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Increase in Prostanoid Formation in Rat Liver Macrophages (Kupffer Cells) by Human Anaphylatoxin C3a

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Human anaphylatoxin C3a increases glycogenolysis in perfused rat liver. This action is inhibited by prostanoid synthesis inhibitors and prostanoid antagonists. Because prostanoids but not anaphylatoxin C3a can increase glycogenolysis in hepatocytes, it has been proposed that prostanoid formation in nonparenchymal cells represents an important step in the C3a-dependent increase in hepatic glycogenolysis. This study shows that (a) human anaphylatoxin C3a (0.1 to 10 μg/ml) dose-dependently increased prostaglandin D_2, thromboxane B_2 and prostaglandin $F_{2\alpha}$ formation in rat liver macrophages (Kupffer cells); (b) the C3a-mediated increase in prostanoid formation was maximal after 2 min and showed tachyphylaxis; and (c) the C3a-elicited prostanoid formation could be inhibited specifically by preincubation of C3a with carboxypeptidase B to remove the essential C-terminal arginine or by preincubation of C3a with Fab fragments of a neutralizing monoclonal antibody. These data support the hypothesis that the C3a-dependent activation of hepatic glycogenolysis is mediated by way of a C3a-induced prostanoid production in Kupffer cells. (HEPATOLOGY 1993;18:1516-1521.)

The human anaphylatoxin C3a is generated by the proteolytic cleavage of the third complement component C3. C3a is under the control of serum carboxypeptidase N, which cleaves the C-terminal Arg-77 and thereby inactivates C3a. The classic anaphylatoxic effects of C3a such as smooth muscle contraction and enhanced vascular permeability are only in part due to direct activation of the effector cells by C3a receptors. Other proinflammatory mediators such as histamine and prostanoids may be involved in the signal chains. Thus the C3a-mediated contraction of guinea pig lung parenchymal strips were inhibited by indomethacin (1), and C3a induced the release of PGE_2 and $PGF_{2\alpha}$ from guinea pig lung (2).

Serum in which the complement system had been activated by incubation with zymosan (3, 4), as well as purified human anaphylatoxin C3a but not C5a (5), has previously been shown to increase glucose and lactate output and reduce and redistribute flow in perfused rabbit (3) and rat (4, 5) liver. Human C3a does not increase glycogenolysis in isolated rat hepatocytes. Its effects in perfused liver on metabolism and hemodynamics are attenuated by inhibitors of prostanoid synthesis or thromboxane (TXB) receptor antagonists. They are accompanied by overflow of prostanoids into the hepatic vein (4). In liver prostanoids can only be formed by nonparenchymal cells (6, 7). PGs stimulated glycogen phosphorylase activity in and the glucose output from parenchymal liver cells (8-10). Therefore it has been proposed that anaphylatoxins probably mediated their effect on hepatic glycogenolysis by way of an increase in prostanoid production in nonparenchymal liver cells (4, 5). To corroborate this hypothesis we investigated the prostanoid production in isolated liver macrophages (Kupffer cells) in response to purified human anaphylatoxin C3a. This study shows that C3a, in a concentration range that can be expected to occur *in vivo*, rapidly and specifically increased the release of PGD_2, TXB_2 and $PGF_{2\alpha}$.

MATERIALS AND METHODS

Materials. All materials were of analytical grade and were from commercial sources. $PGF_{2\alpha}$ and TXB_2 RIAs were provided by Amersham Buchler (Braunschweig, Germany). The antibody against PGD_2 was a generous gift of Dr. Osamu Hayaishi (Osaka, Japan). Pronase and collagenase were purchased from Merck AG (Darmstadt, Germany) and Boehringer (Mannheim, Germany), respectively. Tissue-culture dishes were bought from Greiner (Nürtingen, Germany), Nycodenz was from Immuno GmbH (Heidelberg, Germany), newborn calf serum (NCS) was from Sigma Chemical Co. (Deisenhofen, Germany) and RPMI-1640 was from Serva (Heidelberg, Germany). Whatman CM52-cellulose was provided by Bender & Hobein GmbH (Bruchsal, Germany) and Sephadex G-50 was from Pharmacia (Freiburg, Germany).

