# Identification of De Novo Synthesized and Relatively Older Proteins

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

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**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*-$^{13}C_6$]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*-$^{13}C_6$]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*-$^{13}C_6$]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**

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**See accompanying commentary, p. 2358.**

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

TABLE 1
Characteristics of study participants

| | Nondiabetic subjects | Type 1 diabetic subjects | |
|---|---|---|---|
| Age (years) | 29.7 ± 3 | 30 ± 3 | |
| Body weight (kg) | 81 ± 6.4 | 78.2 ± 5 | |
| BMI (kg/m$^2$) | 25 ± 1.1 | 26.2 ± 1.3 | |
| Fat mass (%) | 33.2 ± 4 | 31.6 ± 4.1 | |
| Fat-free mass (kg) | 51 ± 5 | 51 ± 4 | |
| A1C (%) | 5.0 ± 0.05 | 7.2 ± 0.5§ | |
| Duration of type 1 diabetes (years) | | 18.7 ± 4 | |
| | | $I^-$ | $I^+$ |
| Glucose (mmol/l) | 4.9 ± 0.1 | 17.0 ± 0.6§‡ | 5.2 ± 0.2 |
| Insulin (pmol/l) | 23.4 ± 4.52 | 3.9 ± 1.36§‡ | 69.8 ± 17.8§ |
| ApoA-1 (g/l) | 1.34 ± 0.08 | 1.39 ± 0.12 | 1.36 ± 0.07 |

Data are means ± SE; $n = 7$ per group. Statistical analyses by $1 \times 2$ and $1 \times 3$ ANOVA. $I^-$, insulin-deprived state; $I^+$, insulin-treated state. §Different from ND, $P < 0.05$; ‡different from $I^+$, $P < 0.05$.

incorporation into proteins, it is reasonable to assume that during an intravenous infusion of a tracer such as [*ring*-$^{13}C_6$]phenylalanine, both tracer and tracee will be incorporated into proteins synthesized during the course of the infusion. However, no [*ring*-$^{13}C_6$]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 5:00 P.M. and insulin infusion was started at 6:00 P.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-treated 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 arterialized-venous samples for isotopic measurements (IE), a continuous infusion of L[*ring*-$^{13}C_6$]phenylalanine (Cambridge Isotope Laboratories, Andover, MA) was given for 8 h at a rate of 1.0 mg/kg fat-free mass [FFM] per hour preceded by a priming dose (1.0 mg/kg FFM). ND participants were studied only once with normal saline infusion. Blood samples were then drawn at 2-h intervals in anticoagulant tubes and centrifuged, and plasma was stored at −80°C until analysis.

**Plasma concentration of ApoA-1.** An automated turbidimetric method (Roche Diagnostics, Indianapolis, IN) was used to measure ApoA-1 in plasma in 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 antiserum 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 amount of light scatter. 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).

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 10.0 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, dioxidation 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 $6 \times 10$ 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 semidry electro-transfer apparatus (BioRad). A monoclonal antibody against ApoA-1 (Abcam, Cambridge, MA) was used to probe the membrane in 1:200 dilution. Anti-mouse horseradish peroxidase (HRP)-conjugated antibody was used as the secondary antibody in 1:10,000 dilutions. The reaction was detected by 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

