FEBS Letters 372 (1995) 108–112 FEBS 15955

Stimulation of glycogen phosphorylase in rat hepatocytes via prostanoid release from Kupffer cells by recombinant rat anaphylatoxin C5a but not by native human C5a in hepatocyte/Kupffer cell co-cultures

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Received 19 July 1995

Abstract Human anaphylatoxin C3a had previously been shown to increase glycogenolysis in perfused rat liver and prostanoid formation in rat liver macrophages. Surprisingly, human C5a, which in other systems elicited stronger responses than C3a, did not increase glycogenolysis in perfused rat liver. Species incompatibilities within the experimental system had been supposed to be the reason. The current study supports this hypothesis: (1) In rat liver macrophages that had been maintained in primary culture for 72 h recombinant rat anaphylatoxin C5a in concentrations between 0.1 and 10 μg/ml increased the formation of thromboxane A_2, prostaglandin D_2, E_2 and $F_{2\alpha}$ 6- to 12-fold over basal within 10 min. In contrast, human anaphylatoxin C5a did not increase prostanoid formation in rat Kupffer cells. (2) The increase in prostanoid formation by recombinant rat C5a was specific. It was inhibited by a neutralizing monoclonal antibody. (3) In co-cultures of rat hepatocytes and rat Kupffer cells but not in hepatocyte mono-cultures recombinant rat C5a increased glycogen phosphorylase activity 3-fold over basal. This effect was inhibited by incubation of the co-cultures with 500 μM acetylsalicyclic acid. Thus, C5a generated either locally in the liver or systemically e.g. in the course of sepsis, may increase hepatic glycogenolysis by a prostanoid-mediated intercellular communication between Kupffer cells and hepatocytes.

Key words: Anaphylatoxin C5a; Rat liver macrophage; Kupffer cell; Hepatocyte; Co-culture; Prostaglandin; Thromboxane A_2; Glycogen phosphorylase

1. Introduction

Anaphylatoxins are small 74 to 78 amino acid proteins, that are generated as N-terminal cleavage products during the activation of the complement factors C3, C4 and C5 either through the classical or the alternative pathway [1]. These anaphylatoxins have previously been shown to activate glycogenolysis in perfused rat liver: a low molecular mass fraction (10 to 17 kDa) of complement-activated rat serum [2] as well as purified human anaphylatoxin C3a [3] increased glucose output. This increase in hepatic glycogenolysis was attenuated by inhibitors of prostanoid synthesis and it was preceded by an elevation in prostaglandin and thromboxane overflow [2]. Since in liver prostanoids are formed almost exclusively in non-parenchymal liver cells, i.e. endothelial, Kupffer and Ito cells [4–6] and since prostaglandins $F_{2\alpha}$, E_2 and D_2 can activate glycogen phosphorylase activity in hepatocytes [7–11], these results suggested that anaphylatoxins stimulated hepatic glycogenolysis by an eicosanoid-mediated intercellular communication between non-parenchymal cells and hepatocytes. Such a mechanism had been shown before for other mediators that reach the liver during inflammatory processes such as endotoxins [9], immune complexes [12], platelet activating factor [13–15] and also for zymosan [16], a yeast cell wall preparation. In line with this hypothesis human C3a increased prostanoid formation in isolated cultured rat Kupffer cells [17]. Surprisingly, human C5a, which in most systems elicits much stronger effects than C3a, did not increase glycogenolysis in perfused rat liver [3]. It was assumed that the lack of an effect of human C5a in perfused rat liver was due to a species incompatibility [3].

