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Short Communication

Identification of plasma haptoglobin forms which loosely bind hemoglobin

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Abstract

Haptoglobin (Hpt) is known to capture circulating free hemoglobin (Hb) and bind apolipoprotein (Apo) A-I or E. Here, we report that Hb can be tightly bound by most of Hpt molecules (TB-Hpt, 80%), whereas loosely bound by a minor part of them (LB-Hpt, 20%). LB-Hpt amount was significantly increased (over 60%) in patients with acute coronary syndrome. LB-Hpt bound ApoA-I and ApoE less efficiently than TB-Hpt (8- and 4-fold less, respectively) and did not affect their activity of stimulating the enzyme lecithin-cholesterol acyltransferase. LB-Hpt and TB-Hpt displayed comparable levels of nitrotyrosine residues, but differences in glycan chains. Changes in LB-Hpt level might be associated with changes in Hpt functions.

Keywords: affinity chromatography; apolipoprotein A-I; apolipoprotein E; lectin-binding.

The acute phase α_2-glycoprotein haptoglobin (Hpt) is formed by monomers, each containing one subunit α and one subunit β, and present in humans as three major phenotypes by a genetic polymorphism (Langlois and Delanghe, 1996).

Hpt binds free hemoglobin (Hb) with extremely high affinity (Giblett, 1968; Nagel and Gibson, 1971; Langlois and Delanghe, 1996), and the complex is then uptaken by macrophages and hepatocytes for elimination from circulation (Langlois and Delanghe, 1996; Kristiansen et al., 2001). This function of Hpt is recognized to prevent iron loss and to represent the primary defense mechanism against free Hb (Sadrzadeh et al., 1984; Gutteridge, 1987; Langlois and Delanghe, 1996; Alayash, 2004). Such a function is believed to be very important in the acute phase of inflammation, when Hpt circulates at high levels and increased amounts of free Hb must be removed to impair oxidative stress and kidney damage (Schaer and Alayash, 2010). The Hpt β-chain contains the Hb-binding domain, and N-linked glycan chains are involved in the binding (Katnik, 1984; Kaartinen and Mononen, 1988; Katnik et al., 1994). We previously demonstrated that Hpt also binds apolipoprotein A-I (ApoA-I) (Balestrieri et al., 2001; Spagnuolo et al., 2005) and apolipoprotein E (ApoE) (Cigliano et al., 2009; Salvatore et al., 2009). ApoA-I and ApoE promote cholesterol efflux from peripheral cells, stimulate the enzyme lecithin-cholesterol acyltransferase (LCAT) to esterify cholesterol, transport the lipid cargo into the circulation, and finally deliver cholesterol and cholesteryl esters to hepatocytes for bile formation (Xu et al., 1997; Greenow et al., 2005). Hpt targets and protects both the ApoA-I and ApoE structures against hydroxyl radicals, thus saving the apolipoprotein function in physiological conditions (Salvatore et al., 2007, 2009). By contrast, high levels of Hpt, as those circulating during inflammation, impair both ApoA-I and ApoE activity of stimulating LCAT activity (Balestrieri et al., 2001; Spagnuolo et al., 2005), or mediating cholesterol uptake by hepatocytes (Cigliano et al., 2009).

The Hb-binding activity of Hpt is widely used to isolate Hpt from plasma, by affinity chromatography with Hb-coupled stationary phase. However, we observed that the yield of this procedure failed to come up to expectations, even when an excess of Hb was coupled to stationary phase. In addition, the reproducibility of the purification procedure was poor and differences in ApoA-I or ApoE binding activity, among different Hpt preparations, were often found. We supposed that this phenomenon might be owing to Hpt structure changes, affecting the protein interaction with Hb. To verify this hypothesis, we aimed to evaluate whether circulating Hpt contains forms displaying different ability to bind Hb.

