The Effects of Cholesterol-3-sulfate (CH-3S) on the Phosphorylation of Human C3a (hC3a) in Vitro and on the Ability of hC3a to Induce Vascular Permeability in Rats

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The phosphorylation of human C3a (hC3a, anaphylatoxin) by two distinct protein kinases (PKA and CK-I) and the effect of cholesterol-3-sulfate (CH-3S) on this phosphorylation were biochemically investigated in vitro. It was found that (i) hC3a functions as a phosphate acceptor for PKA and CK-I, but not for CK-II; (ii) the CK-I-mediated phosphorylation of hC3a requires the presence of 3 μM CH-3S in a manner similar to the phosphorylation of HMG1 (CH-3S-binding protein) by CK-I; and (iii) CH-3S inhibits the PKA-mediated phosphorylation of hC3a in a dose-dependent manner (ID₅₀ = approx. 2 μM). As expected, hC3a containing high levels of Arg- and Lys-residues stimulated approx. 3-fold CK-II activity (phosphorylation of α-casein) in vitro. However, no significant effect of hC3a on CK-II activity was observed when hC3a was preincubated with CH-3S or fully phosphorylated by PKA in vitro. Furthermore, preincubation of hC3a with CH-3S diminished the ability of hC3a to induce vascular permeability in rats. The results provided here suggest that (i) hC3a is a CH-3S-binding protein; and (ii) CH-3S functions as a potent inhibitor for its physiological activities, including phosphorylation by PKA and CK-I, in vitro.

Key words: cAMP-dependent protein kinase; casein kinase I; casein kinase II; human C3a; cholesterol-3-sulfate; sulfatide

Serum complement (C) plays an important role in many acute inflammatory processes. C3 is the most common complement protein in human serum (approx. 1.2 mg/ml) and is phosphorylated in vitro by typical serine (Ser)/threonine (Thr)-protein kinases, such as cAMP-dependent protein kinase (PKA), Ca²⁺/calmodulin-dependent protein kinase (Ca²⁺/CaM-kinase), and two casein kinases (CK-I and CK-II), which have different biological effects. The C3α moiety of C3 is known to be phosphorylated by both PKA and CK-I in vitro.1) This phosphorylation renders C3 more resistant to cleavage at the Arg⁷⁷-Ser⁷⁸ bond either by trypsin or both the classical and alternative pathway convertases.4) In addition, casein kinase (with different sensitivities to specific inhibitors and specific antibodies of CK-I and CK-II) released from activated human platelets phosphorylates both α- and β-polypeptide chains of C3 and increases in its susceptibility to cleavage by elastase.5) A subsequent effect of C3 phosphorylation by platelet casein kinase is to enhance the opsonization of its immune complexes.5) Recently, we reported that (i) both human complement C3 and C3α (anaphylatoxin: 77 amino acid residues) are glycyr rhizin (GL)-binding proteins; (ii) both GL and glycyr rhetic acid (GA) induce conformational changes of hC3; and (iii) the GL-induced inhibition of the physiological activities, including phosphorylation, of hC3 may be involved in the anti-inflammatory effect of GL in vivo.7)

CK-I is a ubiquitous and highly conserved second messenger-independent Ser/Thr-protein kinase with a molecular weight of 25—55 kDa.8,9) At least five isoforms (α, β, γ, δ, and e) have been identified from a variety of cell sources.8,9) It is well-known that these CK-I isoforms are implicated in a diverse number of cellular functions, including DNA replication,10) DNA repair,11) nuclear shuttling of transcriptional factors,12) Wnt signaling,13) and circadian rhythms.14) However, the exact details of these CK-I-mediated regulatory mechanisms are unclear at present. Recently, we reported that (i) CK-I preferentially phosphorylates Thr-residues on high mobility group protein 1 (HMG1)15,16) and a isoform η of C-kinase (C-kinase η)17) in the presence of cholesterol-3-sulfate (CH-3S), an effective activator of C-kinase η,18) in vitro; and (ii) CK-I mediates an activation of C-kinase η in the presence of CH-3S in vitro.17) In addition, both HMG1 and C-kinase η are identified as CH-3S-binding proteins, since (i) CH-3S directly induces drastic conformational changes of HMG1,15) and (ii) CH-3S directly stimulates autophosphorylation of CK-I in vitro.7)