The aim of the current study was to corroborate the apparent species specificity of anaphylatoxin. C5a and, moreover, to demonstrate the C5a-elicited intercellular communication via prostanoids in Kupffer cell/hepatocyte co-cultures. It was found that recombinant rat C5a but not native human C5a increased prostanoid formation in cultured rat Kupffer cells and stimulated glycogen phosphorylase activity in rat Kupffer cell/hepatocyte co-cultures via an aspirin-inhibitable mechanism.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and from commercial sources. The radioimmuno assays for TXB_2, PGD_2, $PGF_{2\alpha}$ and PGE_2 were bought from Amersham (38110 Braunschweig, Germany). Hydroluma was purchased from Baker (Deventer, The Netherlands), pronase from Merck AG (64293 Darmstadt, Germany) and collagenase and DNase from Boehringer (68305 Mannheim, Germany). Tissue culture dishes were obtained from Nunc (65203 Wiesbaden, Germany), Nycodenz from Life Technologies GmbH (76344 Eggenstein, Germany), Percoll from Pharmacia Biotech GmbH (79021 Freiburg, Germany), newborn calf serum (NCS) and bis-benzimide from Sigma Chemical Co. (82041 Deisenhofen, Germany), RPMI 1640 from Biochrom KG (12247 Berlin, Germany) and M199 from Gibco BRL (76344 Eggenstein, Germany). The monoclonal antibody QD7 was generated against a C-terminal 20 amino acid peptide of C5a coupled to bovine serum albumin (Sonntag G. and Götze O., unpublished).

2.2. Preparation of recombinant rat C5a and native human C5a

Recombinant rat C5a was prepared by synthesis of a cDNA from rat liver RNA and a subsequent PCR using degenerate 5′ and 3′ primers that were designed according to sequence data published in the Swiss-Prot protein sequence data bank (accession no: P08650, ID: C05A_RAT) [18]. The primers covered the amino acids 1 to 13 (5′-primer) and 56 to 77 (3′-primer) of this sequence. The PCR-product was cloned into the bacterial expression vector pQE-30 (Qiagen, 40724, Hilden, Germany) with the additional N-terminal sequence MRGJHHHHHHGI

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SSDI 0014-5793(95)00883-7

coded for by the polylinker. The recombinant protein was purified from bacterial lysates by Ni^{2+}-chelate chromatography and refolded in a glutathion-containing renaturation buffer. Native human C5a was prepared from complement activated human serum as described elsewhere [19]. *N*-Acetyl-β-D-glucosaminidase release from human granulocytes was determined by an optical test [20].

2.3. Animals

Male Wistar rat (420–500 g for the preparation of Kupffer cells and 200–250 g for the isolation of hepatocytes) were bought from Winkelmann (33178 Borchen, Germany) and kept on a 12-h day–night rhythm (light from 07.00 to 19.00 h) with free access to water and a rat standard diet from Ssniff (59494 Soest, Germany) at least 2 weeks before the experiments.

2.4. Kupffer cell preparation

Non-parenchymal cells were prepared by a combined collagenase/pronase perfusion [21,22]. Kupffer cells were then purified by Nycodenz density-gradient centrifugation and subsequent centrifugal elutriation using a Beckman JE-6 elutriation rotor in a J-21 Beckman centrifuge.

2.5. Hepatocyte preparation

Hepatocytes were isolated according to Meredith [23] without the use of collagenase as described previously [11]. The liver was perfused without recirculation via the portal vein with a Ca^{2+}-free Krebs-Henseleit buffer containing 15 mM glucose, 2 mM lactate, 0.2 mM pyruvate and 2 mM EDTA. Detritus was removed by two subsequent washing steps, sedimenting the viable hepatocytes at $50 \times g$. Viable hepatocytes were further purified by centrifugation through a gradient with 58% Percoll.

2.6. Kupffer cell culture

Kupffer cells were plated (4×10^6/plate) on 3.5 cm tissue culture plates with or without collagen coating in RPMI 1640 in the presence of 1% penicillin/streptomycin and 30% neonatal calf serum for 72 h before the experiment. Medium was changed every 24 h.

2.7. Hepatocyte culture

Hepatocytes were plated (5×10^5/plate) on collagen coated, 3.5 cm tissue culture plates in M199 with 0.5 nM insulin, 1% penicillin/streptomycin and 30% NCS for 24 h before the experiment with a medium change after 4 h.

