Identification of De Novo Synthesized and Relatively Older Proteins

Accelerated Oxidative Damage to De Novo Synthesized Apolipoprotein A-1 in Type 1 Diabetes

Abdul Jaleel,1 Gregory C. Henderson,1 Benjamin J. Madden,2 Katherine A. Klaus,1 Dawn M. Morse,1 Srinivas Gopala,1 and K. Sreekumaran Nair1

OBJECTIVE—The accumulation of old and damaged proteins likely contributes to complications of diabetes, but currently no methodology is available to measure the relative age of a specific protein alongside assessment of posttranslational modifications (PTM). To accomplish our goal of studying the impact of insulin deficiency and hyperglycemia in type 1 diabetes upon accumulation of old damaged isoforms of plasma apolipoprotein A-1 (ApoA-1), we sought to develop a novel methodology, which is reported here and can also be applied to other specific proteins.

RESEARCH DESIGN AND METHODS—To label newly synthesized proteins, [ring-13C6]phenylalanine was intravenously infused for 8 h in type 1 diabetic participants (n = 7) during both insulin treatment and 8 h of insulin deprivation and in nondiabetic participants (n = 7). ApoA-1 isoforms were purified by two-dimensional gel electrophoresis (2DGE) and assessment of protein identity, PTM, and [ring-13C6]phenylalanine isotopic enrichment (IE) was performed by tandem mass spectrometry.

RESULTS—Five isoforms of plasma ApoA-1 were identified by 2DGE including ApoA-1 precursor (pro-ApoA-1) that contained the relatively highest IE, whereas the older forms contained higher degrees of damage (carbonylation, deamidation) and far less IE. In type 1 diabetes, the relative ratio of IE of [ring-13C6]phenylalanine in an older isoform versus pro-ApoA-1 was higher during insulin deprivation, indicating that de novo synthesized pro-ApoA-1 more rapidly accumulated damage, converting to mature ApoA-1.

CONCLUSIONS—We developed a mass spectrometry–based methodology to identify the relative age of protein isoforms. The results demonstrated accelerated oxidative damage to plasma ApoA-1, thus offering a potential mechanism underlying the impact of poor glycemic control in type 1 diabetic patients that affects a patient’s risk for vascular disease. Diabetes 59:2366–2374, 2010

There is substantial evidence to indicate that oxidative stress and subsequent oxidative damage is a major factor in the pathogenesis of diabetic complications (1,2). Oxidative damage of plasma proteins has been reported in patients with type 1 diabetes (3–5), and the accumulation of these damaged proteins is associated with many chronic complications of type 1 diabetes (4,6,7). Each protein does not exist as a homogenous pool within a tissue, but rather exists as a heterogeneous mix of isoforms of the same protein at different ages for which different amounts of time have passed following translation. A key determinant of the composition of the proteome is the rate at which the proteins are synthesized and removed (degraded) from a tissue, and this turnover, which replaces aged, damaged proteins with de novo synthesized proteins, is likely to maintain a relatively healthy composition of the proteome. A relative increase in isoforms of oxidatively damaged proteins can occur because of accelerated oxidative damage if these damaged proteins are not removed by degradation and replaced by de novo synthesized proteins. A better understanding of the oxidative damage to proteins, and their accumulation in proteins such as lipoproteins, is critical to further understand the pathophysiology of diabetic complications, especially since atherosclerosis and cardiovascular complications are common in people with diabetes. However, there has been no methodology to measure the relative age of proteins, and thus, hypotheses related to the accumulation of aged proteins could not yet be tested.

In two-dimensional gel electrophoresis (2DGE) of tissues or body fluids, numerous protein gel spots can be identified, each representing the same protein identity (8–19). Often these numerous spots exist as charge variants and thus present as a train of spots horizontally adjacent to one another on the gel (8–19), each with similar molecular weights but with different isoelectric points (pI). This phenomenon has been observed for various proteins that have known relevance to disease, such as prostate-specific antigen (PSA) (18), heat shock proteins (15), fibrinogen (14), apolipoprotein A-1 (ApoA-1) (9–14), and many others. We hypothesized that the different protein isoforms in spot trains are related to different ages of proteins, and we hoped that experiments to further understand the nature of these proteins would improve the understanding of diseases that are related to an accumulation of aged proteins. Based on principles of tracer...
incorporation into proteins, it is reasonable to assume that during an intravenous infusion of a tracer such as \(^{1-13}\text{C}_6\)phenylalanine, both tracer and tracee will be incorporated into proteins synthesized during the course of the infusion. However, no \(^{1-13}\text{C}_6\)phenylalanine would be incorporated into proteins that were synthesized before the infusion, such as hours, days, or even years earlier. Thus, the young de novo synthesized proteins that were most recently translated will have relatively higher amounts of isotopic label than the protein isoforms that have primarily accumulated from the past. It is likely that high levels of oxidative damage and other modifications would be detected in older protein isoforms because they are exposed to the environmental stresses over longer periods of time.