Purification of Human Anaphylatoxin C3a. C3a was purified from blood samples of healthy voluntary donors essentially as described previously (5). The complement system was

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TABLE 1. C3a- and zymosan-induced prostanoid and superoxide formation in Kupffer cells

Product	Control	C3a	Zymosan
ng × $(10^6$ cells$)^{-1}$ × 30 min^{-1}			
PGD_2	0.6 ± 0.1[a]	4.4 ± 1.8[b]	57.0 ± 10.0[b]
TXB_2	0.8 ± 0.1	2.9 ± 1.0[b]	18.0 ± 6.9[b]
$PGF_{2\alpha}$	0.8 ± 0.2	2.5 ± 0.6[b]	17.9 ± 1.1[b]
nmol × $(10^6$ cells$)^{-1}$ × 30 min^{-1}			
O_2^-	1.1 ± 0.1	2.8 ± 0.5[b]	10.4 ± 1.5[b]

Kupffer cells were prepared by means of a combined collagenase/pronase perfusion and purified by centrifugal elutriation. They were cultured on 24-well tissue-culture plates for 72 hr in RPMI-1640 with 30% NCS. The medium was removed by washes with HBSS. The cells were then preincubated for 10 min at 37° C in HBSS. The experiment was started by addition of C3a or zymosan to final concentrations of 25 μg/ml and 0.5 mg/ml, respectively. After 30 min, supernatants were removed and frozen in liquid nitrogen for the determination of prostanoid concentrations in specific RIAs. Superoxide formation was determined on the basis of cytochrome c reduction.

[a]Data expressed as mean ± S.E.M. of four experiments.

[b]Student's *t* test for unpaired samples: significantly different from control ($p < 0.05$).

activated in human serum by incubation with zymosan in the presence of ε-aminocaproic acid to inhibit serum carboxypeptidase N. The bulk of the proteins was removed by means of acid precipitation (pH 4.5) with 10% (mass/vol) polyethylene glycol 6000. The supernatant was subjected to chromatography on CM52-Cellulose and Sephadex G-50 and a rechromatography on CM52-Cellulose (all from Bender and Hubein Ag, Zurich, Switzerland). The purified C3a was subjected to dialysis against the HBSS used in the cell experiments (see below). C3a and C5a were detected with ELISA procedures using neoepitope-specific monoclonal antibodies (11). The C3a preparation obtained with this procedure was essentially free of contamination by other proteins as judged on the basis of SDS-PAGE results. Contamination with C5a was below the detection limit (200 pg/ml) of the assay employed.

Kupffer Cell Preparation. Kupffer cells were prepared as described previously (12, 13) by means of combined pronase/collagenase perfusion. They were purified with Nycodenz density-gradient centrifugation and subsequent centrifugal elutriation. Kupffer cells were plated on 24-well plates (1×10^6 cells/well) or 3.5-cm tissue-culture plates (5×10^6 cells/plate) in RPMI-1640 in the presence of 30% NCS and 1% penicillin/streptomycin for 72 hr before the experiment. Medium was changed daily. After 72 hr, 25% of the cells were attached; 95% of these cells were Kupffer cells, as judged on the basis of phagocytosis of latex beads at least 1 μm in diameter. For cocultures with hepatocytes, Kupffer cells were cultured in the same manner for the first 48 hr, except that collagen-coated, 3.5-cm tissue-culture plates were used and only 4×10^6 Kupffer cells were seeded. After 48 hr, hepatocytes (7×10^5 cells/plate) were plated on top of about 1×10^6 Kupffer cells. Cocultures were incubated in M199 with 0.5 nmol/L insulin, 1% penicillin/streptomycin and 30% NCS for 24 hr with one medium change after 4 hr.