2.8. Co-culture

For co-culture with hepatocytes, Kupffer cells were seeded (3×10^6/plate) on collagen coated, 3.5 cm culture plates in RPMI 1640 in the presence of 1% penicillin/streptomycin and 30% NCS for the first 48 h. Medium was changed every 24 h. After 48 h, freshly prepared hepatocytes (5×10^5/plate) were plated on top of the Kupffer cells. Co-cultures were incubated in M199 with 0.5 nM insulin, 1% penicillin/streptomycin and 30% NCS for another 24 h with one medium change after 4 h.

2.9. Determination of prostanoid formation in Kupffer cells

After 72 h cells were washed three times with Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 0.34 mM Na_2PO_4, 0.44 mM KH_2PO_4, 20 mM Hepes and 5 mM glucose, pH 7.4) to remove all residual serum and medium and then preincubated for 10 min at 37°C in 1.35 ml of the same buffer. Then the anaphylatoxins were added to the final concentrations indicated. Samples of 200 μl of the supernatant were taken at different times and frozen immediately in liquid nitrogen for the later determination of prostanoid concentrations. TXB_2, PGD_2, $PGF_{2\alpha}$ and PGE_2 were determined in the cell supernatants without further purification by radioimmuno assay according to the instructions of the manufacturer. At the end of the experiment Kupffer cells were scraped off the dish for DNA determination in a fluorescence assay based on the intercalation of bis-benzimide into DNA [24].

2.10. Determination of glycogen phosphorylase activity in mono- and co-cultures

Hepatocyte, Kupffer cell and hepatocyte/Kupffer cell cultures were washed three times with Hepes buffered saline (20 mM Hepes, 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate, pH 7.4). Cells were incubated for 10 min at 37°C in the same buffer. The medium was then discarded and replaced with 200 μl Hepes buffered saline containing recombinant rat C5a (final concentration 1 μg/ml), native human C5a (final concentration 1 μg/ml) or noradrenaline (final concentration 1 μM). After 2 min of incubation the buffer was removed and the plates were frozen in liquid nitrogen. To inhibit the cyclooxygenase (PGH-synthase) cell cultures were pretreated with 500 μM acetylsalicylic acid (ASA) in M199 30 min prior to the experiment. Control plates were incubated with M199 without ASA. Glycogen phosphorylase activity was determined with a standard assay [25].

3. Results and discussion

3.1. Stimulation of prostanoid formation by recombinant rat C5a in rat liver macrophages

Recombinant rat C5a increased prostanoid formation in cultured rat Kupffer cells rapidly and transiently (Fig. 1). After a single application of 10 μg recombinant rat C5a/ml the concentration of TXB_2, PGD_2, $PGF_{2\alpha}$ and PGE_2 increased over the following 5 min and remained almost constant for another 5 min thereafter. The maximal rate of prostanoid formation was reached at 1 min, the first point taken (Fig. 1). Thus, the recombinant rat C5a-mediated increase in prostanoid formation occurred sufficiently rapid to explain the increase in glycogenolysis in the perfused rat liver, which reached a maximum 2 min after the onset of infusion of complement activated serum [2].

Recombinant rat C5a increased the formation of TXB_2, PGD_2, $PGF_{2\alpha}$ and PGE_2 in a concentration range between 0.1 and 10 μg/ml (Fig. 2). At 0.001 μg/ml recombinant rat C5a there was already a slight increase in TXB_2 formation, which did not

