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The 2010 ESPEN Sir David Cuthbertson Lecture: New and old proteins: Clinical implications

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SUMMARY

The past century had witnessed vast advances in biomedical research, particularly in the fields of genomics and proteomics, yet the translation of these discoveries into clinical practice has been hindered by gaps in mechanistic understanding of variability governing disease susceptibility and pathogenesis. Among the greatest challenges are the dynamic nature of the proteome and the imperfect methodologies currently available to study it. Here, we review key recently developed proteomic techniques that have allowed for dynamic characterization of protein quality, as well as quantity, and discuss their potential applications in understanding aging and metabolic disorders including diabetes. These methodologies revealed that senescence is characterized, in part, by decreased rates of *de novo* protein synthesis and potentially also degradation, in addition to concomitantly increased levels of oxidative stress, ultimately resulting in excessive accumulation of damaged and dysfunctional proteins. Insulin may be a key mediator in these pathologies, as hyperinsulinemia has been shown to hinder protein degradation while transient insulin deficiency may accelerate oxidative damage. We also discuss two interventions that have been proposed to delay, and possibly reverse, senescence by augmenting protein degradation: chronic caloric restriction and aerobic exercise.

Keywords

Proteomics; Senescence; Caloric restriction; Aging

1. Introduction

Discovery of DNA structure and sequence nearly sixty years ago heralded in an era of genomic research and promise of individualized medicine, but the translation of these discoveries into clinical practice to improve the diagnosis, prevention, and treatment of disease remains imperfect. Genomic biological research has focused almost exclusively on the interplay between genes, time, and environment. Still, DNA encodes for less than 30,000 unique genes and is thus unlikely to account for the full phenotypic diversity of the human condition.^{1–3} Individual gene expression is further modulated by epigenetic changes modulated by the environment and other organism-specific factors. Genetic loci associated with type 2 diabetes mellitus, for example, appear to offer no additional benefit in predicting diabetes risk than clinical risk factors and family history.⁴

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Conflict of interest

The authors have no conflicts of interest to declare.

The clinical phenotype is primarily a reflection of the proteome, which encompasses the entire set of proteins expressed by the genome at specific points in time. By way of alternative splicing and intricate transcriptional regulation, a relatively small number of genes can yield more than 100,000 structurally distinct proteins (Fig.1). Because complete sequencing of the proteome is a daunting task, traditional proteomics has focused on the comparative study of protein expression across different conditions and points in time. However, this approach fails to capture the dynamic nature of the proteome, with temporal and spatial variations in protein function. The number of distinct protein functionalities exceeds the number of protein sequences and concentrations, as the proteome is defined not only by the amino acid composition of each individual protein, but also by the relative concentration, location, and post-translational modification of each isoform. Measuring concentration alone is therefore not sufficient to characterize protein function.

Several methodologies have been established to estimate *de novo* whole body and skeletal muscle protein synthesis; these have been reviewed elsewhere and are outside the scope of this review.⁵⁻⁷ Commonly used methodologies rely on *in vivo* isotopic tagging of newly synthesized proteins with labeled essential amino acids, such as leucine, lysine, phenylalanine, or tyrosine. These tracers are administered by continuous intravenous infusion following a priming dose, which creates a steady state concentration of the isotope and allows its rates of appearance and disappearance to be used in calculation of whole-body amino acid flux, estimating whole body protein degradation and synthesis using the single-pool steady-state model.⁸⁻¹² Synthesis of mixed plasma, muscle, and individual proteins can also be measured after the protein(s) is purified and identified using one- or two-dimensional gel electrophoresis followed by mass spectrometry.^{6,7}

Measuring the degradation of individual proteins is more challenging, and there is no effective and efficient methodology currently in widespread use. Protein degradation is integral to maintaining a healthy and functional proteome, particularly for turning over misfolded and otherwise damaged proteins. Degradation of a specific protein can be indirectly inferred from its total concentration and *de novo* synthesis rate, but this assumption is fraught with inaccuracy. Most studies rely on surrogates of protein degradation such as estimates of whole-body or regional protein flux.^{9,12} However, individual protein metabolism does not necessarily follow that of the proteome as a whole. Several methodologies were recently proposed to measure individual protein turnover. In a reporter-dependent approach, the protein of interest is expressed as a fusion protein with a fluorescent protein or epitope tag; protein degradation is subsequently measured by tag detection.¹³ Alternatively, reporter-independent approaches tag proteins with isotopically labeled amino acids, and measure the loss of these amino acids after addition of excess unlabeled amino acid to prevent label reutilization (pulse-chase approach).¹⁴ Neither technique has been validated to provide robust and accurate measure of protein degradation, and they were recently shown to provide widely disparate results upon side-by-side comparison.¹⁵

