Serum Prostacyclin Stabilizing Factor Is Identical to Apolipoprotein A-I (Apo A-I)

A Novel Function of Apo A-I

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Abstract

Serum PGI₂ stabilizing factor (PSF) was purified from human serum to a single protein with a molecular weight of 28,000 D by SDS-PAGE. Analyses of NH₂-terminal sequence (32 residues), COOH-terminal sequence (3 residues) and the composition of amino acids disclosed its homology with human apolipoprotein A-I (Apo A-I), a major apolipoprotein of HDL. Apolipoprotein A-II, C-I, C-II, C-III, D and E, as well as LDL, and VLDL did not possess this activity. The alpha-helix structure of Apo A-I is necessary for the binding of PGI₂-HDL and nascent HDL reconstituted from Apo A-I and phospholipid significantly prolonged the half-life of PGI₂. PGI₂ stabilization by HDL and Apo A-I may be an important protective action against the accumulation of platelet thrombi at sites of vascular damage. The beneficial effect of HDL in the prevention of coronary artery disease may be partly due to this action.

Introduction

PGI₂ (prostacyclin) is synthesized by the vascular endothelium and smooth muscle. PGI₂ is a potent vasodilator and inhibitor of platelet aggregation (1). Two factors in serum are reported to regulate PGI₂ metabolism: PGI₂ synthesis stimulating factor and PGI₂ stabilizing factor (PSF)(2). PGI₂ is an unstable substance. However, it binds to PSF resulting in a more stable substance. Recently, decreased PSF activity has been reported in thrombotic disorders (acute myocardial infarction (2), thrombotic thrombocytopenic purpura (3) and ischemic stroke) (4) and PSF is suspected to play an important role in the pathogenesis of the thrombus formation in these diseases. Albumin has been considered as a candidate for the stabilizing factor (5–7). However, PSF has not been analyzed biochemically.

In this study, we purified this factor and found that this factor is identical to Apo A-I, a major apolipoprotein of HDL.

We also found that Apo A-I is present in the albumin preparations. Our finding suggests that the antiatherogenic action of HDL or Apo A-I (8–11) may work partly through PGI₂ stabilization.

Methods

Evaluation of PSF activity. The activity of PSF was evaluated by gel filtration using [3H]PGI₂ sodium and a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column as previously described (3). [3H]PGI₂ methyl ester (12 Ci/mmol, New England Nuclear, Boston, MA) was reduced to [3H]PGI₂ sodium and purified by TLC (12). [3H]6-keto-PGF₁α can not bind PSF. Protein was determined by the method of Lowry et al. (13).

Electrophoresis. SDS-PAGE was performed using the 2/16 gradient gel (Pharmacia) for Fig. 2. For Fig. 5, Phast-system (Pharmacia) was used with 10–15% gel. Procedures were performed according to the manufacturer’s directions. Western blotting was performed using the Bio-Rad system (Bio-Rad Laboratories, Richmond, CA) and the blotting detection kit (Amersham Corp., Arlington Heights, IL). The antibody for human Apo A-I was obtained from Daiichi Chemical Pharmaceutical Company (Tokyo, Japan).

NH₂-terminal, COOH-terminal and amino acid analysis. Subunits were separated by reversed-phase HPLC (Cosmosil 5TMS-300 column, 4.6 mm × 50 mm, Nakarai Chemical Co., Kyoto, Japan) eluted with a linear gradient from 20% B buffer to 80% B buffer in 30 min at a flow rate of 0.8 ml/min at 35°C. The column was monitored at 220 nm (buffer A: 10% CH₃CN, 90% water, 0.1% trifluoroacetic acid (TFA), buffer B:90% CH₃CN, 10% water, 0.1% TFA). For the collected peak, automated Edman degradation was performed on a gas vapor sequencer (model 470 A; Applied Biosystem, Foster City, CA). This instrument is equipped with an on-line isocratic phenylthiohydantoin amino acid analysis system using a Spectra-Phyisc HPLC and a Senshu-Pak SEQ-4 column (4.6 mm × 30 cm; Senshu Chemical Company, Tokyo, Japan). The COOH-terminal analysis was performed by the digestion of carboxypeptidase Y. The amino acid composition of HPLC-purified protein was obtained after 20 h of hydrolysis (4 N methanoulsofonic acid, at 110°C) followed by analysis on an Arika model A-5500 analyzer (Irika Co., Tokyo, Japan).