Cell Experiments. During the cell-culture experiments cells were maintained at 37° C. Most experiments were performed in 24-well plates. After 72 hr of primary culture, Kupffer cells were counted, washed three times with HBSS (137 mmol/L NaCl, 5.4 mmol/L KCl, 1.3 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 4.2 mmol/L $NaHCO_3$, 0.34 mmol/L Na_2PO_4, 0.44 mmol/L KH_2PO_4, 20 mmol/L HEPES and 5 mmol/L glucose [pH 7.4]) to remove all residual serum and medium and then preincubated for 10 min in the same buffer. Then C3a, C3a(desArg), C3a preincubated with C3a Fab antibody fragments or zymosan were added to the final concentrations indicated. Samples of the supernatant were taken at different times and frozen immediately in liquid nitrogen for later determination of prostanoid concentrations with RIAs. The experiments for the dose response curves were performed similarly except that 3.5-cm culture plates were used and that Kupffer cells were scraped off the dish at the end of the experiment for DNA determination in a fluorescence assay (14).

Biochemical Assays. Glycogen phosphorylase activity was determined by means of a standard procedure measuring the release of inorganic phosphate from glucose-1-phosphate, which is incorporated in glycogen in the inverse natural reaction (15). Superoxide production was determined on the basis of cytochrome c reduction (16). Prostanoids were determined in the cell supernatants without further purification by means of RIA. TXB_2 and $PGF_{2\alpha}$ were measured with commercial test kits (Amersham, Braunschweig, Germany). The linear ranges of the calibration curves were about 150 to 2,800 pmol/L. PGD_2 was determined with an RIA based on an antibody provided by Dr. Hayaishi with a linear range of 700 to 14,000 pmol/L.

RESULTS

Increase in Prostanoid and Superoxide Formation in Kupffer Cells Caused by C3a. After 72 hr in primary culture, Kupffer cells were stimulated with zymosan (0.5 mg/ml) or C3a (25 μg/ml). After 30 min, supernatants were removed for prostanoid and superoxide determination. C3a increased the production of PGD_2, TXB_2 and $PGF_{2\alpha}$ three to eight times basal values. However, the zymosan-induced increase in prostanoid formation after 30 min of incubation was five to 10 times larger than that observed with C3a (Table 1). Similarly, we noted a small increase in superoxide formation by C3a; however, the zymosan-mediated increase in superoxide formation was more pronounced (Table 1). The relative amounts of PGD_2, TXB_2 and $PGF_{2\alpha}$ released into the medium after stimulation with C3a or zymosan, as determined on RIA, were confirmed with HPLC profiles of supernatants of Kupffer cells that had been prelabeled with [^{3}H]arachidonic acid for 24 hr before the experiment (data not shown).

Time Course of C3a-mediated Prostanoid Formation. The C3a-mediated increase in prostanoid formation in Kupffer cells was rapid in onset and not sustained. Prostanoid concentration was already significantly increased 2 min after addition of C3a; it reached a plateau