Importantly, changes in either synthesis or degradation have identical effects on final protein concentration despite vastly different functional states. Specifically, protein concentrations increase with both decreased degradation and increased synthesis, yet while decreased degradation results in accumulation of “old” proteins, increased synthesis does not. This is evident in the normal aging process, which is associated with rising fibrinogen levels despite an overall reduction in *de novo* protein synthesis.¹⁶ This implies that the surplus of circulating fibrinogen, a clinical marker of cardiovascular disease, is caused by protein accumulation in the setting of inadequate degradation. It is possible that such “old” fibrinogen is structurally and functionally distinct from *de novo* synthesized, “new”, fibrinogen, contributing to the pathogenesis of cardiovascular disease in the elderly. Protein

quality, in addition to *quantity*, may ultimately account for manifestations of health and disease.

2. Functional implications of protein aging

Functional proteomics is an intricate interplay between *de novo* protein synthesis (translation) and subsequent 3-dimensional folding, post-translational modification (PTM), and degradation (Fig. 2). Misfolded proteins are dysfunctional and typically targeted for degradation. Proteins are also subject to extensive posttranslational modification by the covalent addition of functional groups, both reversible and irreversible. More than 140 types of PTMs have been identified, though their functional significance is often uncertain.¹⁷ Most commonly studied PTMs occur by way of specific, targeted, and closely regulated enzymatic activity. These include protein phosphorylation, glycosylation, acetylation, myristoylation, methylation, and ubiquitination.^{5,17,18} Such PTMs dictate how proteins interact with surrounding structures, whether protein, lipid, carbohydrate, or nucleic acid. They also aid in cellular and extracellular protein localization, ensure protein stability, and target proteins for metabolism or degradation.

Other PTMs occur spontaneously after exposure to environmental stressors. Reactive oxygen species (ROS) can generate more than 35 distinct PTMs, including oxidation, methylation, deamidation, and carbonylation.¹⁹ Cellular anti-oxidant defense systems normally target these modified proteins for degradation, but pathological levels of oxidant stress can overwhelm these defense systems. Elevated levels of reactive oxygen species have been detected with aging,^{20–22} diabetes mellitus,²³ neurodegenerative diseases,²⁴ inflammatory and allergic diseases,²⁵ atherosclerosis,²⁶ and cancer.²⁷ Similarly, nitrosative stress can irreversibly modify proteins and has been implicated in aging,²² diabetes,^{28,29} atherosclerosis,²⁹ and others.

Timely and efficient removal of damaged proteins by degradation, followed by replacement with nascent proteins by *de novo* protein synthesis, maintains proteomic functional integrity. In the absence of timely degradation, damaged and misfolded proteins can accumulate, interfere with normal physiologic processes, and even be cytotoxic (Fig. 2). We propose that the pathologies of aging, diabetes, insulin resistance, and metabolic syndrome are caused, at least in part, by disproportionate accumulation of damaged and dysfunctional proteins, rather than their increased concentration *per se*.

3. Detecting post-translational protein damage

Separation or purification of individual proteins within a proteome is typically accomplished by 2-dimensional gel electrophoresis (2D-GE), a high resolution technique which separates proteins by mass and isoelectric focusing (charge). However, many proteins do not localize to a single train spot, but rather separate into several adjacent yet isoelectrically distinct spots. We hypothesized that these discrete train spots represent different PTM densities that accrue as proteins age, which we first demonstrated with apolipoprotein A-1 (ApoA-1). Fig. 3 depicts the five distinct train spots of ApoA-1 after it was purified by 2D-GE from plasma of a healthy sedentary adult; all proteins were labeled *in vivo* by an 8 h infusion of a [*ring*-¹³C₆]-phenylalanine (¹³C₆-Phe) prior to collection.³⁰ Sequencing of peptides localized to spots D and E revealed presence of the ApoA-1 propeptide, indicating that these train spots are composed of *de novo* synthesized proteins (pro-ApoA1).³⁰ Conversely, spots A, B, and C contained more mature ApoA-1 isoforms. Newly synthesized ApoA-1 peptides also contained higher levels of the ¹³C₆-Phe tracer, with ¹³C₆-Phe isotopic enrichment (IE) increasing in direct proportion to ¹³C₆-Phe infusion duration and inverse proportion to peptide age (Fig. 4A). Indeed, the IE of each train spot was suggestive of peptide age, with highest IE in the most recently synthesized and thus “youngest” spots D and E, and lowest

