Increase of glucose and lactate output and decrease of flow by human anaphylatoxin C3a but not C5a in perfused rat liver

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The complement fragments C3a and C5a were purified from zymosan-activated human serum by column chromatographic procedures after the bulk of the proteins had been removed by acidic polyethylene glycol precipitation. In the isolated in situ perfused rat liver C3a increased glucose and lactate output and reduced flow. Its effects were enhanced in the presence of the carboxypeptidase inhibitor DL-mercaptomethyl-3-guanidinoethylthio-propanoic acid (MERGETPA) and abolished by preincubation of the anaphylatoxin with carboxypeptidase B or with Fab fragments of an anti-C3a monoclonal antibody. The C3a effects were partially inhibited by the thromboxane antagonist BM13505. C5a had no effect. It is concluded that locally but not systemically produced C3a may play an important role in the regulation of local metabolism and hemodynamics during inflammatory processes in the liver.

Hepatic glucose balance; Hepatic lactate balance; Hepatic hemodynamics; Complement system; Anaphylatoxin

1. INTRODUCTION

Pyrogenic substances such as cell wall particles from yeast (zymosan) [1,2] and lipopolysaccharides from Gram negative bacteria [3] have been shown to increase glucose and lactate output and to reduce perfusion flow or increase portal pressure, respectively, in the perfused rat liver. Similar alterations were observed after infusion of heat-aggregated immunoglobulins which resemble immune complexes in their biological properties [4]. Rabbit serum that had been complement activated by incubation with zymosan was shown to induce the release of thromboxane from rabbit livers [5]. The complement fragments C3a and C5a have been reported to cause contraction of smooth muscle [6] and human C5a was shown to elicit vasoconstriction in the kidneys of Munich Wistar rats [7].

It was the aim of this study to evaluate whether these peptides might affect the metabolism and the hemodynamics of the liver. Complement-activated human serum and the purified anaphylatoxin C3a but not C5a were found to increase glucose and lactate output and to decrease flow in the perfused rat liver.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals were reagent grade and from commercial sources. The Merck glucose system was purchased from Merck (D-6100 Darmstadt). Lactate dehydrogenase and glutamic pyruvic transaminase were from Boehringer (D-6800 Mannheim). The thromboxane antagonist BM13505 was a gift of this company. ϵ-Amino caproic acid and carboxypeptidase B were obtained from Sigma (D-8024 Deisenhofen). MERGETPA was purchased from Calbiochem (D-6000 Frankfurt) and polyethylene glycol 6000 (PEG) from Serva (D-6900 Heidelberg). CM-52 Cellulose was obtained from Whatman (UK-ME 14 2LE Kent) and Sephadex G50 fine from Pharmacia (D-7800 Freiburg). Dialysis tubing with a cut off of 3.5 kDa was bought from Reichelt (D-6900 Heidelberg).

2.2. Animals

Male Wistar rats (140–180 g, Winkelmann, D-4791 Borchen) were kept on a 12 h day-night rhythm, light from 7 a.m. to 7 p.m., with free access to water and food (standard rat diet, Ssniff, D-4770 Soest). All experiments were started between 9 and 10 a.m.

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2.3. Isolation of human C3a and C5a

Human C3a and C5a were purified using a modified method based on a previous brief description of the C5a purification [8]. Human serum obtained from healthy blood donors through the transfusion service of the university hospital was activated with boiled yeast cells (zymosan) in the presence of 1 M ε-aminocaproic acid for 1 h at 37°C. All subsequent steps were carried out at 0–4°C. The bulk of the proteins was precipitated by adding 10% (w/v) PEG and titrating the incubation mixture to pH 4.0 with 8 N HCl. Zymosan and precipitated proteins were removed by centrifugation at 27000 × g for 30 min. The supernatants were then frozen at −20°C overnight and the centrifugation repeated after thawing the sample the next morning. The clear supernatant was diluted about fivefold with 50 mM ammonium formate, pH 4.5, to reach a conductivity of about 10 mS/cm. This sample was applied to a CM-52 column that was equilibrated with the same buffer. The column was washed with 400 ml of the equilibration buffer and eluted with 800 ml of a linear NaCl gradient from 0 to 0.8 M in the same buffer (fig.1). C5a and C3a were detected in the eluate employing ELISA procedures [8,9]. The fractions with the highest contents of the respective peptides were pooled for further purification.