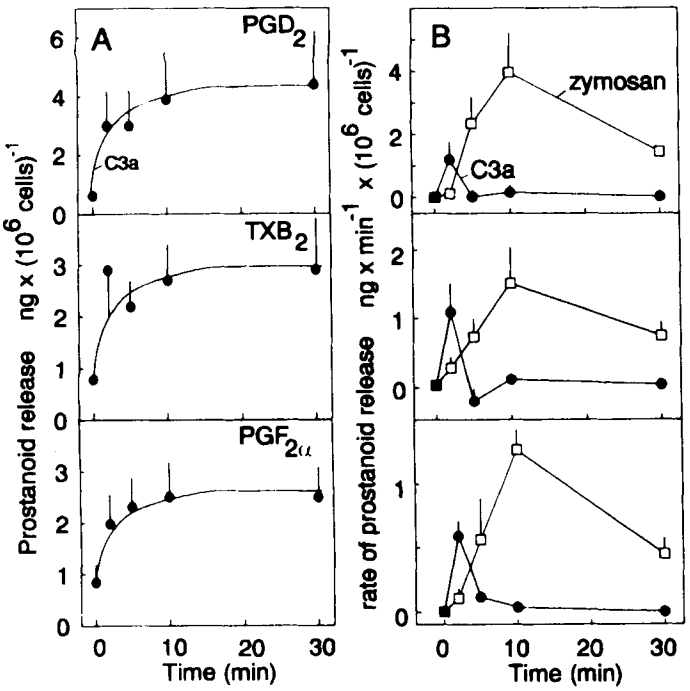

FIG. 1. Kinetics of C3a- and zymosan-induced prostanoid release from Kupffer cells. Kupffer cells were cultured for 72 hr as described in Materials and Methods. The medium was removed by three washes with HBSS. After 10-min preincubation of cells at 37° C in HBSS, the experiment was started by addition of C3a or zymosan to final concentrations of 25 μg/ml and 0.5 mg/ml, respectively. At the times indicated, supernatants were removed and frozen in liquid nitrogen for determination of prostanoid concentrations. (A) Cumulative C3a-induced prostanoid formation. Zymosan is not shown because values are off the scale after 5 min. (B) Rate of zymosan- and C3a-induced prostanoid formation. Data expressed as mean ± S.E.M. of four experiments each. The rate was calculated as difference in the amount of prostanoids formed between two time points divided by the time difference.

at 10 min (Fig. 1A). The maximum rate of prostanoid release was reached at 2 min; the rate returned to zero at 10 min (Fig. 1B). By contrast, the zymosan-induced increase in prostanoid formation was sustained; the rate of prostanoid formation reached a maximum at 10 min and remained increased at a lower value throughout an incubation period of 30 min (Fig. 1B).

The C3a-mediated increase in prostanoid formation showed the phenomenon of tachyphylaxis. If the medium was replaced 15 min after the first stimulation of Kupffer cells with C3a and C3a was added a second time, no increase in prostanoid formation could be seen in the 15 min after the second stimulation (Table 2).

Specificity of C3a Action. The biological activity of C3a depends on the presence of its C-terminal arginine. C3a(desArg) is inactive in other biological systems (17). C3a, which was converted to its desArg form by incubation with carboxypeptidase B (1 hr at 37° C), did not stimulate prostanoid formation in Kupffer cells (Fig. 2). Preincubation (12 hr at 4° C) of C3a with Fab fragments of a monoclonal antibody that neutralized the biological activity of C3a (5) almost abolished the C3a-mediated increase in prostanoid formation (Fig. 2). These data—together with the phenomenon of tachyphylaxis, which is typical for anaphylatoxins (18)—indicate that the stimulation by C3a of prostanoid production in Kupffer cells was specific.

Dose-dependence of C3a-mediated Prostanoid Formation. The increase in prostanoid formation in Kupffer cells by C3a was dose dependent. C3a increased PGD_2, TXB_2 and $PGF_{2\alpha}$ formation half-maximally at concentrations between 0.1 and 1 μg/ml (Fig. 3). Maximal stimulation of prostanoid formation was evoked by 10 μg C3a/ml. These concentrations are in the range that can be expected to occur *in vivo*.

Increase in Glycogen Phosphorylase Activity by C3a in Kupffer Cell–hepatocyte Cocultures. C3a did not enhance glycogen phosphorylase activity in monocultures of hepatocytes or Kupffer cells (Table 3). However, in cocultures of Kupffer cells and hepatocytes C3a increased glycogen phosphorylase activity by about 100% (Table 3). Supernatants of C3a-treated Kupffer cells did not augment glycogen phosphorylase activity in hepatocyte cultures (data not shown).

