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Effects of selective phosphodiesterase 3 inhibition in the perfused liver of the rat after endotoxin treatment

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1 This study was designed to investigate the role of rat phosphodiesterase 3 (RPDE3) in regulation of liver metabolism in sepsis. We studied the effects of the phosphodiesterase 3 inhibitor (PDI), enoximone, alone and in combination with regulating factors of hepatic carbohydrate metabolism and bile secretion in the perfused liver of rats treated 4 h earlier with endotoxin. In addition, cyclic AMP and cyclic GMP levels were determined in the effluate and bile by radio immunoassay methods.

2 After endotoxin treatment, infusion of enoximone at three concentrations (1 μM, 10 μM) resulted in an increased glucose output from -1.4 ± 0.9 to 7.8 ± 2.5 μmol l^{-1} 20 min^{-1}. Bile acid-independent bile flow increased also, in a dose-dependent manner.

3 In untreated livers, cyclic AMP release increased in the effluate from 1000 ± 73 fmol g^{-1} min^{-1} to 1710 ± 143 fmol g^{-1} min^{-1} when enoximone (10 μM) was administered. In bile from untreated livers, the level of cyclic AMP was also significantly increased by enoximone. After endotoxin treatment, the enoximone (10 μM) effect on cyclic AMP levels in effluate and bile was greatly reduced. Levels of cyclic GMP in the effluate and bile appeared unchanged in the presence of enoximone.

4 During co-infusion of glucagon (1 nM) and enoximone (10 μM), cyclic nucleotide levels in the effluate and bile of livers after endotoxin treatment were determined. In the effluate, cyclic AMP release increased from 827 ± 144 fmol g^{-1} min^{-1} to 17802 ± 2821 fmol g^{-1} min^{-1} when glucagon was administered. The presence of enoximone enhanced cyclic AMP further to 41696 ± 920 fmol g^{-1} min^{-1}. The same changes in cyclic AMP release were found in bile. Levels of cyclic GMP in the effluate and bile were not significantly affected by the administration of glucagon and the PDI.

5 Glucose release was determined during glucagon, sympathetic nerves stimulation and phenylephrine administration in the presence and absence of enoximone. The addition of enoximone to glucagon increased glucose release by 8.2 ± 2.8 μmol g^{-1} 20 min^{-1}, without alteration of lactate balance. The PDI enhanced the glycogenolytic effects of nerve stimulation and of phenylephrine, accompanied by a reduction in lactate production.

6 Enoximone significantly enhanced the bile acid independent bile flow after glucagon, nerves stimulation and after administration of phenylephrine. Bile acid secretion was unaffected by the PDI. The vasoconstrictor effect of nerve stimulation was reduced by the PDI.

7 We conclude that endotoxin treatment reduces the ability of the PDI, enoximone, to increase cyclic AMP release in the perfused liver. The significant increase in cyclic AMP release after stimulation with glucagon and enoximone favours the view that RPDE3 is involved in the degradation of cyclic AMP in the liver after exposure to endotoxin. Additionally, the inhibition of the RPDE3 results in glucose release, vasodilatation and choleresis in endotoxin pretreated livers.

Keywords: Cyclic AMP phosphodiesterase 3; enoximone; endotoxin; liver; cyclic AMP; carbohydrate metabolism; bile flow

Introduction

In septic patients, characteristic metabolic alterations are detected which induce increased glucose turnover and impaired glucose utilisation (Shangraw *et al.*, 1989). In particular, these alterations of the carbohydrate metabolism have been implicated as playing an important role for the immune function (Bagdade *et al.*, 1971), neurological recovery, and for overall mortality (Longstreth *et al.*, 1985).

The cellular and molecular basis of this clinical entity is a matter of debate-which biochemical pathways are affected, can they be reversed, and by what means? Various authors have postulated that the changes in catecholamine levels and receptors are responsible for the metabolic and haemodynamic changes observed under septic conditions (Parker & Parrillo, 1983; Liu & Ghosh, 1983). For the liver, dynamic changes of α and β receptors in sepsis have been reported (Hwang *et al.*, 1994). The α-adrenoceptor-mediated increase in glucose output from the liver is caused mainly by a calcium-dependent mechanism, where the β-adrenoceptor response is mainly mediated by the second messenger, adenosine 3':5'-cyclic monophosphate (cyclic AMP), via the adenylyl cyclase system. The level of cyclic AMP is controlled by the activity of adenylyl cyclase and by cyclic nucleotide phosphodiesterases (PDE), which hydrolyse cyclic AMP to adenosine 5' monophosphate.

Many PDE isozymes have been characterized in mammalian cells. On the basis of their substrate selectivity, kinetic properties, regulatory control and inhibition by selective inhibitors, PDE are classified into at least seven families (Verghese *et al.*, 1995). The type 3 PDE (PDE3) family is characterized by its high affinity for cyclic AMP, its low K_m, the competitive inhibition by cyclic GMP, and certain positive inotropic drugs. PDE3 has been isolated from a number of tissues, including adipose, myocardium, smooth muscles and liver. In the liver, activation of the PDE3 isozyme by insulin and glucagon is well established (Pyne *et al.*, 1988).

