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Potential biomarkers of an exaggerated response to endotoxemia

R. S. KASTHURI[1], M. WROBLEWSKI[2], B. JILMA[3,4], N. S. KEY[5], & G. L. NELSESTUEN[2]

[1]*Division of Hematology, Oncology and Transplantation,* [2]*Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota, USA,* [3]*Department of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria,* [4]*Department of Pharmaceutics, University of Florida, Gainesville, Florida, USA and* [5]*Division of Hematology and Oncology, University of North Carolina, Chapel Hill, North Carolina, USA*

Abstract
Serial plasma protein analysis was used to study the acute plasma proteome response to endotoxemia (presence of toxic bacterial products called endotoxins in the blood stream). Plasma samples from healthy volunteers before and multiple time points up to 24 h following administration of low-dose endotoxin were evaluated. Plasma protein profiles were obtained by rapid extraction of whole plasma followed by analysis with matrix-assisted laser desorption ionisation-time of flight mass spectrometry. The profiles were unique to each individual and stable over the time of the experiment. Administration of low-dose endotoxin caused profound change in six of 18 individuals. At 8 h many proteins showed quantitative oxidation, in addition to the appearance of new components and disappearance of common baseline components. An exceptionally intense new component at 4154 mass units was identified as the activation peptide of C1 esterase inhibitor. While recovery of baseline protein structure was nearly complete by 24 h, serum amyloid A, an acute-phase reactant, was still increasing and minor profile changes persisted. Clinical features did not distinguish these extreme responders from others, suggesting that plasma proteome changes offered unique insights into and potential biomarkers of subclinical events following endotoxin exposure.

Keywords: *Biomarkers, acute-phase reactants, complement, human, lipopolysaccharide*

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Introduction

Several animal and human models have been developed to try to replicate some of the clinical and/or laboratory changes of sepsis (Witcherman et al. 1980, Fink & Heard 1990, Deitch 1998, Schultz & van der Poll 2002). An important goal of these efforts is better understanding of the pathophysiology of sepsis syndromes and identification of potential biomarkers of susceptibility and outcomes in these patients. Sepsis syndromes encompass the body's systemic response to the presence of a pathogen and the sequelae there of, and as such are a significant cause of morbidity and

Correspondence: G. L. Nelsestuen, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 6-155 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455, USA. Tel: 1-612-624-3622. Fax: 1-612-625-2163. E-mail: nelse002@umn.edu

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mortality. One of these models is the human endotoxemia model, which involves administration of low-dose endotoxin intravenously to healthy volunteers. Endotoxins are potentially harmful bacterial components and the human endotoxemia model uses lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria. The human endotoxemia model has provided the bulk of knowledge on the pathophysiology of systemic inflammation resembling the early phases of sepsis and the early events leading to activation of cytokines and other inflammatory pathways (Suffredini et al. 1999, Fiuza & Suffredini 2001). While this model has distinct advantages over some animal models, extrapolation to sepsis remains tentative. One limitation is that endotoxin circulates only for a brief period after bolus infusion. This contrasts with typical sepsis syndromes where there is usually a local focus of infection from which systemic dissemination of pathogens occurs repeatedly over a long period of time.

Emerging proteomic technologies may offer the ability to gain insight on disease pathophysiology and identification of potential biomarkers that may lead to improved diagnosis and treatment (Adam et al. 2002, Liao et al. 2004, Alessandro et al. 2005, Grossman et al. 2005, Jacquemier et al. 2005, Poon et al. 2005). Approaches range from global analysis of thousands of proteins per sample (Adkins et al. 2002, Pieper et al. 2003) to identification of a single protein (Nelsestuen et al. 2005a). One experimental paradigm termed 'proteomic pattern diagnostics' examines proteome patterns or signatures, generally by using high throughput technologies (Petricoin et al. 2002a,b). Application of pattern analysis without identification of individual components in the pattern has been criticised on a number of grounds (Baggerly et al. 2004, Check 2004, Baggerly et al. 2005). Despite this controversy, the general approach of rapid extraction followed by protein profile analysis can allow high throughput at low expense, critical properties for eventual clinical application.

Recently, we described a simple method to obtain plasma protein profiles (Nelsestuen et al. 2005b). While similar approaches have reported detection of several hundred components after rapid extraction of plasma (Dayal & Ertel 2002, Gillespie 2003, Le Bihan et al. 2003), our study focused on 15 very intense peaks that were well characterised and present in all healthy individuals. The proteins consisted primarily of apolipoproteins CI, CII, CIII (apoCI, apoCII, apoCIII, respectively) and transthyretin (TTr) plus various isoforms and proteolytic digestion products of each. A striking quality of profile analysis was reproducibility, both with respect to replicate measurements of one sample and to serial samples from each individual. While a broad range of peak intensity ratios was observed among healthy individuals, each showed exceptional stability with respect to their own profile. This paradigm for proteome analysis might be characterised by sacrifice of large numbers of components for ease of analysis, precision and robustness, and the use of a smaller cohort of components to report events occurring on a larger scale.