DISCUSSION

Increase by C3a in Hepatic Glycogenolysis by Way of C3a-mediated Prostanoid Formation in Kupffer Cells. Peptides of the activated complement system (3, 4), in particular purified human C3a (5, 19), increased glucose output from perfused rabbit or rat liver. The anaphylatoxin-mediated increase in hepatic glycogenolysis was inhibited by inhibitors of the prostanoid synthesis and by TXB receptor antagonists. Human C3a did not stimulate glycogenolysis in hepatocytes directly. Therefore C3a must have stimulated hepatic glycogenolysis indirectly, probably by increasing the prostanoid synthesis in nonparenchymal cells. Prostaglandins in turn could have activated glycogenolysis in hepatocytes (8-10). Among the nonparenchymal liver cells the liver macrophages (Kupffer cells) semed to be the best candidates to take part in this signal chain; peritoneal macrophages have previously been shown to respond with enhanced prostanoid formation to stimulation with anaphylatoxin C3a (20). This investigation showed that C3a indeed increased prostanoid formation in Kupffer cells. The increase in prostanoid formation was rapid in onset and reached its maximum after only 2 min. It thus preceded the maximum glucose output observed in perfused liver 3 to 4 min after infusion of C3a (5), which would be a prerequisite for being an early link in a putative signal chain. The concentrations of C3a that evoked prostanoid formation in Kupffer cells were in the same range as those that stimulated hepatic glycogenolysis (5, 19).

The concentrations of PGD_2 and $PGF_{2\alpha}$ detected in the culture medium of the Kupffer cells were much lower (around 1 nmol/L) than those needed to increase glycogenolysis in hepatocytes (2 to 10 μmol/L; 9, 21, 22). Yet *in situ* the Kupffer cells are in close contact with the hepatocytes and the prostanoids are being released into the small space of Disse between the Kupffer cells and

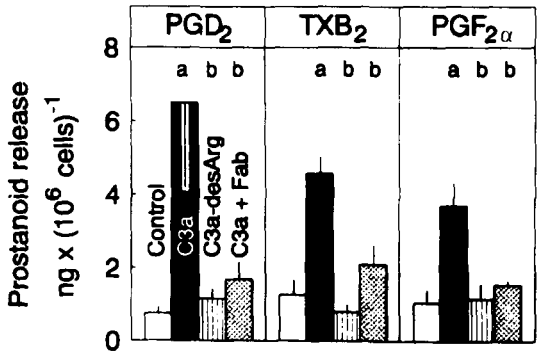

FIG. 2. Specificity of C3a-induced prostanoid formation. Kupffer cells were cultured as described in Materials and Methods. The medium was removed by three washes with HBSS. After 10-min preincubation of cells at 37° C in HBSS, the experiment was started by addition of C3a, C3a(desArg) or C3a preincubated with Fab fragments of a monoclonal C3a antibody to a final concentration of 6 μg/ml. After 15 min, supernatants were removed for determination of prostanoid concentrations. Data expressed as mean ± S.E.M. of four experiments. Student's *t* test for unpaired samples: *a*, $p < 0.05$, significantly different from control; *b*, $p < 0.05$, significantly smaller than C3a-stimulated values. *TXB*$_2$ = TXB$_2$.