IE in the remotely synthesized “old” spots A, B, and C (Fig. 4A).³⁰ To confirm this hypothesis, we repeated the plasma collection and 2D-GE separation of ApoA-1 ten days later, without another ¹³C₆-Phe infusion. This time, ¹³C₆-Phe tracer shifted to “old protein” train spots A through C, as it was incorporated into proteins synthesized ten days earlier (Fig. 4B).³⁰

“Old” protein isoforms also have higher PTM density when measured by tandem mass spectrometry (MS/MS), decreasing progressively from spots A to E (Fig. 5A). Staining for carbonyl groups, the most commonly used method for measuring PTMs, confirmed that PTM density is highest in spots A to C, decreasing incrementally in the newer spot trains (Fig. 5B). We demonstrated that this methodology can be applied to study protein damage in real time during a specific intervention, in this case insulin infusion in insulin-deprived individuals with type 1 diabetes (Fig. 5C and D). Protein aging, manifest by the extent of PTM, is reflected by the ratio of IE within the “old” and “new” train spots (ApoA-1 C/E), with higher C/E isotopic enrichment ratios indicative of older protein age and/or more extensive PTM. It is therefore evident that insulin deprivation in type 1 diabetes results in more extensive PTM of ApoA-1, with higher C/E isotopic enrichment ratio (Fig. 5C) and lysine-to-allysine conversion (Fig. 5D), the latter being a more traditional method for estimating protein PTM. This was the first successful demonstration that protein *quality*, not necessarily *quantity*, is altered in a disease state and is reversed by appropriate treatment.

4. Insulin and protein metabolism

Although insulin is most known for its role in glucose metabolism, insulin also has a profound anti-catabolic effect. Prior to the introduction of insulin, individuals with type 1 diabetes experienced rapid muscle wasting and cachexia, which was reversed by insulin replacement.^{31,32} Insulin is known to inhibit protein degradation,^{9,33–36} but despite its key role in translational regulation of transcripts, insulin’s effect on *de novo* protein synthesis in humans was, until recently, uncertain.^{9,34,35} In a series of studies conducted among healthy volunteers and insulin-deficient individuals with type 1 diabetes, we found that insulin exerts different vastly effects on protein synthesis and degradation depending on the protein and tissue type.³⁴

As a whole, insulin infusion in the post-absorptive (fasting) state lowers both whole-body protein degradation and synthesis in dose-dependent manner.³⁷ In healthy fasting individuals, when insulin levels are low, skeletal muscle is in a catabolic state and protein degradation exceeds *de novo* synthesis. At the same time, the splanchnic bed is in an anabolic state where protein synthesis exceeds degradation. This results in net transfer of amino acids from skeletal muscle to the splanchnic bed, allowing splanchnic tissues such as the liver to maintain synthesis of essential proteins without continued influx of amino acids. Insulin, which normally rises in response to a nutritional load, selectively decreases protein degradation in skeletal muscle and synthesis in the splanchnic bed, without changing protein synthesis in skeletal muscle or degradation in the splanchnic bed. The concomitant rise in circulating amino acids decreases degradation and increases protein synthesis within skeletal muscle (Fig. 6). Thus, in the post-prandial state, skeletal muscle experiences a net accretion of proteins to allow for the later amino acid loss that invariably follows in the post-absorptive state.^{37,38}

It is possible, therefore, that the hyperinsulinemic state that characterizes obesity, type 2 diabetes, and metabolic syndrome interferes with protein degradation and ultimately leads to accumulation of damaged proteins that would otherwise be destined for degradation. Indeed, when insulin is infused in individuals with type 2 diabetes, baseline hyperinsulinemia is aggravated and protein degradation is decreased.^{39,40} In animal studies of diabetes,

lysosomal degradation systems are down-regulated, despite a concomitant increase in oxidative and nitrosative stress.^{23,28,29} While cross-sectional comparisons of healthy individuals and those with type 2 diabetes failed to detect significant differences in post-absorptive whole-body protein degradation,^{39–41} such studies overlook tissue-specific variability of insulin action and disease-associated changes in body composition, both of which invariably influence protein flux when normalized for fat free mass or body weight.

