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DIFFERENTIAL EFFECTS OF HUMAN ANAPHYLATOXIN C3A ON GLUCOSE OUTPUT AND FLOW IN RAT LIVER DURING ORTHOGRADE AND RETROGRADE PERFUSION: THE PERIPORTAL SCAVENGER CELL HYPOTHESIS

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1) During orthograde perfusion of rat liver human anaphylatoxin C3a caused an increase in glucose and lactate output and reduction of flow. These effects could be enhanced nearly twofold by co-infusion of the carboxypeptidase inhibitor MERGETPA, which reduced inactivation of C3a to C3adesArg. 2) During retrograde perfusion C3a caused a two- to threefold larger increase in glucose and lactate output and reduction of flow than in orthograde perfusions. These actions tended to be slightly enhanced by MERGETPA. 3) The elimination of C3a plus C3adesArg immunoreactivity during a single liver passage was around 67%, irrespective of the perfusion direction and the presence of the carboxypeptidase inhibitor MERGETPA; however, less C3adesArg and more intact C3a appeared in the perfusate in the presence of MERGETPA in orthograde and retrogade perfusions

It is concluded that rat liver inactivated human anaphylatoxin C3a by conversion to C3adesArg and moreover eliminated it by an additional process. The inactivation to C3adesArg seemed to be located predominantly in the proximal periportal region of the liver sinusoid, since C3a was less effective in orthograde perfusions, when C3a first passed the proximal periportal region before reaching the predominant mass of parenchyma as its site of action, than in retrograde perfusions, when it first passed the perivenous area. These data may be evidence for a periportal scavenger mechanism, by which the liver protects itself from systemically released mediators of inflammation that interfere with the local regulation of liver metabolism and hemodynamics.

Anaphylatoxins are potent mediators of inflammation formed during the activation of the complement system (1). They have short half lives and normally are inactivated in serum by the action of carboxypeptidase N [anaphylatoxin inactivator (1)], which removes their C-terminal arginine residues (2). During severe sepsis with a generalized systemic activation of the complement system, however, this inactivation system might not be sufficient to inactivate all of the anaphylatoxins. It has been shown previously that human anaphylatoxin C3a increased glucose and lactate output and decreased

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flow in perfused rat liver (3). These effects were inhibited by preincubation of C3a with either Fab-fragments of neutralizing monoclonal antibodies or with carboxypeptidase B to remove the C-terminal arginine.

It was the purpose of this study to investigate whether the liver itself can inactivate and eliminate C3a and where in the liver the inactivation might take place. For this purpose rat livers were perfused either in the orthograde or retrograde direction and purified human C3a was infused with or without the carboxypeptidase inhibitor MERGETPA. It was found that C3a increased glucose and lactate output and perfusion resistance to a clearly larger extent in retrograde than in orthograde perfusions and that the C3a actions were enhanced in the presence of MERGETPA. Apparently C3a inactivation was mainly localized in the proximal periportal region which thus could help to protect the liver by scavenging circulating inflammatory mediators.

MATERIALS AND METHODS

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). DL-Mercaptomethyl-3-guanidinoethylthio-propanoic acid (MERGETPA) was purchased from Calbiochem (D-6000 Frankfurt), polyethylene glycol 6000 (PEG) from Serva (D-6900 Heidelberg). CM-52 Cellulose was obtained from Whatman (UK-ME 14 2LE Kent). Dialysis tubing with a cut off of 3.5 kD was bought from Reichelt (D-6900 Heidelberg). The C3adesArg specific monoclonal antibody H466 was a generous gift from Professor R. Burger (D-1000 Berlin).

Male Wistar rats (160-200 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 begun between 9 and 10 a.m..

Human C3a was purified as described previously by fractionated acid precipitation and cation exchange chromatography from complement activated serum of healthy blood donors (3). The purity of the C3a preparation was ascertained by standard SDS-polyacrylamid gel electrophoresis (SDS-PAGE) (4).