The present study analysed plasma proteome changes among healthy adult volunteers in the human endotoxemia model. This offered an opportunity to detect specific changes associated with endotoxemia and to test the hypothesis of profile change associated with inflammatory disease. In fact, pronounced and individual responses were found. Individuals with 'high response' showed extensive oxidation of proteins and the appearance of an exceptionally intense potential marker of complement activation. The clinical implications for those demonstrating these severe changes are unclear as only low doses of endotoxin are used in these studies and the

resulting changes detected in the plasma proteome were largely subclinical. However, the implications could range from heightened sensitivity to infections to greater protection from systemic microbial invasion. The specific proteins and protein changes described in this study may represent biomarkers useful for a number of clinical conditions.

Materials and methods

Materials

Sinipinic acid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Carboxypeptidase A (product 11075-17-5) was obtained from MP Biomedicals, Inc. (Aurora, OH, USA). C4 ZipTips were from Millipore, Inc (Billerica, MA, USA).

The endotoxemia model

This study was approved by the Institutional Review Boards of the University of Minnesota and the Medical University of Vienna. Prior informed consent was obtained from all subjects. Blood samples were collected from 18 adult male volunteers (ages 18–35 years) at 3 h before and at 1, 2, 4, 8 and 24 h following administration of low-dose endotoxin. Details of the model and conduct of the endotoxin challenge have been provided in other studies (Hollenstein et al. 2002, Jilma et al. 2005). Blood samples were also obtained from six healthy adults (five male, one female). Whole blood was immediately anti-coagulated by mixing 9 volumes of blood with 1 volume of 0.1 M sodium citrate. Platelet-free plasma was obtained by centrifugation at 12 000g. Aliquots were frozen at -70°C for later assay.

Sample analysis

The samples were extracted by a carefully controlled ZipTip rapid extraction protocol and MALDI-TOF profiling was performed as previously described (Nelsestuen 2005b). Briefly, plasma was thawed, 0.5 μl were diluted into 15 μl of a reconstitution solution (5% ACN in 0.1% TFA), and incubated for 1 h at room temperature. They were then extracted using a ZipTip. The adsorbed proteins were eluted with 1.6 μl of 75% ACN in 0.1% TFA and 0.75 μl of the extract was applied to the MALDI target and mixed with an equal amount of a saturated solution of sinipinic acid (in 50% ACN and 0.1% TFA). After drying, analysis was conducted with the Bruker Biflex III MALDI-TOF mass spectrometer in the linear mode with collection of 500 laser shots per analysis. The raw data were processed with the Bruker Xtof processing software version 5.1.1. The peaks were labelled and peak intensity lists were generated.

Profile analysis

Comparison of two samples was accomplished using peak intensity ratios within a profile. The ratios were compared to those of another sample or subject. Significance was based on replicate values that differed at the $>95\%$ confidence level ($p < 0.05$) as estimated by Student's 2-tail test.

Enzyme digestion

Plasma samples were extracted by ZipTip method as described above. The solution was neutralised by addition of 20 μl of 0.1 M ammonium bicarbonate. Carboxypeptidase A (0.005 or 0.01 units) was added and the solution was incubated at 37°C for 1 h. Samples were acidified by addition of 10% TFA solution, extracted with a C4 ZipTip and profile analysis was carried out. New peaks appeared in the spectrum corresponding to loss of carboxy-terminal residues.

Edman degradation

The component appearing at $m/z = 4154$ was identified using manual Edman sequencing and mass spectrometry in a sample from an individual who displayed a polymorphism for this component with equal intensity peaks at $m/z = 4154$ and 4184. Each sequential Edman degradation step was performed by desalting the sample using a C18 ZipTip (Millipore, Bedford, MA, USA) and drying the eluate with a speed vacuum. The dried sample was reconstituted in 10 μl of 90:5:5 methanol:phenylisothiocyanate:pyridine by vigorous mixing. The samples were incubated at 30°C for 90 min and speed vacuumed. The dried sample was reconstituted in 1:1 TFA:water and incubated for 60 min at 50°C. The first cycle started with 0.5 μl of plasma in each sample. Multiple samples were started in parallel fashion so that material produced in two samples of the first cycle could be pooled as the starting material for the next cycle and so on. This was necessary to maintain signal intensity of subsequent MALDI-TOF profiles. Mass loss after each cycle of degradation was used to identify the amino acid removed. The components at $m/z = 4154$ and $m/z = 4184$ were also subjected to direct MS/MS fragmentation in the QStar o-MALDI mass spectrometer using standard methods. Daughter ions arising from these components were identified by the fact of identical mass loss from both forms of the protein.