An additional area of interest is whether, and to what degree, insulin resistance to glucose metabolism extends to protein metabolism. Insulin infusion in individuals with type 2 diabetes results in significantly smaller decrements of protein degradation compared to individuals without diabetes, supporting the presence of proteomic insulin resistance.⁴² Furthermore, insulin resistance is associated with increased oxidative stress,^{43,44} with accelerated oxidative post-translational protein damage. It seems logical that in the setting of increased protein damage and impaired protein degradation, type 2 diabetes and other insulin resistant states experience greater accumulation of damaged and potentially dysfunctional proteins.

5. Protein accumulation and the aging process

Oxidative stress and protein PTMs increase with age,^{20–22} and this appears to translate into clinical pathology. Senile cataracts contain higher levels of oxidized α -crystallin proteins.⁴⁵ *In vitro* studies detected nearly a linear relationship between carbonyl content of cultured fibroblasts and the donor's age.⁴⁶ We believe that this is the result of impaired cellular degradation systems, whereby oxidized proteins that would normally be targeted for degradation accumulate due to age-related slowing of degradation pathways that are easily overwhelmed by excess post-translational stress.^{17,46–49}

Sarcopenia of aging, a geriatric syndrome characterized by progressive loss of muscle mass and function, results in frailty and causes significant morbidity in the rapidly expanding aging population. We demonstrated that there is a progressive age-related decline in *de novo* synthesis of mixed muscle protein, myosin heavy chain, and mitochondrial protein.^{8,50,51} However, large epidemiologic studies convincingly demonstrated that age-related loss of muscle strength exceeds the concomitant decline in muscle mass.^{52,53} This mass-independent decline in muscle strength and muscle quality may be indicative skeletal muscle protein damage and subsequent functional impairment.

Nearly all protein degradation pathways slow down with age. Glutathione, the major non-enzymatic oxidant defense species, decreases in aging mice and rats, resulting in a corresponding rise of oxidized and nitrosylated proteins.^{54,55} Mice deficient in protein isoaspartate methyltransferase (PIMT), which reverses asparagine deamidation, have greater intra-cellular accumulation of deami-dated proteins and decreased life span.⁵⁶ In humans, cross-sectional comparison of young healthy, elderly healthy, and elderly adults with at least one chronic disease showed that glutathione levels progressively decrease, and lipid peroxidation products (e.g. damaged proteins) progressively increase, both with age and comorbidities.⁵⁷

There are also two proteolytic systems that are responsible for the majority of intracellular protein turnover, the lysosomal system and the ubiquitin-proteasome system, and both become progressively less robust with age. In animal and human studies, the ubiquitin-proteasome system slows significantly with age, resulting in accumulation of modified and misfolded proteins (reviewed by Chondrogianni et al.,⁵⁸ Martinez-Vicente et al.,⁵⁹ and Carrard et al.⁶⁰). This has been shown, *in vivo*, in a variety of species and tissues, including mouse hepatocytes; rat cardiac myocytes, hepatocytes, and kidney cells; and human fibroblasts, erythrocytes, neurons, keratinocytes, skeletal myocytes, and cells of the lens,

among others.^{59,60} The naked mole rat, the longest-living rodent, differs from other rodents only by a high proteasome activity that persists throughout its life span.²⁰ Despite being subject to the same degree of oxidative stress, old naked mole rats have significantly fewer oxidized and ubiquitinated proteins, suggesting they are more efficiently degraded. Naked mole rats also do not exhibit the age-related changes in body composition and metabolic function that are seen in other rodents.²⁰