Our investigation was carried out with Hpt 1-1 phenotype, to rule out the influence of polymorphism and structure heterogeneity on the protein binding activity. Indeed, polymorphism affects the subunit α, which is present in Hpt 2-2 oligomers as α2, in Hpt 2-1 oligomers as α1 together with α2, and in dimeric Hpt 1-1 as α1 (Langlois and Delanghe, 1996). The phenotypes Hpt 2-1 and 2-2 are actually known to bind Hb less than Hpt 1-1 (Saeed et al., 2007). Moreover, in both Hpt 2-1 and Hpt 2-2, the relative abundance of each isoform was found affected by individual variability and physical effort (Cigliano et al., 2003). Hpt was isolated from plasma of healthy subjects or patients with acute coronary syndrome (ACS) by a multistep purification procedure, based on a gel filtration and an anion exchange chromatography, followed by an affinity chromatography using a column of Sepharose coupled with rabbit anti-Hpt IgG (Cigliano et al.,

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2009). In this study, ACS patients include subjects with unstable angina, non-ST segment elevation myocardial infarction, or ST segment elevation myocardial infarction. Isolated Hpt (approximately 1 mg) was further fractionated by affinity chromatography on a column of Sepharose (1 ml prepacked column) coupled with a saturating amount (approximately 10 mg) of human Hb (Hb-Sepharose). In particular, the column was loaded with Hpt in 130 mM NaCl, 20 mM Tris-HCl, pH 7.4, and the flow through (with loosely binding material, namely LB-Hpt) was collected. After extensive washing at pH 7.4, the retained material (tightly binding, namely TB-Hpt) was eluted with 0.1 M glycine at pH 2.8. The amounts of LB-Hpt and TB-Hpt from normal plasma were measured by ELISA, and found to be 24%±1% and 76%±2% (n=4, $p<0.01$) of loaded Hpt, respectively. This result suggests that the population of circulating Hpt contains both forms with high affinity for Hb and forms with poor or no ability to bind Hb.

Surprisingly, the amount of LB-Hpt from ACS patients was approximately 1.6-fold higher than that from healthy subjects (38%±1% of total circulating Hpt vs. 24%±1%; $p=0.001$). This finding suggests that the increased level of LB-Hpt might reflect conditions associated with the acute phase response in ACS.

LB and TB-Hpt abilities of binding Hb were also compared by incubating human Hb (20 nM) into LB or TB-Hpt-coated wells for ELISA. Rabbit anti-Hb and GAR-HRP IgGs were used to detect Hpt-bound Hb. As expected, TB-Hpt was found to bind Hb more efficiently than LB-Hpt. In particular, the percentage of Hb bound to TB-Hpt was approximately 20-fold higher than that bound to LB-Hpt ($p<0.0001$; Figure 1). LB-Hpt forms, although negatively selected by affinity chromatography, were able to bind Hb in ELISA experiments. This finding was probably owing to the experimental conditions used in ELISA (soluble Hb at low concentration), which are more favorable than those of flow-assisted chromatography in stabilizing the Hpt-Hb interaction (Liau et al., 2003). No differences in the Hb binding efficiency were found between TB-Hpt from patients and healthy subjects.

LB-Hpt and TB-Hpt were also analyzed, by ELISA, for their ability to bind ApoA-I or ApoE. ApoA-I- or ApoE-coated wells were separately loaded with different amounts of Hpt (25–200 nM). Rabbit anti-Hpt and GAR-HRP IgGs were used to detect apolipoprotein-bound Hpt. TB-Hpt bound ApoA-I or ApoE more efficiently than LB-Hpt (Figure 2). In particular, the amount of ApoA-I-bound TB-Hpt was over 8-fold higher than that of LB-Hpt ($p<0.0001$), and the amount of ApoE-bound TB-Hpt was approximately 4-fold higher than that of LB-Hpt ($p<0.0001$). No significant differences in either apolipoprotein binding ability were found between the Hpt forms isolated from patients and healthy subjects.