Preparation of HDL, LDL, APO C, D, and E. HDL and LDL were prepared from pooled human serum (10 normal men) by the method of Havel et al. (14). Apolipoproteins A, C, D, and E were prepared from human (very) low density lipoprotein or HDL by the methods of Edelstein (15), Shore (16), and Shellburne (17), respectively. Cholesterol, phospholipid, and triglyceride were determined with the analyzing system by Wako Pure Chemical Co. (Tokyo, Japan).

Nascent HDL. Nascent HDL was reconstituted from Apo A-I (1.2 mg; Sigma Chemical Co., St. Louis, MO), bovine brain sphingomyelin (2.5 mg) and dipalmitoylphosphatidylcholine (2.5 mg, both from Sigma Chemical Co.) (18, 19).

Determination of Apo A-I levels. Apo A-I level was determined by a single radial immunodiffusion (SRID) method (Daichi Chemical Pharmaceutical Co.). This antisera did not bind to albumin.

Evaluation of PGI₂ half-life. The stability of PGI₂ was determined by a quantitative HPLC method (5, 20). Samples were injected onto a YMC A-314 C18 column (6 mm × 30 cm; Yamamura Chemical Industry, Kyoto, Japan). The mobile phase was a methanol/boric acid buffer (40 mM, pH 8.9) = 55/45 (vol/vol) with a flow rate of 1.0 ml/min. This method separates and quantitates both PGI₂ and 6-keto-...
Apo A-I immunos affinity column. The Apo A-I immunos affinity column (21) was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan). Serum (0.25 ml) was applied to the column (1 ml of gel volume), which was equilibrated with 0.01 M PBS (pH 7.2). After washing with 20 ml of 0.01 M PBS (pH 7.2), the Apo A-I particles were eluted with 7.5 ml of 0.5 M NaCl/1 M acetic acid. After the neutralization by 3.5 M Tris·HCl (pH 7.4), the eluent was dialyzed against 0.01 M PBS (pH 7.2).

Results

Human serum (50 ml) was applied to the Blue-Sepharose CL-6B (Pharmacia) column. PSF appeared by the elution of 0.2 M sodium thiocyanate (NaSCN) in 50 mM Tris-HCl, pH 7.4 (Fig. 1 A). Albumin was eluted in the second peak. The recovery rate was 60%. The eluent was concentrated using a PM-10 membrane in a stirred cell (Amicon Corp., Danvers, MA). The concentrate was subjected to gel-permeation chromatography (Sephacryl S-300; Pharmacia) (Fig. 1 B). The recovery rate was 73%. The fractions of PSF were pooled and concentrated, and then subjected to high-performance adsorption and gel-filtration chromatography (GS-620P; Asahi Chemical Industry, Tokyo, Japan) (Fig. 1 C). The activity was present in the last tailing peak. The recovery rate was 62%. SDS-PAGE of that portion showed a single band of 28,000 D (Fig. 2). The native molecular weight was determined by gel-permeation chromatography with a Diol-300 column (1 cm × 50 cm; Yamamura Chemical Industry). The estimated value was 224 kD. The NH2-terminal amino acid sequence of this protein was H2N-Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg-Val-Lys-Asp-Leu-Ala-Thr-Val-Tyr-Val-Asp-Val-Leu-Lys-Asp-Ser-Gly-Arg-Asp-Val-Tyr-Val-Ser-Gln. This sequence was identical to that of human Apo A-I (22). The COOH-terminal of this protein was HOOC-Gln-Thr-Asn. This sequence was also identical to that of Apo A-I (22). Comparison of the amino acid composition of PSF with that of human Apo A-I (22) revealed strong similarities (Table I). Thus, we concluded that PSF is identical to Apo A-I. The chemical composition of the active fractions from GS-620P was as follows; 42% phospholipids 1% triglycerides, 1% cholesterol, and 56% protein.