The knowledge of the changes in the adrenoceptor and the cyclic AMP second messenger system in septic conditions provides the pathophysiological basis for the use of PDE3 inhibition in the low-output cardiac syndrome (Vincent *et al.*, 1988). The PDE3 inhibitor (PDI), enoximone, is an inotropic

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substance which is used for the treatment of cardiac output failure. It has been proposed that enoximone is a selective inhibitor of the high-affinity cyclic AMP phosphodiesterase type 3 (Colucci *et al.*, 1986; Silver *et al.*, 1989). The enhanced level of cyclic AMP also mediates the vasodilator properties of the PDI enoximone. Inhibition of cyclic AMP hydrolysis by PDI in vascular smooth muscle results in vasodilatation which is mediated by phosphorylation of myosin light chain kinase and changes in free intracellular Ca^{2+} levels (Lugnier *et al.*, 1986; Kauffman *et al.*, 1987). We and others have shown that enoximone is not only acting on cardiac or vascular tissue, but also on liver tissue (Youssef *et al.*, 1994; Weidenbach *et al.*, 1995b).

The present study was designed to investigate the effects of the PDI, enoximone, on glycogenolytic and vasoactive stimuli following endotoxin treatment in a liver perfusion model. The adenylyl cyclase pathway was stimulated with glucagon. The α-adrenoceptor stimulus was applied by perfusion of phenylephrine or stimulation of sympathetic nerves to the liver. Cyclic AMP release, glucose production and bile acid-independent bile flow increased following stimulation of adenylyl cyclase pathway and RPDE3 inhibition. Vasoconstriction after stimulation of the sympathetic liver nerves was modulated by the PDI, enoximone.

Methods

Animals and experimental protocol

Male Wistar rats (200–250 g body weight) from the breeding colony of Ulm University Animal Facilities were used for all experiments. Animals were kept on an alternating 12 h light-dark cycle with free access to water and standard laboratory chow. Animals were used after treatment with endotoxin (10 mg kg^{-1} body weight i.p. in 0.9% saline, controls were injected with saline i.p.) 4 h before the start of the perfusion. All experiments were performed between 11 h 00 min and 12 h 00 min, to ensure that liver glycogen levels were equal. After animals had been anaesthetized with phenobarbitone (60 mg kg^{-1} body wt), the abdomen was opened through a midline and subcostal abdominal incision. The portal vein and common bile duct were cannulated with a PVC tube (1.4 mm i.d.) and a polyethylene tube (0.4 mm i.d.), respectively. Livers were perfused *in situ* with a Krebs-Henseleit-bicarbonate-buffer equilibrated with 95% O_2 and 5% CO_2 (v/v). The perfusion pressure was kept constant at 10 ± 1 cmH_2O with a flow rate of 4 ml min^{-1} g^{-1} liver using a non recirculating perfusion system. The perfusion flow was measured every minute by fractionating the effluate into calibrated glass vials. Bile was collected at 5 min intervals and determined gravimetrically. After an equilibration period of 30 min, the perfusion effluent was collected at 1 min intervals. Compounds were added to the perfusion buffer using micro pumps, entering the perfusion system directly before the liver. Two different types of perfusion protocols were used in this study. In the first protocol, enoximone was administered over 35 min at concentrations of 1, 10 and 100 μM. To study the effect of PDI together with glycogenolytic effectors, a second protocol applied a 5 min pulse of glucagon (1 nM), nerve stimulation (frequency of 20 Hz, monophasic impulses of 20 V, duration of 2 ms), or phenylephrine (10 μM) with and without enoximone (10 μM), respectively. Compounds were tested alone in individual experiments with or without the PDI. Hepatic nerves were stimulated electrically with a bipolar platinum electrode placed in the liver hilus perivascularly at the portal vein and hepatic artery (Beckh & Arnold, 1991). Hepatic glycogen content was determined in endotoxin-treated and control animals.

All experiments were approved by the Institutional Animal Care Committee and executed according to the National Animal Welfare Law of Germany.

Analytical procedures

Glucose and lactate concentrations were measured photometrically with standard enzymatic methods using glucose dehydrogenase or lactate dehydrogenase (Bergmeyer, 1984). Bile acids were quantitated using the 3α-hydroxy-steroid-dehydrogenase method (Turley & Dietschy, 1978). Measurements were related to the period before substances were administered. Liver glycogen was determined after hydrolysing with amyloglucosidase from *Aspergillus niger* (Bergmeyer, 1984).

Measurement of cyclic nucleotide

Cyclic nucleotide levels were determined in the effluent and bile by radioimmunoassay methods. Commercially available kits for cyclic AMP and cyclic GMP (Amersham Buchler, Braunschweig Germany) were used. The assay is based on the competition between unlabelled cyclic nucleotide and a fixed quantity of ^{125}I-labelled cyclic AMP or cyclic GMP for a limited number of binding sites on a specific antibody. The antibody-bound cyclic AMP or cyclic GMP reacts with the second antibody reagent which contains second antibody that is bound to magnetizable polymer particles. Separation of the antibody bound fraction is then performed by magnetic separation and decanting of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled cyclic AMP or cyclic GMP in the bound fraction to be calculated. The concentration of unlabelled cyclic nucleotides in the sample is then determined from the standard curve via linear regression analysis.

