Pancreatic bile-salt-dependent lipase activity in serum of diabetic patients: is there a relationship with glycation?

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2. In the present study, we investigated the effect of glycation and particularly that of human serum albumin on the activity of bile-salt-dependent lipase. In vitro, bile-salt-dependent lipase activity decreased in the presence of human serum albumin; however, this was less pronounced in the presence of glycated human serum albumin. In vivo, bile-salt-dependent lipase specific activity was about 2-fold higher in the sera of diabetic patients than in the sera of normal subjects.

3. A significant increase in the specific activity of bile-salt-dependent lipase related to the serum level of glycation was observed. The increase in bile-salt-dependent lipase specific activity was not related to the glucose concentration in serum suggesting that glycation of bile-salt-dependent lipase could not be involved in the observed effects. Although the stability of serum bile-salt-dependent lipase was important enough to allow a systemic action of the enzyme on lipoproteins, it could not explain the higher activity of the enzyme in diabetic serum.

4. We concluded that bile-salt-dependent lipase could be helpful against the premature development of atherosclerosis in diabetes.

INTRODUCTION

Pancreatic bile-salt-dependent lipase (BSDL), also referred to as carboxyl ester hydrolase [1] or bile-salt-dependent cholesterol ester hydrolase, hydrolyses cholesteryl and lipid-soluble vitamin esters, triacylglycerols and (lyso)phospholipids [2, 3]. Although the action of BSDL is thought to be confined to the duodenum, BSDL activity and/or mRNA have been reported in human breast [4] and placenta [5], in rat heart [6], aorta [7] and liver [8, 9], and in rabbit heart [10] and aorta [7]. BSDL is also found in the blood plasma of humans [11] where it hydrolyses low-density lipoprotein (LDL)-cholesterol esters [12, 13]. Of physiological importance is the fact that BSDL reduces the atherogenicity of oxidized LDL by decreasing its lysophospholipid content [14].

Atherosclerosis develops rapidly in patients with diabetes. Individuals with diabetes suffer a 3–4-fold increased risk for developing atherosclerotic complications and vascular insufficiency [15]. Since diabetes afflicts at least 30 million people in western countries, the contribution of diabetes to the overall mortality of heart disease and stroke is significant. Reactions involving glycation and oxidation of proteins and lipids are believed to contribute to atherogenesis. Glycation, a non-enzymic attachment of glucose to protein amino groups, can increase the atherogenic potential of certain plasma constituents including LDL [16]. Glycation of LDL, which is significantly increased in diabetic patients, induces multiple metabolic abnormalities. These include interaction modifications between LDL and the LDL-receptor [17, 18] and stimulation of foam cell formation [19, 20]. It is also believed that protein glycation generates free radicals and subsequent oxidative damage [21]. In addition, glycation of LDL can be extensive enough to provoke an immune response [22].

Besides the glycation of apolipoprotein (Apo) B-100 of LDL particles and Apo A-I of high-density lipoprotein (HDL) particles, both of which contribute to the dyslipidaemia of diabetes, glycation of human serum albumin (HSA) has also been observed [23]. Glycation modified the catabolism of HSA which appeared accelerated in rabbit [24] and decreased in rat [25]. This indicated that HSA glyca-
tion may modulate the cellular capture of this protein. Glycation of HSA induces conformational changes [26] and consequent modifications of binding properties. In part, the binding capacity of glycated albumin decreased for ligands such as bilirubin and salicylates. However, the HSA of diabetic patients had a better binding capacity for fatty acids than that of normal subjects [27]. Consequently, modifications of HSA properties after glycation may modulate the activities of serum lipolytic enzymes, such as BSDL, and in turn be responsible for secondary pathologies of diabetes. The aim of this study was primarily to determine the modifications of the binding capacity of HSA after in vivo and in vitro glycation and, secondly, to examine whether HSA glycation could affect BSDL activity present in the serum of diabetic patients.