Results

Protein profile description

The plasma protein profile from a healthy subject shows 15 major components found in all healthy subjects ($n > 1500$, peak identity in Table I) examined to date (Nelsestuen et al. 2005b). These few components demonstrated a surprising number of protein features. For example, apoCIII is present as several glyco-isoforms and shows partial C-terminal degradation. ApoCI shows N-terminal truncation, presumed to arise from the action of dipeptidylpeptidase IV (Bondarenko et al. 1999). TTr contains a free sulfhydryl group and is also present in disulfide linkage to cysteine. The levels of TTr versus its disulfide linked form reflect the oxidation state of the blood.

Analysis was accomplished by comparing the ratio of one peak intensity to another in the same profile (Nelsestuen et al. 2005b). The peak ratios reflect the relative availability or abundance of one protein to another in a profile and can be used for comparison to the same ratio in another profile. The range of values for a given peak ratio among healthy individuals was often 3–4-fold but each individual showed a very consistent ratio with variations less than 10% over several years (Nelsestuen et al. 2005b).

Table I. Peak identification (From Nelsestuen et al. 2005b).

Subjects	Theoretical *m/z*
Healthy subjects	
ApoCI minus amino terminal ThrPro	6434
ApoCI	6632
+2 Transthyretin (TTr)	6883
+2 TTr-Cys	6943
Residues 1–64 of apoCIII	7157
ApoCII mature form	8206
ApoCIII0 (no CHO)	8766
Pro-apoCII	8916
ApoCIII+GalNAc/Gal	9132
ApoCIII1 minus C-terminal Ala	9352
ApoCIII1 (GalNAc/Gal/SA)	9423
ApoCIII2 minus C-terminal Ala	9643
ApoCIII2 (apoCIII1+additional sialic acid)	9714
Transthyretin (TTr)	13 762
Cysteinylated TTr	13 881
Diseased subjects	
SAA-1 minus amino-Term ArgSer and C-Term Tyr	11 277
SAA-2 minus ArgSer	11 386
SAA-1 minus ArgSer	11 440
SAA-2 minus Arg	11 473
SAA-1 minus Arg	11 528
SAA-2	11 629
SAA-1	11 683
Beta-2 microglobulin	11 732
Sulfonylated TTr	13 842

Controls for this study consisted of six individuals (five male, one female) from whom blood samples were obtained at intervals similar to the endotoxemia study. As expected, changes in the peak ratios of these controls were minimal and approximately equal to the precision for replicate measurements (see below).

High responders to endotoxin administration

The endotoxemia study allowed every individual to serve as his own control. That is, the sample taken 3 h before endotoxin administration provided the baseline for comparison with subsequent samples from that individual. This allowed very accurate measurement of profile changes and the ability to detect minor as well as major change. A subset of individuals (six out of 18) demonstrated extreme qualitative as well as quantitative changes at 8 h. These were termed 'high responders' (Figure 1B). Nearly all of the common peaks were missing and replaced by other peaks with lower intensity, as shown by higher signal to noise levels. Two approaches were used to rationalise these extreme profiles. The first was to note that several of the new peaks had *m/z* values that were 16 and/or 32 amu above a normal component, consistent with addition of one or two oxygens per protein. Examples included a peak at $m/z = 6450$, 16 amu higher than truncated apoCI, a peak at $m/z = 9455$, 32 amu higher than apoCIII1, and a peak at $m/z = 8948$, 32 amu higher than apoCII. This type of oxidation did not arise from sample handling. For example, hundreds of samples that have been examined after storage at -70°C for 10 and even 25 years showed almost

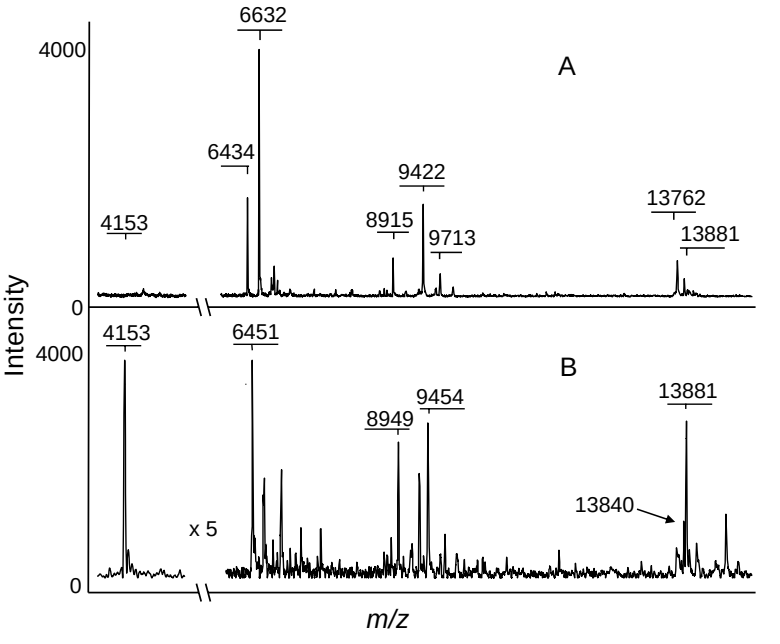

Figure 1. MALDI-TOF protein profiles. (A) The baseline protein profile of a representative high responder. ApoCI ($m/z = 6632$) is the most dominant peak. (B) Profile of the same individual at 8 h. The x-axis is split to better illustrate the appearance of a new dominant peak at m/z 4153. Please note that the peak identities shown in the figures are the observed experimental values. In order to avoid confusion, the text describes all peaks using their theoretical values as listed in Table I.