As regards the physiological significance of CH-3S in cholesterol metabolism and in the clinical conditions involved in recessive X-linked ichthyosis and liver cirrhosis, it has been reported that CH-3S (i) is widely distributed in various body fluids (e.g. the circulating concentration of CH-3S in human plasma: approx. 2.0 μM19) and in tissues and cells, including erythrocytes, platelets, skin, hair, adrenals, lung and brain;20,21) (ii) is a potent inhibitor of several enzymes, such as 3-hydroxyl-3-methylglutaryl CoA reductase,22) phosphatidyl inositol-3-kinase,23) proteases (trypsin, chemotrypsin and pronase),24) and plasma thrombin and plasmin,25) (iii) acts as an endogenous regulator of mammalian cholesterol biosynthesis,26) and (iv) selectively inhibits steroidogenesis in steroid-sulfatase deficient fibroblasts derived from patients with recessive X-linked ichthyosis27) and liver cirrhosis.28) Furthermore, the sulfation of cholesterol may play a role in the differentiation of epithelial cells,29,30) the integrity of lipid membrane and the capacititation of serum.31) Although CH-3S may function as an effective activator for the CK-I-mediated phosphorylation of these two CH-3S-binding proteins (HMG1 and C-kinase η) in vitro,15—17) the physiological correlation between hC3a (anaphylatoxin) and CH-3S, and identification of the protein kinases responsible for the physiological regulation of hC3a remain to be elucidated.

The present study was carried out to characterize the phosphorylation of hC3a by PKA and CK-I, and to determine the

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effect of CH-3S on the physiological activities of hC3a in vitro and in rats. Here, we describe (i) the further characterization of hC3a as a CH-3S-binding protein; and (ii) the inhibitory effect of CH-3S on the phosphorylation of hC3a by PKA and CK-I in vitro, and on the ability of hC3a to induce vascular permeability in rats.

MATERIALS AND METHODS

Chemicals [γ-32P]ATP (3000 Ci/mmol) and [125I]-labeled hC3a (500 mCi/ml) were obtained from Amersham Pharmacia Biotech (Arlington Heights, U.S.A.). Human C3a (96% purity) was obtained from CN biosciences, Inc. (La Jolla, CA, U.S.A.); dithiothreitol (DTT), dephosphorylated α-casein (an effective substrate for both CK-I and CK-II in vitro), heparin and CH-3S from Sigma Chemical Co. (St. Louis, U.S.A.); and CK-I-7 [N-(2-amino-ethyl)-5-chloro-iso-quinoline-8-sulfonamide (a specific CK-I inhibitor)] from Seikagaku Kogyo (Tokyo, Japan). A synthetic human lactoferricin (hLFcin, 47 amino acids) was obtained from Tana Laboratories (Texas, Tokyo, Japan). Recombinant human CK-II (rhCK-II) [a heterodimer of αβ2 (α-subunit = 44 kDa and β-subunit = 26 kDa); specific activity 400 kunits/mg] was obtained from Promega Co. (Madison, U.S.A.). GL (20β-carboxyl-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranosuronosyl-3β-o-glucopyranosidouronic acid) and GA (olean-12-en-3β-ol-11-one-30-oic acid) were kindly supplied by Minophagen Pharmaceutical Co. Ltd. (Tokyo).

Protein Kinases Rat liver CK-I (specific activity: 17500 units/mg) was determined from Promega Co. (Madison, U.S.A.). Recombinant human CK-II (rhCK-II) [a heterodimer of αβ2 (α-subunit = 44 kDa and β-subunit = 26 kDa); specific activity 400 kunits/mg] was obtained from Tana Laboratories (Texas, U.S.A.). GL (20β-carboxyl-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranosuronosyl-3β-o-glucopyranosidouronic acid) and GA (olean-12-en-3β-ol-11-one-30-oic acid) were kindly supplied by Minophagen Pharmaceutical Co. Ltd. (Tokyo).

Phosphorylation of hC3a by PKA and CK-I in Vitro Phosphorylation of hC3a by PKA was assayed in reaction mixtures comprising 40 mM Tris–HCl (pH 7.6), purified PKA (approx. 50 ng), 5 μM [γ-32P]ATP (500 cpm/pmol), 1 mM MnCl2, 2 mM DTT, 1 μM AMP and hC3a (approx. 4 μg). Phosphorylation of hC3a by CK-I was carried out by incubating purified hC3a (approx. 4 μg) with 5 μM [γ-32P]ATP (500 cpm/pmol) in reaction mixtures comprising 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 3 mM MnCl2, CK-I (approx. 20 ng) and 3 μM CH-3S, as reported previously. After incubation for 20 min at 30 °C, 32P-labeled hC3a in the reaction mixtures was detected directly by autoradiography after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), as reported previously.