Materials

Enoximone was kindly provided by Marion Merrell Dow GmbH, Ruesselsheim, Germany. Bacterial lipopolysaccharide (*E. coli* serotype 026:B6), glucagon, phenylephrine, and all other chemicals used were reagent grade and from commercial sources (Sigma Chemical Co St. Louis, MO, U.S.A.). For hepatic nerve stimulation, an HSP Stimulator P (Hugo Sachs Electronic March Hugstetten Germany) was used.

Statistical analysis

The results represent means of four or more experiments. Vertical error bars denote s.e.mean. Experimental data from different groups were compared by one-way analysis of variance (ANOVA) and by pairwise multiple comparison by pairs (Student-Newman-Keuls Test). Differences were considered significant at $P<0.05$ and are marked with asterisks in the figures.

Results

Effect of enoximone on glucose release in rat liver following endotoxin treatment

After an equilibration period of 30 min, infusion of enoximone into the portal vein for 35 min resulted in an increase in glucose release. Basal levels appeared negative due to the regular baseline shift. In untreated livers, glucose output increased from basal levels of -2.0 ± 0.6 μmol g^{-1} 20 min^{-1} to 30.2 ± 9 μmol g^{-1} 20 min^{-1} when 100 μM enoximone was administered. After endotoxin treatment, infusion of enoximone into the portal vein at concentrations ranging from 1 μM to 100 μM resulted in a dose-dependent increase of glucose output from basal levels of -1.4 ± 0.9 μmol g^{-1} 20 min^{-1} to 7.8 ± 2.5 μmol g^{-1} 20 min^{-1}. The stimulatory effect of enoximone on glucose output was lower after endotoxin treatment (Figure 1). Since hepatic glucose release is dependent on liver glycogen, glycogen content was determined 4 h after endotoxin treatment and was reduced to a level of 29% ± 5%

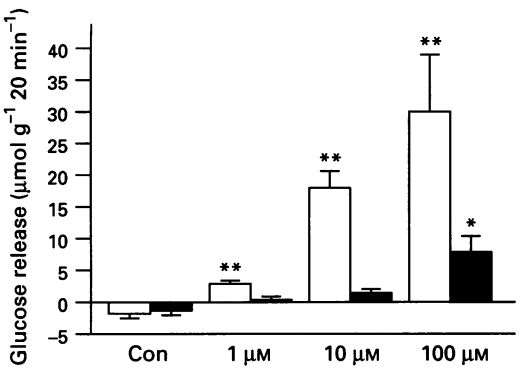

Figure 1 Effect of different concentrations of enoximone on the cumulative amount of glucose production over 20 min in the *in situ* perfused liver. Open columns, livers without endotoxin pretreatment; shaded columns, livers from endotoxin pretreated animals. Values shown are mean ± s.e.mean of four or more experiments (*$P<0.05$, **$P<0.01$ versus control).

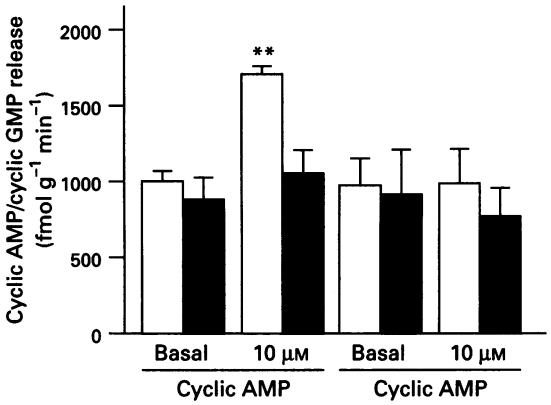

Figure 2 Effects of 10 μM enoximone on the cyclic AMP and cyclic GMP release in the effluent from rat perfused liver. Open columns, livers without endotoxin pretreatment, shaded columns, livers from endotoxin pretreated animals. Values shown are mean ± s.e.mean of four experiments (**$P<0.01$ versus basal).

compared to control values. For comparison, data for untreated livers were taken from a previous study (Weidenbach *et al.*, 1995b).

Effect of enoximone on lactate release and bile flow in rat liver following endotoxin treatment

After an equilibration period and during perfusion with enoximone (1, 10, 100 μM) over a period of 35 min, lactate output slightly decreased in the perfusate of endotoxin pretreated livers. Simultaneously, bile flow increased from -0.03 ± 0.08 μl g^{-1} 15 min^{-1} to 0.69 ± 0.11 μl g^{-1} 15 min^{-1} without stimulation of bile acid secretion, indicating a stimulation of the bile acid independent bile flow. The effects of enoximone on lactate release and bile flow are dose-dependent (Table 1).