We studied insulin deprivation and treatment in type 1 diabetes, as it is known that insulin deprivation and subsequent hyperglycemia in type 1 diabetes leads to oxidative stress and an increased accumulation of oxidized plasma proteins (3–5). We focused our investigation on ApoA-1, which is a key protein component of HDL and is involved in cholesterol clearance from arteries. Considering preliminary knowledge of ApoA-1 isoforms (10,11), the importance of ApoA-1 in vascular health, the increased risk of macrovascular disease in type 1 diabetic patients (20), and our experience with resolving its isoforms on 2DGE, we chose ApoA-1 for the current study. We hypothesized that withdrawal of insulin treatment in type 1 diabetes would result in increased oxidative damage to the newly synthesized proteins and that these proteins with damage-induced charge alteration would be found at the locations in 2DGE where the older proteins are typically found.

**RESEARCH DESIGN AND METHODS**

**Study protocol and sample collection.** Seven participants with type 1 diabetes (3 women, 4 men) with an average diabetes duration of 18.7 (range 7–35) years were matched with 7 nondiabetic healthy participants (ND) (3 women, 4 men). The characteristics of study participants are shown in Table 1. The study protocol and sample collections were approved by the Institutional Review Board and have been reported previously (21,22). On the day before tracer infusion, participants were admitted at 8:00 P.M. and insulin infusion was started at 6:00 A.M. in type 1 diabetic participants on each of two separate visits. The insulin regimen of type 1 diabetic participants changed during the 3 days before the study in those who were taking long-acting insulin (n = 4 of 7 participants). They were instructed to use ultra rapid-acting insulin (aspart or lispro, recombinant insulins) before each meal and bedtime based on their blood glucose. Participants on an insulin pump (n = 3) using ultra rapid-acting insulin continued their regimen. All participants maintained their blood glucose between 4.4 and 6.6 mmol/L. On the evening before the study, all participants were admitted to the Clinical Research Unit. On the insulin-treatment day in type 1 diabetic participants (type 1 diabetes insulin-treated state [I±]; performed first), an intravenous insulin infusion was administered and plasma glucose was maintained between 4.44 and 5.56 mmol/L. On the insulin-deprived day (type 1 diabetes insulin-deprived state [I–]; performed second, 10 days after the first study day), insulin infusion was discontinued from 4:00 A.M. to 12:00 P.M. (8 h) and replaced with normal saline. At 4:00 A.M., after taking baseline arterial-venous samples for isotope measurements (IE), a day in type 1 diabetic participants (I±) (type 1 diabetes insulin-treated state) was performed. A1C (%) was determined from the final sample drawn (after 8 h of tracer infusion). The sample was first incubated with an antibody diluent. After initial incubation and measurement of the sample, blank, undiluted antisera specific to human ApoA-1 was added. When sample solution is mixed, insoluble antigen-antibody complexes begin to form. These complexes produce turbidity in the mixture and increase the light transmittance. The decrease in light transmittance resulting from the antigen-antibody reaction was measured as a function of the concentration of ApoA-1. Measurements were taken at 700 and 340 nm.

**Purification of ApoA-1 isoforms.** Each plasma sample was subjected to affinity chromatography for the depletion of six highly abundant proteins (albumin, IgG, antitrypsin, IgA, transferrin, and haptoglobin) in a single step, with a Multiple Affinity Removal Column Hu-6 (Agilent Technologies, Santa Clara, CA) using a BioLogic HR Chromatography System (BioRad, Hercules, CA) running buffers A and B according to specifications of the column manufacturer. This selective immuno-depletion process provides one enriched pool of low-abundant plasma proteins containing ApoA-1 in the column flow-through. The flow-through was later concentrated using a 5-kDa molecular weight cutoff filter, and protein estimation was done by Bradford’s method (BioRad) before being subjected to 2DGE.

ApoA-1 was isolated from the depleted plasma by performing large, high-resolution 2DGE, using 24-cm, pH 3–10, 4–7, and 4.7–5.9 immobilized pH gradient (IPG) strips (BioRad). The ApoA-1 gel spots were visualized by silver staining. Duplicate 2DGEs for each sample were done for IE analyses and one gel per sample for the analysis of posttranslational modifications (PTMs). The method to identify proteins by mass spectrometry is described below.