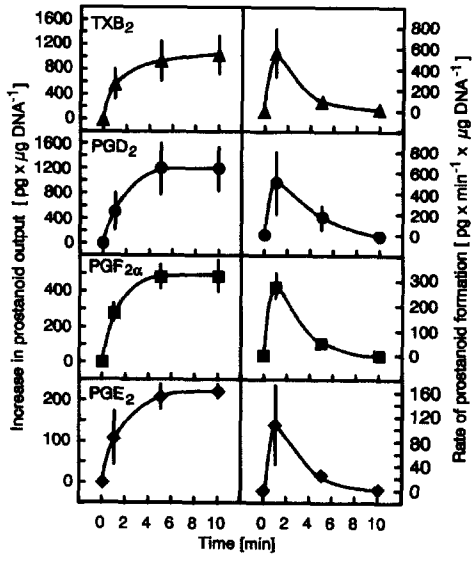

Fig. 1. Time dependence of prostanoid formation elicited by recombinant rat C5a in rat liver macrophages. Rat Kupffer cells were isolated by a combined collagenase/pronase perfusion and purified by density gradient centrifugation and centrifugal elutriation. They were cultured in RPMI medium containing 30% newborn calf serum for 72 h prior to the experiments. Medium was removed and replaced by Hanks' balanced salt solution. Cells were stimulated with 10 μg recombinant rat C5a/ml (final concentration). Aliquots of the supernatant were removed at the times indicated. The increase in eicosanoids over basal levels at 0 min, was determined by radioimmuno assay. Values are means ± S.E.M. of 4 to 6 independent experiments.

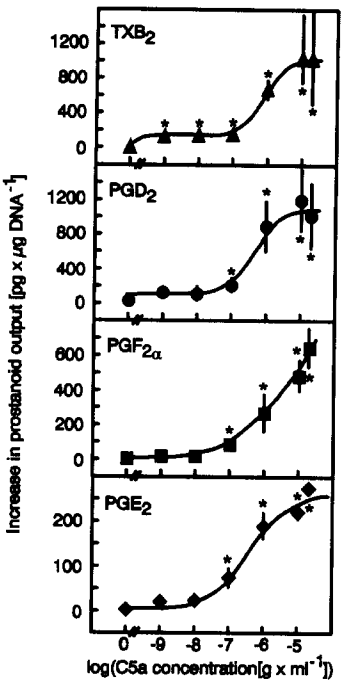

Fig. 2. Dose dependence of prostanoid formation elicited by recombinant rat C5a in rat liver macrophages. Kupffer cells were isolated and cultured for 72 h as described in Fig. 1. Medium was then replaced by Hanks buffered salt solution. Cells were stimulated with the concentrations of recombinant rat C5a indicated. After 10 min supernatants were removed and the increase in eicosanoid concentration over basal levels at 0 min, was determined by radioimmuno assay. Values are means ± S.E.M. of 4 to 6 independent experiments. Statistics: Student's *t*-test for paired samples $*P < 0.05$.

increase further up to 0.1 µg/ml. With the low recombinant rat C5a concentrations no significant increase in PGD_2, $PGF_{2\alpha}$ or PGE_2 formation was observed. The concentrations of recombinant rat C5a, that were needed to increase prostaglandin formation, were in the same range as the concentrations of human C3a, that were needed to increase prostanoid formation in rat Kupffer cells [17], and the concentrations of C5a, that were reached in human serum after complete activation of the complement system [19]. However, in other biological systems native rat C5a elicited half maximal effects already at lower concentrations. Thus, the ED_{50} in a leukocyte chemotaxis assay was about 0.01 µg/ml [26], and the ED_{50} for the contraction of guinea pig ileum was even as low as 0.3 ng/ml [26]. Most likely these variations in the biological efficiency of rat C5a reflect differences in the sensitivity of the cell types or tissues used in the biological assay systems. However, the recombinant rat C5a used in the current investigation was synthesized according to sequence data in the Swiss-Prot protein sequence data bank. It differed from the recently published sequence [26] in amino acid position 55 (K- > N), 63 (P- > H), 68 (Q- > E) and contained 12 additional N-terminal residues (see section 2). In addition the recombinant protein was not glycosylated. Thus it is possible, that the differences in the biological efficiency of the recombinant rat C5a preparation from the native rat C5a [26] may also partially reflect sequence differences and the lack of glycosylation. However, differences in sequence and glycosylation probably play only a minor role, since recombinant rat C5a and native human C5a were almost equally potent in the release of *N*-acetyl-β-D-glucosaminidase from human granulocytes (see below).