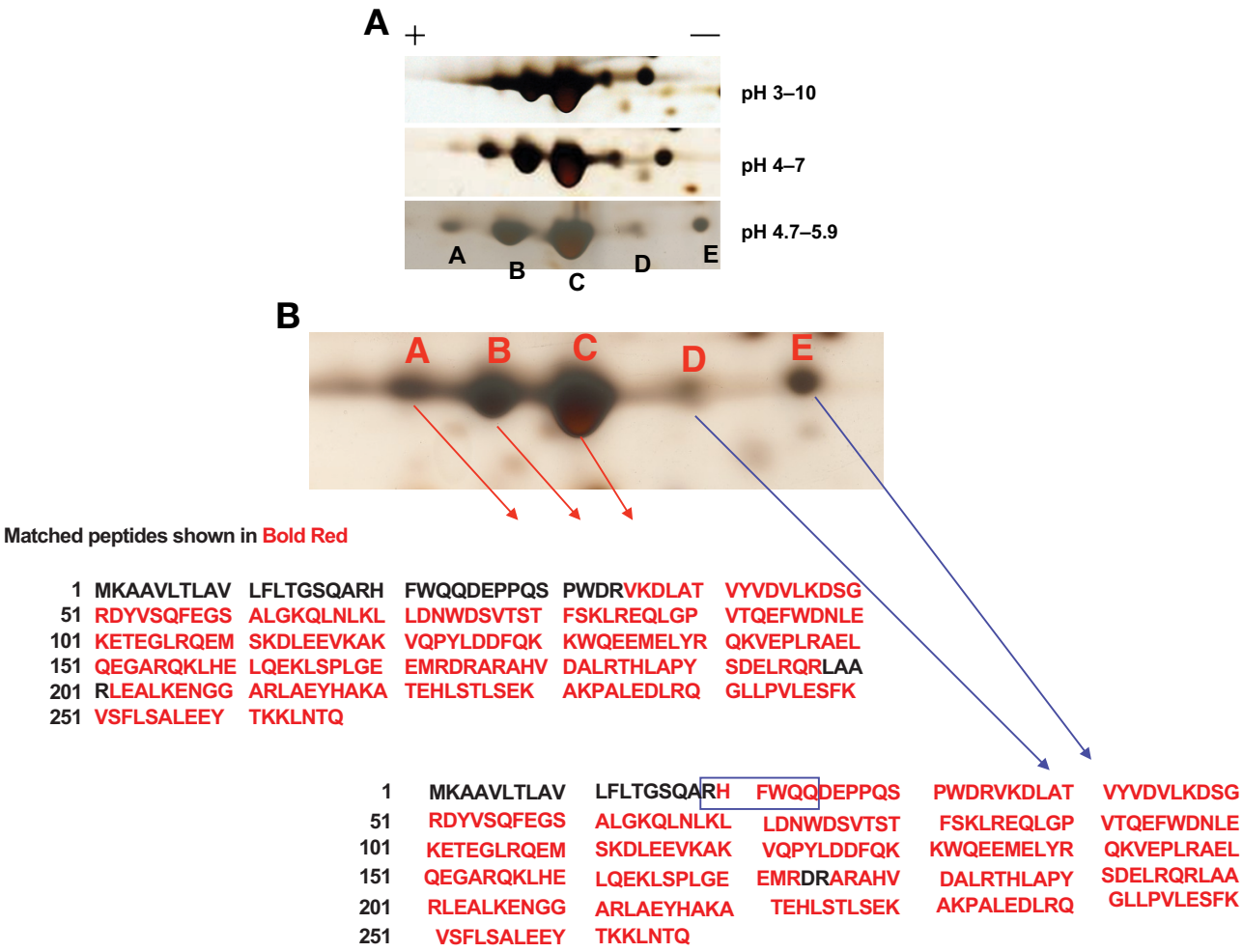



**FIG. 1. Isoform separation.** ***A*****: Two-dimensional gel electrophoresis of human plasma to separate ApoA-1. IPG strips of 24-cm length having various pH ranges (3–10, 4–7, and 4.7–5.9) were used for IEF. The isoforms were named as A to E (as relatively negative to positive in charge).** ***B*****: Amino acid sequence of ApoA-1 spots. Propeptide RHFWQQ (in the blue box) for spots D and E, which defines those as ApoA-1 precursors. The propeptide portion was absent in spot-trains A, B, and C, and thus those are mature forms. Red indicates sequence coverage. (A high-quality digital representation of this figure is available in the online issue.)**

IPG strips were incubated in 1× DNPH reagent for 10 min, followed by the second dimension gel separation. The transfer of protein was done as described above. The remaining steps were done per the instructions in the Oxyblot kit.

**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 [*ring*-$^{13}C_6$]phenylalanine was successfully performed. The IE in units of moles percent excess (MPE) for [*ring*-$^{13}C_6$]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).