Assay for the Stimulatory Effect of hC3a on CK-II Activity in Vitro To determine the stimulatory effect of hC3a on CK-II activity (phosphorylation of α-casein), α-casein (approx. 5 μg) was incubated with CK-II (approx. 20 ng) and 5 μM [γ-32P]ATP (500 cpm/pmol) in reaction mixtures comprising 40 mM Tris–HCl (pH 7.6), 2 mM DTT and 3 mM MnCl2, as reported previously. After incubation for 20 min at 30 °C in the presence or absence of the indicated doses of CH-3S, 32P-labeled α-casein (p35) in the reaction mixtures was detected directly by autoradiography after SDS-PAGE, as reported previously.

Assay for the Ability of hC3a to Induce Vascular Permeability in Rats Male 8-weeks-old SD rats (Japan SLC Inc., Shizuoka, Japan) were anaesthetized with ether. Five minutes after i.v. injection of pontamine sky blue (50 mg/kg, i.p.), 0.5 μg/site of hC3a were injected intradermally (i.d.) into three sites of the shaved dorsal skin. The rats were killed by exsanguination 20 min after i.d. injection of hC3a for the determination of leaked serum. Blood was taken before exsanguination to obtain serum. The exuded dye in the skin at each site was extracted by the method reported previously. In brief, excised skin was incubated overnight in 0.5 ml of 1 M KOH at 37 °C, neutralized with 1.25 ml of 1.2 M phosphoric acid, and then extracted with 3.75 ml acetone. After centrifugation for 20 min at 1200×g, the optical density of supernatant and serum was measured at 620 nm with a spectrophotometer.

RESULTS

Characterization of the Phosphorylation of hC3a by PKA and CK-I in Vitro hC3a was incubated with either PKA or CK-I and 5 μM [γ-32P]ATP (500 cpm/pmol) under their optimum assay conditions. It was found that PKA phosphorylates hC3a in vitro (lane 8) and this phosphorylation is significantly enhanced in the presence of 1 μM cAMP (lane 4, Fig. 1A). The PKA-mediated phosphorylation of hC3a in the presence of 1 μM cAMP increased time-dependently up to 90 min and reached a plateau within 120 min (Fig. 1B). This phosphorylation was inhibited by CH-3S in a dose-dependent manner (ID50 = approx. 2 μM, but a relatively high dose of CH-3S was required to inhibit autophosphorylation of PKA (ID50 = approx. 50 μM; Fig. 1C). In contrast, CK-I phosphorylated hC3a in the presence of CH-3S (lane 3, Fig. 2A) and this phosphorylation was sensitive to heparin (lane 4) and CK-I-7 (lane 5, Fig. 2A). In the presence of 3 μM CH-3S, the CK-I-mediated phosphorylation of hC3a increased time-dependently up to 60 min and reached a plateau within 90 min (Fig. 2B). The effect of CH-3S on the CK-I-mediated phosphorylation of hC3a in vitro was biphasic: increasing in a dose-dependent manner of CH-3S up to an optimum dose of CH-3S at approx. 3 μM and decreasing dose-dependently at higher doses above 10 μM (Fig. 2C). Autophosphorylation of CK-I was slightly stimulated at lower doses (0.1—3 μM) of CH-3S, whereas it was dose-dependently inhibited at higher doses above 10 μM (Fig. 2C). However, no significant effect of 1 μM CH-3S on the CK-I-mediated phosphorylation of α-casein was observed and CH-3S, at higher doses above 1 μM, inhibited it in a dose-dependent manner (Fig. 2C). These results suggest that (i) both PKA and CK-I can phosphorylate hC3a in vitro; and (ii) CH-3S may induce the extreme phosphorylation of hC3a by CK-I in a manner similar to the CK-I-mediated phosphorylation of HMGl (CH-3S-binding protein) in vitro.