Effect of enoximone on cyclic AMP and cyclic GMP release in rat liver following endotoxin treatment

The 10 μM concentration of enoximone was used for determination of cyclic nucleotide levels in the effluent and bile using radioimmunoassay methods. In untreated livers, cyclic AMP release increased from basal levels of 1000 ± 73 fmol g^{-1} min^{-1} to 1710 ± 143 fmol g^{-1} min^{-1} when 10 μM enoximone was administered. Four hours after endotoxin treatment, infusion of the same dose of enoximone resulted in a minor increase from basal levels of 880 ± 45 fmol g^{-1} min^{-1} to 1049 ± 155 fmol g^{-1} min^{-1} cyclic AMP. Levels of cyclic GMP appeared unchanged in the effluent of both groups when 10 μM enoximone was administered (Figure 2).

Table 1 Effect of different doses of enoximone on the lactate output and bile flow

	Lactate (μmol g^{-1} 20 min^{-1})	*Bile flow* (μl g^{-1} 15 min^{-1})
4h Endotoxin	-1.4 ± 0.6	-0.03 ± 0.08
4h Endotoxin + enoximone 1 μM	-1.6 ± 0.7	0.0 ± 0.1
4h Endotoxin + enoximone 10 μM	-2.6 ± 0.9	0.28 ± 0.17
4h Endotoxin + enoximone 100 μM	-3.2 ± 0.4*	0.69 ± 0.11*

Values shown are mean ± s.e.mean of four or more experiments (*$P<0.05$ versus control).

In bile from untreated livers, the basal level of cyclic AMP increased from 41.2 ± 7 fmol g^{-1} min^{-1} to 87 ± 10 fmol g^{-1} min^{-1} when 10 μM enoximone was administered. After endotoxin treated only a minor rise from 23.8 ± 10 fmol g^{-1} min^{-1} to 44 ± 14 fmol g^{-1} min^{-1} after administration of PDI was detected. Basal levels of cyclic GMP in bile were at the detection limit of the assay (1.5 fmol g^{-1} min^{-1}) whether or not animals were pretreated with endotoxin or during perfusion with 10 μM enoximone (Figure 3).

Effect of glucagon and enoximone on glucose- and lactate release in the rat perfused liver following endotoxin treatment

To study the nature of the glycogenolytic effects after endotoxin treatment in the perfused liver, glucagon was used to activate the cyclic AMP pathway via activation of adenylyl cyclase. During perfusion with glucagon (1 nM) for 5 min, hepatic glucose and lactate output did not change significantly in the perfusate of livers pretreated with endotoxin (Figure 4a). The 10 μM concentration of enoximone for 35 min was used for all co-infusion protocols. The addition of enoximone to glucagon induced a dramatic increase in glucose release, whereas lactate was unaffected (Figure 4b).

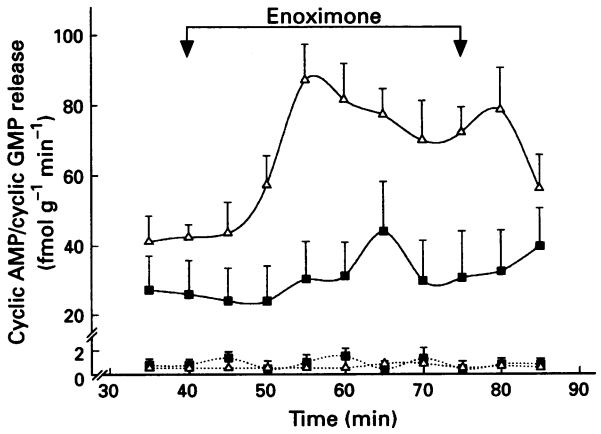

Figure 3 Effects of 10 μM enoximone on the cyclic AMP and cyclic GMP release in bile of rat perfused rat livers. Bile of untreated livers (△); (■) bile from endotoxin pretreated animals. Cyclic AMP levels shown by solid lines in the upper part of the graph. Cyclic GMP levels shown by dotted lines in the lower part of the graph. Data are expressed as mean ± s.e.mean of four experiments.

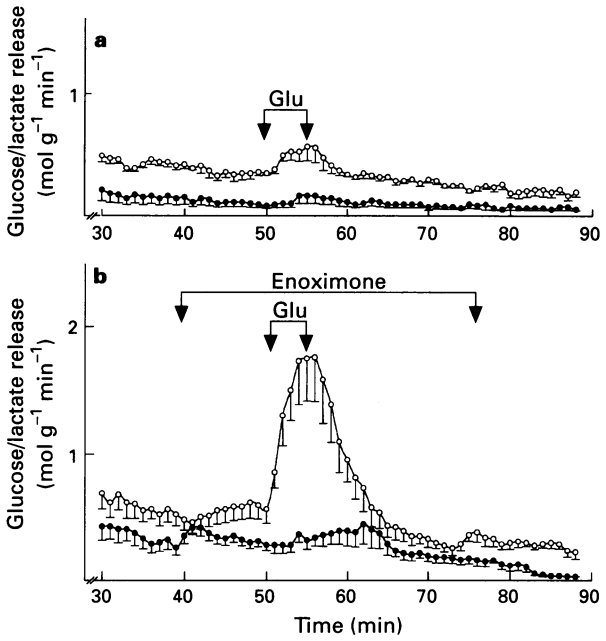

Figure 4 Effects of glucagon (Glu, 1 nM, 5 min) on glucose (○) and lactate (●) output in the effluent from rat perfused liver 4 h after the administration of endotoxin (a) and perfusion with enoximone (10 μM, 35 min) and glucagon (Glu, 1 nM) together after administration of endotoxin (b). Data are expressed as mean ± s.e.mean of four experiments.