**Statistical analyses.** Results are presented as mean ± SE. Results were analyzed by ANOVA with a Fisher protected least significant difference post hoc test. For mixed-model ANOVAs comparing ND, type 1 diabetes $I^+$, and type 1 diabetes $I^-$, the study participant's identity was set as a random effects variable. Analyses were performed using JMP 7.0 statistical software. Statistical significance was set at $P = 0.05$.

## 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 isoforms (toward the positive end of gel [*left*]) to the more positively charged isoform spots [toward the negative end of gel (*right*)] (Fig. 1*A*), 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 RHFWQQ was present only in spots D and E (Fig. 1*B*). The propeptide was absent in spots A, B, and C, indicating that these spots were mature versions of ApoA-1.

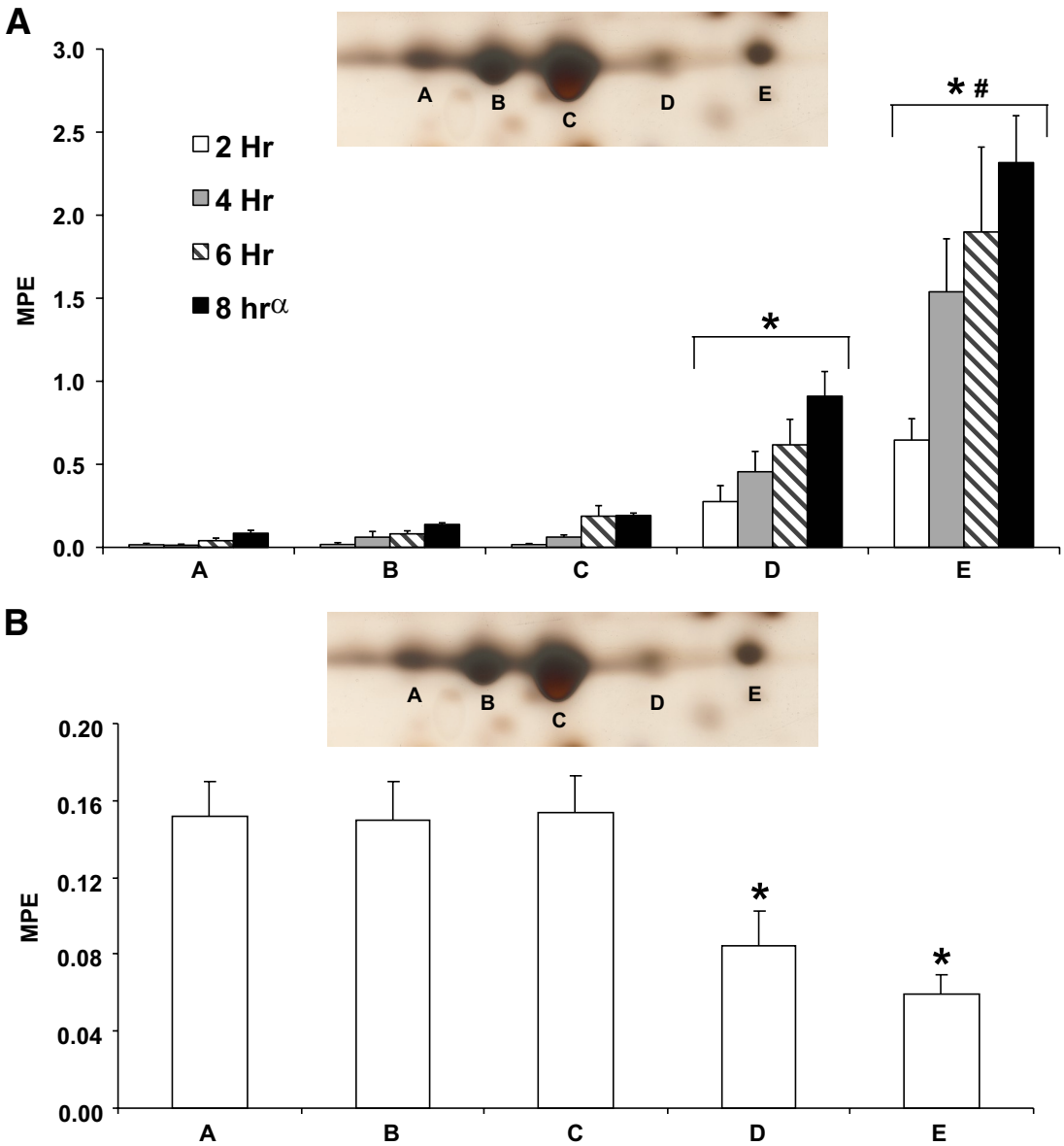
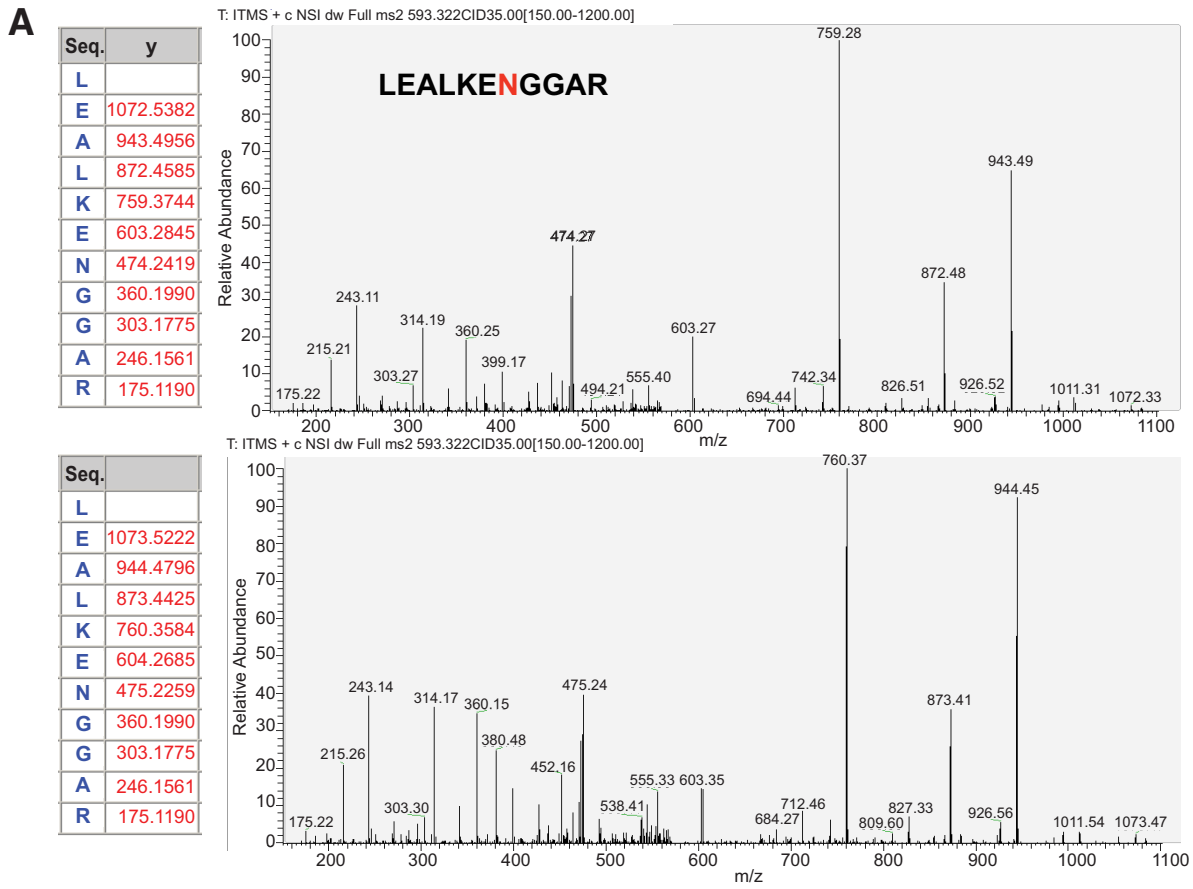
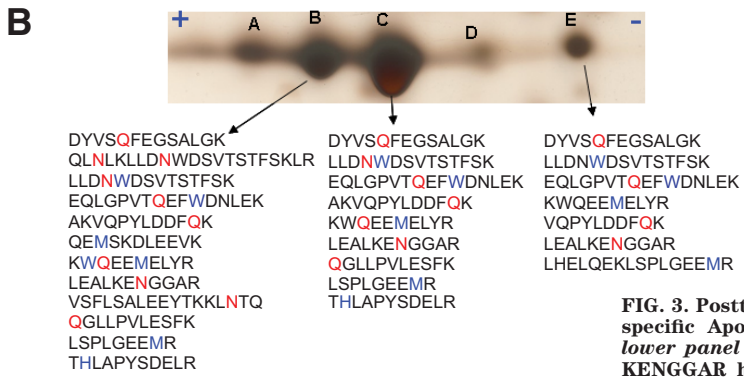