C3a was quantified in the eluate and in the perfusate fractions (see below) employing ELISA procedures using anti-C3a monoclonal antibodies (5) and the neoantigen specific monoclonal antibody H466 that recognizes C3adesArg but does not crossreact with C3a (6).

Rats were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body weight). The liver was perfused in situ as described previously (7) either in the orthograde direction (inflow via the vena portae, outflow via the vena cava inferior) or in the retrograde direction (inflow via the vena cava, outflow via the vena portae) 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). To prevent swelling of the organ due to high pressure at the outflow site the outflow was kept below the level of the organ. If swelling occured at any time during the perfusion, which rarely happened, the experi-

ment was discarded. After a preperfusion period of 30 min the experiment was started. At the times indicated purified C3a or C3a together with MERGETPA appropriately diluted in 20 mM HEPES buffer pH 7.4 with 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate were infused for 30 s at a final concentration of about 220 nM and 100 µM, respectively. The flow was determined by quantitating the perfusate fractions collected at 1 min intervals.

Glucose was determined in the perfusate using a commercial test kit (Merck Glucose System) based on the glucose dehydrogenase method (8). Lactate was measured in a combined optical test using lactate dehydrogenase and glutamic pyruvic transaminase (9).

RESULTS AND DISCUSSION

Actions of C3a on liver functions in orthograde perfusions

Rat livers were perfused in the orthograde direction from the vena portae via the vena hepatica to the vena cava. Human anaphylatoxin C3a, infused for 30 s at a final concentration of 220 nM (2 µg/ml), increased glucose and lactate output and reduced flow. Metabolite output started to increase during the infusion period. It reached a maximum 3 min after the infusion onset and returned to basal levels after 7 min (Fig. 1A). During this period a total of 2.5 µmol extra glucose and 1.5 µmol lactate were released per gram liver (area under the curve = AUC) (Fig. 2A). The flow rate decreased also with the onset of infusion, the maximal flow reduction was observed already after 2 min. Flow returned to prestimulation levels after 5 min (Fig. 1A). The total reduction of flow during this period amounted to 0.7 ml per gram liver (AUC) (Fig. 2A).

The carboxypeptidase inhibitor MERGETPA (10) had no effect on liver metabolism or hemodynamics when infused at a final concentration of 1 mM (not shown), which is a 10-fold higher concentration than used in any subsequent experiment. When MERGETPA was co-infused together with C3a to final concentrations of 0.1 mM and 220 nM, respectively, the maximal increases in glucose and lactate output were about 2-times and 1.5-times and maximal flow reduction 2.5-times larger than the changes in the controls. The kinetics of the alterations were not affected by MERGETPA (Fig 1B). The relative increases in total substrate output and flow reduction (AUC) corresponded roughly to the change in peak values (Fig. 2B vs. 1B).

This indicated that the liver was capable of inactivating C3a via a mechanism that could be inhibited by the carboxypeptidase inhibitor. These results also suggested that the inactivating process should be located in the portal vascular bed of the liver or within the first sections of the sinusoids, since partial inactivation must have occurred before C3a reached its site of action (Fig. 3). To further test this hypothesis livers were perfused in the retrograde