no oxidation as detected by addition of oxygen to the major components of the profile. In addition, minimal oxidation was detected after ten or more freeze–thaw cycles (Nelsestuen et al. 2005b). Samples stored at room temperature for 6 days showed extensive oxidation but the components at +16 and +32 amu were still minor when compared with those of the sample in Figure 1B. Of more than 3000 samples we have examined to date, only one showed a profile similar to that seen among the high responders (Figure 1B). That profile occurred in an individual with suspected autoimmune disease (data not shown).

To further confirm the identity of the new component at $m/z = 9455$, a sample was digested with carboxypeptidase A. Profiles run following different incubation times showed disappearance of the peak at $m/z = 9455$ with successive appearance of new components at minus 71, 142, and 241 amu. These mass changes were consistent with sequential loss of Ala-Ala-Val, the known C-terminal sequence of apoCIII. Thus, assignment of the peak at $m/z = 9455$ as an oxidised form of apoCIII appeared correct (Figure 1B). Oxidation was transient and the components returned to the baseline m/z values at 24 h.

The alpha and beta chains of haemoglobin ($m/z = 15\,126$ and $15\,866$) were easily detected in samples with minor haemolysis. One high responder showed detectable haemoglobin at 8 h. The double peak for the +2 charge states of alpha and beta haemoglobin were observed ($m/z = 7564$ and 7934, data not shown) along with a second peak for each protein at m/z of +8 amu, consistent with addition of oxygen (+16 amu) to the intact protein. Once again, oxidation was reversed at 24 h.

TTr also showed evidence of oxidation. In controls and baseline samples, the major species was the reduced protein ($m/z = 13762$) with a less abundant peak corresponding to cysteinylated transthyretin (TTr-Cys, $m/z = 13881$, Figure 1A). The six high responders showed nearly quantitative loss of TTr at 8 h (Figure 1B, inset and Figure 2A), consistent with extensive oxidation of free sulfhydryls. Some of the 'low responders' also displayed change in the TTr to TTr-Cys peak ratio relative to controls (Figure 2B).

Oxidation and appearance of acute-phase proteins (serum amyloid A (SAA), see below) could not explain all of the changes observed in Figure 1B. For example, there was little or no peak corresponding to either the normal ($m/z = 6632$) or an oxidised form of full-length apoCI, suggesting selective and complete degradation of this protein. This was especially striking since full-length apoCI is commonly the most intense peak of the profile (Figure 1A). While the products formed from degradation of full-length apoCI were not identified as a part of this study, loss may involve more extensive degradation by dipeptidylpeptidase IV. Truncation by plasma and cell surface enzyme is suggested to be the basis for appearance of truncated apoCI ($m/z = 6434$) in the circulation (Figure 1A, Nelsestuen et al. 2005b).

Evidence of complement activation in high responders

An exceptionally intense new peak at $m/z = 4154$ was observed among all of the high responders at the 8 h time point (Figure 1B). This peak was either absent or present at only nominal levels among the low responders or at other time points among the high responders. A search of standard protein databases for a blood protein of $m/z = 4154$ amu did not reveal a candidate, suggesting a heretofore unidentified protein or, more likely, a protein fragment. Protein identification was pursued using a modification of the Edman degradation protocol and tandem mass spectrometric analysis.

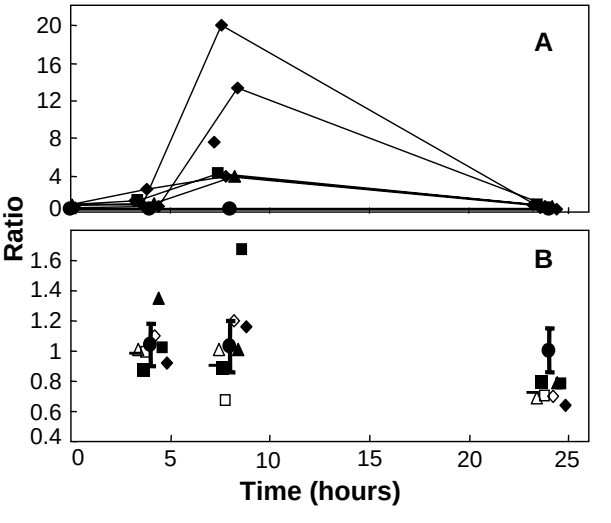

Figure 2. Changes in transthyretin following exposure to endotoxin. The ratio of TTr to TTr-Cys ($m/z = 13\,881/13\,762$) of subjects is shown along with the ratios of a healthy control. (A) The absolute change in ratio at 4, 8 and 24 h among the high-responder subset. (B) The fold change in the ratio among low responders. The y-axis scale differs in the two panels to better depict the changes.

