Immunochemical Analysis of Common Antigenic Determinants in Insulin and Apoprotein B Molecules

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A study is performed of the mechanism underlying stress diabetes, which develops in human beings and animals under stress. Dot-immunoanalysis shows the presence of common antigenic determinants in insulin, apoprotein B, and apoprotein B-containing low density and very low density lipoproteins isolated from human and rat serum. Electrophoresis, immunoelectroblotting, and immunoenzyme analysis reveal 5-6 peptides belonging to apoB, which specifically react with anti-insulin and anti-very low density lipoprotein antibodies. Insulinlike immunoreactivity is also identified in human serum supernatant obtained after precipitation of the total low density and very low density lipoprotein fraction and after removal all lipoproteins from it.

Key Words: *lipoproteins; apoproteins; insulin; antigenic determinants*

Human and animal blood contains substances with pro- and contra-insulin activities [3,4]. The ultimate effect of insulin itself depends on the ratio between these substances. Some insulin-resistant forms of diabetes are directly related to the prevalence of substances with a contra-insulin activity in the blood [2]. Many of these substances have so far not been identified, and their nature remains unknown. Previously, we showed that tension diabetes develops in human beings and animals exposed to stress and we attributed this kind of diabetes to an increase in the blood content of a substance or substances with contra-insulin activity. Clinically this disease is manifested in reduced blood concentrations of sugar and insulin, diminished sensitivity of the pancreatic islet apparatus to glucose, a lowered renal barrier for sugar, and an elevated concentration of sugar in the urine [13]. The mechanism responsible for the development of tension diabetes remains unclear.

In the present study we identified antigenic determinants common for insulin and other serum proteins using immunochemical methods, the assumption being that these proteins contribute greatly to the development of both the pro- and contra-insulin effect of serum and modify the severity of tension diabetes.

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MATERIALS AND METHODS

The development of tension diabetes proceeds against the background of increased blood contents of low density (LDL) and very low density (VLDL) lipoproteins containing apoprotein b (apoB) [1]. Therefore, the possibility of there being common antigenic determinants in insulin and apoB molecules was studied by dot-immunoanalysis [7]. The total LDL/VLDL fraction obtained from donor serum and from Wistar rats weighing 250-300 g was employed as a source of apoB. ApoB-containing lipoproteins (LP) were precipitated with $MgCl_2$ in a dextran sulfate solution [5]. ApoB was titrated assuming its mean content to be 100 μg/ml in rat serum and 1000 μg/ml in human serum. The apoB solutions were then applied to a nitrocellulose membrane (Schleiher und Schuell, Germany) with a pore diameter of 0.45 μ. In order to block

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apoB-free sites the membranes were incubated with a mixture containing 0.2% ovalbumin, 0.5% bovine serum albumin, and 0.5% lactalbumin at 37°C for 1 h with vortexing. The nitrocellulose was then washed three times with 0.1 M phosphate-buffered saline (PBS) together with 0.05% Tween-20 and incubated with rabbit anti-insulin antibodies for 1 h and washed three times with PBS containing Tween-20. Incubation with the second antibodies (1:1000, goat anti-rabbit antibodies conjugated with horseradish peroxidase) was performed at 37°C for 1 h. After washing (PBS/Tween-20, five times), the enzyme reaction was conducted in 0.1 M phosphate buffer (pH 7.4) containing 0.6 M diaminobenzidine and 0.006% H_2O_2. The reaction was terminated by washing the nitrocellulose with water. Nitrocellulose plates with developed bands were photographed. It is known that in the blood apoB can undergo limited proteolysis or chemical modifications that disrupt the primary structure of protein molecules [8,12]. The integrity of such molecules is maintained by disulfide bonds. Reduction of these bonds with different compounds leads to fragmentation of an apoB molecule. In other experimental series apoB fragments containing antigenic determinants common with those of insulin were identified immunochemically: polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was followed by immunoblotting. Precipitate of apoB-containing LP was obtained as described previously [5]. It was dissolved in 0.5 M sodium oxalate and dialyzed against 0.05 M Tris-HCl (pH 7.6). For the removal of VLDL the mixture of apoB-containing LP was centrifuged in a KBr solution (d=1.006 g/cm³) for 18 h at 140,000 g (an L5-75 Beckman centrifuge, USA, rotor 75Ti). The density of the KBr solution was then adjusted to 1.063 g/cm³, and centrifugation was continued for the next 20 h to isolate LDL. LDL were dialyzed against 0.15 M NaCl with 0.001 M Na-EDTA. Lipids were extracted with an ethanol-diethyl ether mixture cooled to -16°C. The purity of apoB was controlled with PAGE as described elsewhere [10]. For reduction of the disulfide bonds and more complete "exposure" of the antigenic determinants the apoB solution was treated with 0.5 M Tris-