**METHODS**

**Materials**

Globulin-free human serum albumin (batch 93H9345), containing less than 0.005% fatty acid (mol/mol) and unlabelled palmitic acid (>99% pure) were obtained from Sigma (St. Louis, MO, U.S.A.). [1-3H]Palmitic acid >98.7% radiochemically pure (60 Ci/mmol) was from NEN (Dupont de Nemours, Dreieich, Germany). Microdialysis apparatus was locally toolefd from Plexiglass; dialysis chambers had a volume of 570 μl on each side of a cellulose triacetate membrane (cut-off 20 kDa; type 14549 from Sartorius, Goettingen, Germany). Acetylcellulose membranes give measurable dialysis rates for a range of standard albumin concentrations from 10 to 600 μmol/l with total recovery of radioactivity from left and right compartments ranging from 70 to 90% [28, 29]; the use of acetylcellulose membranes has been documented previously [30].

**Patients**

Sera (n = 21, 13 women and 8 men, age range 24–85 years) used in this study were blind-collected from a cohort of patients consulting the Department of Metabolic Investigation (Hôpital de la Conception, Marseille, France). None had liver disease or insulin treatment, was taking corticosteroids, oral contraceptives or lipid-lowering agents. All were normoalbuminaemic (>540 μmol/l) and normolipidaemic (triacylglycerols<1.5 mmol/l, cholesterol<5.2 mmol/l). Among these sera, five (three women and two men, age range 24–77 years), glycated HbA1c <5%, glucose<5.6 mmol/l, fructosamine<285 μmol/l, creatinine<120 μmol/l) were randomly chosen and used as control. In addition, these five sera had normal lipid profiles (triacylglycerols, 0.9±0.2 mmol/l; cholesterol, 4.4±0.5 mmol/l). Blood citrated samples were drawn at 09.00 hours after an overnight fast while subjects had been resting for at least 20 min. Samples were used for HbA1c measurement which was routinely determined by HPLC (Variant II, BioRad). The study protocol was approved by our Ethics Committee and written informed consent was obtained from all subjects. Five sera from sex- and age-matched patients (three women and two men, 18–60 years old) with type I diabetes (glycated HbA1c, 11.4±0.4%; cholesterol, 5.5±1.3 mmol/l) came from the Laboratoire de Biochimie, Hôpital Nord, Marseille. These patients had received insulin (60 units/day) for 1 year. They did not present with nephropathy nor have any complication due to diabetes. They had no glycosuria, normal proteinuria (<100 mg/24 h) and normal microalbuminuria (<30 mg). Serum creatinine was normal (86±11 μmol/l).

**Microdialysis method**

The albumin/fatty acids binding equilibrium can be assessed quantitatively in relative terms utilizing the reserve albumin concentration (p), defined as the concentration of a reference albumin preparation which, in a buffered solution, binds a trace amount of fatty acids (e.g. [3H]palmitate) as tightly as it is bound to albumin in a plasma sample [31]. p is determined by measuring the rate of dialytic exchange between two identical plasma samples after addition of the labelled tracer on one side of the membrane as already described [29]. The dialysis exchange rate for a given plasma, which depends upon the free ligand concentration, is compared with rates measured with varying concentrations of standard albumin; p value is the albumin concentration which gives the same rate as that measured with the plasma sample. From p, the palmitate availability C/p can be deduced, where C is the concentration of bound palmitate. In practice, the p value for one given fatty acid (e.g. palmitate) has been extended to p*, the mean value for the major long-chain fatty acids circulating in human plasma [32]. This is acceptable since pilot studies have shown that the variation of p does not exceed 10% for saturated and monounsaturated fatty acids of 16- or 18-carbon chain length [33]. Consequently, we can let C* represent the total fatty acid concentration in a sample and measure p ( = p*) for palmitate. It follows that C*/p* is the total fatty acid availability in the plasma sample. The relative binding affinity, L*, of albumin for plasma fatty acids can be calculated using the formula: L* = (p*/P) − α(C*/P), where P is the albumin concentration in the sample; the numerical value of α is −0.05, as published [32] and verified here. Exchange rates were plotted against albumin concentrations, supplying the linear reference curve for the determination of p*. Triplicate determinations of p* for control serum (269±70 μmol/l) were in good agreement with those reported for 33 healthy women (351±50 μmol/l) [33]. A plot of observed values of p*/P versus C/P represents a
relative Scatchard plot [32] giving the ordinate intercept, \( L^* \). The extrapolation of this plot to abscissa intercept gives the relative molar amount of bound fatty acids to albumin, \( C/P \).