Owing to the low Hb-binding efficiency of LB-Hpt to either apolipoprotein, it was expected that LB-Hpt might not inhibit ApoA-I and ApoE in stimulating the enzyme LCAT. To verify such a hypothesis, the effects of different amounts of LB- or TB-Hpt on enzyme activity were compared *in vitro*. TB-Hpt/apolipoprotein molar ratios similar to those circulating in inflammatory conditions (>5) significantly reduced (down to 50%) both the ApoA-I and the ApoE-dependent enzyme activity ($p\leq0.001$; Figure 3A and B). Conversely, LB-Hpt, used at the same levels of TB-Hpt, did not impair ApoA-I or ApoE-dependent LCAT activity, at any concentration assayed. These data suggest that LB-Hpt is not involved in the formation of the Hpt-apolipoprotein complex, thus not influencing the amount of apolipoprotein available

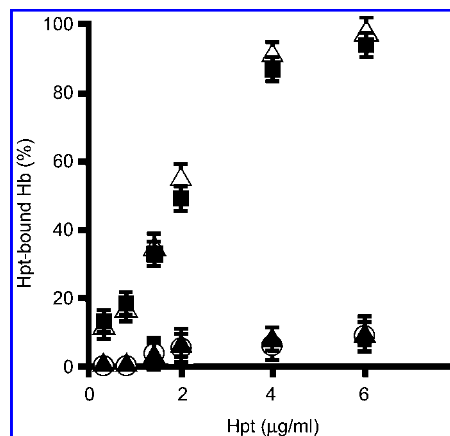

Figure 1 Binding of LB-Hpt or TB-Hpt to Hb.
Hpt was isolated from plasma of Hpt 1-1 subjects by a multistep purification procedure (Cigliano et al., 2009). Isolated Hpt (approximately 1 mg) was further fractionated by affinity chromatography on a column of Sepharose (1 ml prepacked column; GE Healthcare, Buckinghamshire, UK) coupled with approximately 10 mg of Hb, according to the manufacturer's instructions. The volume containing non-retained protein was analyzed for Hpt concentration by ELISA (Balestrieri et al., 2001). Hpt recovered in the column flow through (Hpt loosely binding Hb), was termed LB-Hpt. Hpt tightly binding Hb on the stationary phase, and eluted by 0.1 M glycine-HCl at pH 2.8 (Cigliano et al., 2009), was termed TB-Hpt. LB-Hpt and TB-Hpt were further purified by affinity chromatography using rabbit anti-human Hb IgG coupled with Hi-Trap NHS-activated Sepharose, to obtain a form freed of plasma Hb. The binding of LB-Hpt from patients (▲) or healthy subjects (○), and of TB-Hpt from patients (■) or healthy subjects (△) to Hb was analyzed by ELISA. The following methods were used: microtiter plate wells were coated with aliquots (50 µl) of different amounts (0.4–6 µg/ml) of LB-Hpt or TB-Hpt, diluted in coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6). After washes by 130 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.4 (T-TBS), and by high salt TBS (500 mM NaCl in 20 mM Tris-HCl, pH 7.4), possible sites of protein absorption were blocked with 130 mM NaCl, 20 mM Tris-HCl, pH 7.4 (TBS) containing 0.5% BSA. The wells were then incubated (2 h, 37°C) with aliquots of human Hb (20 nM in T-TBS supplemented with 0.25% BSA). Bound Hb was detected by incubation with rabbit anti-human Hb IgG (1:4000 dilution in T-TBS supplemented with 0.25% BSA; 1 h, 37°C) followed by Goat anti rabbit-horseradish peroxidase-linked IgG (GAR-HRP; 1:6000 dilution; 1 h, 37°C). Color development was monitored at 492 nm, as previously described (Spagnuolo et al., 2003). Non-specific binding of Hb was determined in wells processed without Hpt. The data are reported as percent of the value obtained by incubating Hb with 6 µg/ml of Hpt, and expressed as means values of three replicates±SEM. The program 'GraphPad Prism 3' (GraphPad Software, San Diego, CA, USA) was used to perform the *t*-test.