3.2. *Specificity of recombinant rat C5a-mediated formation in rat liver macrophages*

The increase in prostanoid formation by 0.1 µg/ml recombinant rat C5a was inhibited by the neutralizing monoclonal antibody QD7, if recombinant rat C5a was incubated with a 5-fold molar excess of the antibody 2 h prior to the experiment (Fig. 3). Incubation of C5a with buffer for the same period did not result in a loss of activity (not shown). The increase in prostanoid formation elicited by human C3a was not inhibited by QD7 (not shown). These results clearly indicate, that the action of recombinant rat C5a on Kupffer cells is due to a specific action of the peptide and cannot be attributed to a contamination with e.g. endotoxin. In addition, the endotoxin concentration in the recombinant rat C5a preparation used was in the range of 1 µg/ml. This would yield a concentration of 0.04 µg/ml in the incubation mixture at the highest recombinant rat C5a concentration tested. This endotoxin concentration is far

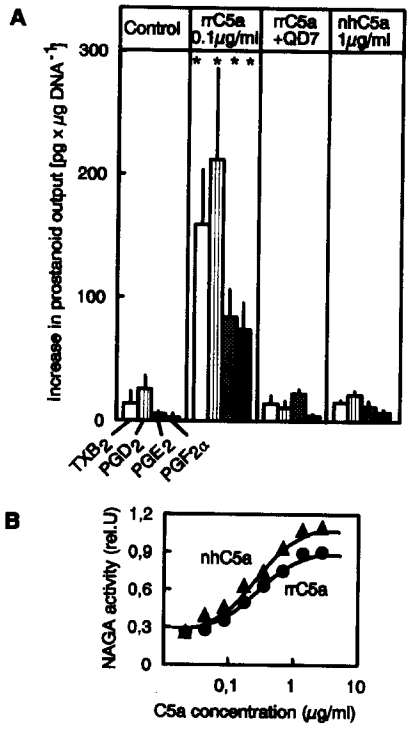

Fig. 3. Specificity of prostanoid formation elicited by recombinant rat C5a in rat liver macrophages (A) and human granulocytes (B). (A) Kupffer cells were isolated and cultured as described in Fig. 1. Medium was replaced by Hanks buffered salt solution. Cells were stimulated with 0.1 µg recombinant rat C5a/ml (final concentration, rrC5a), that had been pre-incubated with a 5-fold molar excess of the neutralizing antibody QD7 for 2 h in a 500 µg/ml stock solution where indicated, or with 1 µg native human C5a/ml (nhC5a). After 10 min supernatants were removed and the increase in prostanoid concentration over basal levels at 0 min, was determined by radioimmuno assay. Values are means ± S.E.M. of 4 to 6 independent experiments. Statistics: Students *t*-test for paired samples $*P < 0.01$. (B) Freshly isolated human granulocytes where stimulated with the concentration of native human C5a (nhC5a) or recombinant rat C5a (rrC5a) indicated and the release of *N*-acetyl-β-D-glucosaminidase was determined in an optical test. Values represent arbitrary units of *N*-acetyl-β-D-glucosaminidase activity determined in duplicate points in a single representative experiment.