**Immunoprecipitation**

Polyclonal antibodies (\( \alpha Ab\) L64) specific for human pancreatic BSDL were obtained, purified and immobilized on agarose beads as previously described [34]. The specificity of these antibodies for BSDL was established using human pancreatic juice, human pancreatic tissue homogenate and human liver microsomes. BSDL was the unique protein detected in material from pancreas whereas it was not detected in human liver [35]. To 100 \( \mu l \) of serum sample, 20 \( \mu l \) of a suspension of immobilized \( \alpha Ab\) L64 (corresponding to 100 \( \mu g \) of antibodies) were added (assays) and incubated for 4 h at 25°C under agitation. Beads were then pelleted by centrifugation at 10,000 \( g \) for 20 min. The esterolytic activity was recorded in the supernatant. Controls were performed under the same conditions omitting immobilized antibodies. In these conditions, the difference in activity between controls and assays was representative of the BSDL activity in sera. Note that this procedure allowed us to precipitate 1–2 pg \( (or 1–2 \text{units}(\text{s})) \) of pure human pancreatic BSDL, which represents, at least, a 1000-fold molar excess of antibodies to serum BSDL [11].

**Enzyme assay**

Esterolytic activity of serum samples was determined on 4-nitrophenyl hexanoate [36]. Initial hydrolysis rate determinations were performed at 404 nm in a thermostatic cell at 30°C using a computer-driven 8452A Hewlett Packard spectrophotometer. Substrate (0.1 mmol/l, final concentration) was dissolved in 0.1 mol/l Tris/HCl, pH 7.4, containing NaCl (150 mmol/l). The serum sample (100 \( \mu l \)) was added at time 0. Assays were performed in the absence or presence of a saturating concentration of sodium taurocholate [1] as activator of BSDL. When assays were performed in the presence of HSA, the blank medium had the same composition as that of assays, except for BSDL which was omitted. Diisopropyl-fluorophosphate (DFP)-inhibited esterolytic activity was obtained after sample incubation (1 h at 25°C) in the presence of 4 mmol/l DFP (stock solution 1 mol/l in dry isopropyl alcohol) or of the vector alone. The esterolytic activity of serum, given as \( \mu \text{mol of substrate hydrolysed per min and ml (units/ml)} \), was then recorded as above. Unless otherwise stated, the specific activity of BSDL represents the DFP-inhibited esterolytic activity determined in each sample and normalized to the concentration of BSDL (units/g) determined by a specific ELISA [11, 14] in the same sample.

**In vitro glycation of HSA**

Glassware used in these experiments was sterile disposable material. Fatty-acid-free HSA was incubated in the absence or presence of glucose in a 66 mmol/l sodium phosphate buffer, pH 7.4. Before incubation at 37°C, samples were sterilized by filtration over hydrophilic filters (cut-off 0.22 \( \mu m \)). At the required time of incubation, samples (1–2 ml) were dialysed at 4°C (Spectra/Por tube, cut-off 12–14 kDa) against 11 of 66 mmol/l sodium phosphate buffer, pH 7.4, containing 0.1 mol/l NaCl and analysed for protein [37] and fructosamine content.

**Other procedures**

Serum albumin was measured by the Bromocresol Green protocol (VBC) from Sigma Diagnostics (St. Louis, MO, U.S.A.). Serum concentrations of fatty acids, triacylglycerol, cholesterol, fructosamine and glucose were measured by enzymic methods (Biomerieux, Charbonnières-les-Bains, France; Roche Diagnostic Systems, Neuilly, France; Boehringer, Mannheim, Germany and Sigma Diagnostics).

**Statistical analysis**

Results were expressed as means ± S.D. The significance of difference was examined using Student's \( t \)-test. Regression lines were calculated by the least-squares method from individual data. Statistical comparisons between regression lines were performed by analysis of covariance. Probability values of \( P < 0.05 \) were considered significant.