Effect of glucagon and enoximone on cyclic AMP and cyclic GMP release in the rat perfused liver following endotoxin treatment

During infusion of 1 nM glucagon and during co-infusion of glucagon and enoximone, cyclic nucleotide levels in the effluent and in the bile of livers after endotoxin treatment were determined. In the effluent cyclic AMP release increased clearly from basal levels of 827 ± 144 fmol g^{-1} min^{-1} to 17802 ± 2821 fmol g^{-1} min^{-1} when glucagon was administered. The presence of enoximone enhanced cyclic AMP further to 41696 ± 920 fmol g^{-1} min^{-1}. Similar increases in cyclic AMP release were found in bile. Cyclic AMP increased from 21 fmol g^{-1} min^{-1} to 640 ± 139 fmol g^{-1} min^{-1} when 1 μM glucagon was infused. The PDI enhanced cyclic AMP level in bile to 938 ± 38 fmol g^{-1} min^{-1} (Figure 5). Basal levels of cyclic GMP in the effluent were not significantly affected by the administration of glucagon or the PDI. Only an insignificant decrease from 545 fmol g^{-1} min^{-1} to 357 after glucagon and to 331 ± 69 fmol g^{-1} min^{-1} after glucagon and enoximone appeared. Basal levels of cyclic GMP in bile after glucagon and enoximone were at the detection limit of the assay (Figure 5).

Effects of enoximone, glucagon, sympathetic nerves stimulation and phenylephrine on glucose- and lactate release in the rat perfused liver following endotoxin treatment

Enoximone increased hepatic glucose output by 1.37 ± 0.6 μmol g^{-1} 20 min^{-1}, lactate decreased to −2.59 ± 0.9 μmol g^{-1} min^{-1}. The addition of glucagon to enoximone increased glucose release to 8.2 ± 2.8 μmol g^{-1} 20 min^{-1}. Perivascular nerves stimulation for 5 min resulted in an increase in glucose (5.7 ± 0.4 μmol g^{-1} 20 min^{-1}) and of lactate output (5.5 ± 1.1 μmol g^{-1} 20 min^{-1}). The presence of enoximone enhanced glucose release to 9.5 ± 1.5 μmol g^{-1} 20 min^{-1} and decreased lactate release by 2.3 ± 0.6 μmol g^{-1} 20 min^{-1}. Administration of phenylephrine (10 μM) increased glucose output to 5.4 ± 0.9 μmol g^{-1} 20 min^{-1} and lactate output to 3.6 ± 0.9 μmol g^{-1} 20 min^{-1}. Combined infusion of enoximone and phenylephrine further enhanced the glucose release to 11.5 ± 1.9 μmol g^{-1} 20 min^{-1} with a reduction of lactate output to −0.29 ± 0.9 μmol g^{-1} 20 min^{-1} (Figures 6 and 7).

Effects of sympathetic nerves stimulation and enoximone on bile flow and bile acid secretion in the rat perfused liver following endotoxin treatment

Stimulation of hepatic nerves bundles by a platinum electrode placed around the hepatic artery and portal vein resulted in a decrease of bile flow and bile acid secretion. Enoximone in-

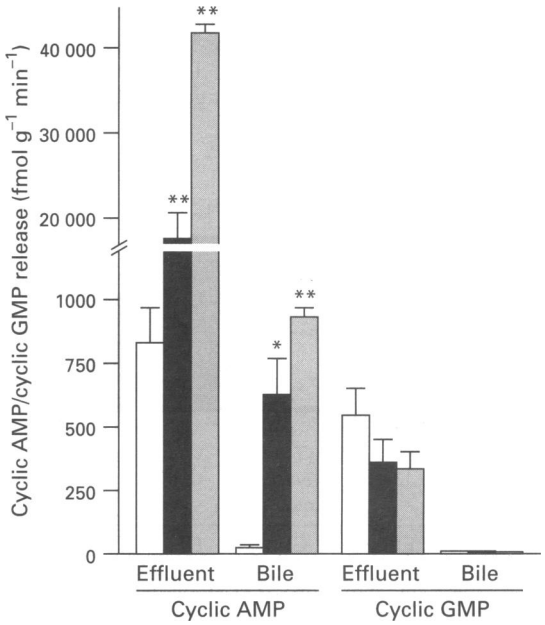

Figure 5 Effects of glucagon (1 nM) and enoximone (10 μM) on cyclic nucleotide levels in the effluent and bile from rat perfused liver after endotoxin treatment. Open columns, basal levels of cyclic nucleotide; solid columns, cyclic nucleotide release after glucagon was administered; shaded columns, glucagon together with the PDI, enoximone. Levels of cyclic GMP in bile were at any time close to the detection limit of the assay. Data are expressed as mean ± s.e.mean of four experiments (*$P<0.05$, **$P<0.01$ versus basal).