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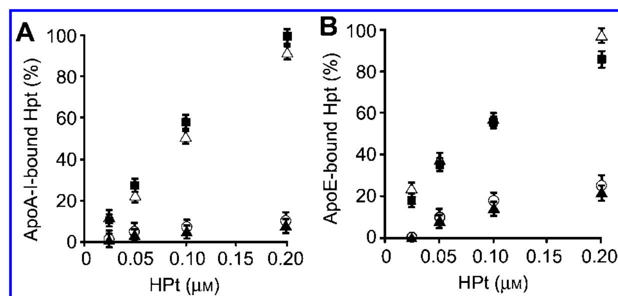

Figure 2 Binding of LB-Hpt or TB-Hpt to ApoA-I or ApoE.
Microtiter plate wells were coated with aliquots of 0.008 mg/ml ApoA-I (A) or Apo E (B), diluted in coating buffer. Aliquots (50 μl) of LB-Hpt (▲: patients; ○: healthy subjects), or of TB-Hpt (■: patients; △: healthy subjects) were then incubated in the wells (2 h, 37°C). Different amounts of Hpt (25–200 nM in T-TBS supplemented with 0.25% BSA) were used. Bound Hpt was detected by treatment with rabbit anti-human Hpt IgG (1:3000 dilution; 1 h, 37°C) followed by GAR-HRP-linked IgG (1:6000 dilution; 1 h, 37°C). Non-specific binding of Hpt was measured in wells processed without ApoA-I or ApoE. The data are reported as percent of the value obtained by incubating the apolipoprotein with 200 nM Hpt, and expressed as means values of three replicates±SEM. The program 'GraphPad Prism 3' (GraphPad Software) was used to perform the *t*-test.

for LCAT stimulation, both in physiological and inflammatory conditions. In addition, it cannot be excluded that LB-Hpt, owing to the lower ability of binding to Hb, ApoA-I and ApoE, might act as a less effective antioxidant agent in preventing toxic effects caused by free Hb, or apolipoprotein oxidative structure modification.

Structure differences in N-linked complex glycan chains might explain why LB-Hpt and TB-Hpt display different Hb-binding activity and support the hypothesis that specific carbohydrate residues are required for their interaction with the apolipoproteins. Indeed, carbohydrate structure is essential for the functionally active form of Hpt and is known to influence its interaction with Hb (Katnik, 1984; Kaartinen and Mononen, 1988). Therefore, a preliminary characterization of LB-Hpt and TB-Hpt glycosylation was carried out by Western blotting and ELISA. In detail, we analyzed the reactivity of these protein forms to biotin-linked lectins. *Sambucus nigra* agglutinin (SNA), which reacts preferentially with sialic acid α2–6 linked to terminal galactose, and *Maakia amurensis* agglutinin (MAA), which reacts with sialic acid α2–3 linked to galactose, were used to obtain information on the amounts of the two distinct types of α-glycosidic bonds in LB-Hpt and TB-Hpt glycans. The lectin *Concanavalin A* (ConA), which detects the mannose core of N-linked complex glycans, was used to evaluate the extent of glycan branching. *Lotus tetragonolobus* agglutinin (LTA), which reacts with fucose α1–6 or α1–3 linked to N-acetylglucosamine in antenna or dichitobiose, respectively, was used to evaluate the extent of glycan fucosylation. As several contaminants (i.e., albumin, IgG) might be purified together with Hpt, Western blot analysis was carried out to investigate possible binding of lectins to proteins other than Hpt in our preparations. We found that ConA, SNA, LTA, and MAA weakly reacted (<5–8%) with contaminants, as assessed by densitometry of the stained bands (Figure 4). As the lectin reactivity with these contaminants was much lower than that with Hpt, we assumed that lectins, in ELISA, reacted to TB-Hpt and LB-Hpt. ELISA analysis was then carried out to quantify and to compare the lectin reactivity of TB-Hpt and LB-Hpt. The different Hpt forms were coupled to wells of microtiter plates, and lectin binding was detected by Avidin-HRP. TB-Hpt reactivity to ConA was significantly higher than that of LB-Hpt, both in healthy subjects and patients (approximately 1.4- and 1.9-fold, $p \leq 0.01$ and $p \leq 0.001$, respectively; Figure 5A). The reactivity of TB-Hpt to SNA was higher than that of LB-Hpt (approximately 2- and 1.4-fold in healthy subjects and patients, respectively; $p \leq 0.02$; Figure 5B), whereas the reactivity to MAA did not differ between the two forms (Figure 5C). We also found that the reactivity of TB-Hpt to LTA was approximately 1.2-fold higher than that of LB-Hpt in healthy subjects ($p \leq 0.02$; Figure 5D), whereas no difference was observed between the two forms from patients. This result suggests that LB-Hpt,