The C5a pool was lyophilized and redissolved in 8 ml of 25 mM Tris-HCl, pH 7.4. It was chromatographed on Sephadex G50 fine equilibrated with the same Tris-HCl buffer containing 154 mM NaCl which was also used for elution (fig.1). The C5a-containing fractions were pooled and stored at −20°C.

The C3a pool was dialyzed against 10 mM sodium-acetate, pH 5.0, using a dialysis tubing with a 3.5 kDa cutoff and applied to CM-52 cellulose equilibrated with the same buffer. The proteins were eluted with the equilibration buffer containing 0.6 M NaCl. The C3a-containing fractions were pooled and stored at −20°C.

The purity of the C3a and C5a preparations was ascertained by standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [10].

2.4. Antibodies against C3 and C5a

Monoclonal antibodies specific for C3a or C5a, respectively, were produced as described [8,11]; Fab fragments from the anti-C3a antibodies were obtained by papain-cleavage. They were purified by FPLC anion-exchange chromatography on Mono-Q [12].

2.5. Liver perfusion

Rats were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body wt). The liver was perfused in situ as described previously [13] using an erythrocyte-free Krebs-Henseleit bicarbonate buffer, which contained 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate and was equilibrated with 95% O_2/5% CO_2 (v/v). After a preperfusion period of 30 min the experiment was started. At the times indicated purified C3a or C5a were infused. The flow was determined by quantitating the perfusate fractions collected at 1 min intervals.

2.6. Metabolite assays

Glucose was determined in the perfusate using a commercial test kit (Merck Glucose System) based on the glucose dehydrogenase method [14]. Lactate was measured in a combin-

ed optical test using lactate dehydrogenase and glutamic pyruvic transaminase [15].

3. RESULTS

3.1. Purification of the anaphylatoxins

Chromatography of zymosan-activated human serum on CM-52 cellulose after acidic PEG precipitation resulted in a good separation of C3a and C5a. C5a co-eluted with the major protein fraction at 0.25 M NaCl; C3a eluted, separated from the major protein peak, at about 0.6 M NaCl (fig.1A). The C5a fractions were lyophilized, redissolved in a small volume of buffer and chromatographed on Sephadex G-50 fine. On this column most of the protein eluted before the C5a
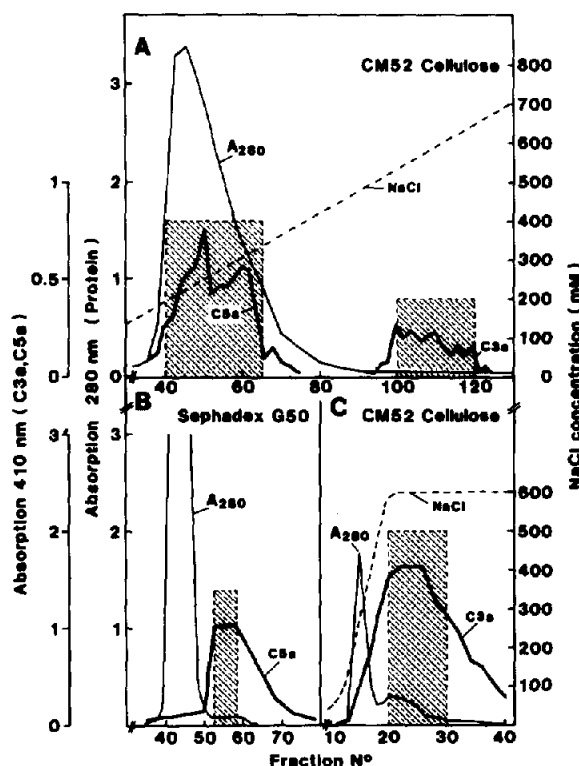