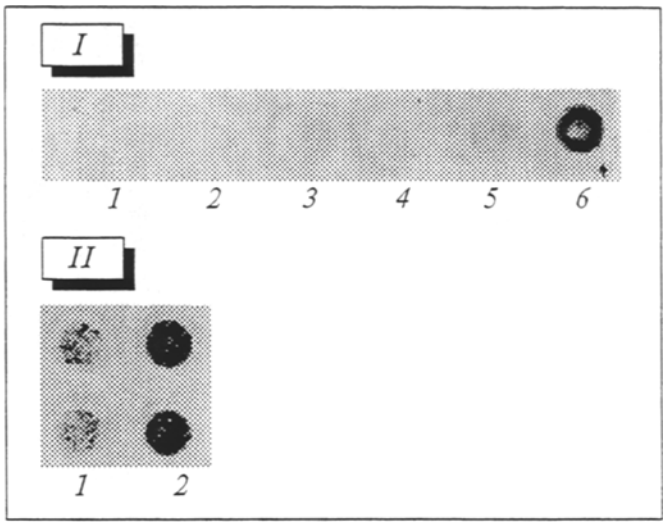

Fig. 1. Insulinlike immunoreactivity of apoB isolated from human (*I*) or rat (*II*) blood in dot–immunoanalysis. *I*) 0, 10, 50, 100, 200, or 500 μg apoB were added to the 1st, 2nd, 3rd, 4th, 5th, and 6th well, respectively. *II*) 200 and 500 μg apoB were added to the 1st and 2nd well, respectively.

HCl (pH 6.8) containing 5% β-mercaptoethanol and 2% SDS for 2 min at 100°C. Special Pharmacia kits were employed as marker proteins. Proteins and peptides were separated by electrophoresis in 9% polyacrylamide gel. The total protein concentration was determined by the method of Lowry [11]. After electrophoresis, the protein fractions were transferred to nitrocellulose using a semi-dry technique and flat carbon electrodes [14]. Transfer was performed for 1 h at 0.8 mA/cm². Protein-free sites were blocked as described above, after which the nitrocellulose was treated with either anti-insulin or anti-human LDL and antibodies conjugated with horseradish peroxidase (the second antibody). Diaminobenzidine was used as a substrate. Plates with visualized bands were photographed and compared. The effect of apoB-containing LP on insulin production was studied *in vitro* on persistent islets of Langerhans, which were isolated from rat pancreas using 100% collagenase. After washing, 5 islets were incubated with Krebs-Ringer bicarbonate buffer containing 20 mM glucose for 30 min at 37°C with vortexing. The insulin content in the incubation medium was determined by radioimmunoenzyme technique. Lipoproteins were isolated from rat serum by ultracentrifugation in a

TABLE 1. Effect of Serum LP on Insulin Production (μU/ml) by Langerhans Islets ($n = 11$)

LP	Control		Protein, mg/ml	
	without LP	with LP	without LP	with LP
VLDL	16.90±1.74	10.92±1.61*	0.17±0.016	0.15±0.017
LDL	16.79±1.26	34.49±7.82*	0.16±0.016	0.13±0.011

Note. Asterisk indicates $p<0.05$.