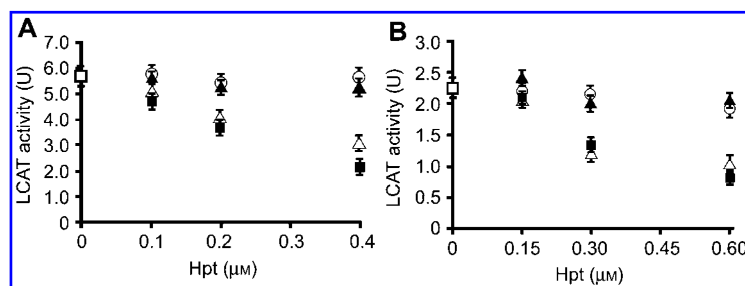

Figure 3 Effect of LB-Hpt or TB-Hpt on ApoA-I- or ApoE stimulation of LCAT activity.
The LCAT activity was assayed in the presence of different concentrations of LB-Hpt (▲: patients; ○: healthy subjects) or TB-Hpt (■: patients; △: healthy subjects). A pool of plasma samples (treated with 0.65% dextran sulfate, M_W=50 kDa, in 0.2 M $CaCl_2$, to remove lipoproteins) was used as source of LCAT, whereas a proteoliposome (ApoA-I or ApoE: lecithin:cholesterol=1.5:200:18, molar ratio) was used as substrate. The assay was carried out essentially according to Spagnuolo et al. (2005). The ApoA-I concentration in the assay was 0.03 μM (A), the ApoE concentration in the assay (B) was 0.045 μM. The Hpt/apolipoprotein molar ratios used were: 3.3, 6.6, and 13.3. As control, a sample without Hpt was processed (□). The LCAT activity was expressed as nmol of cholesterol esterified per hour per ml of plasma (units). The samples were analyzed in triplicate, and the data are expressed as mean values±SEM.

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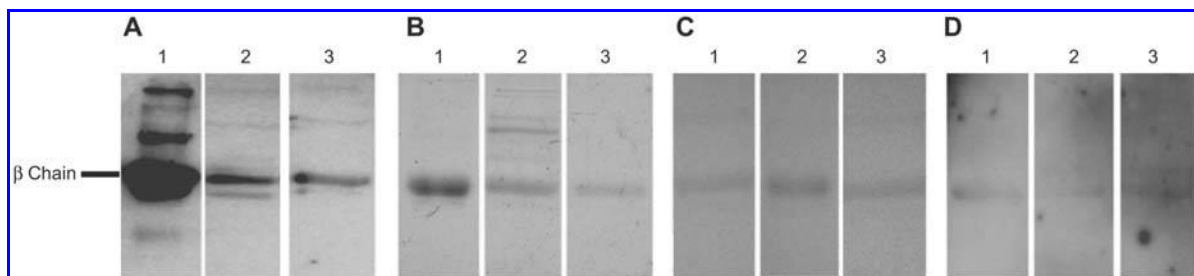