**RESULTS**

**BSDL activity in serum**

The activity of pancreatic BSDL was reported to be undetectable in serum, partly due to the possible presence of other esterases or lipases of various origin [3–10, 38, 39], the presence of putative BSDL substrates within lipid–protein complexes [14, 40] and the strong background using synthetic substrates such as 4-nitrophenyl acetate (D. Lombardo, unpublished work). In this study, we attempted to assay BSDL in serum using 4-nitrophenyl hexanoate, a specific and sensitive substrate for pancreatic BSDL [36], before and after treatment of sera with 4 mmol/l DFP (an inhibitor of BSDL). The difference in activities obtained under these conditions was referred to as DFP-inhibited esterolytic activity. To assert that the measured esterolytic activity was representative of that of BSDL, we first recorded the esterolytic activity in serum which was activated by bile salts by the determination of the difference of activity measured with 4-nitrophenyl hexanoate in the presence and absence of sodium taurocholate (an activator of BSDL). Finally, the esterolytic...
activity of serum due to BSDL was precipitated with polyclonal antibodies specific for human pancreatic BSDL (pAb L64) immobilized on Sepharose beads [34]. Results of these experiments, performed on blind-collected sera (see the Methods section), show that the fraction of esterolytic activity which is inhibited by DFP (0.14 ± 0.02 units/ml) is, within experimental error, very similar to that activated by sodium taurocholate (0.15 ± 0.02 units/ml) and to that immunoprecipitated by immobilized pAb L64 (0.11 ± 0.05 units/ml). Consequently, the fraction of esterolytic activity determined on 4-nitrophenyl hexanoate occurred.

For this purpose, 21 serum samples were blind-collected from a group of consulting patients. From these sera, five parameters (i.e. BSDL specific activity, HSA, glucose, triacylglycerols and fructosamine concentrations) were recorded. Examination of the sera showed that BSDL activity did not differ between males and females and did not vary with patient age. Although no correlation was found between BSDL specific activity, glucose and HSA concentrations (Figs. 1, A and B) or triacylglycerols and protein concentrations (results not shown), the relationship between BSDL specific activity and fructosamine concentration or the molar ratio of fructosamine to HSA was evident (Figs. 1, C and D). The slope of the regression lines was positive and significant (P < 0.05, r = 0.49 and P < 0.05, r = 0.56 respectively). These data indicated that glycation or glycated proteins, but not glucose, may affect BSDL activity in diabetic serum. We therefore returned to the sera from normal subjects and diabetic patients and examined them for glycation and fatty acid binding parameters (Table 2). In serum from normal subjects, the concentration of glucose, fructosamine and the ratio of fructosamine to HSA was significantly lower (P < 0.01) than that determined in diabetic serum. With respect to free-fatty-acid binding parameters, we noticed that the concentration of reserve HSA for the binding of fatty acids, p*, was significantly decreased in the serum of normal subjects (P < 0.05) while the availability of fatty acid, C*/p*, was not significantly increased. The HSA concentration, P, was also comparable between the two groups. Interestingly, the values for the relative binding affinity, L*, of HSA for plasma fatty acids were significantly different between the two groups with P < 0.05. The L* value for diabetic patients was increased by approximately 40%.

**Table 1. BSDL in sera of normal subjects and diabetic patients.** The esterolytic activity of the serum was recorded as described in the text and normalized with the BSDL concentration of serum determined by ELISA. Values are means ± S.D. of determinations performed on sera from five normal donors and five diabetic patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>DFP-inhibited</th>
<th>Bile-salt-activated</th>
<th>Immunoprecipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>46 ± 22</td>
<td>54 ± 28</td>
<td>24 ± 16</td>
</tr>
<tr>
<td>Diabetic</td>
<td>94 ± 23</td>
<td>95 ± 13</td>
<td>76 ± 23</td>
</tr>
</tbody>
</table>