To improve the ability to identify daughter peptides from Edman degradation of the $m/z = 4154$ peak, plasma from a person who showed a polymorphism for this component was used. Among a much larger cohort of subjects from other studies (unpublished) who possessed the $m/z = 4154$ component, we found approximately 20% had an apparent polymorphism that resulted in two, equally intense ions at $m/z = 4154$ and $m/z = 4186$ (Figure 3). An expected number of individuals appeared to be homozygous for the $m/z = 4186$ component. Sequencing of a heterozygote allowed daughter peaks to be clearly identified by the characteristic doublet (Figure 3, inset). Mass loss at each turn of sequence gave four possible N-terminal sequences of: TIIVF, TILVF, TLIVF and TLLVF. A computer program was written to search the NCBI non-redundant protein database for any human peptide of $m/z = 4154$ amu that contained any of these four combinations at the amino terminal plus a C-terminal sequence giving rise to a MS/MS fragment of 341 Da. The 341 Da C-terminal fragment was suggested by direct MS/MS fragmentation of intact $m/z = 4154$ and $m/z = 4186$ peaks in a QStar o-MALDI mass spectrometer. An intense ion representing loss of 341 amu occurred for both forms (data not shown). Theoretical MS/MS fragmentation from the amino terminal did not show potential loss of 341 amu and it was assumed to arise from the C-terminal. The tolerance for the searches was set at 1.5 Da. Only C1 protease inhibitor gave a sequence that matched these criteria. Furthermore, the $m/z = 4154$ fragment corresponded to the C-terminal 34 residue activation fragment of the C1 protease inhibitor. Loss of 341 amu from the C-terminal of this peptide corresponded to the tripeptide, Pro-Arg-Ala. This was expected since cleavage at proline residues gives rise to especially intense ions. Finally, the ZipTip extract of a person who was polymorphic for the $m/z = 4154$ and $m/z = 4186$ components was subjected to a series of proteolytic digestions (Trypsin, Glu-C, Arg-C and Asp-N proteases) and the MS peptide fingerprints of each were analysed for the expected peptides. Without detail, the m/z values for peptides released by these proteases were in complete agreement with the C-terminal 34 residues of C1 esterase inhibitor. The presence or absence of the polymorphic relationship among these

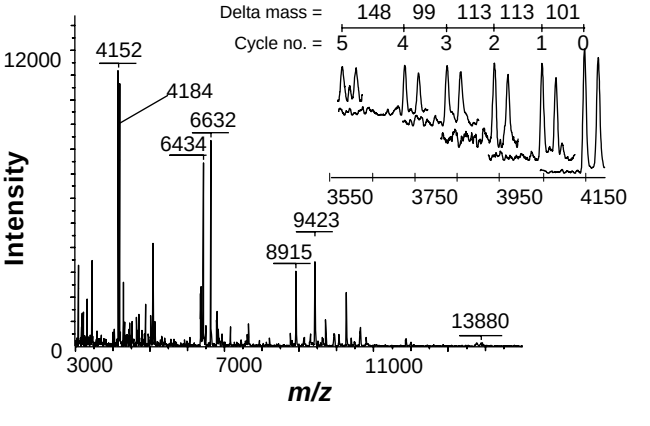

Figure 3. Identification of C1 esterase inhibitor. The new peak ($m/z = 4152$) at 8 h following administration of endotoxin was sequenced by Edman degradation. Plasma of a person heterozygous for a polymorphism of this protein was used ($m/z = 4152$ and 4186). The mass losses from five consecutive Edman sequencing steps are shown in the inset. Once again, note that the peak identities shown in the figures are the observed m/z values while those used in the text and Table I are the theoretical m/z values.

peptides indicated that the polymorphism occurred between residues 467 and 482. A known modification in this region with a 32 mass unit difference is V480M (Stein & Carrell 1995).

The profiles of high-responding subjects at 8 h showed a number of other components not detected in samples from healthy individuals. Major new peaks in Figure 1B include those between $m/z = 10\,000$ and 11 000 and the peak at $m/z = 9305$. These peaks were not identified. Future studies are required to determine whether they may also be useful for analysis of inflammation or other events associated with low-dose endotoxin infusion.

Serum amyloid A and C-reactive protein

As a part of our standard protocol, C-reactive protein (CRP) was measured at 24 h. Levels were 4–6-fold higher (Table II) than the reported upper limit for normal individuals (5 mg l^{-1}). Since baseline CRP levels were not measured, the fold increase in CRP could not be calculated. However, infusion of 2 ng kg^{-1} of endotoxin has been shown to increase CRP levels about 100-fold, mainly by interleukin-6 release (Derhaschnig et al. 2004).