Moreover, *in vitro* studies of mouse and rat tissues, and *in vivo* studies of human epidermal cells and lymphocytes, demonstrated that the proteasome and lysosome themselves are subject to greater oxidative PTM and dysfunction with age, resulting in the accumulation of damaged proteins.^{58–61} At the same time, age-related mitochondrial dysfunction promotes lipofuscin (age pigment) formation, which has been shown to inhibit the proteasome and lysosome in human lung fibroblast cell lines.⁶¹ Lipofuscin also exacerbates mitochondrial dysfunction and reactive oxygen species formation (shown in rat cardiac myocytes and neurons), resulting in a vicious cycle of post-translational protein oxidation, protein accumulation, and ultimately cell death.⁶²

The direct relationship between proteasome activity and longevity was recently demonstrated in *Saccharomyces cerevisiae*.⁶³ Mutant yeast strains lacking any one of several proteasome components had decreased replicative lifespans that were independent of incurred oxidative stress. Conversely, strains designed to have increased proteasome activity, degraded damaged proteins more efficiently, and had longer replicative lifespans.⁶³

Although no prospective human studies have demonstrated a clear causal relationship between proteasome activity and longevity, observational studies support this hypothesis. Fibroblasts cultured from centenarians were found to have exceptionally robust proteasome activity that did not decrease linearly with age, as expected, and approaches the levels measured in their children.⁶⁴ The degree of oxidative damage to fibroblast proteins was concomitantly decreased in centenarians, resembling those of significantly younger people.⁶⁴

6. Can aging be delayed or reversed?

Popular culture abounds with claims of easy “fountains of youth”, but only two interventions have held promise as possible strategies to delay, if not reverse, the pathologies of aging and age-associated disorders. These are chronic caloric restriction and moderate-intensity aerobic exercise. Both must be distinguished from acute weight loss *per se*, which is not the goal of either caloric restriction or exercise. We propose that they do so by augmenting protein degradation and thereby lowering the degree of damaged protein accumulation.

Chronic caloric restriction without malnutrition, which reduces energy intake to low-normal levels while maintaining adequate protein and micronutrient levels, raises endogenous anti-oxidant systems such as superoxide dismutase, catalase, and glutathione peroxidase, in mice^{65,66} and rats.⁶⁷ The age-related decline in lysosome-mediated autophagy has been reported to reverse with caloric restriction in rats.⁶⁸ As a result, protein carbonylation, which typically increases with age, is also lowered by caloric restriction in mice⁶⁹ and rat models.⁶⁷ Ultimately, calorically-restricted mice have longer mean and maximal life-spans, with greater benefit derived from greater caloric restriction.⁷⁰

Trials of caloric restriction in rhesus monkeys seem to suggest that similar health benefits may extend to long-lived mammals, although recent studies have not yielded consistent results in terms of life span.⁷¹ The Wisconsin National Primate Research Center reported increased life span and decreased incidence of age-associated diseases, including diabetes, cancer,

cardiovascular disease, and brain atrophy, in calorically-restricted rhesus monkeys.⁷² In the Baltimore rhesus monkey study, risk of death was 2.6-fold lower in calorically restricted monkeys compared to ad-lib fed controls. Importantly, the greatest benefit appeared to stem from reduction in insulin levels, with hyperinsulinemia alone linked to 3.7-fold differences in mortality.⁷³

Chronic caloric restriction may similarly delay the progression of age-associated chronic diseases in humans.⁷⁴ Okinawan adults, who in the early 20th century traditionally ate approximately 30% less than other Japanese adults, had nearly 35% lower rates of coronary artery disease and cancer, and longer life-spans.⁷⁵ More recently, the Caloric Restriction Society, a group of lean and healthy adults who eat 30% less than the Western society norm, were shown to have significantly lower rates of obesity, dyslipidemia, hypertension, type 2 diabetes, and left ventricular diastolic dysfunction.^{76,77}

We propose that the beneficial effects of caloric restriction are due, in part, to improved insulin sensitivity and lack of hyperinsulinemia. A prospective study of 40% caloric restriction in lean healthy men revealed a progressive decline in basal metabolic rate, insulin concentration, and protein oxidation.⁷⁸ Our group has shown that insulin levels and insulin sensitivity are affected primarily by adiposity, not chronological age, but that aging is associated with an independent increase in visceral adiposity.⁷⁹ We also detected a significant age-related decline in muscle mitochondrial function and maximal endurance capacity,⁷⁹ suggesting that the elderly may also be exposed to more oxidative stress irrespective of concomitant hyperinsulinemia similar to what was previously observed in type 1 diabetes.⁸⁰