Effect of glycated HSA on BSDL activity in vitro

We then focused our studies on HSA (which is the most represented protein in human serum) and studied the effect of glycated HSA on BSDL activity. For this purpose, we initially examined properties of glycated HSA. Incubation of HSA (approx. 600 μmol/l) with physiological (i.e. approx. 5.6 mmol/l) and pathological concentrations (>16.7 mmol/l) of glucose showed that HSA glycation increased with time until a plateau value was reached after approximately 10 days of incubation. However, the level of HSA glycation significantly increased for glucose concentrations above 16.7 mmol/l (results not shown). Glycaemia in diabetic patients was frequently >28 mmol/l. In *vitro*, the level of HSA glycation after 15 days of incubation in the presence of approx. 33 mmol/l of glucose, reached 1.39 ± 0.06 mol of fructosamine/mol of proteins (glycated HSA) and was about two to three times that of control HSA (0.59 ± 0.01 mol of fructosamine/mol of proteins) incubated with a physio-
logical concentration of glucose (5.6 mmol/l). During the time-course of HSA incubation in the presence or absence of glucose, we observed that relative binding affinity for non-esterified palmitic acid to HSA, \( L^* \), decreased with time while that of glycated HSA was not modified (Fig. 2). It was observed that a plot of \( p^*/P \) versus \( C/P \), where \( p^*/P \) denotes the ratio of albumin bound to non-esterified fatty acid, \( P \), the total concentration of HSA and \( C/P \) the molar ratio of bound palmitate to HSA, was a relative Scatchard plot [32]. We further investigated the properties of glycated HSA by determining the relative amount of binding sites susceptible to bind palmitate. Results indicated that the molar ratio of bound palmitate to HSA was identical (\( C/P \) approx. 3) for control and glycated HSA. Taken together these data indicated that glycated HSA has a higher capacity to bind non-esterified fatty acids, which corroborates data obtained in serum.

Indeed, glycated HSA may modulate the activity of lipolytic enzymes such as blood-circulating BSDL [14]. Consequently, the activity of BSDL was deter-
mined in the presence of increasing amounts of HSA. When control HSA was used in assay, the BSDL activity decreased as a function of increasing HSA concentrations (Fig. 3). Alternatively, in the presence of glycated HSA, the effect was less pronounced. Kinetic analysis indicated that glycated HSA increases affinity of BSDL for its substrate and consequently the activity. However, when compared with control HSA, glycated HSA increases $V_{max}$ which in turn partially compensates for the effect on the affinity ($K_m$, not shown). Hence, these data indicated that glycation attenuated the loss of BSDL activity promoted by HSA. The net result was that, for the same albumin concentration, glycated HSA can stimulate the apparent activity of BSDL.

Stability of BSDL in normal versus diabetic serum

The stability of BSDL, which is a prerequisite for its relatively long-term action in serum [14], may be different in diabetic patients compared with normal subjects. A difference in BSDL stability can also explain data obtained therein. To examine this possibility, pure BSDL obtained from human pancreatic juice [1] was added (approx. one enzyme unit) separately to serum (Table 2) from normal subjects ($n = 5$) and diabetic patients ($n = 5$). Sera were then incubated at 30°C and BSDL activity was recorded over time. In all sera, the activity of added BSDL did not decrease with time and 100% of added activity was recovered after 72 h incubation. Indeed, BSDL activity in serum was remarkably stable when compared with BSDL in buffer (1 enzyme unit in 66 mmol/l sodium phosphate buffer, pH 7.4, containing 100 mmol/l NaCl), which lost more than 75% of its original activity in 3 days (Fig. 4). Interestingly, the presence of glucose (approx. 33 mmol/l) in the buffer did not modify the loss of BSDL activity (results not shown). This suggested that even though BSDL can be glycated under these conditions, glycation does not affect BSDL activity.