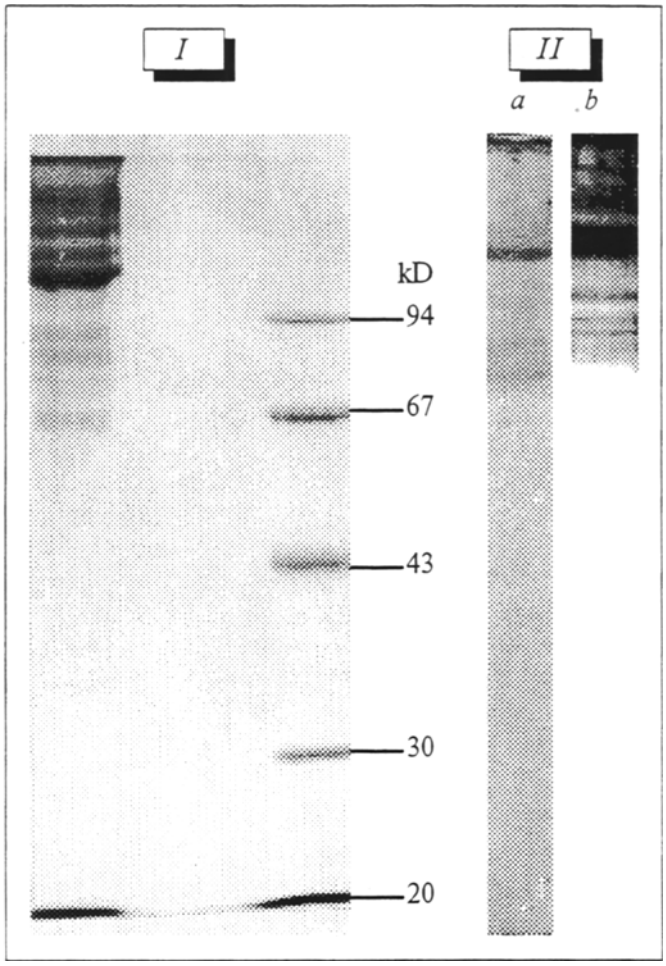

Fig. 2. Immunochemical identification of common antigenic determinants in the insulin and apoB molecule by immunoblotting. *I*) apoB treated with β-mercaptoethanol and electrophoresed in 9% polyacrylamide gel; *II*) electrophoretic transfer of apoB to nitrocellulose membrane followed by treatment with anti-insulin (*a*) and anti-LDL (*b*) antibodies.

KBr density gradient [6] and added after dialysis to the incubation medium (final concentration 0.2 mg/ml protein). The identity of isolated islets was assessed by the total protein concentration. For this purpose the islets were homogenized in 0.04 M Tris-HCl (pH 7.4), and the protein concentration in the homogenate was measured after Lowry.

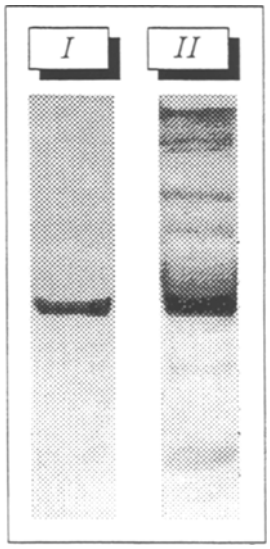

Fig. 3. Insulinlike immunoreactivity in serum supernatant after precipitation of the total LDL/VLDL fraction. Electrophoretic transfer of supernatant to nitrocellulose membrane followed by treatment with anti-insulin (*a*) and anti-LDL (*b*) antibodies.

RESULTS

We speculated that apoB carries antigenic determinants (AD) common with those of insulin. LDL and VLDL contain not less than 95% and 30% apoB of total protein, respectively. In addition to apoB, VLDL contain apoE and apoC. The presence of other proteins in apoB-containing LP does not hamper the identification of AD common for insulin and apoB. With this in mind, the total LDL/VLDL fraction was precipitated from human serum, the precipitate was solubilized in 0.5 M sodium oxalate, titrated by protein, and subjected to dot-immunoanalysis with anti-insulin antibodies. The proteins of the total LDL/VLDL fraction proved to contain AD common with insulin. The number of these AD linearly depends on the protein content in each well (Fig. 1).

Similar studies were performed with apoB purified from rat LDL. Anti-insulin antibodies reacted with the AD of apoB. The dependence between the intensity of nitrocellulose staining and the apoB content in a well (by protein) revealed by immunoanalysis is shown in Fig. 1.

The next series of experiments was performed with human LDL. After treatment with β-mercaptoethanol, LDL were electrophoresed in polyacrylamide gel, which showed that reduction of the disulfide bonds leads to the formation of 10-12 peptides with a molecular weight ranging from 14 to >94 kD. Five or six of these peptides reacted with anti-insulin and anti-LDL antibodies, as evidenced by immunoelectroblotting (Fig. 2).

We think this fact to be of key importance, since apoB can serve as a source of peptides with contra-insulin effect directly in the bloodstream. This was confirmed by our results.

After precipitation of the total LDL/VLDL fraction from human serum, proteins in the supernatant were analyzed by immunoblotting. The blood serum proved to contain a certain amount of proteins and peptides which are not constituents of LDL or VLDL and which cross-react with anti-insulin and anti-LDL antibodies (Fig. 3).

Thus, apoB-containing LP and products of apoB degradation present in the blood can elicit a contra-insulin (or pro-insulin?) effect, an effect which may be associated both with sugar absorption by peripheral tissues and with alterations in

insulin production by β-cells of the pancreatic islets. Our experiments show that incubation of Langerhans islets with VLDL leads to a more than 40% drop in insulin production in response to the addition of glucose (Table 1). The mechanism of this inhibition remains unclear. It may be associated with decreased absorption of glucose by β-cells. This phenomenon is being studied. By contrast, the addition of LDL to the incubation medium increased insulin production 2-fold.

Thus, immunochemical analysis revealed common AD in insulin, apoB, and apoB-containing LP (LDL and VLDL). After the removal of apoB-containing LP from serum, there remain three or four protein and peptide fractions cross-reacting with anti-insulin and anti-LDL antibodies. VLDL, which contain up to 30% apoB, markedly inhibit insulin production by β-cells of islets isolated from rat pancreas, whereas LDL stimulate it.

Our results are useful not only for understanding the mechanisms of diabetes developing under stress but also for elucidating risk factors and for their control in patients suffering from diabetes mellitus, in whom a prevalence of VLDL over LDL may aggravate the disease.

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