Figure 4 Analysis of lectin reactivity of Hpt by Western blotting.
The lectin reactivity of TB-Hpt or LB-Hpt was analyzed by Western blotting. Equal amounts (8 μg) of Hpt were fractionated by electrophoresis on 15% polyacrylamide gel in denaturing and reducing conditions, and blotted onto PVDF membrane, as described previously (Cigliano et al., 2003). After protein blotting, the membrane was rinsed in T-TBS and incubated with TBS containing 2% Tween 20 (1 h, 37°C). After extensive washing with T-TBS, the membrane was incubated (2 h, 37°C) with biotinylated ConA (A) (1:10 000 dilution), or biotinylated SNA (B) (1:8000 dilution), or biotinylated LTA (C) (1:1000 dilution), or biotinylated MAA (D) (1:500 dilution). The lectin dilution buffer was T-TBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After extensive washing, the membrane was incubated (1 h, 37°C) with Avidin-HRP (1:12 000 dilution in T-TBS), to detect Hpt-bound lectins. The complexes were detected by the ECL detection system, using luminol as substrate, according to the manufacturer protocol. Lane 1: typical lectin binding to TB-Hpt from healthy subjects; line 2: typical lectin binding to TB-Hpt from patients (lane 2); lane 3: typical lectin binding to LB-Hpt.

both in patients and healthy subjects, might contain more glycans with high branching and lower content of α2–6 linked sialic acid residues than TB-Hpt.

Lectin reactivity of Hpt from healthy subjects and patients was also compared, and significant differences in the composition of glycan chains were observed (Figure 5). The reactivity of TB-Hpt and LB-Hpt, isolated from healthy subjects, to SNA and ConA was significantly higher than that of the corresponding forms from patients ($p \leq 0.003$ and $p \leq 0.001$, respectively). Furthermore, the reactivity of both TB-Hpt and LB-Hpt isolated from patients with LTA was significantly higher than that of the corresponding form isolated from healthy subjects ($p \leq 0.03$ and $p \leq 0.006$, respectively). Therefore, Hpt forms purified from patients seem to be characterized by glycan chains more branched, more fucosylated, and with a lower amount of sialic acid α2–6 linked. These