Fig.1. Purification of the anaphylatoxins C3a and C5a. (A) Chromatography of the supernatant after acidic PEG precipitation of zymosan-activated human serum on CM52-cellulose (5 cm diameter, 200 ml bed volume, 200 ml/h flow rate, 4 ml fractions). (B) Chromatography of the pooled and lyophilized C5a fractions from panel A on Sephadex G50 (2.5 cm diameter, 440 ml bed volume, 16 ml/h flow rate, 4 ml fractions). (C) Rechromatography of the dialyzed C3a fractions from panel A on CM52-cellulose (2.5 cm diameter, 30 ml bed volume, 50 ml/h flow rate, 2.5 ml fractions).

peak (fig.1B). The peak fractions of C5a were pooled; the preparation contained 6 μg C5a/ml and one major contaminant, which migrated in the SDS-PAGE as a 69 kDa band and was assumed to be albumin. Rechromatography of the dialyzed C3a fractions on CM-52 cellulose in a small column (fig.1C) yielded a C3a preparation that had a concentration of 35 μg C3a/ml and was free of contaminants detectable by SDS-PAGE.

3.2. Actions of C3a and C5a on liver metabolism and hemodynamics

In perfused rat livers infusion of human serum at a rate of about 7% of the perfusion flow for 2 min caused a slight increase in glucose and lactate output and a slight decrease in perfusion flow. Preincubation of the serum with zymosan for 1 h at 37°C led to a marked enhancement of these effects (not shown).

Infusion of purified human C3a for 2 min to a final concentration of 3 μg/ml (fig.2A) caused an increase in glucose and lactate output that reached a maximum 3 min after the start of the infusion and returned to preinfusion levels during the following 3 min. C3a also elicited a decrease in flow which reached its lowest values 2 min after the start of the infusion. After cessation of the infusion the flow slowly recovered to reach preinfusion levels within 4 min.

C5a (0.5 μg/ml) did not influence liver metabolism or hemodynamics in either the absence or the presence of 0.1 mM of the carboxypeptidase inhibitor MERGETPA [16] (not shown). The biological activity of the C5a used had been ascertained by its induction of ATP release from guinea-pig platelets.

3.3. Increase of the metabolic and hemodynamic actions of C3a by inhibition of hepatic carboxypeptidase

The alterations caused by C3a could be almost totally prevented by preincubation of C3a at 37°C for 1 h with carboxypeptidase B, which inactivates C3a through the removal of the C-terminal arginine residue [17] generating C3a-desArg (fig.2B). Infusion of C3a together with a final concentration of 0.1 mM of MERGETPA resulted in a marked enhancement of both glucose and lactate output and flow reduction (not shown). In the presence of MERGETPA the increase in glucose

and lactate release and the decrease in flow caused by 1 μg C3a/ml infused for only 30 s were larger than the changes elicited by 3 μg C3a/ml applied for 2 min in the absence of MERGETPA (fig.3A vs fig.2A).

In the presence of MERGETPA infusion of about 0.4 μg C3a/ml for 30 s caused already maximal alterations, while the minimal concentration of C3a that was found to reduce flow and to increase lactate output was 0.08 μg/ml. An increase in glucose output could still be observed at 0.04 μg/ml (not shown).