**Protein Identification and PTM analyses.** To identify proteins in each gel spot and test for PTMs, ApoA-1 spot trains isolated from blood samples drawn at 8 h of isotopic tracer infusion were analyzed. The silver-stained SDS-PAGE gel spots were excised, destained, and subjected to in-gel trypsin digestion. The extracted peptides were analyzed for protein identification by nano-flow liquid chromatography electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS) using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer (ThermoElectron, Bremen, Germany) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA). The tandem mass spectra (MS/MS) were extracted by BioWorks, version 3.2. All MS/MS spectra were analyzed using Mascot (Matrix Science, London, U.K., version 2.2.04), set to search the Swissprot database (699,052 entries) designating the digestion enzyme as trypsin and searching with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.8 parts per million. Variable modifications chosen were oxidation of methionine and tryptophan, deamidation of asparagine and glutamine, lysine to allysine, lysine to aminoadipic acid, formylation of lysine, dioxygenation of tryptophan and methionine, and arginine to glutamic semialdehyde. Mascot-generated data files were manually studied, and any modifications with an ion score below 20 were not considered. We report the level of a particular modification as the ratio of that modified peptide to that of the total number of unmodified peptides.

**Western blot analyses to detect protein identity and oxidative damage.** ApoA-1 from plasma samples were separated by 2DGE. The 6 x 10 cm gel areas corresponding to the location of ApoA-1 spot trains (as identified by the mass spectrometry analyses described above) were cut and transferred to polyvinylidene fluoride membrane by a semidy-electro transfer apparatus (BioRad). A monoclonal antibody against ApoA-1 (Abcam, Cambridge, MA) was used to detect ApoA-1 at 200 dilutions. A horseradish peroxidase (HRP)-conjugated antibody was used as the secondary antibody in 1:10,000 dilutions. The reaction was detected with chemiluminescence (ECL-Plus, GE Health Care). For the detection of carbonylation, an Oxyblot kit (Millipore, Billerica, MA) was used. Briefly, after the IEF step in 2DGE, the
IE of ApoA-1 isoforms in spot trains. IE measurements were conducted using gas chromatography/tandem mass spectrometry (GC/MS/MS). ApoA-1 spot trains isolated from plasma samples obtained at 2, 4, 6, and 8 h were used for GC/MS/MS analysis. Plasma protein gel spots were excised and hydrolyzed using 6 M HCl overnight at 110°C to obtain free amino acids. The free amino acids were isolated using BioRad AG-50W × 8 cation exchange resin and excess solvent evaporated to dryness. The amino acid residues were derivatized to their N-heptafluorobutyryl methyl esters and analyzed by tandem mass spectrometry using either a ThermoFinnigan TSQ 7000 (ThermoElectron, Waltham, MA) or a Quattro-Micro GC (Waters Corporation, Milford, MA) under negative ion chemical ionization conditions using isobutane as a reactant gas as described previously (23). Free amino acids were extracted from plasma samples using acetic acid as described (24) and analyzed as their t-butyldimethylsilyl ester derivative (25). In the proposed method, plasma amino acid IE is not used in calculations to determine relative protein age, but simply confirms that steady infusion of the \[\text{ring}^{13}\text{C}_6\]phenylalanine was successfully performed. The IE in units of moles percent excess (MPE) for \[\text{ring}^{13}\text{C}_6\]phenylalanine (M + 6) was calculated above background (subtracting preinfusion IE) as previously described (24). The relative age of protein isoforms was determined by comparing relative IE between isoforms of the same protein, such as mature isoforms versus precursor isoform (e.g., IE ratio of C vs. E). Plasma ApoA-1 concentrations were not different among the three groups (Table 1).

**RESULTS**

Plasma ApoA-1 as a spot train in 2DGE. In 2DGE with IPG strips having full pH range (3–10 pH) down to the narrowest range possible (4.7–5.9 pH), ApoA-1 produced five distinct protein gel spots horizontally separated from one another based on the pI (a spot train). We named them spots A, B, C, D, and E from relatively negatively charged spots left to the more positively charged isoform spots [toward the positive end of gel (right)] (Fig. 1A), with the center spot C at a pI of 5.4. We also further verified the identity of the spots by Western blot, which demonstrated immunologic reactivity of each protein isoform (data not shown). Amino acid sequence obtained from the Mascot search revealed that the propeptide RHFQWQ was present only in spots D and E (Fig. 1B). The propeptide was absent in spots A, B, and C, indicating that these spots were mature versions of ApoA-1.
Identification of the relative age of protein isoforms using labeled amino acids. Figure 2A shows the IEs, corrected for preinfusion background IE, measured from all of the five spots of ApoA-1 isolated from blood samples collected at times 2, 4, 6, and 8 h during the tracer infusion period. It demonstrates that spot E is the newest (higher IE than all other spots, \( P < 0.05 \)), followed by spot D (higher IE than A, B, and C, \( P < 0.05 \)), and then spots A, B, and C are the oldest. Furthermore, it shows real-time incorporation of label, since the IEs at 8 h of infusion were significantly higher than those at 2 h (\( P < 0.05 \)). In ApoA-1 spots 10 days after the 8-h infusion of labeled amino acid, we found that spots A, B, and C, which are mature forms of ApoA-1, had significantly higher levels of [ring-\(^{13}\)C\(_6\)]phenylalanine enrichment (IE) than the newer versions D and E (\( P < 0.05 \)) (Fig. 2B), which indicates that mature forms were synthesized in the past, back when the propeptide contained a higher IE.