Characterization of Other Sulfated Derivatives on the CK-I-Mediated Phosphorylation of hC3a as Effectors in Vitro Previously, we reported that the CK-I-mediated phosphorylation of C-kinase η is significantly stimulated by CH-3S or sulfatide in vitro. As expected, a similar stimulation of the CK-I-mediated phosphorylation of hC3a was observed when incubated with sulfatide (cerebroside sulfate) instead of CH-3S (Fig. 3). However, no phosphorylation of hC3a by CK-I was detected when incubated with three sulfated derivatives (dehydro-i-androsterone-3-sulfate, 5-pregnen-3β-o-
20-one-sulfate and cholesterol p-toluene-sulfate) or five other homologous compounds (cholesteryl phosphoryl choline, bufaline, bassicasterol, glycyrrhetinic acid and cholesteryl 3-phosphate), as has been previously reported.

The Stimulatory Effect of hC3a on CK-II Activity in Vitro

Since hC3a is a basic protein, like HMG110,11 and human lactoferricin (hLFcin),33 the stimulatory effect of hC3 on CK-II activity (phosphorylation of α-casein) was examined in vitro. CK-II was incubated with 5 μM [γ-32P]ATP (500 cpm/pmol) and α-casein in the presence of the indicated doses of hC3a or hLFcin as a CK-II activator in vitro. Although both hC3a and hLFcin did not function as phosphate acceptors for CK-II under the given experimental conditions, hC3a stimulated CK-II activity about 3-fold at the optimum dose of 1.0 μg/ml (Fig. 4A). In contrast, hLFcin at 10 μg/ml stimulated it about 8-fold (Fig. 4A). No stimulation of CK-II activity was observed when hC3a was preincubated with 1 μM CH-3S (lane 5), whereas no reduction in this stimulation was observed upon preincubating hC3a with GA (lanes 3—5, Fig. 4B). No significant effect of CH-3S at 1 μM on CK-II activity was observed (Fig. 2C). These results suggest that the direct binding of CH-3S to hC3a may diminish to stimulate CK-II activity in vitro.

After full phosphorylation of hC3a by PKA (incubation for 120 min at 30°C), hC3a previously phosphorylated by PKA did not significantly stimulate CK-II activity in vitro.
The PKA-mediated reduction of hC3a was prevented in vitro by a specific PKA inhibitor (gallory pedunculagin). In addition, only Thr-residues on the hC3a phosphorylated by PKA in the presence of 1 mM cAMP and 5 mM \(^{32}\)P-ATP (500 cpm/pmol) were detected (Fig. 5B). These results suggest that hC3a may diminish to stimulate CK-II activity after its full phosphorylation by PKA in vitro.

The Inhibitory Effect of CH-3S on the Ability of hC3a to Induce Vascular Permeability in Rats

It is well known that hC3a can induce vascular permeability in rats. This was confirmed to occur in a dose-dependent manner of hC3a (Fig. 6A). The preincubation of hC3a with CH-3S reduced the ability of hC3a to induce vascular permeability in a manner dose dependent upon CH-3S (Fig. 6B). These results show that the direct binding of CH-3S to hC3a may reduce its ability to induce vascular permeability in vivo.

DISCUSSION

In the present study, it was found that the CK-I-mediated phosphorylation of hC3a requires CH-3S as an effector in vitro (Figs. 2A, C) and, in the presence of 3 \(\mu\)M CH-3S, the maximum phosphorylation of hC3a by CK-I is observed (Fig. 2C). This optimum dose (3 \(\mu\)M) is close to a physiological circulating concentration (approx. 2.0 \(\mu\)M) of CH-3S in normal human plasma. The phosphorylation kinetics of hC3a by CK-I in the presence of 3 \(\mu\)M CH-3S in vitro were that hC3a can induce vascular permeability in rats. This was confirmed to occur in a dose-dependent manner of hC3a (Fig. 6A). The preincubation of hC3a with CH-3S reduced the ability of hC3a to induce vascular permeability in a manner dose dependent upon CH-3S (Fig. 6B). These results show that the direct binding of CH-3S to hC3a may reduce its ability to induce vascular permeability in vivo.
similar to those observed with HMG1 (CH-3S-binding protein) containing high levels of Arg- and Lys-residues. In deed, the significant stimulation of the CK-I-mediated phosphorylation of hC3a was observed when incubated with sulfatide instead of CH-3S (Fig. 3), as has been shown in the phosphorylation of C-kinase η by CK-I in vitro. However, no phosphorylation of hC3a was observed with other sulfated derivatives and their related compounds, except CH-3S and sulfatide, in vitro.