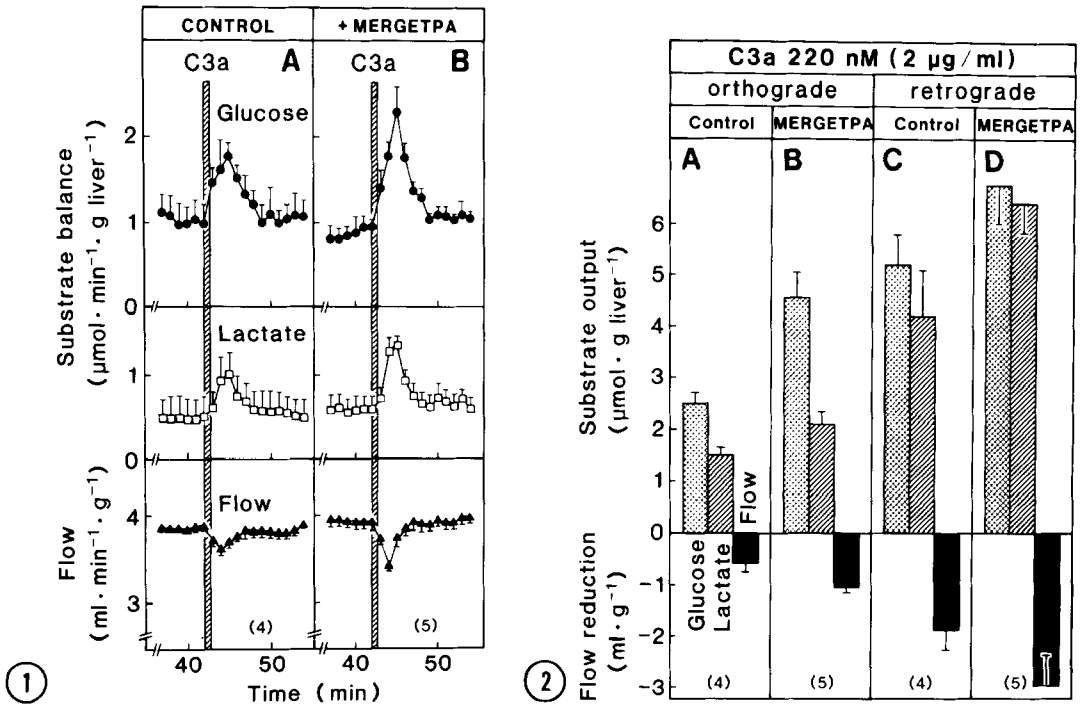

Fig. 1. **Enhancement of the C3a-mediated increase in glucose and lactate output and reduction of flow by the carboxypeptidase inhibitor MERGETPA.** Livers were perfused in situ in the orthograde direction at constant pressure with a Krebs-Henseleit buffer containing 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate. At the times indicated C3a was infused for 30 s at $1/15^{th}$ of the perfusion rate to a calculated final concentration of about 220 nM either alone (panel A) or together with MERGETPA in a final concentration of 0.1 mM (panel B). Substrate balance is given by (posthepatic concentration - prehepatic concentration in µmol * ml^{-1}) * flow in ml * min^{-1} * g $liver^{-1}$. Values are means ± SEM of the number of experiments given in parentheses.

Fig. 2. **Comparison of metabolic and hemodynamic effects of C3a in orthograde and retrograde perfusions.** Rat livers were perfused in situ with a Krebs-Henseleit buffer containing 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate either in the orthograde direction with medium inflow via the vena portae and outflow from the vena cava or in the retrograde direction with inflow via the vena cava and outflow from the vena portae. C3a or C3a together with MERGETPA were infused for 30 s each to final concentrations of 220 nM and 0.1 mM, respectively. The increases in substrate output and flow reduction are given as areas under the curve during the periods in which substrate outputs were above and flow rate below the mean levels. Significant differences were calculated with Student's t-test for unpaired samples: A versus B: Glucose $p<0.01$, lactate $p<0.05$, flow $p<0.05$; A versus C: Glucose $p<0.025$, lactate $p<0.025$, flow $p<0.01$; A versus D: Glucose $p<0.0025$, lactate $p<0.001$, flow $p<0.01$; B versus C: Glucose ns, lactate $p<0.025$, flow $p<0.025$; B versus D: Glucose $p<0.05$; lactate $p<0.001$, flow $p<0.01$; C versus D: Glucose ns, lactate $p<0.05$, flow ns. ns = not significant.

direction. Then, according to the hypothesis, C3a should reach its site of action before passing through areas of high inactivating potency. Thus, metabolic and hemodynamic effects should be larger.