**ApoA-1 charge variants are formed because of PTM.** Since the first dimension separation of proteins in 2DGE is based on their pI (isoelectric pH, or net charge of a protein), the separation of a specific protein into a spot train is the result of charge variants in the same protein. PTMs are the main reason for variation in the charge of protein amino acid residues and were detected by changes in the mass spectra such as that shown for deamidation in Fig. 3A. As shown in Fig. 1B, spots D and E include the propeptide extension RHFWQQ. The presence of histidine (H) in the propeptide gives an additional strong positive charge for spots D and E. We believe that this may

diabetes.diabetesjournals.org
be the main reason why spots D and E are shifted to the positive side of the gel that attracts negatively charged proteins. Spot E was purely propeptide in all samples, but D sometimes contained some mature peptide in addition to propeptide. Thus, D could be a transition stage between the newer and mature form of ApoA-1. The IE results support the idea that D represents a transition stage, since the IE for spot D was between that of spots E and C.

The protein coverage for spots B, C, and E was comprehensive enough to allow for comparison of the number of peptides containing PTMs present in each spot, whereas spots A and D were not analyzed for all PTMs because of their lower sequence coverage. Deamidation of asparagine and glutamine are two modifications that are believed to represent biologically deleterious protein damage. These PTMs can be detected by a shift of 1 Da in the parent ion.
spectrum, and the position of deamidation in the MS/MS spectra can be determined as shown in Fig. 3A for the ApoA-1 peptide LEALKENGGR. The number of peptides with deamidated amino acids (asparagine and glutamine) was higher in spots B and lowest in spot E (B > C > E), as shown in Fig. 3B. Deamidation adds a negative charge under IEF conditions, which contributes to the shift of spots within a train toward the positive side of the gel. Similarly, oxidation of tryptophan, histidine, and methionine was determined by the shift of 16 Da in the ion fragments of modified peptides. We observed that oxidation of these amino acids (letters in bold blue) in spot B was highest and spot E was lowest (B > C > E) (Fig. 3B). Although these oxidations do not contribute to the difference in the charge of protein isoforms, oxidation is considered a maker for protein aging and damage that corresponds to the fact that the older proteins showed more of this modification.

By Western blot analysis, we found that spots A, B, and C reacted for the presence of carbonylation (irreversible oxidation), whereas spots D and E did not (Fig. 3C, panel B). Carbonyl formation in certain amino acids (e.g., lysine conversion to allysine) removes their positive charge to make the protein more negative, which in turn will make the spot shift toward the positive end of the 2DGE. Together, all these data suggest that PTMs are likely responsible for the development of spot trains of ApoA-1. Higher oxidative damage and deamidation are present in the spots of ApoA-1 that are relatively older and to the positive side of the gel, which is consistent with the concept that older proteins have experienced more opportunity over time for chemical insults.

**Insulin deficiency in type 1 diabetes alters IE and modification in isoforms of ApoA-1.** The old/new IE ratio for spots C versus E (Fig. 4A) showed higher value during insulin deficiency (P < 0.05), demonstrating that insulin deficiency triggers the newly formed proteins to be modified into the mature, damaged versions of the protein. A similar trend, although not statistically significant, was shown for protein spots B/E (type 1 diabetes I^+, 0.060 ± 0.016; type 1 diabetes I^−, 0.053 ± 0.015; ND, 0.057 ± 0.010) and for spots A/E (type 1 diabetes I^+, 0.023 ± 0.008; type 1 diabetes I^−, 0.050 ± 0.014; ND, 0.023 ± 0.007). The pattern reveals that the ApoA-1 isoforms that are more negatively charged are older and that insulin withdrawal makes the mature isoforms chronologically younger, but more damaged because of rapid modification of the de novo synthesized isoform. It should be reiterated here that when applying this methodology, the ratio of IE in aged versus newer proteins is important rather than the absolute IEs of any individual spot.

As there were no differences between groups (type 1 diabetes I^+ vs. type 1 diabetes I^− vs. ND) for the relative amount of each isoform (percentage of total ApoA-1 represented by each spot; data not shown), we analyzed for PTMs in spot C, the most abundant isoform. We did not find any difference in deamidation between groups. However, we found a significant increase in oxidation as shown by the ratio of carboxylated to total peptides (modification of lysine to allysine, P < 0.05) (Fig. 4B). Allysine formation is an intermediate step in the irreversible oxidative damage of carboxylation in the form of aminoacidic peptide. Allysine formations also remove the positive charge from the amino acid residue, so that the protein becomes more negatively charged and moves to the left toward the positive side of the gel. We demonstrated an association between the higher level of oxidative damage in spot C with the old/new IE ratio (C/E) in type 1 diabetes during insulin deprivation. Hence, this shows that in application of this novel methodology, important knowledge can be gained by combining a static measure of the amount of damage with a kinetic measurement of the conversion of relatively undamaged de novo synthesized protein into damaged, mature protein isoforms.