**FIG. 2. Isotopic enrichments. *A*: IE during labeled amino acid infusion was measured in ApoA-1 spot trains isolated from plasma. Spot E had the highest levels of IE, indicating that it was the newest isoform, whereas A was the oldest. MPE, moles percent excess; Hr, hours of tracer infusion. Means ± SE for $n = 7$ ND participants. Statistical analysis by ANOVA. Main effect of isoform, $P < 0.0001$; main effect of time, $P < 0.0001$; αDifferent from 2-h time point, $P < 0.05$; #different from isoform D, $P < 0.05$; *different from isoforms A, B, and C; $P < 0.05$. *B*: IE in the ApoA-1 spots 10 days after the 8-h infusion of labeled amino acid. The figure demonstrates that the label accumulated in isoforms A, B, and C. Greater decrease or disappearance of the label in spots D and E indicates that proteins representing those isoforms were replaced by de novo synthesis. Means ± SE for $n = 7$ type 1 diabetic participants. Statistical analysis by ANOVA. Main effect of isoform, $P < 0.0001$. *Different from isoforms A, B, and C; $P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)**

**Identification of the relative age of protein isoforms using labeled amino acids.** Figure 2*A* 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 isotopic infusion, 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. 2*B*), 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. 3*A*. As shown in Fig. 1*B*, 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



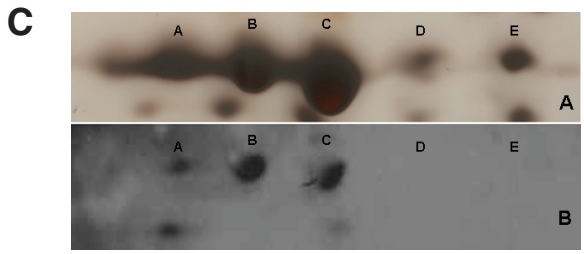




**FIG. 3. Posttranslational modifications. *A*: The MS/MS spectrum of a specific ApoA-1 peptide showing deamidation of asparagine. The *lower panel* shows the fragment ions of the specific peptide LEAL KENGGAR having a 1-Da shift starting from the Asp residue (N) where it is deamidated. Similar addition of 1 Da in y ions is shown in the table on the left starting from Asp residue N. *B*: Level of deamidation and reversible oxidation in isoforms of ApoA-1. ApoA-1 spots B, C, and E were analyzed by tandem mass spectrometry for deamidation of asparagine (N) and glutamine (Q) and oxidation of tryptophan (W), histidine (H), and methionine (M). Spot B showed higher levels of both deamidation and oxidation when compared with spot C, and C showed higher levels of these modifications than E (B > C > E). *C*: Extent of protein carbonylation among the isoforms of ApoA-1. [A]The same protein sample ran in parallel, visualized by silver stain. [B]Immunoblot showing the extent of carbonylation that was detectable in spots A, B, and C of ApoA-1 as opposed to spots D and E, which are newly synthesized and showed complete absence of carbonyl reaction. (A high-quality digital representation of this figure is available in the online issue.)**

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. 3*A* for the ApoA-1 peptide LEALKENGGAR. 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. 3*B*. 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. 3*B*). 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. 3*C*, 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. 4*A*) showed higher value during insulin deficiency ($P < 0.05$), demonstrating that insulin deficiency triggers the newly formed proteins to be modified into the matured, 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 $I^-$, 0.083 ± 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 new 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 carbonylated to total peptides (modification of lysine to allysine, $P < 0.05$) (Fig. 4*B*). Allysine formation is an intermediate step in the irreversible oxidative damage of carbonylation in the form of aminoadipic acid. 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.