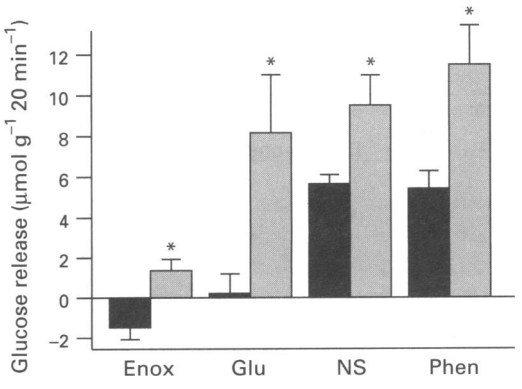

Figure 6 Effect of enoximone (Enox, 10 μM) on cumulative amounts of glucose release in the perfused liver. Solid columns, glucose release from endotoxin-treated liver not stimulated and after glucagon (Glu, 1 nM), sympathetic nerves stimulation (NS) and phenylephrine (Phen, 10 μM) stimulation showed columns, PDI alone and together with glucagon, nerve stimulation and phenylephrine. Values shown are mean ± s.e.mean of four or more experiments (*$P<0.05$ versus control).

creased bile flow for the period of the administration with a short interruption during the period of nerves stimulation. This increase of bile flow was not associated with an effect on bile acid output. The time course of the alterations induced by sympathetic nerves stimulation alone and combination with the PDI is given in Figure 8.

Effect of enoximone, glucagon, sympathetic nerve stimulation and phenylephrine on the bile flow and bile acid secretion in the rat perfused liver endotoxin treatment

Infusion of enoximone (10 μM) for 35 min induced an increase in bile flow from -0.03 ± 0.08 μl g^{-1} 15 min^{-1} to 0.28 ± 0.17 μl g^{-1} 15 min^{-1}. Bile acid secretion was unchanged in comparison to controls. Glucagon by itself changed hepatic bile flow (0.07 ± 0.026 μl g^{-1} 15 min^{-1}) and bile acid secretion insignificantly (-310 ± 80 nmol g^{-1} 15 min^{-1}). The addition of enoximone in the presence of glucagon increased bile flow to 0.38 ± 0.13 μl g^{-1} 15 min^{-1}, but left bile acid secretion unchanged (-365 ± 60 nmol g^{-1} 15 min^{-1}). Nerve stimulation decreased bile flow to -0.34 ± 0.19 μl g^{-1} 15 min^{-1} and bile acid secretion (-425 ± 45 nmol g^{-1} 15 min^{-1}). Combined infusion of the PDI and hepatic nerves stimulation enhanced bile flow to 0.27 ± 0.16 μl g^{-1} 15 min^{-1}, whereas bile acid secretion was unchanged (-465 ± 50 nmol g^{-1} 15 min^{-1}). Phenylephrine (10 μM) decreased bile flow to -0.4 ± 0.29 μl g^{-1} 15 min^{-1} and bile acid secretion (-655 ± 150 nmol g^{-1} 15 min^{-1}). Comparing phenylephrine alone, to the additional presence of the PDI, the bile flow was altered significantly to -0.01 ± 0.11 μl g^{-1} 15 min^{-1} while the bile acid (-670 ± 75 nmol g^{-1} 15 min^{-1}) secretion remained unchanged (Figure 9).

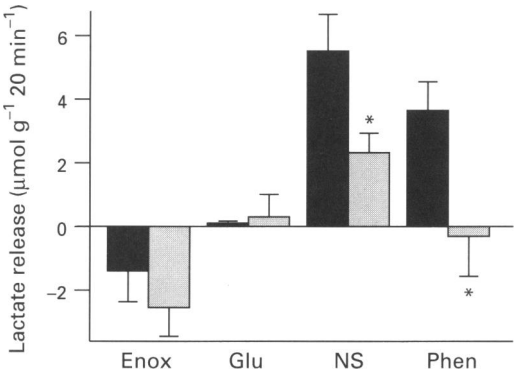

Figure 7 Effect of enoximone (Enox) on lactate release in the perfused liver. Solid columns, lactate release from endotoxin-treated liver not stimulated and after glucagon (Glu), sympathetic nerves stimulation (NS) and phenylephrine (Phen) stimulation. Shaded columns, PDI alone and together with glucagon, nerve stimulation and phenylephrine. Values shown are mean±s.e.mean of four or more experiments (*$P<0.05$ versus control).

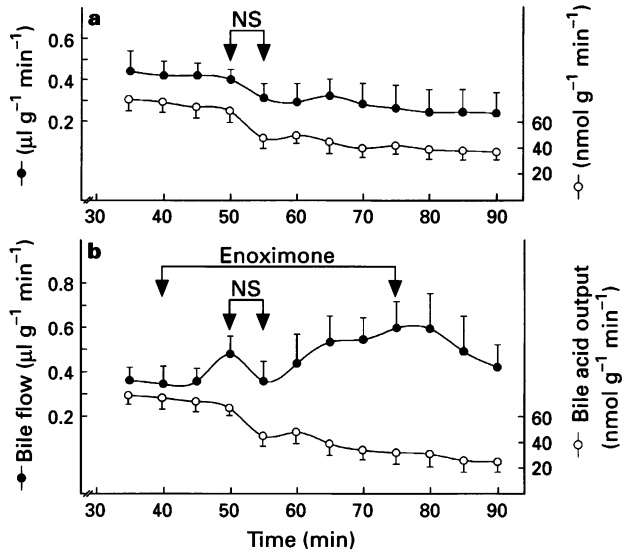

Figure 8 Effect of sympathetic nerves stimulation (NS) and enoximone in bile flow (●) and bile acid secretion (○). Stimulation of hepatic nerves resulted in a mild decrease of bile flow and bile acid secretion (a). Enoximone (10 μM) administration increased bile flow (●) without a major effect on the bile acid output (○, b). Data are expressed as mean±s.e.mean of four experiments.