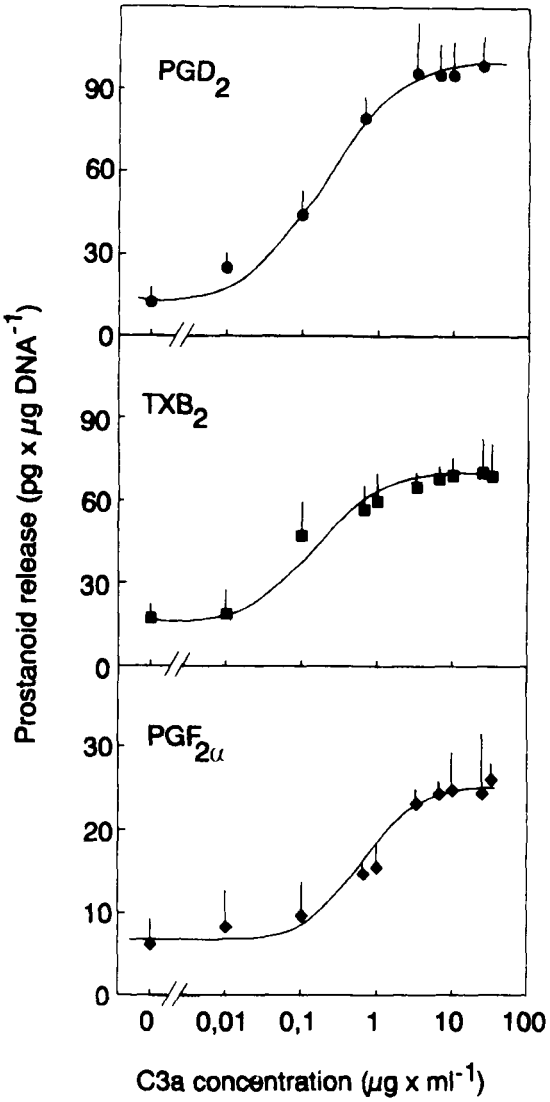

FIG. 3. Dose-dependence of the C3a-induced prostanoid formation in Kupffer cells. Kupffer cells were cultured on 3.5-cm tissue-culture plates for 72 hr. The medium was removed by three washes with HBSS. After 10-min preincubation of cells at 37° C in HBSS, the experiment was started by addition of C3a to the final concentration indicated. Supernatants were removed after 10 min for the determination of prostanoid concentrations. At the end of the experiment, cells were scraped off the dishes for DNA determination. Data expressed as mean ± S.E.M. of four to 10 experiments per concentration.

the hepatocytes instead of the large space of medium under the experimental conditions. Thus, *in situ*, high local concentration of prostanoids can be built up. If the production of PGD_2 is increased by C3a to 2.5 ng/min × 10^6 cells (Fig. 1) and this prostaglandin were evenly (nonvectorially) released into a surrounding space of 2 μm around a Kupffer cell with an 8-μm diameter, then the local concentration of PGD_2 would amount to about 25 μmol/L after 1 min, sufficient to stimulate glycogenolysis in hepatocytes (9, 21, 22). If in Kupffer cells prostanoid release were not evenly distributed over the entire cell but, as has been shown for polarized Madin-Darby canine kidney cells (23), occurred vectorially predominantly at the site facing the space of Disse, local concentrations there would clearly exceed the calculated value.

The importance of the small space separating hepatocytes and Kupffer cells is further supported by the fact that C3a increased glycogen phosphorylase activity only in Kupffer cell–hepatocyte cocultures, whereas supernatants of C3a-treated Kupffer cells had no influence on hepatocyte glycogen phosphorylase activity. The volume of medium overlaying the separate Kupffer cell cultures and the Kupffer cell–hepatocyte cocultures was the same. However, in the cocultures prostanoids were released from Kupffer cells not only into the large space of medium but also into a much smaller space between Kupffer cells and hepatocytes. This smaller space was still less confined and thus larger than the space of Disse *in vivo*. Therefore it may have been impossible to reach maximal efficiency in coculture.

Physiological Significance of the C3a-mediated Prostanoid Formation in Kupffer cells. The concentration of C3a that elicited half-maximal stimulation of prostanoid formation in Kupffer cells (0.1 to 1 μg/ml) was 10 to 100 times higher than the normal circulating C3a concentration in human beings, which is about 10 ng/ml (24). However, in patients subjected to hemodialysis or connected to extracorporeal circuits for other reasons, C3a concentrations of 5 to 10 μg/ml may be found (11, 24). These pathophysiologically increased C3a concentrations are 10 times greater that what would suffice to stimulate prostanoid synthesis in Kupffer cells. It is interesting to note here that the liver apparently has a periportally located inactivation system for C3a (19) that can protect the local intrahepatic regulation of liver metabolism and hemodynamics from a signal input of