In the human, SAA exists as three isoforms, two acute-phase forms, SAA1 ($m/z = 11\,683$ (Kiernan et al. 2003)) and SAA2 ($m/z = 11\,629$ (Kiernan et al. 2003)) and a constitutive form (SAA4 or C-SAA (Uhlar & Whitehead 1999, Hari-Dass et al. 2005)). SAA was generally not detected in the baseline protein profiles (Figure 1A) and was first detectable between 4 and 8 h with a rising trend to 24 h. SAA1 ($m/z = 11\,683$) was the more dominant form and appeared earlier than SAA2, which was only detected at the 24 h time point. A peak with an m/z value of SAA4 was not detected in any sample. Several degradation forms of SAA1 were suggested by the profile patterns. While the full-length protein ($m/z = 11\,683$) was the only form detected at the earliest time points (4 and 8 h), a second peak appeared by 24 h that was 156 amu

Table II. Clinical response to endotoxin.

Subject	T baseline (°F)	T max (°F)	CRP (@24 h)	Adverse effect
1[a]	98.4	99.1	2.20	Diarrhoea, emesis, queasiness
2	98.4	99.8	2.00	Stomach pain
3	97.7	100	n/a	None
4[a]	97.5	100.2	2.60	Emesis
5[a]	97.5	100.6	2.9	None
6[a]	97.0	99.9	2.6	None
7	96.8	99.7	3.7	Feeling cold, headaches
8	97.5	99.5	2.91	Weakness, headaches
9[b]	97.2	100.2	1.57	None
10	97.5	99.9	1.7	None
11	97.7	100.6	3.23	Dizziness, headaches
12[a]	97.9	99.9	3.4	None
13	98.1	100.4	2.34	Headaches
14	97.5	99.1	2.9	Dysphagia
15[a]	98.1	100	2.81	Headaches
16	96.6	99	3.0	Stomach pain
17	97.5	99.7	2.1	None
18	98.2	99.7	2.4	None

[a]High-responder phenotype; [b]elevated SAA levels at baseline

lower ($m/z = 11\,527$). This peak had the correct mass for loss of C-terminal Arg from intact SAA1. Smaller peaks corresponding to loss of Arg-Ser were also detected in a minority of subjects. This trend in appearance followed prior reports and is presumed to arise from carboxypeptidase action (Kiernan et al. 2003).

Appearance of SAA was expressed as the SAA peak intensity relative to other components of the profile and/or as a fold change from baseline. Since all but one individual had undetectable SAA before endotoxin, baseline intensity consisted of signal to noise at $m/z = 11\,683$ (see Methods). A small SAA peak was detected in most subjects by 4 h with an average increase of 2.3-fold over baseline. By 8 h, SAA was present in all but one subject with an average increase of sixfold. This increased to 15-fold by 24 h (Figure 4). In agreement with CRP, no significant difference was found in the fold change in SAA between low and high responders.

One of the 18 subjects showed a high level of SAA at baseline. Although detected occasionally in other studies of healthy individuals, it was extremely unusual. This individual was apparently healthy and satisfied eligibility criteria to participate in the study. Regardless of the basis for SAA presence at baseline, this individual offered the unusual opportunity to observe the impact of elevated SAA expression at baseline. The result was striking. That is, SAA levels actually declined in this person and reached non-detectable levels at 24 h (Figure 4, inset), the only example of this result. While only a single case, the result suggested that expression of acute-phase proteins prior to exposure to endotoxin may be protective or serve to blunt the response. This phenomenon deserves future evaluation.

Changes in ApoCIII glycosylation

Virtually all peak ratios of the profile changed in response to low-dose endotoxin. Changes in glycosylation of apoCIII provide an illustration. ApoCIII1 ($m/z = 9423$) and apoCIII2 ($m/z = 9714$) differ by a single sialic acid residue and changes in their ratio could result from altered rates of biosynthesis or degradation. Figure 5 shows this peak ratio for 13 individuals. The baseline ratios varied from about 0.17 to 0.65 (Figure 5, inset), similar to the range observed for healthy individuals in all samples we have examined to date (Nelsestuen et al. 2005b). Following endotoxin infusion, the ratios changed but, with the exception of the high-responder cases, they all

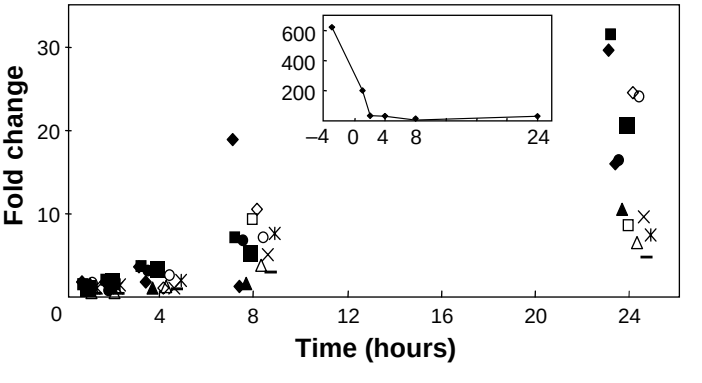

Figure 4. Serum amyloid A (SAA). With one exception, SAA was not detected at baseline and signal to noise was used to estimate fold increases over baseline. Inset, the subject with detectable SAA at baseline. Signal to noise was used to indicate change following endotoxin.