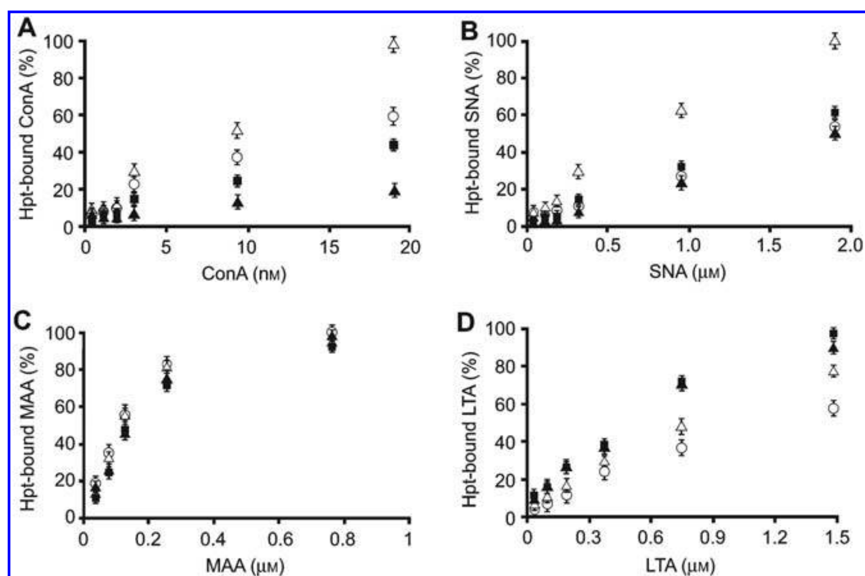

Figure 5 Analysis of lectin reactivity of Hpt by ELISA.
The reactivity of LB-Hpt (▲: patients; ○: healthy subjects) or TB-Hpt (■: patients; △: healthy subjects) to biotin-linked lectins was evaluated by ELISA. Microtiter wells were coated with aliquots (50 μl) of Hpt diluted in coating buffer (0.002 mg/ml). Blocking was carried out with TBS containing 2% Tween 20. The wells were then incubated (2 h, 37°C) with aliquots of biotinylated ConA (0.64–19 nM) (A), or SNA (0.48–1.90 μM) (B), or MAA (0.039–0.770 μM) (C), or LTA (0.060–1.480 μM) (D). The lectin dilution buffer was T-TBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After extensive washing, the wells were incubated (1 h, 37°C) with Avidin-HRP (1:6000 dilution in T-TBS). The amount of Hpt-bound lectins was determined measuring color development at 492 nm. The absorbance values were converted to the percentage of the value obtained by incubating isolated Hpt with the higher concentration of biotinylated lectin used in each assay and expressed as means values of three replicates±SEM. The program 'GraphPad Prism 3' (GraphPad Software) was used to perform the *t*-test. Wells processed without Hpt or without lectin were used as controls.

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Table 1 Titration of nitrotyrosine (N-Tyr).

N-Tyr levels (nmol/mg of Hpt)	Healthy subjects	Patients
LB-Hpt	2.273±0.364	2.362±0.627
TB-Hpt	2.241±0.377	1.573±0.213

N-Tyr level in LB-Hpt and TB-Hpt was calculated by ELISA (Spagnuolo et al., 2001). Microtiter plate wells were coated with different amounts of Hpt (1.8–20 ng/μl in coating buffer). N-Tyr was detected by incubation with rabbit anti-N-Tyr IgG (1:1500 dilution in T-TBS supplemented with 0.25% BSA; 1 h, 37°C) followed by GAR-HRP-linked IgG (diluted 1:5000 as the primary antibody; 1 h, 37°C). Color development was monitored at 492 nm. Data are reported as nmol of N-Tyr per mg of protein and expressed as means values of three replicates±SEM.

results are in agreement with previous data on characteristic changes in glycan profile associated with several diseases (Thompson et al., 1992; Katnik et al., 1994; Piva et al., 2002; Okuyama et al., 2006; Fujimura et al., 2008) or to modifications of Hpt function (Oh et al., 1990; Sharpe-Timms et al., 2002). Thus, we suppose that the characteristic glycan structure of Hpt forms isolated from ACS patients might be owing to the activation of specific glycosyltransferases, and might be associated with a shift of Hpt physiological functions toward other activities, which are part of the organism response to the disease. We cannot exclude that such an increased level, as well as the observed changes in Hpt glycans between patients and controls, might also be associated with other diseases including angina pectoris and acute inflammatory disease. These changes might actually reflect alteration of glycoform synthesis during inflammation. However, a more detailed analysis of the glycan structures in TB-Hpt and LB-Hpt is required to assess whether the observed changes differ from those reported in other diseases, and to hypothesize that such changes might be specifically associated with ACS.

Oxidative damages might also explain the observed difference between LB-Hpt and TB-Hpt in the ability to bind Hb, ApoA-I, and ApoE. A major protein alteration under oxidative stress comes from the attack of peroxynitrite, an oxidant species formed at increased levels in cardiovascular disease (Szabó et al., 2007), to tyrosine residues resulting in their conversion into nitrotyrosines (N-Tyr). The level of N-Tyr in LB-Hpt and TB-Hpt was measured essentially according to a published procedure (Spagnuolo et al., 2001). This level was found similar in LB-Hpt and TB-Hpt, in both healthy subjects and patients (Table 1), thus suggesting that differences between the forms tightly and the forms loosely binding Hb in their ability to bind Hb, ApoE, and ApoA-I might not be ascribed to different protein nitration.

In conclusion, we report, for the first time, information on Hpt forms with reduced Hb binding capacity, just the dimer β-α1, claimed to more efficiently bind Hb (Delanghe et al., 2000; Saeed et al., 2007). Our results suggest that changes in the LB-Hpt levels might reflect alteration of cardiovascular conditions, even though it cannot be excluded that LB-Hpt might be associated with a specific physiological function. It remains to be assessed whether such changes represent a physiological response to the disease rather than just a dysfunction in Hpt synthesis, and whether LB-Hpt is more abundant than TB-Hpt also in other pathological conditions.

Acknowledgments

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