TABLE 2. Tachyphylaxis of the C3a-induced prostanoid formation in Kupffer cells

Product	First stimulation		Second stimulation	
	Control	C3a	Control	C3a
PGD_2	2.2 ± 0.8[a]	10.8 ± 0.9[b]	0.4 ± 0.2	0.4 ± 0.2
TXB_2	1.4 ± 0.5	5.0 ± 0.9[b]	0.7 ± 0.1	0.7 ± 0.2
$PGF_{2\alpha}$	1.0 ± 0.1	4.1 ± 0.7[b]	0.6 ± 0.1	0.7 ± 0.2

Kupffer cells were cultured for 72 hr as described in Materials and Methods. The medium was removed by three washes with HBSS. The cells were then preinculabed for 10 min at 37° C in HBSS. The experiment was started by addition of C3a to a final concentration of 6 μg/ml. After 15 min, supernatants were frozen in liquid nitrogen, new buffer was added and cells were restimulated with C3a. After another 15 min, the medium was removed and frozen in liquid nitrogen. Prostanoid concentration was determined with specific RIAs.

[a]Data expressed as mean ± S.E.M. of four experiments (ng × 10^6 cells^{-1} × 15 min^{-1}).

[b]Student's *t* test for unpaired samples: significantly greater than control value ($p < 0.05$).

TABLE 3. C3a-mediated increase in glycogen phosphorylase activity in Kupffer cell–hepatocyte cocultures

Culture condition	Additions	Glycogen phosphorylase activity	Change in glycogen phosphorylase activity
Kupffer cells	None (n = 6)	23 ± 8[a]	–
	C3a, 25 μg/ml (n = 6)	18 ± 6	−5 ± 3
Hepatocytes	None (n = 6)	26 ± 2	–
	C3a, 25 μg/ml (n = 7)	35 ± 5	+9 ± 5
Kupffer cells + hepatocytes	None (n = 6)	26 ± 2	–
	C3a, 25 μg/ml (n = 7)	57 ± 8[b]	+31 ± 8[b]

Kupffer cells (1 × 10^6), prepared as described in Materials and Methods, were cultured on collagen-coated, 3.5-cm tissue-culture dishes for 48 hr in RPMI-1640 with 30% NCS. Then hepatocytes (7 × 10^5) were seeded on top of the Kupffer cells, and culture was continued for another 24 hr in M199 with 30% NCS and 0.5 nmol/L insulin. Control Kupffer cells and hepatocytes were cultured accordingly. After 72 hr, medium was removed by three washes with HBSS. Cells were preincubated for 10 min at 37° C in HBSS; then C3a was added to a final concentration of 25 μg/ml. After 2 min, supernatants were removed and the plates were frozen in liquid nitrogen for later determination of glycogen phosphorylase activity.

[a]Data expressed as mean ± S.E.M. (nmol × min^{-1} × gm protein^{-1}).

[b]Student's *t* test for paired values ($p < 0.01$).

systemically generated C3a that escaped inactivation by carboxypeptidase N.

The liver is one of the main sites of complement protein synthesis. Most serum complement proteins are produced by parenchymal liver cells. Yet monocytes and macrophages produce complement proteins in culture, and it has been discussed that these complement proteins are used in the direct environment of these cells (25). Thus, during local hepatic inflammation, C3 synthetized by liver macrophages (Kupffer cells) or hepatocytes might be cleaved to yield C3b and C3a. C3a (and possibly other locally released anaphylatoxins) may cause vasoconstriction (i.e., narrowing of the sinusoids) and reduce or even stop blood flow in the area of inflammation. This might facilitate the invasion of inflammatory cells. At the same time, the infiltrated cells might stimulate prostanoid production in Kupffer cells by way of an autocrine mechanism. Prostaglandins in turn could activate hepatocytes to release glucose that would be required for the anaerobic energy metabolism at the poorly perfused site of inflammation.

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