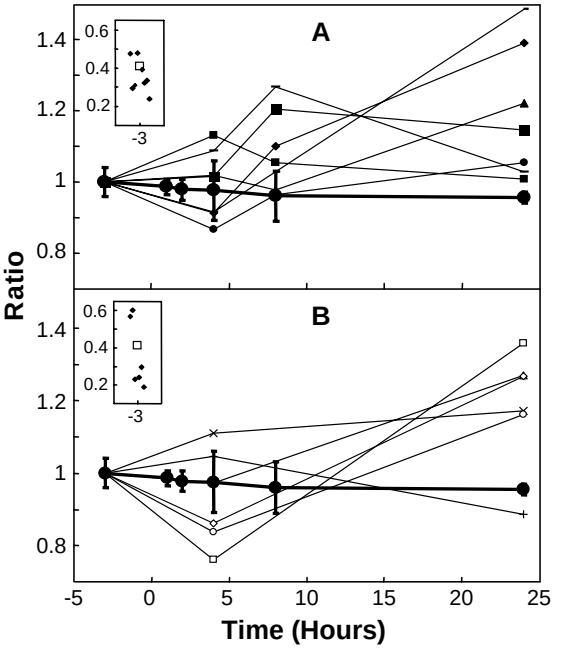

Figure 5. Changes in the apoCIII2 ($m/z = 9714$) to apoCIII1 ($m/z = 9423$) ratio. (A) Fold change among the low-responder subset. For clarity, only eight of the 12 subjects are plotted. (B) Fold change for six high responders. In both panels, the actual ratios before endotoxin are shown in the insets. The plots show ratios expressed relative to the zero time point of each individual.

remained within the range of values for healthy individuals. Thus, from any single reading it was not possible to determine that any of these individuals had been subject to a health challenge. However, when ratios were expressed relative to each individual's baseline peak ratio, it was evident that most of these individuals experienced significant change (Figure 5). The 8 h time points are not plotted for the high responders due to quantitative loss from oxidation (Figure 5B). The changes at 24 h ranged from 0 to 30% ($p < 0.002$) with no evident difference between high and low responders. In contrast, healthy individuals undergo little change in this peak ratio over the same time interval (heavy line, Figure 5). The range of values among controls approximated the standard deviation for determination of this peak ratio.

Clinical response to low-dose endotoxin

Clinical data following the administration of endotoxin showed that all 18 subjects developed a low-grade elevation of body temperature that peaked between 4–6 h (Table II). A number developed other mild symptoms as presented in Table II. On this basis, low-dose endotoxin exposure seemed to be well tolerated by all volunteers. There was no apparent correlation between the clinical symptoms and the high-responder subset. Therefore, the dramatic changes in protein profile appeared to be a subclinical phenomenon.

Discussion

Proteome analysis identified early changes in the plasma protein milieu following exposure to low-dose endotoxin in all individuals studied. Of note, an extreme response to low-dose endotoxin was observed in a subset of healthy individuals and potential biomarkers of this altered response were identified. Rapid extraction and MALDI-TOF analysis is a simple method that provides a surprisingly large amount of information about acute, as well as chronic, changes in the plasma proteome. This study illustrates how a perturbation in homeostasis was dramatically reflected in the plasma protein profiles and in proteins that may serve as surrogate markers of events occurring on a larger scale. Of special note was that most of these changes would not be fully appreciated by more conventional proteome analysis consisting of protease digestion, peptide isolation and MS/MS analysis. For example, these steps would obliterate many specific protein fragments that arise from biological processes, cause reduction of methionine sulfoxide or its artificial production, and they seldom detect specific glycopeptides or present quantitative evaluation of mixtures of different glycoisoforms. In addition, two-dimensional gel electrophoresis depends on protein charge and molecular weight, and may not be sensitive to methionine oxidation. Thus, direct observation of intact proteins by mass spectrometry was necessary to fully appreciate the extent of protein changes and oxidation following low-dose endotoxin infusion.

One-third of the subjects who were exposed to low-dose endotoxin demonstrated extensive changes at the 8 h time point and were designated 'high responders'. The most notable changes were: appearance of C1 esterase inhibitor as an exceptionally dominant peak in the profile, widespread oxidation of plasma lipoproteins, increased proteolytic activity, and altered glycosylation states of apolipoprotein C. Each of these changes offered unique information about events in the plasma.