Similar to caloric restriction, regular aerobic exercise has been shown to lower the rates of cardiovascular disease and all-cause mortality irrespective of age.^{81–85} Exercise likely exerts its beneficial effects through a variety of mechanisms. In young and middle-aged rats, aerobic exercise lowers carbonylated protein content and augments proteasome activity in skeletal muscle,⁸⁶ liver,⁸⁷ and brain.^{88,89} In humans, aerobic exercise improves insulin sensitivity and lowers plasma insulin levels.⁹⁰ Lean elderly individuals who maintain high levels of aerobic physical activity do not exhibit the age-associated decline in muscle mitochondrial function or protein content, and maintain insulin sensitivities that are comparable to young adults.⁹¹ Both resistance and aerobic exercise have been shown to augment protein synthesis and degradation,^{92,93} but they appear to have different effects on muscle mass such that net protein turnover may vary between these two forms of physical activity. Indeed, aerobic exercise ameliorates age-related decline in mixed muscle protein synthesis without increasing lean tissue mass, suggesting that protein degradation is also increased.⁹³ In contrast, resistance training in the elderly increases both synthesis and mass of skeletal muscle, implying that *de novo* synthesis must exceed degradation.⁹⁴ The impact of specific exercise programs on protein synthesis, modification, and degradation therefore requires further investigation.

7. Conclusion

Shifting demographics with continued rise in population age and increasing prevalence of obesity shifted focus to the prevention and treatment of chronic diseases including diabetes and related metabolic disorders. Genomic and early proteomic approaches failed to fully explain individual variability in disease susceptibility and pathogenesis. However, recently developed proteomic methodologies have enabled the dynamic study of the proteome, encompassing both the *quantity* and *quality* of the available protein pool. Aging, diabetes, and other chronic diseases are characterized by a relative decline in protein degradation, potentially resulting in the accumulation of proteins that were subject to post-translational

modification by oxidizing cellular milieu. These damaged proteins may lose their function, aggregate, and interfere with normal cellular processes. Observational studies in animals suggest that enhancing protein degradation by caloric restriction and aerobic exercise may delay aging and reverse age-associated pathologies. Whether moderate caloric restriction, aerobic physical activity, or potential pharmacological interventions can promote similar improvements in people remains to be shown.

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Statement of authorship

The manuscript was conceived by RGM and KSN, who also co-wrote the manuscript. The corresponding author (KSN) is the guarantor for the manuscript and takes responsibility for integrity of the data presented in the manuscript. The work is supported by RO1 DK41973 and RO1 AG09531 grants from the National Institutes of Health, the David Murdock – Dole Professorship, and institutional funds from the Mayo Clinic.

Abbreviations

¹³C₆-Phe	[<i>ring</i> - ¹³ C ₆]-phenylalanine
2D-GE	2-dimensional gel electrophoresis
ApoA-1	apolipoprotein A-1
IE	isotopic enrichment
MS	mass spectrometry
PTM	post-translational modification
ROS	reactive oxygen species.

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Fig. 1.
Translation of limited genomic information into infinitely diverse biological function.
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Fig. 2.

Life cycle of a dynamic proteome. Composition of the proteome is determined by the interplay between *de novo* protein synthesis, post-translational modification (PTM), and degradation. After irreversible PTM, including by oxidative and/or nitrosative stress, proteins are targeted for degradation via the lysosome or proteasome pathways, with subsequent recycling of free amino acids. However, when degradation is impaired or overwhelmed by the degree of accrued protein damage, these modified proteins can aggregate and/or accumulate. Pathology ultimately insures as a result of decreased degradation with increased aggregation and/or accumulation.