3.4. Inhibition of the metabolic and hemodynamic actions of C3a

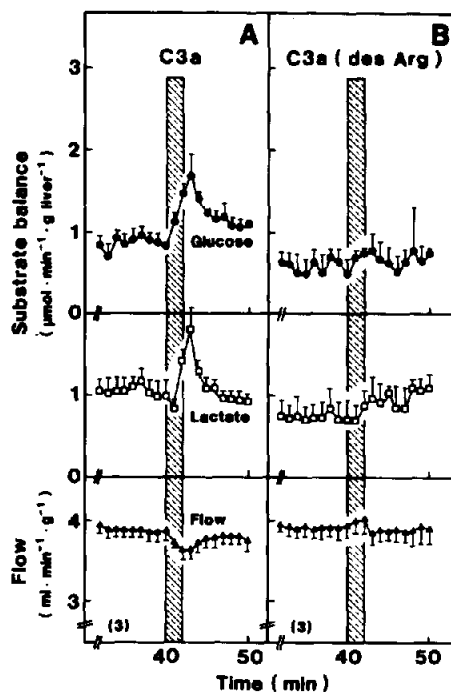Preincubation of C3a at pH 7.4 in the presence of 1 mM MERGETPA for 12 h at 4°C with a

Fig.2. Metabolic and hemodynamic alterations in perfused rat liver after infusion of the anaphylatoxins C3a and C3a-desArg. Livers were perfused in situ with Krebs-Henseleit bicarbonate buffer containing 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate. Substrate balance is given by (posthepatic concentration − prehepatic concentration in μmol × ml⁻¹) × flow in ml × min⁻¹ × g liver⁻¹. (A) C3a in a 20 mM Hepes buffer, pH 7.4, with 5 mM glucose and 2 mM lactate was infused to a final concentration of about 3 μg/ml for 2 min. (B) Same experiment as in panel A, except that the C3a solution was preincubated for 1 h at 37°C with carboxypeptidase B (10 μg/ml). Values are means ± SE of the number of experiments given in parentheses.
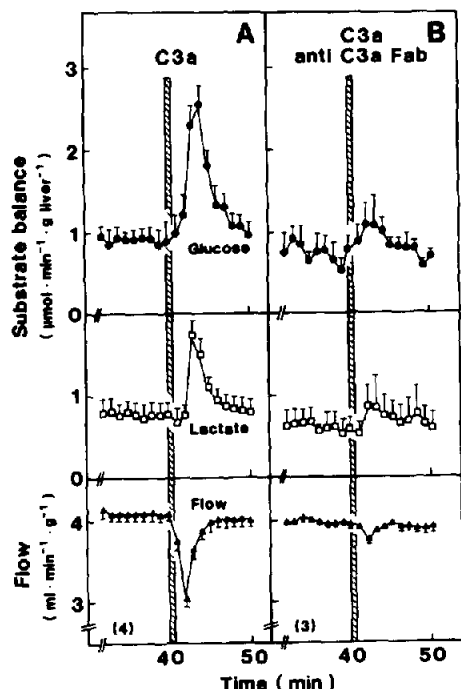
Fig.3. Metabolic and hemodynamic alterations in perfused rat liver after infusion of the anaphylatoxin C3a in the presence of the carboxypeptidase inhibitor MERGETPA with and without pretreatment of C3a with anti-C3a Fab fragments. The experimental conditions were the same as in fig.2. (A) C3a was incubated overnight at 4°C in 20 mM Hepes buffer, pH 7.4, containing 5 mM glucose, 2 mM lactate and 1 mM MERGETPA. This mixture was infused to a final concentration of 1 µg C3a/ml and 0.1 mM MERGETPA for 30 s. (B) Same conditions as in panel A, except that C3a was preincubated in a Hepes buffer containing in addition a threefold molar excess of Fab fragments of a neutralizing monoclonal antibody against C3a. Values are means ± SE of the number of experiments given in parentheses.

threefold molar excess of Fab fragments of a monoclonal antibody, which neutralized C3a activity in a guinea-pig platelet ATP-release test (Oppermann, unpublished results), abolished almost entirely the C3a-mediated stimulation of glucose and lactate output as well as the reduction in perfusion flow (fig.3B). C3a incubated overnight under the same conditions without Fab fragments remained biologically active (fig.3A).