C1 esterase inhibitor is the only known inhibitor of complement protein 1 and is an important regulator of the classic pathway (Moore 2004). Detection of a C1 esterase inhibitor fragment could provide direct evidence that alterations in the classic complement pathway had occurred. Gram-negative bacterial lipopolysaccharide (LPS, endotoxin) can activate all the complement pathways (Moore 2004). However, previous studies of endotoxin infusion (2 ng kg^{-1}) have not demonstrated activation of complement as measured by C3a-des-arg levels, FXIIa-C1 inhibitor complexes, kallikrein-C1 inhibitor complexes or modified C1 inhibitor (van Deventer et al. 1990). Plasma samples taken at 1, 2, 3 and 4 h did not show the presence of the activation peptide of C1 inhibitor. Thus, it stands to reason that an event other than the infusion of endotoxin itself was responsible for this alteration in the complement pathway. Activation of the classic pathway of complement is an antibody-dependent process (Walport 2001a,b). For this activation to be directly mediated by endotoxin itself, past exposure would be essential. None of the high responders had participated in a previous endotoxin trial, although we cannot rule out previous exposure, e.g. during urinary tract infections. However, even if this were the case, complement activation should be an immediate event and we would expect C1 esterase inhibitor degradation immediately following exposure. Infusion of endotoxin induces leukocyte degranulation and release of proteolytic enzymes such as elastase that peak at 4 h and last for at least 12 h (Jilma et al. 2002, Derhaschnig et al. 2003). Elastase and other leukocyte proteinases are known to degrade C1 esterase inhibitor (Brower & Harpel 1982, Caliezi et al. 2000). Increased levels of inactive C1 esterase inhibitor have been found

in septic patients, particularly those with poor prognosis (Nuijens et al. 1989). Thus, proteolytic degradation is another possible explanation for the appearance of the C1 esterase inhibitor fragment. In any event, future studies may find that appearance of the $m/z = 4154$ peak constitutes a useful, early biomarker that may foreshadow more profound subsequent events such as development of sepsis syndromes.

A second event that defined the high-responder subset was the widespread oxidation of plasma proteins. This is likely to correspond to oxidation of methionine (Met) residues. In keeping with a cataclysmic and quantitative oxidative event, addition of 16 or 32 mass units corresponded to the number of methionines per protein for apoCI (1 Met, +16 amu), apoCII (2 Met, +32) and apoCIII (2 Met, +32). Protein oxidation may result from the 'oxidative burst', which has been observed in the endotoxemia model (Pleiner et al. 2004). Additional evidence in support of this is the fact that oxidation occurred 8 h after administration of endotoxin and in concert with the appearance of C1 inhibitor activation peptide rather than as an immediate response to endotoxin. Whatever the basis for oxidation, this study showed that it can be a profound event with the potential to alter every protein in the plasma. Further studies are needed to determine the extent of oxidation of other proteins and the impact of such radical events on cells and cell membranes. That it occurred in a subset of individuals suggests the need to compare a larger population and correlate the outcomes of protein oxidation to other characteristics of these individuals.

Oxidative changes had completely resolved by 24 h. Methionine sulfoxide is reduced/repaired by a family of proteins called methionine sulfoxide reductases (MSRs), antioxidant enzymes that protect against oxidative stress (Hansel et al. 2005). Resolution of the oxidative changes may result from the action of MSRs or from protein turnover.

Lipoproteins play an important role in the host defense against bacterial endotoxin and HDL has been shown to decrease the response to endotoxin and to play a protective role in sepsis (Pajkrt 1996, 1997, Chien et al. 2005). In this study, the administration of endotoxin resulted in an altered ratio of the different glycoisoforms of apolipoprotein CIII. Apolipoprotein C plays an important role in the synthesis and metabolism of HDL and VLDL. Future characterisation of changes in this protein may offer valuable insight into pathophysiological mechanisms and warrants further study.

Our definition of high- and low-responder phenotypes was based on changes at the level of the plasma proteome. There was no significant difference between the two groups from a clinical standpoint. As the study conditions and eligibility criteria were the same and all subjects were healthy at the time of the study, an exaggerated response to low-dose endotoxin may be genetically determined. A recent study of gene expression profiling identified high- and low-responder genotypes among healthy individuals following exposure to endotoxin *ex vivo* (Wurfel et al. 2005). Indeed, there is increasing evidence that genetic polymorphisms in key signalling pathways play a role in the response/susceptibility to certain pathogens (Villar et al. 2004, Schroder & Schumann 2005). The identification of a high-responder phenotype by a proteomic approach may reflect these genetic phenomena or be related to others as yet unidentified. Further studies are needed to determine any link with known or future genetic properties of high responders.

In conclusion, simple and rapid proteomic techniques can provide powerful tools for studying disease pathophysiology and in biomarker discovery. This study

illustrated how a very simple method of rapid extraction and MALDI-TOF protein profiling can identify a number of changes in the plasma proteome following exposure to low-dose endotoxin. A subset of individuals demonstrated a heightened response to endotoxin that was characterised by delayed alterations of the complement pathway as well as widespread protein oxidation and increased proteolytic activity, a phenomenon not previously described. Monitoring of these events deserves further investigation for identification of surrogate biomarkers in a number of candidate disease conditions.

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