Effects of enoximone, glucagon, sympathetic nerves stimulation and phenylephrine on portal flow in the rat perfused liver following endotoxin treatment

The alterations of glucose release and bile secretion after perivascular nerve stimulation and phenylephrine administration were accompanied by a decrease of portal flow. For nerve stimulation, the reduction of portal flow was 6.7 ± 0.9 ml g^{-1} 5 min^{-1}. This effect was diminished to 3.4 ± 1 ml g^{-1} min^{-1} in the presence of enoximone. The portal flow decreased after phenylephrine (-1.9 ± 0.3 ml g^{-1} 5 min^{-1}) and was not significantly influenced by enoximone (Table 2). In this system of perfusion, portal flow was not influenced by enoximone and glucagon alone or in combination (data not shown).

Discussion

It has been shown that bacterial endotoxin, a lipopolysaccharide component of the outer membrane of gram-negative bacterial, its a pivotal trigger of gram-negative sepsis and that its biological effects are mediated by inflammatory com-

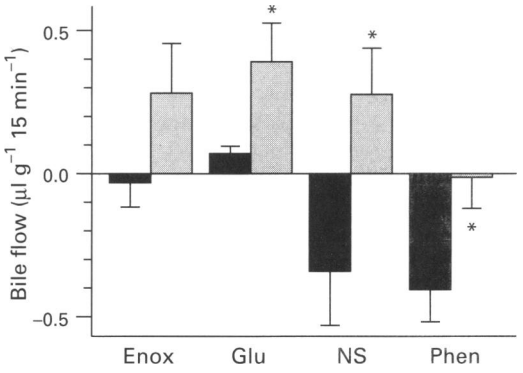

Figure 9 Effect of enoximone (Enox) on bile flow in the perfused liver. Solid columns, bile flow from endotoxin-treated liver not stimulated and after glucagon (Glu), sympathetic nerve stimulation (NS) and phenylephrine (Phen) stimulation. Shaded columns, PDI alone and together with glucagon, nerve stimulation and phenylephrine. Values shown are mean±s.e.mean of four or more experiments (*$P<0.05$ versus control).

Table 2 Effect of enoximone on portal flow in the perfused rat liver after endotoxin treatment

Effectors	*4h Endotoxin (ml g^{-1} 5 min^{-1})*	*+ Endotoxin (ml g^{-1} 5 min^{-1})*
Nerve stimulation (20 Hz, 20V, 2 ms)	-6.7 ± 0.9	-3.4 ± 1.0*
Phenylephrine (10 μM)	-1.9 ± 0.3	-2.0 ± 0.4

Data are expressed as mean±s.e.mean of four or more experiments (*$P<0.05$ versus control).

petent cells (Raetz *et al.*, 1991). In the liver, endotoxin causes hepatic alterations of the liver macrophages, namely the Kupffer cells. After endotoxin contact, Kupffer cells release various endogenous factors such as prostaglandins, tumour necrosis factor (TNF) or nitric oxide (Luster *et al.*, 1994; Weidenbach *et al.*, 1995a). Prostaglandin E_2 and prostaglandin $F_{2\alpha}$ are known for their glycogenolytic, cholestatic properties (Utili *et al.*, 1976; Castelijn *et al.*, 1988; Beckh *et al.*, 1994). The glycogenolytic effects of these mediators are associated with the observed hyperglycaemia in sepsis (Shangraw *et al.*, 1989; Ceppi *et al.*, 1992). Several prostaglandins exert strong inhibitory interference with hormone-induced cyclic AMP accumulation in rat hepatocytes. (Bronstad & Christoffersen, 1981).

Cytokines such as TNF can affect levels of cyclic AMP by increasing PDE activities. For cultured endothelial cells a 1.5 to 2.5 fold increase of PDE2 and PDE4 activity has been recently suggested (Koga *et al.*, 1995). For mouse cultured macrophages, elevation of cyclic AMP and a decrease of the low K_m PDE by 42% 6 h after endotoxin treatment has been shown (Okada *et al.*, 1995). These data are consistent with observations in a human monocytic cell line. Four hours after endotoxin treatment 2–3 fold increased PDE4 activity without concomitant increase of PDE3 activity was detected (Verghese *et al.*, 1995). The nitric oxide/cyclic GMP pathway inhibits PDE3 (Eckly & Lugnier, 1994). Factors such as insulin and glucagon increase PDE3 activity.

