regulation of expression of iNOS, but not through a modulation of NF-κB activity. The protective effect of glucagon may also be dependent on its stimulatory effect on cAMP synthesis, which showed a hepatoprotective effect in different models of hepatic injury.

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