There was a linear relationship between HDL concentration and PSF activity (data not shown). Apo A-I is a major apolipoprotein of HDL. Fig. 3 A shows the relationship between Apo A-I concentration and PGII binding activity. PSF activity varies in parallel with the concentration of Apo A-I (solid circles, Fig. 3 A). However, by delipidation of HDL by ethanol-diethyl ether (23), the activity of PSF was lost. Nascent HDL reconstituted from Apo A-I, sphingomyelin, and dipalmitoylphosphatidylcholine showed [3H]PGII binding activity. Delipidated Apo A-I and delipidated nascent HDL can not bind [3H]PGII. Thus, an alpha helix structure of Apo A-I seems to be necessary for the binding of PGII. No binding was observed with other apolipoproteins of HDL such as apolipoprotein A-II (reconstituted as Apo-AII, CI, CII, CIII, D, and E, or LDL and VLDL).

In d > 1.21 g/ml fraction and d < 1.21 g/ml fraction (lipoprotein fraction) of human serum, the percent PSF activity was 1.7 and 98.3%.

Fig. 4 shows the effect of the addition of HDL (50 mg as cholesterol) on the prolongation of PGII half-life at 24°C. By

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Figure 1. (A) Blue-Sepharose CL-6B chromatography. Affinity chromatography was performed by applying 50 ml of serum to a Blue-Sepharose CL-6B column (5 cm × 20 cm) that had been equilibrated with a 50 mM Tris·HCl, pH 7.4. The column was washed until the 280 nm absorbance became stabilized. After the successive elution with 500 mM CaCl2, 50 mM Tris·HCl, pH 7.4, and 2 M NaCl, 50 mM Tris·HCl, pH 7.4, the activity appeared by the washing with 200 mM NaSCN, and 50 mM Tris·HCl, pH 7.4. The active fractions were collected and concentrated using an Amicon stirred ultrafiltration cell with a PM-10 membrane, and passed through a PD-10 column (Pharmacia), using 50 mM Tris·HCl, pH 7.4. (B) Gel-permeation chromatography by Sephacryl S-300. The concentrated fraction was loaded onto a column (3 cm × 84 cm) of Sephacryl S-300 (Pharmacia) equilibrated in 50 mM Tris·HCl, pH 7.4 containing 100 mM NaCl. The flow rate was 1.0 ml/min. The fractions of binding activity were pooled and concentrated. (C) GS-620P column chromatography. The concentrated protein was injected to the high-performance adsorption and gel-permeation chromatographic column (2 cm × 50 cm, GS-620P). The flow rate was 5 ml/min.
the addition of HDL, the half-life was prolonged from 4.8 min (○) to 22.3 min (■).

Albumin has been reported to bind PGI₂ and to prolong PGI₂ activity (3–7). Indeed, the PSF activity was found in human albumin (Cohn fraction V albumin, Sigma). The contaminated band of Apo A-I was detected by SDS-PAGE with silver staining (protein band 7 in Fig. 5 B). The No. 7 protein in Fig. 5 B was identified as Apo A-I by the same purification method. This protein was also identified as Apo A-I using Western blotting. After the purification of albumin with Blue-Sepharose CL-6B as described in Fig. 1 A, the purified albumin showed no band of Apo A-I (Fig. 5 A) and was without activity.

There was a linear relationship between Apo A-I concentration in albumin (Cohn fraction V) and albumin concentration (open circles, Fig. 3 B). PSF activity also varied in parallel with concentration of Apo A-I in albumin (open circles, Fig. 3 A). Moreover, we were able to eliminate this PSF activity from human serum using an Apo A-I immunoaffinity column. From the above results, PSF in serum was considered to be Apo A-I.

Table I. Amino-acid Composition of PSF and Apo-AI

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<td>Thr</td>
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<tr>
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Asp + Asn was calculated as 21.