In the present investigation some PDE3 modulating factors were excluded. The perfusion buffer was free of insulin and the nitric oxide substrate L-arginine. Normally, levels of L-arginine are sufficient for continuous nitric oxide biosynthesis, because the by-product of nitric oxide synthesis, L-citrulline is recycled back to L-arginine. In the perfused liver, the rate of nitric oxide synthesis appears to be in part limited by L-arginine availability in the perfusion buffer (Wettstein *et al.*, 1994; Weidenbach *et al.*, 1995b). Under these conditions, major effects of nitric oxide mediation on PDE3 are unlikely. A second argument for this proposal is the very low level of cyclic GMP measured in the effluent and bile. The missing modulation of cyclic GMP suggests, that the PDI, enoximone, is not altering other cyclic GMP PDE isozymes.

The question remains, what key enzymes and second messenger are involved in the impaired regulation of glucose metabolism in sepsis? In the liver, the intracellular second messenger cyclic AMP controls the glucose metabolism. PDE3 is one of the relevant key isoenzymes regulating cyclic AMP levels in different cell systems (Colucci *et al.*, 1986; Grous & Barnette 1994). The present study addresses the question whether the liver responds after endotoxin treatment to inhibition of the RPDE3. Is there evidence for a substantial role of RPDE3 in cyclic AMP breakdown in the septic liver metabolism?

The intracellular cyclic AMP pool depends on synthesis of cyclic AMP and its degradation. Inhibition of the RPDE3 results in a signal-independent accumulation of intracellular cyclic AMP. Cyclic AMP mediates protein phosphorylation by protein kinase A, which activates glycogen phosphorylase and inactivates pyruvate kinase. These changes in enzyme phosphorylation provide more glucose for metabolism in peripheral organs. In this study, the PDI, enoximone, was able to mobilise glucose from the liver in a concentration-dependent manner. Due to a lower content of liver glycogen and a reduction in cyclic AMP release, the glucose response was reduced after endotoxin treatment. This result supports the assumption that in the liver, the biological activity of the RPDE3 is influenced by endotoxin treatment.

In this model, glucagon was found to be only a weak stimulator of glucose release. The observation of responsiveness of glucagon administration after endotoxin treatment is in line with previous studies on glucagon and fructose 2,6-bisphosphate metabolism. Fructose 2,6-bisphosphate is known as the major controlling element of glycolysis and gluconeogenesis in the liver (for review see, Van Schaftingen, 1990). It was suggested that the inhibition of gluconeogenesis after endotoxin treatment is the result of a 2–3 fold increase in fructose 2,6-bisphosphate and that this effect is resistant to the presence of glucagon (Ceppi *et al.*, 1992). Interestingly in the present study, glucagon elevated cyclic AMP release in the effluent and bile. But it was quite remarkable that in the presence of PDI, enoximone and glucagon, the cyclic AMP level was raised more than forty-five times. These data suggest that RPDE3 is involved in regulating cyclic AMP turnover in the experimental conditions employed.

The decrease of lactate release after administration of the PDI is consistent with the inactivation of pyruvate kinase. After the stimulation of sympathetic nerves, the significant vasodilatation induced by the PDI may also contribute to the reduction of lactate release.

There is much evidence of blood flow redistribution among different organs in sepsis. Hepatic microcirculatory disorders were observed following endotoxaemia *in vivo*. Microscopic studies of the liver tissue demonstrated relevant perfusion failure of liver sinusoids after endotoxin treatment (McCluskey *et al.*, 1993). In the perfused liver, endotoxins are known to induce a dose-dependent immediate vasoconstriction and intrahepatic cholestasis (Utili *et al.*, 1976). This effect is mediated by prostaglandins released from non-parenchymal cells (Castelijn *et al.*, 1988; Beckh *et al.*, 1994). In the present investigation, we further influenced the circulation by electrical stimulation of the hepatic nerves. We have recently demonstrated that stimulation of autonomic hepatic nerves reduces bile flow and bile acid secretion (Beckh & Arnold, 1991). The drastic decrease in portal flow might play a part in the reduction of bile flow and bile acid secretion after stimulation of autonomic hepatic nerves. In addition, the vasodilator properties of the PDI might also have been involved in the increase of bile flow during nerves stimulation.

A variety of studies have investigated the role of cyclic AMP levels on bile flow. Substances that increase intracellular cyclic AMP such as glucagon, forskolin or non-specific PDIs increase bile acid-independent bile flow. It has been demonstrated that cyclic AMP-mediated choleresis is canalicular in origin, involves bicarbonate secretion and requires an intact microtubular system (Lenzen *et al.*, 1990; Tomihiro *et al.*, 1990).

In conclusion, we have demonstrated that endotoxin treatment reduces the ability of the PDI, enoximone, to raise cyclic AMP levels in the effluent and bile of perfused rat liver. The significant increase of cyclic AMP release after stimulation with glucagon and enoximone favours the view that RPDE3 is still involved in the degradation of cyclic AMP in the liver after endotoxin exposition. Inhibition of the RPDE3 and stimulation of adenylyl cyclase or the α-adrenergic pathway resulted in a distinct increase in glucose release and bile acid-independent bile flow. Additionally, vasoconstriction after stimulation of the sympathetic liver nerves was reduced by the PDI, enoximone.

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