The reduction of perfusion flow by C3a could be inhibited almost completely by the thromboxane antagonist BM13505 (fig.4). The increase in glucose and lactate output were also reduced by this inhibitor, to about 45% of the maximal effect.
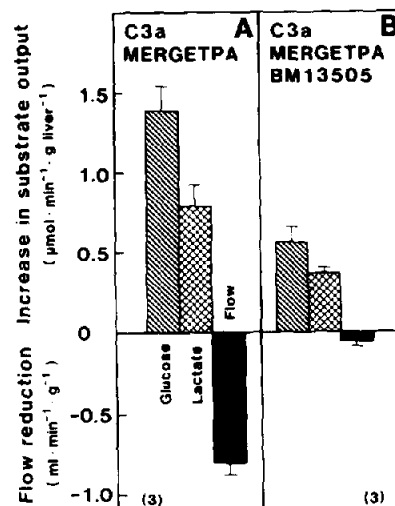

Fig.4. Inhibition of the C3a-mediated increase of glucose and lactate outputs and the reduction of flow by the thromboxane A$_2$ antagonist BM13505. (A) Livers were perfused as described in fig.2. C3a was infused to a final concentration of 0.4 µg/ml. The bars indicate the maximal increase in substrate output or decrease in flow. (B) Same conditions as in panel A, except that BM-13505 was infused to a final concentration of 20 µM from 5 min prior to C3a infusion until the end of the experiments. Values are means ± SE of the number of experiments given in parentheses.

The time course of the change of glucose output was not altered by BM13505; however, the lactate output remained increased until the end of the experiment (not shown).

4. DISCUSSION

This study showed that the purified human C3a anaphylatoxin, the activation peptide of the third component of complement, induced an increase in glucose and lactate outputs and a reduction in flow in perfused rat liver (figs 2A, 3A). It further demonstrated that these actions could be antagonized by the Fab fragments of a C3a-specific monoclonal antibody (fig.3B).

4.1. Mechanism of action of the anaphylatoxin C3a

The thromboxane antagonist BM13505 was found to interfere with the action of C3a on liver metabolism and hemodynamics (fig.4). This indicates that the action of C3a was indirect, at least in part. The anaphylatoxin might cause a release of

thromboxane from non-parenchymal cells that in turn could cause the increase in glucose and lactate outputs and the reduction of flow. This interpretation is in accord with the previous finding that zymosan-activated rabbit serum caused a release of thromboxane from the perfused rabbit liver [5]. The interpretation also conforms to the previous observation that the thromboxane A_2 analogue U46619 enhanced gluocose [18–21] and lactate [19,20] outputs and reduced flow [19,20] or increased portal pressure [18,21], respectively. It has been reported that guinea-pig peritoneal macrophages respond to C3a with a release of thromboxane [22]. Thus Kupffer cells which also belong to the mononuclear phagocyte system [23] might be responsible for the production of thromboxane in the perfusion system used here.

4.2. Function of the metabolic and hemodynamic actions of the anaphylatoxin C3a

Treatment of C3a with carboxypeptidase to remove the C-terminal arginine abolished (fig.2B), and the presence of the carboxypeptidase inhibitor MERGETPA increased (fig.3A), the observed effects of C3a. Apparently the rat liver itself is highly capable of inactivating the peptide. This inactivation must have occurred very rapidly within the vessels of the liver or at the very beginning of the sinusoids resulting in the degradation of most of the active peptide before it reached the sites of its metabolic and hemodynamic actions. The inactivation of C3a by the liver might play an important role in its self-protection during systemic generation of the anaphylatoxin. The cells at the entrance of the sinusoids might act as protecting scavengers as proposed previously [24]. Thus, it seems unlikely that C3a generated systemically in the circulation has an effect on liver metabolism or hemodynamics. In contrast, C3a formed within the liver, that is at the site of synthesis of C3, its precursor, during inflammatory processes might lead to pronounced local metabolic and hemodynamic alterations.

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