**DISCUSSION**

The current experiment clearly demonstrates that the different spots within spot trains of 2DGE represent different ages of ApoA-1 by differences in the stable isotope-labeled amino acid ([ring-13C]phenylalanine) enrichment and the presence of posttranslational protein modifications.
tions. The highest $[^{13}C_6]$phenylalanine enrichment is shown in pro-ApoA-1, which is newly synthesized, whereas the isoforms of ApoA-1 with modifications such as carbonylation and deamidation have lower $[^{13}C_6]$phenylalanine enrichment, supporting the theory that they were synthesized earlier. In support of our hypothesis, the results show that in type 1 diabetes during insulin deficiency, in comparison with insulin treatment, greater oxidative damage occurred to ApoA-1 and results in a rapid shifting of de novo synthesized protein to the location of the aged proteins within the spot train in 2DGE.

ApoA-1 is produced by the liver and intestines (26) and is released into the circulation as a propeptide (spots D and E), which is converted into the mature isoform while in circulation (spots A, B, and C) (9,27,28). Principles of this proposed method dictate that proteins that were most recently synthesized (i.e., during the infusion of $[^{13}C_6]$phenylalanine) should contain higher amounts of $[^{13}C_6]$phenylalanine as was shown for the propeptides, especially isoform E. In contrast, spots D, C, B, and A showed progressively lower IE, indicating that they were more synthesized before the stable isotope exposure (Fig. 2A). The half-life of the mature form is much longer than the propeptide form (27), which is consistent with the fact that we found higher isotopic enrichment in the mature forms than propeptides in blood samples drawn 10 days after tracer administration (Fig. 2B). When we tested spots B, C, and E, the spots with sufficient protein amount for reliable analysis, the spots with lower pI (toward the positive side of the gel) showed higher degrees of deamidation and oxidation (spot B > C > E) (Fig. 3B). This higher degree of damage further supported the isotopic results showing different ages of the isoforms, with mature forms having more time to age and thus more time for environmental insults causing damage. Furthermore, the results for deamidation and carbonylation may provide a mechanism for the phenomenon of spot trains. Both of these PTMs produce a relative decline of pI (the charge becomes more negative) and thus would shift the protein spots toward the left, which is positive and attracts negatively charged species. With regard to the physiologic relevance of these modifications, carbonylation has been shown to reduce enzyme activities (29,30), and the ability to sufficiently repair deamidation is important for proper physiologic functions and survival (31,32). Accumulations of these types of damage have been shown in many pathologic states, such as in the brains of individuals with Alzheimer disease (8,33,34) and in the aging human lens that is prone to cataracts (35,36).

For this isotope-based approach of measuring relative protein age, the important comparison is between the IEs (the ratio) for any certain isoform versus the most newly synthesized form (ideally the precursor isoform). Thus, for example, we compared between groups spot C’s IE divided by spot E’s IE. To reiterate, when comparing different individuals, this ratio of IEs in older protein versus newest protein isoform (old/new) was calculated and analyzed. In people with type 1 diabetes, insulin deprivation increased the ratio of IEs in spots A, B, and C versus E, and results for spot C versus E are shown in Fig. 4A. This pattern of results clearly indicates that the young form of ApoA-1 more rapidly becomes a damaged, aged form. Coincident with this faster pI shift for ApoA-1 was a greater accumulation of carbonylation during insulin deprivation (e.g., conversion of lysine to allylsine as shown in Fig. 4B). Thus, the ApoA-1 proteins acquired damage acutely during insulin deprivation. This provides a mechanism for the association between poor glycemic control chronically and higher levels of protein oxidation in diabetes (37). Admittedly, we did not study insulin deprivation in ND participants, so we cannot know with certainty that protein damage was caused directly by impacts of insulin or glucose changes per se, as it is possible that long-term diabetes in type 1 diabetic individuals somehow leads to greater susceptibility to the detriments of insulin withdrawal. Nonetheless, apparently each glycemic excursion in people with type 1 diabetes causes an increment of oxidative damage to important proteins such as ApoA-1, and certainly to others as well. A potential reason for this higher oxidative damage is increased oxygen consumption (21,38) involving increased fatty acid oxidation (39), glucose oxidation (40), and amino acid oxidation (41) potentially resulting in increased reactive oxygen species production. It certainly may be that this rapid aging of ApoA-1, a key protein in lipoprotein metabolism, causes the known higher risk for macrovascular disease in people with type 1 diabetes (20). However, the current study did not directly address whether oxidative damage of ApoA-1 causes alterations of ApoA-1 function.