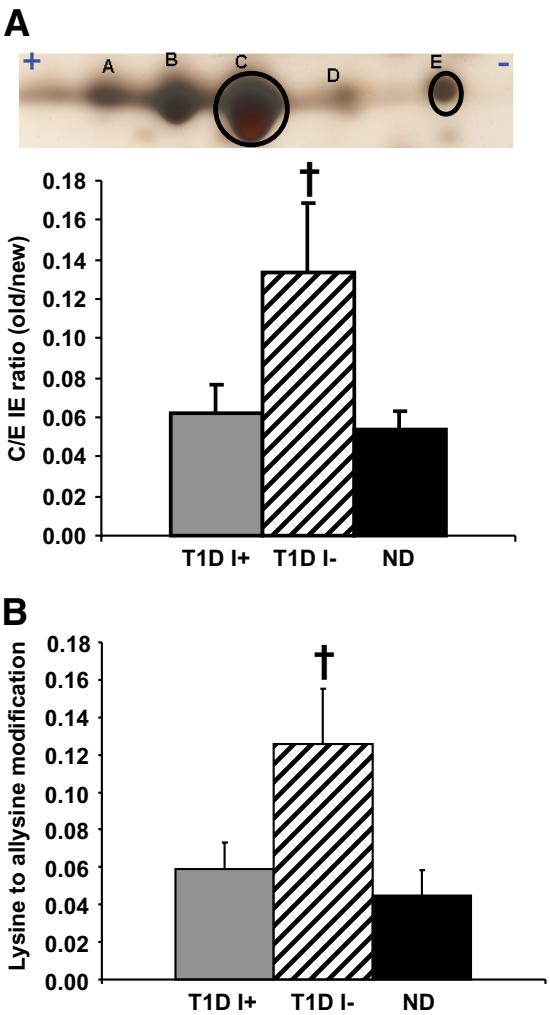



**FIG. 4. Protein aging. *A*. The ratio of isotopic enrichment (IE) in older versus newer (C/E) ApoA-1 spots demonstrates that the ratio is higher in type 1 diabetic individuals during insulin deprivation. This shows that during insulin deprivation, newly synthesized ApoA-1 is more rapidly modified and thus matures and ages more quickly. Means ± SE for $n = 7$ per group. Statistical analysis by ANOVA. Main effect of group, $P < 0.05$. †Different from type 1 diabetes $I^+$ and ND; $P < 0.05$. *B*: Extent of carbonylation (oxidation) in gel spot C of ApoA-1 as measured by mass spectrometry after 8 h of insulin deprivation or treatment in type 1 diabetic individuals and no hormone administration in ND. The ratio of allysine to lysine was higher in the specific ApoA-1 peptides of spot C in type 1 diabetic individuals during insulin deprivation. Means ± SE for $n = 7$ per group. Statistical analysis by ANOVA. Main effect of group; $P < 0.05$. †Different from ND and type 1 diabetes $I^+$; $P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)**

## 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*-$^{13}C_6$]phenylalanine) enrichment and the presence of posttranslational protein modifica-

tions. The highest [*ring*-$^{13}C_6$]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 [*ring*-$^{13}C_6$]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 methodology dictate that proteins that were most recently synthesized (i.e., during the infusion of [*ring*-$^{13}C_6$]phenylalanine) should contain higher amounts of [*ring*-$^{13}C_6$]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. 2*A*). 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. 2*B*). 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. 3*B*). 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. 4*A*. 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 allysine as shown in Fig. 4*B*). 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 underlying 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 damage 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.

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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. S.G. analyzed the results. K.S.N. conceived of and designed the experiment, interpreted the findings, and wrote the manuscript.

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