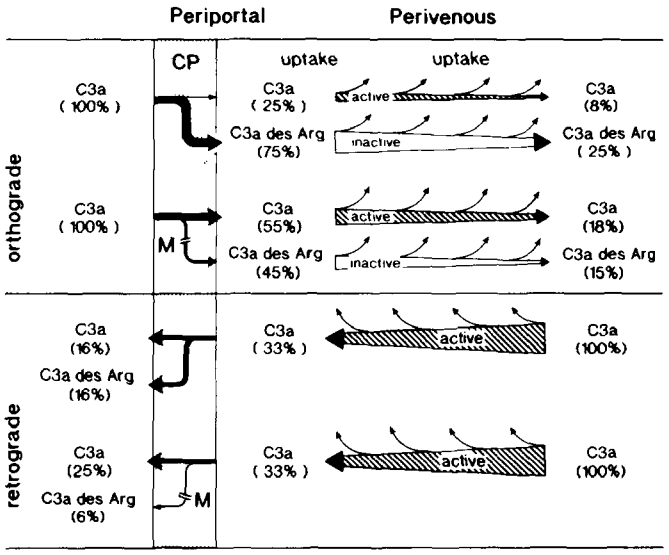

Fig. 3. **Model for the inactivation and elimination of c3a by the liver.** The carboxypeptidase (CP)-mediated inactivation of C3a to C3adesArg is located in the proximal periportal region, the probably uptake-dependent elimination of C3a and C3adesArg is situated in the distal periportal and the perivenous area. M = MERGETPA, a carboxypeptidase inhibitor. The percentage C3a and C3adesArg present in the perfusate at a particular site is indicated in parentheses. For details see text.

Actions of C3a on liver functions in retrograde perfusions

When rat livers were perfused in the retrograde direction from the inferior vena cava via the vena hepatica to the vena portae, C3a elicited, under otherwise identical conditions, an about twofold larger glucose output and threefold larger lactate output and flow reduction than in orthograde perfusions (Fig. 2C versus Fig 2A). The alterations in substrate balance and flow in retrograde perfusions without MERGETPA were even larger than those observed in orthograde perfusions in the presence of MERGETPA (Fig. 2C versus 2B). The metabolic and hemodynamic effects of C3a in retrograde perfusions tended to be further enhanced in the presence of MERGETPA (Fig. 2C versus 2D). MERGETPA increased the C3a-dependent alterations more strongly in orthograde than in retrograde perfusions. These results support the view that the inactivation mechanism that could be inhibited by MERGETPA was predominantly located in the branches of the portal vein or the proximal periportal zone of the liver acinus, and not in the perivenous region or the branches of the central vein.

Inactivation and elimination of C3a in the liver

The inhibition of C3a-inactivation by MERGETPA provided some indirect evidence that a carboxypeptidase N might be the inacti-

vating principle. This enzyme removes the C-terminal arginine from C3a to form C3adesArg and thereby inactivates the anaphylatoxin.

Two different ELISA procedures were used to dicriminate between the inactivation of C3a to C3adesArg and the elimination of C3a plus C3adesArg during passage through the liver.

The first ELISA reacted equally well with C3a and C3adesArg. With this ELISA it was found that during a single passage through the liver about 67% of the infused C3a were eliminated and about 33% appeared in the effluent as immunoreactive C3a plus C3adesArg independent of the perfusion direction and of the presence of MERGETPA (Table 1, Fig. 3).

With the second ELISA procedure C3adesArg but not C3a could be detected specifically, so that a discrimination between effluent C3a and C3adesArg was possible. In orthograde perfusions in the absence of MERGETPA only 8% C3a left the liver; in the presence of MERGETPA this value was increased to 19%. In retrograde perfusions without MERGETPA 15% and with MERGETPA 25% C3a passed through (Table 1, Fig. 3).