DISCUSSION

The physiological role of BSDL present in plasma has not been clearly defined. It has been shown to increase uptake of HDL-associated cholesteryl esters by hepatoma cells [41], to increase sterol transfer between small unilamellar vesicles [42] and recently, to modify normal human LDL and HDL composition and structure [14, 43] and to reduce the atherogenicity of oxidized LDL [14]. Since LDL glycation precedes and enhances LDL oxidation which
contributes to atherogenicity [15], it was natural to investigate whether glycation affects systemic BSDL activity. The first step of this study was to determine the properties of HSA (the major circulating protein) upon glycation. During the time-course of HSA glycation, we observed that the relative binding affinity, $L^*$, of non-esterified palmitate to HSA increased by approximately 40\% when compared with non-glycated HSA. These data suggested that glycated HSA had a better binding capacity for non-esterified fatty acids than the non-glycated form. This appeared to be due to the increased stability of the glycated HSA/fatty acids complexes, as the binding properties of glycated HSA did not decrease compared with those of non-glycated HSA. The structural modification of HSA upon glycation, as shown by fluorescence studies [26], may be the cause for the change in binding properties as observed here. Murtiashaw and Winterhalter [27] reported that non-glycated and glycated HSA presented no difference for the binding of palmitic acid while Shaklai et al. [26] found that glycated HSA binds 10-fold less fluorescent parinaric acid than normal HSA. The apparent discrepancy between these results is probably inherent in the techniques used for the fatty acid binding determination. However, other studies have found that albumin from diabetic patients was able to bind approximately twice as many fatty acids than that of normal control subjects, which is confirmed by these in vivo studies. Vorum et al. [44] examined diabetic patients under continuous insulin treatment and healthy adults. Their results indicated that the albumin fatty acid binding properties, $L^*$, were on average identical in diabetic and control donors, although the individual variation of $L^*$ was wider in the former. However, these authors concluded that the individual differences may well be of consequence for the transfer of fatty acids from plasma to tissues.

A likely target for consecutive modification of lipid metabolism by glycation could be lipases that circulate in plasma. The effect can be either direct or indirect modification of enzyme properties or lipase cofactor properties respectively. For example, activation of the lecithin:cholesterol acyltransferase by glycated Apo A-I was lower than the activation by normal Apo A-I [45]. Modification of fatty acid albumin binding properties could also affect enzymic lipolysis. Our in vitro investigations presented here have shown that BSDL activity decreased in an HSA-concentration-dependent manner while glycated HSA had a less pronounced effect. Furthermore, we examined the BSDL activity in serum. One point that we needed to consider was that the activity of BSDL has never been detected in serum. It has been shown that LDL inhibits BSDL activity as determined with fluorescent cholesteryl esters and triacylglycerols [40]. This precludes the use of such substrates in serum assays. However, using a very stable and sensitive substrate for BSDL [36], we were able to detect in serum an esterolytic activity sensitive to DFP, and stimulated by sodium taurocholate and immunoprecipitated by polyclonal antibodies specific for BSDL [34, 35]. Using these properties, we have shown that BSDL specific activity is always significantly higher in diabetic patients than in healthy adults. We have also shown that the specific activity of BSDL in serum significantly increases with the level of glycation as determined by the fructosamine assay. No correlation was found between BSDL activity and protein, HSA, triacylglycerol or glucose concentrations. This increased BSDL activity could be due to the positive effect of glycated HSA compared with normal HSA as observed in in vitro experiments. The better binding capacity of glycated HSA for fatty acids liberated by enzymic lipolysis could be an explanation for this positive effect. Nevertheless, specific studies will be necessary to understand the relationships between BSDL activity and glycated HSA.

Considering the systemic action of BSDL, particularly on oxidized LDL [14], one relevant point was the stability of the protein which could be different in diabetic patients and normal subjects. Evidence indicated that in the absence of serum protein turnover, BSDL was stable for at least 3 days (roughly the serum half-life of LDL and HDL) in the serum of control and diabetic patients, while in buffered solution BSDL lost most of its activity during the same time. This exceptional stability of BSDL in serum could partly be due to the formation of a complex with HSA, either glycated or not. More likely, BSDL could be associated with lipoproteins forming a stable complex as described for paraoxonase, another serum esterase [46]. Consequently, the stability of BSDL in serum was important enough to allow for the long-term action of the enzyme but could not explain the higher specific activity of the enzyme in diabetic serum. Taking into account the wide specificity of BSDL [1–3], it may be risky to predict whether the higher specific activity of BSDL is advantageous or pernicious for diabetic patients. Nevertheless, BSDL is active on oxidized LDL [14] and its activity was positively correlated to LDL-cholesterol and Apo B-100 concentration in the serum of normolipidaemic donors [N. Caillol et al., unpublished work]. It is worth noting that HDL-associated paraoxonase, which has been shown to prevent LDL oxidation, is reduced in diabetes [46]. Therefore, the decrease in paraoxonase activity in diabetes can be partly balanced by the higher activity of BSDL, which consequently could be advantageous for diabetic patients.

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