Figure 2. SDS-PAGE of the purified PSF. For the last tailing peak of GS-620P, SDS-PAGE was performed using the 2/16 gradient gel (Pharmacia) according to the manufacturer’s directions. The protein band was stained with Coomassie Brilliant Blue. A single 28-kD band was obtained.

Figure 3. (A) A relationship between Apo A-I concentration and PGI₂ binding activity. (○) Apo A-I in HDL prepared from human serum (as already stated in the method section); (■) Apo A-I in the albumin preparation in B. Apo A-I levels were determined by the SRID method. (B) A relationship between Apo A-I concentration in albumin and albumin concentration.

Figure 4. Effect of HDL on the prolongation of PGI₂ half-life. Each plot shows the mean±SD of five experiments at 24°C. (○) HDL (50 mg/dl as cholesterol level) in 50 mM Tris·HCl, pH 7.4. (■) 50 mM Tris·HCl, pH 7.4.
Thus, we carefully tested the activity of this fraction in 100 mM Tris· HCl buffer instead of 50 mM. However, PSF activity was not also found in albumin fraction.

NH₄-terminal analysis of this 28-kD protein revealed its identity with that of Apo A-I (22). Data on COOH-terminal analysis, and the analysis of amino acid composition were also compatible with those of Apo A-I (22). Apolipoprotein A-II, another apolipoprotein of HDL was not found in the SDS-PAGE shown in Fig. 2. This protein was separated from Apo A-I with Sephacryl S-300.

The chemical composition of HDL is as follows (24): cholesterol 20%, phospholipid 23%, triglyceride 5%, protein 50%, and others 2%. By the purification method which we employed (Blue-Sepharose CL-6B, gel-permeation chromatography by Sephacryl S-300 and GS-620P), PSF was purified from serum to Apo A-I, and other apolipoproteins in HDL, such as A-II, C-I, II, and III, D and E were excluded. During this purification, cholesterol content was decreased to 1% and phospholipid content was increased to 42%.

Nascent HDL reconstituted by Apo A-I and the phospholipid revealed PSF activity. However, delipidated Apo A-I has no binding activity. Thus, the alpha-helix structure of Apo A-I seems to be necessary for this activity. There was a linear relationship between HDL concentration and PSF activity. By delipidation of HDL, the activity of PSF was also lost.

By adding HDL, the half-life of PGI₂ prolonged significantly from 4.8 min (at 24°C, pH 7.4) to 22.3 min. The mechanism of this prolongation is unknown at this moment. LDL and LDL have only trace amounts of Apo A-I. These lipoproteins had no effect on the prolongation of the PGI₂ half-life.

PGI₂ stabilization by albumin has been studied using commercially available albums (3–7). However, in their papers, the purity of albumin was not examined by SDS-PAGE. As shown in Fig. 5 B, commercially available albumin (Cohn fraction V) has many contaminated bands. The Apo A-I band is also seen in the gel. This protein was identified as Apo A-I by Western blotting. In other types of albumin, such as fatty acid free or globulin-free albumin, many bands were also seen (data not shown). Purified albumin (Fig. 5 A) has no binding activity. Moreover, it is demonstrated that the comparative study in Fig. 3 A and B shows that Apo A-I in albumin contributes to the binding of PGI₂.

HDL levels were reported to be inversely related to the incidence and mortality rate of coronary heart disease (8–11). HDL is considered to promote the removal of free cholesterol from peripheral tissue and its transport to the liver for eventual clearance (25). Apo A-I appears to be a more useful index than HDL cholesterol for identifying patients with coronary artery disease (26). In addition to the activation of lecithin-cholesterol acyl-transferase (LCAT) (27), PGI₂ stabilization is thought to be an important new function of Apo A-I.

PGI₂ is reported to increase cholesteryl ester hydrolysis activity in rabbit aortic smooth muscle cells (28). At high levels of HDL, this hydrolysis activity may increase through PGI₂ stabilization. The increased cholesterol will bind to the high levels of HDL and will be carried to the liver.

Our findings suggest that when HDL and Apo A-I are reduced, the availability of PGI₂ at the sites of vascular damage may be reduced, thus inactivating the protection mechanism against platelet thrombus formation and vasoconstriction.
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References