The results from this isotope-based approach indicate important information about the mechanism underling the higher degree of ApoA-1 damage in type 1 diabetic individuals with poor glycemic control. The higher damage appears to be largely related to a more rapid rate at which the damage is incurred. We have shown for ApoA-1 in type 1 diabetic individuals that de novo synthesized protein is actually rapidly converted into the damaged mature forms, and so the high amount of amino acid label incorporated into the damaged forms indicates that the problem is not simply inadequate clearance of damaged proteins, but rather the problem is that the environment promotes rapid oxidation and deamidation. It could be that there was also some impact upon degradation of damaged protein, but it appears that an increased rate of damage was a primary event in the development of altered PTM distribution during insulin withdrawal. Therefore, potential medical interventions in this type of situation, such as that of type 1 diabetic individuals, would appropriately be aimed at creating a less harsh environment in plasma, rather than solely focusing on increasing rates of protein degradation. We did not find any differences in plasma ApoA-1 concentrations in the diabetic participants under the two study conditions and nondiabetic control subjects. It may also be that long-term changes in circulating insulin levels can affect the absolute concentration of ApoA-1 and its isoform distribution, but we have shown that even short-term changes in glycemic control can alter the degree of damage for a given level of ApoA-1 and can alter the kinetics of the damage process. Moreover, by applying the current approach, we have generated a hypothesis regarding the etiology of type 1 diabetes–related vascular complications, and hopefully this methodology will be useful for studying other conditions as well (such as Alzheimer disease, cataracts, cancer, sarcopenia, etc.) in which oxidative damage and other modifications have been implicated (8,33–36,42,43). The current methodology demonstrates that when new proteins are rapidly damaged, as in type 1 diabetic individuals during insulin deficiency and hyperglycemia, then apparently aged proteins will contain substantial label. In contrast to the accelerated ApoA-1 oxidation observed during insulin deficiency, hyperinsulin-
emia may result in the accumulation of older and damaged proteins, which contain a lower isotope label. Indeed, insulin is a major in vivo regulator of the rate of protein synthesis and degradation, and insulin deprivation is associated with profound changes in protein metabolism in people with type 1 diabetes (41,44 – 46). Although we studied plasma, any tissue can be studied based on this novel methodology.

In conclusion, we have developed a methodology to measure the relative age of ApoA-1 isoforms, oxidative damage, and other modifications. With this new methodology, using stable isotope-labeled amino acid infusion in vivo, 2DGE resolution of spot trains, and mass spectrometry analyses, we demonstrated the effects of acute insulin deficiency and hyperglycemia in type 1 diabetic individuals upon the conversion of the newly synthesized ApoA-1 precursor into mature and damaged forms. This method also allows investigators to identify de novo synthesized protein isoforms, assess the relative age of the older isoforms, and determine whether aged and damaged proteins accumulate in conditions of insulin resistance and hyperinsulinemia, thus contributing to various pathologies and dysfunctions.

ACKNOWLEDGMENTS
This study was supported by a Mayo Clinic Center for Translational Science Activities (CTSA) Novel Methodology Development Award to A.J., National Institutes of Health (NIH) Grants R33-DK-70179 and R01-DK-41793 to K.S.N., and NIH CTSA Grant UL1 RR024150. G.C.H. was supported by NIH Grant T32-DK-07352.

No potential conflicts of interest relevant to this article were reported.

A.J. conceived of and designed the experiment, performed laboratory analyses, analyzed results, interpreted the findings, and wrote the manuscript. G.C.H. analyzed the results, interpreted the findings, and wrote the manuscript. B.J.M., K.A.K., and D.M.M. performed laboratory analyses, interpreted results, and determined whether aged and damaged proteins accumulate in conditions of insulin resistance and hyperinsulinemia, thus contributing to various pathologies and dysfunctions.

REFERENCES

1. Brownlee M. The pathology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615–1625
2. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813–820
3. Dominguez C, Ruiz E, Gussiñe M, Carrascosa A. Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. Diabetes Care 1998;21:1736–1742
4. Martin-Gallan P, Carrascosa A, Gussiñe M, Dominguez C. Biomarkers of diabetes-associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. Free Radic Biol Med 2002;33:1563–1574
5. Telci A, Cakatay U, Salman S, Satman I, Sivas A. Oxidative protein damage in early stage type 1 diabetic patients. Diabetes Res Clin Pract 2000;50:213–223
6. Grattagliano I, Vendemiale G, Boscia F, Micelli-Ferrari T, Cardia L, Altmare E. Oxidative retinal products and ocular damage in diabetic patients. Free Radic Biol Med 1998;25:369–372
7. Cakatay U, Telci A, Salman S, Satman I, Sivas A. Oxidative protein damage in type 1 diabetic patients with and without complications. Endor Res 2000;26:365–379
8. Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Marksbery WR, Butterfield DA. Proteomic identification of oxidatively modified proteins in Alzheimer’s disease brain. Part II: dihydroxyindole-