Fig. 3. ApoA-1 isoform separation. (A) *In vivo* isotopic labeling of *de novo* synthesized proteins by 8-h intravenous infusion of $^{13}\text{C}_6$ -Phe allows for subsequent separation and identification of skeletal muscle and plasma proteins. Skeletal muscle studies typically utilize the vastus lateralis muscle as it contains both type I and II fibers. (B) Two-dimensional gel electrophoresis (2D-GE) of human plasma to separate ApoA-1. The isoforms were named A through E on the basis of isoelectric charge (relatively negative to positive). (C) Amino acid sequencing of peptides contained within each train spot; red indicates sequence coverage. The ApoA-1 propeptide RHFQQ (blue box) was detected only in spots D and E, which defines those as ApoA-1 precursors. The propeptide was absent in spot-trains A, B, and C, identifying those as mature forms. Modified with permission from Jaleel A et al. Diabetes 2010; 59:2366–2374.³⁰ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Isotopic enrichment (IE) as a surrogate marker of protein age. (A) ApoA-1 was isotopically labeled *in vivo* with continuous infusion of $^{13}\text{C}_6$ -Phe (infusion duration 2–8 h) prior to plasma collection and separation by 2D-GE. Extent of IE, measured by molar percent excess (MPE) of labeled $^{13}\text{C}_6$ -Phe within each train spot, rose in direct proportion to duration of $^{13}\text{C}_6$ -Phe infusion. Spots D and E had high IE, indicating that these are the newest isoforms (E newer than D). Conversely, spots A through C had low IE, indicating that these are the oldest isoforms. Hr, hours of tracer infusion. Means $\pm$ SE for $n = 7$ individuals. Statistical analysis by ANOVA. α different 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) ApoA-1 was

subject to repeat isolation and purification 10 days after 8-h $^{13}\text{C}_6$ -Phe infusion. Newly synthesized isoforms do not contain $^{13}\text{C}_6$ -Phe label and therefore have low IE (spots D and E), while old isoforms synthesized during the $^{13}\text{C}_6$ -Phe infusion 10 days previously, have high IE (spots A through C). Means $\pm$ SE for $n = 7$ individuals. Statistical analysis by ANOVA. *Different from isoforms A, B, and C; $P < 0.05$. Reproduced with permission from Jaleel A et al. Diabetes 2010; 59:2366–2374.³⁰

Fig. 5.

ApoA-1 post-translational modification density increases with protein age and insulin deprivation. (A) 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); PTMs are highlighted in red. PTM density, measured by percent modified amino acids among all peptides present, increased in direct proportion with protein age: B > C > E. (B) ApoA-1 carbonylation, the most common PTM, visualized by silver stain (bottom 2D-GE panel). Carbonylation was highest in spots A, B, and C (old isoforms) as compared to spots D and E (newly synthesized isoforms). (C) Degree of ApoA-1 post-translational modification can be estimated by the relative isotopic enrichment (IE) in older vs. newer train spots (C/E). Thus, ApoA-1 damage is highest in individuals with type 1 diabetes (T1D I-) during insulin 8 h of deprivation and is reversed by administration of insulin (T1D I+) when it resembles that of non-diabetic controls (ND). Means $\pm$ SE for $n = 7$ per group. Statistical analysis by ANOVA. †Different from type 1 diabetes I+ and ND; $P < 0.05$. (D) The same trend in ApoA-1 post-translational damage confirmed by measuring the degree of carbonylation (oxidation) in gel spot C of ApoA-1 as measured by mass spectrometry after insulin deprivation or treatment in individuals with type 1 diabetes and untreated non-diabetic controls. The ratio of alllysine to lysine was higher in the specific ApoA-1 peptides of spot C in T1D I-. Means $\pm$ SE for $n = 7$ per group. Statistical analysis by ANOVA. † Different from ND and T1D I+; $P < 0.05$. Reproduced with permission from

Jaleel A et al. Diabetes 2010; 59:2366–2374.³⁰ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Effects of insulin on whole body, skeletal muscle, and splanchnic protein breakdown and synthesis. Whole body protein breakdown decreases with insulin treatment of insulin-deprived individuals with type 1 diabetes, mostly as a result of decreased skeletal muscle protein breakdown. Whole body protein synthesis also decreases with insulin treatment, however this is largely the result of much greater reduction in splanchnic bed protein synthesis with a concomitant increase in skeletal muscle protein synthesis. I-, insulin deprivation; I+, insulin treatment. *Rate of synthesis/ breakdown is significantly less than during insulin deprivation ($P < 0.05$). Reproduced with permission from Charlton M, Nair KSJ. Nutr. 1998; 128:323S–327S.⁹⁵