The following observations: (i) CH-3S at lower doses (0.1—3 μM) significantly stimulates autophosphorylation of CK-I (Fig. 2C); and (ii) CH-3S, at an optimum dose (3 μM), acts as an effective activator for the CK-I-mediated phosphorylation of hC3a (Fig. 2C) in a manner similar to the phosphorylation of HMG1 and C-kinase η by CK-1 in vitro, suggest that the direct activation of CK-I by CH-3S at lower doses (0.1—3 μM) may be involved in the CH-3S-induced extreme phosphorylation of CH-3S-binding proteins (hC3a, HMG1 and C-kinase η) by CK-1 in vitro. Although there are no potential phosphorylation sites (R-R-X-S/T) for PKA on hC3a, only Thr-residues on hC3a was phosphorylated by PKA in vitro (Fig. 5B). These results suggest that the direct binding of CH-3S may induce its conformational changes, leading to provide a novel phosphorylation site (Thr-residue) for CK-I in vitro, as has been shown in the CH-3S-induced extreme phosphorylation of HMG1 by CK-I.

The effect of CH-3S on the phosphorylation of hC3a by PKA and CK-I was determined in vitro. It was found that CH-3S inhibits the PKA-mediated phosphorylation of hC3a in a dose-dependent manner (ID50 = approx. 2 μM) and a relatively high dose (ID50 = approx. 50 μM) is required to inhibit autophosphorylation of PKA in vitro (Fig. 1C). Interestingly, hC3a stimulated CK-II activity (phosphorylation of α-casein) about 3-fold (Fig. 4A), which may be related to the fact that hC3a contains high levels of Arg- and Lys-residues, as in other basic proteins such as HMG1 and hFcIn. It was further concluded that hC3a is a CH-3S-binding protein, because (i) the phosphorylation of hC3a by CK-I requires the presence of CH-3S (lane 3, Fig. 2A); (ii) preincubation of hC3a with CH-3S may diminish its stimulatory effect on CK-II activity in vitro (Fig. 4B); and (iii) CH-3S dose-dependently inhibits the ability of hC3a to induce vascular permeability in rats (Fig. 6B). These results suggest that CH-3S may be a potent inhibitor for the physiological activities of hC3a through its specific phosphorylation by CK-I in the presence of CH-3S or its phosphorylation by PKA in vitro. Since CH-3S dose-dependently inhibits both the CK-I-mediated phosphorylation of hC3a in vitro and the ability of hC3a to induce vascular permeability in rats, the biological effect of hC3a generated from C3 during inflammation and immunological responses may be correlated with the serum level of CH-3S.

Although the CH-3S-binding sites on hC3a have been not yet determined, one of the potential phosphorylation sites for PKA on hC3a (77 amino acid residues) may correspond to the CH-3S-binding site comprising Arg- and Lys-residues, which can interact with the β-subunit of CK-II in vitro. It seems likely that hFcIn (N-terminal positions 1—47 of human lactoferrin) may contain at least a potential CH-3S-binding site comprising four amino acid residues (positions 48—51: RRTR) including a Thr-residue (a potential phosphorylation site for PKA) on hC3a. This possibility is supported by evidence that (i) the phosphorylation of hC3a by PKA is inhibited by CH-3S in a dose-dependent manner (ID50 = approx. 2 μM) in vitro (Fig. 1C); and (ii) no stimulatory effect of hC3a on CK-II activity is observed after its full phosphorylation by PKA in vitro (Fig. 5A). Therefore, the phosphorylation of hC3a at Thr-50 by PKA may alter the ionic strength of an Arg-rich region of hC3a in its interaction with the β-subunit of CK-II in vitro. Wang et al. have demonstrated that (i) the serum level of HMG1 increases after the administration of endotoxin, and injection of HMG1 itself is lethal; (ii) delayed administration of antibodies to HMG1 attenuates endotoxin lethality; and (iii) proinflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), induce the release of HMG1 from pituitary cells in time- and dose-dependent manners. In addition, sulfatide has a high binding affinity with HMG1. Therefore, it seems likely that both HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. Our previous reports concerning HMG1 and hC3a (anaphylatoxin) are basic proteins with similar biochemical properties in vitro: (i) the direct binding of CH-3S or sulfatide to these two basic proteins may result in the induction of their extreme phosphorylation by CK-I in vitro; and (ii) these basic proteins may participate in physiological regulation of the inflammatory response as inflammatory mediators. 

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