nase-related protein 2, α-enolase and heat shock cognate 71. J Neurochem 2002;82:1524–1532
9. Bojanovski D, Gregg RE, Ghiuseppe G, Schaefer EJ, Light JA, Brewer HB Jr. Human apolipoprotein A-I isoform metabolism: proapoA-I conversion to mature apoA-I. J Lipid Res 1985;26:185–193
10. Ghiuseppe G, Rohde MF, Tanenbaum S, Krishnan S, Gotto AM Jr. Origin of apolipoprotein A-I polymorphism in plasma. J Biol Chem 1985;260:15662–15668
11. Zannis VI, Breslow JL, Katz AJ. Isoforms of human apolipoprotein A-I demonstrated in plasma and intestinal organ culture. J Biol Chem 1980;255:8612–8617
12. Kim SS, Kim MH, Shin BK, Na HI, Choi JY, Kee MK, Chong SA, Nam MJ. Different isoforms of apolipoprotein A-I present heterologous post-translational modification in HIV-infected patients. J Proteome Res 2007;6:180–184
13. Yang F, Yin Y, Wang F, Zhang L, Wang Y, Sun S. An altered pattern of liver apolipoprotein A-I isoforms is implicated in male chronic hepatitis B progression. J Proteome Res 2009;9:134–143
14. Mateos-Caceres PJ, Garcia-Mendez A, Lopez FA, Macaya C, Nunez A, Gomez J, Alano-Org, Carrasco C, Burgos ME, de Andres R, Granizo JJ, Farre J, Rico LA. Proteomic analysis of plasma from patients during an acute coronary syndrome. J Am Coll Cardiol 2004;44:1578–1583
15. Elguezgar E, Kenapster L, Wait R, Gosling M, Dunn MJ, Powell JT. F-actin capping (CapZ) and other contractile saphenous vein smooth muscle proteins are altered by hemodynamic stress: a proteomic approach. Mol Cell Proteomics 2004;3:115–124
16. Poth AG, Deeth HC, Alewod PF, Holland JW. Analysis of the human casein phosphopeptide by 2-D electrophoresis and MALDI-TOF/TOF MS reveals new phosphoforms. J Proteome Res 2008;7:5017–5027
17. Pieper R, Gatlin CL, Metzoth AM, Maas, Makusky AJ, Mondal M, Seonarain M, Field E, Schatz CB, Estock MA, Ahmed N, Anderson NG, Steiner S. Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics 2004;4:1150–1174
18. Lee C, Tsai Y, Sensibar J, Oliver L, Grayhack JT. Two-dimensional characterization of prostatic acid phosphatase, prostatic specific antigen and prostate binding protein in expressed prostatic fluid. Prostate 1986;9:135–140
19. Sarigoli H, Lottspeich F, Walk T, Jung G, Eckerskorn C. Deamidation as a widespread phenomenon in two-dimensional polyacrylamide gel electrophoresis of human blood plasma proteins. Electrophoresis 2000;21:2209–2218
20. Krolewski AS, Kosinski EJ, Warram JH, Lelan OS, Busick EJ, Amal AC, Rand LI, Christlieb AR, Bradley RF, Kahn CR. Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. Am J Cardiol 1987;59:750–755
21. Karakaidoses H, Asmann YW, Bigelow MW, Short KR, Dhatriya K, Conen-Schimke J, Kahl J, Mukhopadhyay D, Nair KS. Effect of insulin deprivation on muscle mitochondrial ATP production and gene transcription levels in type 1 diabetic subjects. Diabetes 2007;56:2083–2089
22. Jaleel A, Klaus K, Morse DM, Karakaidoses H, Ward LE, Irving BA, Nair KS. Differential effects of insulin deprivation and systemic insulin treatment on plasma protein synthesis in type 1 diabetic people. Am J Physiol Endocrinol Metab 2009;297:E889–E897
23. Jaleel A, Short KR, Asmann YW, Klaus KA, Morse DM, Ford GC, Nair KS. In vivo measurement of synthesis rate of individual skeletal muscle mitochondrial proteins. Am J Physiol Endocrinol Metab 2008;295:E1255–E1268
24. Ford GC, Cheng KN, Halliday D. Analysis of [1-C-13]leucine and [13-C-1]KIC in plasma by capillary gas chromatography/mass spectrometry in protein turnover studies. Biomed Mass Spectrom 1985;12:432–436
25. Schwenk WF, Berg PJ, Beaufre`re B, Miles JM, Hammond MW. Use of t-butyldimethylsilylation in the gas chromatographic/mass spectrometric analysis of physiologic compounds found in plasma using electron-impact ionization. Anal Biochem 1984;131:101–105
26. Wu AL, Windmuelles HG. Relative contributions by liver and intestine to individual plasma apolipoproteins in the rat. J Biol Chem 1979;254:7316–7322
27. Ghiuseppe G, Gotto AM Jr, Tanenbaum S, Sherrill BC. Proapoprotein A-I conversion kinetics in vivo and in rat. Proc Natl Acad Sci U S A 1985;82:874–878
28. Ghiuseppe G, Bradley WA, Gotto AM Jr, Sherrill BC. Identification of proapo-I in rat lymph and plasma: metabolic conversion to “mature” apoA-I. Biochem Biophys Res Commun 1983;116:704–711
29. Yan L, Sial RS. Mitochondrial adenine nucleotidase translocase is modified oxidatively during aging. Proc Natl Acad Sci U S A 1998;95:12806–12801