These results indicate the existence of two independent systems for the inactivation and elimination of C3a in the liver. The inactivation system was sensitive to inhibition by the carboxypeptidase inhibitor MERGETPA. More intact C3a and less C3adesArg were found in the effluent in the presence of the inhibitor than in its absence. These findings support the view that a carboxypeptidase-

Table 1. Amounts of C3a and C3adesArg leaving the liver during single-pass perfusion after infusion of C3a

Perfusion direction	MERGETPA	C3a plus C3adesArg (3)	C3adesArg (2)	C3a (calculated)
		µg	µg	µg
orthograde	-	10.1±2.5(33%)	7.6±0.9(25%)	2.5(8%)
orthograde	+	10.5±1.5(34%)	4.6±0.9(15%)	5.9(19%)
retrograde	-	9.5±2.0(31%)	4.9±1.5(16%)	4.6(15%)
retrograde	+	9.7±0.7(31%)	1.9±0.3(6%)	7.8(25%)

Livers were perfused as described in the legend to Fig. 1. 31 µg of human C3a were infused (=100%). The concentrations of total C3a and C3adesArg immunoreactivity, respectively, were determined by ELISA procedures. Values are means ± SEM of the number of experiments in parentheses.

like enzyme constituted the inactivation system. The elimination system in contrast to the inactivation system was insensitive to inhibition by MERGETPA. C3a and C3adesArg could be degraded at cell surfaces to fragments, that were no longer detectable in the ELISA. Alternatively, C3a and C3adesArg could be taken up by liver cells for subsequent degradation within the organ. It is not possible to distinguish between these possibilities from the results of the current study. However, the finding of Glovsky that after injection of iodinated C3a radioactivity accumulated in liver, lung and kidney (11) would argue in favor of the second possibility.

The inactivation and elimination systems worked asymmetrically, i.e. in orthograde perfusions less C3a and more C3adesArg was found in the effluent than in retrograde perfusions (Table 1, Fig. 3). This suggests that the carboxypeptidase-mediated inactivation might be located in the proximal periportal area and the probably uptake-dependent elimination in the distal periportal and perivenous regions. In the orthograde perfusions a high proportion of C3a, i.e. three quarters, would be inactivated to C3adesArg in the proximal upstream region and a constant proportion of the remaining 25% C3a and the formed 75% of C3adesArg, i.e. two thirds, would be eliminated in the distal upstream and downstream region, so that 8% C3a and 25% C3adesArg would leave the liver. In retrograde perfusions again a constant proportion of two thirds would be eliminated in the upstream and proximal downstream area and - due to the now less saturating conditions - a medium proportion of the remaining 33% of C3a and C3adesArg, i.e. one half, would leave the liver. Both in orthograde and retrograde perfusions the carboxypeptidase inhibitor MERGETPA would increase the ratio of effluent C3a over C3adesArg (Fig. 3, Table 1).

A protein similar to the catalytically more active small subunit of serum carboxypeptidase N has been purified from hog liver and it has been suggested that liver is the origin of serum carboxypeptidase N (12,13). Thus, it is very likely, that in perfused liver a carboxypeptidase N-related enzyme lining the luminal surface of the vascular and/or sinusoidal endothelium (14,15) participated in the inactivation of C3a.

CONCLUSION

This study showed that rat liver was capable of inactivating human anaphylatoxin C3a probably by a carboxypeptidase-like enzyme which could be located on the surface of the sinusoidal cells in the proximal periportal region. The location of the inactivating prin-

ciple would explain why C3a was more effective in altering metabolism and hemodynamics during retrograde than during orthograde perfusions.

An inactivation system for anaphylatoxin C3a located in the proximal periportal region in the liver might be an example of a scavenger mechanism that protects the liver from systemically generated active signal substances which are not inactivated in the circulation and otherwise would perturb the local regulation of liver metabolism and hemodynamics. A different scavenger system has been proposed to be located in the distal perivenous region (16,17). The role of this system was presumed to be the inactivation of intrahepatically released eicosanoids and adenine nucleotides that have been implicated as signal substances in the intra-organ communication between non-parenchymal and parenchymal liver cells in the regulation of liver metabolism and hemodynamics by humoral factors (18-21) and hepatic nerves (22,23). The liver tissue would thus be enclosed between two scavenger systems. The upstream system guarantees the correct fine regulation of liver metabolism and hemodynamics by preventing the inflow of extrahepatically generated signal substances; the downstream system prevents the efflux of intrahepatically formed mediators and thus a disturbance of the regulation of extrahepatic organs.

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