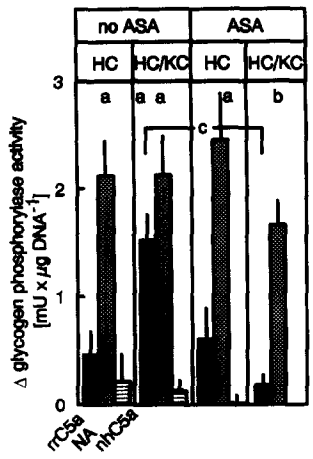

Fig. 4. Increase in glycogen phosphorylase activity elicited by recombinant rat C5a in co-cultures of rat liver macrophages and rat hepatocytes. Rat Kupffer cells were isolated and purified as described in Fig. 1 and cultured in RPMI medium containing 30% newborn calf serum for 48 h on collagen coated tissue culture dishes. Then, hepatocytes that had been isolated by an EDTA perfusion and purified by percoll density gradient centrifugation, were seeded on top of the Kupffer cells or in separate culture dishes. Culture was continued for another 24 h. Where indicated, cells were incubated with 500 μM acetylsalicylic acid (ASA) for 30 min prior to the experiment. Medium was then removed and replaced by Hanks' balanced salt solution. Cells were stimulated by the addition of recombinant rat C5a (rrC5a, 1 μg/ml), noradrenaline (NA, 1 μM) or native human C5a (nhC5a, 1 μg/ml). Supernatants were removed and the increase in glycogen phosphorylase activity over basal levels was determined in the cell homogenates. Values are means ± S.E.M. of the increase over basal activity in 4 or 5 independent experiments. Basal activities were: hepatocyte mono-cultures (HC) 1.6 ± 0.3 mU/mg hepatocyte DNA, hepatocyte/Kupffer cell co-cultures (HC/KC) 0.9 ± 0.1 mU/mg hepatocyte DNA, assuming that 65% of the total DNA was due to hepatocytes [Hespeling, Jungermann, Püschel, unpublished observation]. Statistics: Student's *t*-test for paired samples: significantly greater than basal a: $P < 0.01$, b: $P < 0.05$; increase in glycogen phosphorylase activity in untreated co-cultures significantly greater than in acetyl salicylic acid treated cultures c: $P < 0.05$.

below the concentration that has been described to increase prostanoid formation in rat Kupffer cells (100 μg/ml) [9].

Native human C5a, in contrast to recombinant rat C5a, did not increase prostanoid formation in rat Kupffer cells even when added in concentrations up to 1 μg/ml. Thus, native human C5a was not able to interact with C5a receptors on rat liver macrophages. In line with this hypothesis, native human C5a did not increase glycogenolysis in perfused rat liver [3]. By contrast, recombinant rat C5a and purified human C5a stimulated the release of *N*-acetyl-β-D-glucosaminidase (NAGA) from human leukocytes almost equally well (Fig. 3).

3.3. Stimulation by recombinant rat C5a of glycogen phosphorylase activity in hepatocyte/Kupffer cell co-cultures

In hepatocyte mono-cultures recombinant rat C5a increased glycogen phosphorylase activity only slightly by 1.25-fold (Fig. 4). This increase, though statistically not significant, might reflect the presence of C5a recptors in low density on hepatocytes. The presence of a C5a receptor on hepatocytes would be in line with the recent demonstration of C5a receptors by in situ hybridization in human liver and by binding studies on HepG2 hepatoma cells [27].

In contrast to monocultures, recombinant rat C5a increased glycogen phosphorylase activity to a much larger extent, i.e. about 3-fold, in co-cultures of rat hepatocytes and Kupffer cells (Fig. 4). The difference between mono-cultures and co-cultures was abolished, if cultures were incubated with the irriversible inhibitor of cyclooxygenase acetylsalicylic acid prior to stimulation with recombinant rat C5a. Thus, the increase in glycogen phosphorylase activity in co-cultures must have been largely mediated by the release of prostanoids from Kupffer cells. Prostanoids in turn can be directly activate glycogen phosphorylase in hepatocytes via a phospholipase C linked signal chain [7,8,10,11]. In line with this conclusion, the increase in glycogen phosphorylase activity by noradrenaline, which acts primarily directly on hepatocytes, was not attenuated by incubation with acetylsalicylic acid (Fig. 4). Native human C5a was inactive also in this system (Fig. 4).

4. Conclusion

C5a produced locally within the liver during regional inflammatory processes or produced systemically during severe sepsis may increase glycogenolysis in hepatocytes and thus glucose output via an increase in prostanoid release from liver macrophages, i.e. by intrahepatic intercellular communication. A direct stimulation by C5a of glycogenolysis in hepatocytes may play, if any, only a minor role.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft through the SBF 402, Teilprojekt B4, B5 and B6 and SFB 236, Teilprojekt A11, A24 and B6. The technical assistance of A. Nolte is gratefully acknowledged.

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