diabetes.diabetesscience.org

DIABETES, VOL. 59, OCTOBER 2010 2373
30. Yan L-J, Levine RL, Sohal RS. Oxidative damage during aging targets mitochondrial aconitase. Proc Natl Acad Sci U S A 1997;94:11168–11172
31. Kim E, Lowenson JD, MacLaren DC, Clarke S, Young SG. Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. Proc Natl Acad Sci U S A 1997;94:6132–6137
32. Chavous DA, Jackson FR, O’Connor CM. Extension of the Drosophila lifespan by overexpression of a protein repair methyltransferase. Proc Natl Acad Sci U S A 2001;98:14814–14818
33. Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. Proteomic identification of oxidatively modified proteins in Alzheimer’s disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. Free Radic Biol Med 2002;33:562–571
34. Watanabe A, Takio K, Ibara Y. Deamidation and isoaspartate formation in smeared τ in paired helical filaments. Unusual properties of the microtubule-binding domain of τ. J Biol Chem 1999;274:7368–7378
35. Sharma KK, Santhoshkumar P. Lens aging: effects of crystallins. Biochim Biophys Acta 2009;1790:1095–1108
36. Takemoto L. Deamidation of Asn-143 of gamma S crystallin from protein aggregates of the human lens. Curr Eye Res 2001;22:148–153
37. Cakatay U. Protein oxidation parameters in type 2 diabetic patients with good and poor glycaemic control. Diabetes Metab 2005;31:551–557
38. Nair KS, Halliday D, Garrow JS. Increased energy expenditure in poorly controlled type 1 (insulin-dependent) diabetic patients. Diabetologia 1984;27:13–16
39. Owen OE, Trapp VE, Recihrad GA Jr, Mozzoli MA, Boden G. Effects of therapy on the nature and quantity of fuels oxidized during diabetic ketoacidosis. Diabetes 1980;29:365–372
40. Pehling GB, Tessari P, Gerich JE, Haymond MW, Service FJ, Rizza RA. Abnormal meal carbohydrate disposition in insulin-dependent diabetes. Relative contributions of endogenous glucose production and initial splanchnic uptake and effect of intensive insulin therapy. J Clin Invest 1984;74:985–991
41. Nair KS, Garrow JS, Ford C, Mahler RF, Halliday D. Effect of poor diabetic control and obesity on whole body protein metabolism in man. Diabetologia 1983;25:400–403
42. Orpiakowski J, Schormann N, Kluve-Beckerman B, Liepnieks JJ, Benson MD. Protein aging hypothesis of Alzheimer disease. FASEB J 2000;14:1255–1263
43. Radak Z, Takahashi R, Kumiyma A, Nakamoto H, Ohno H, Ookawara T, Goto S. Effect of aging and late onset dietary restriction on antioxidant enzymes and proteasome activities, and protein carbonylation of rat skeletal muscle and tendon. Exp Gerontol 2002;37:1423–1430
44. Nair KS, Ford GC, Halliday D. Effect of intravenous insulin treatment on in vivo whole body leucine kinetics and oxygen consumption in insulin-deprived type I diabetic patients. Metabolism 1987;36:491–495
45. Pacy PJ, Nair KS, Ford C, Halliday D. Failure of insulin infusion to stimulate fractional muscle protein synthesis in type 1 diabetic patients— anabolic effect of insulin and decreased proteolysis. Diabetes 1989;38:618–624
46. De Feo P, Gan Gaisano M, Haymond MW. Differential effects of insulin deficiency on albumin and fibrinogen synthesis in humans. J Clin Invest 1991;88:833–840

OXIDATIVE DAMAGE TO ApoA-1 IN TYPE 1 DIABETES

2374 DIABETES, VOL. 59, OCTOBER 2010 diabetes.diabetesjournals.org