

UC San Diego

UC San Diego Previously Published Works

Title

Elevated Resting and Postprandial Digestive Proteolytic Activity in Peripheral Blood of Individuals With Type-2 Diabetes Mellitus, With Uncontrolled Cleavage of Insulin Receptors

Permalink

<https://escholarship.org/uc/item/0wq770h0>

Journal

Journal of the American Nutrition Association, 38(6)

ISSN

2769-7061

Authors

Modestino, Augusta E
Skowronski, Elaine A
Pruitt, Chris
[et al.](#)

Publication Date

2019-08-18

DOI

10.1080/07315724.2018.1545611

Peer reviewed



Elevated Resting and Postprandial Digestive Proteolytic Activity in Peripheral Blood of Individuals With Type-2 Diabetes Mellitus, With Uncontrolled Cleavage of Insulin Receptors

Augusta E. Modestino, Elaine A. Skowronski, Chris Pruitt, Pam R. Taub, Karen Herbst, Geert W. Schmid-Schönbein, Michael J. Heller & Paul J. Mills

To cite this article: Augusta E. Modestino, Elaine A. Skowronski, Chris Pruitt, Pam R. Taub, Karen Herbst, Geert W. Schmid-Schönbein, Michael J. Heller & Paul J. Mills (2019): Elevated Resting and Postprandial Digestive Proteolytic Activity in Peripheral Blood of Individuals With Type-2 Diabetes Mellitus, With Uncontrolled Cleavage of Insulin Receptors, Journal of the American College of Nutrition, DOI: [10.1080/07315724.2018.1545611](https://doi.org/10.1080/07315724.2018.1545611)

To link to this article: <https://doi.org/10.1080/07315724.2018.1545611>



Published online: 09 Apr 2019.











Submit your article to this journal [↗](#)



View Crossmark data [↗](#)



Elevated Resting and Postprandial Digestive Proteolytic Activity in Peripheral Blood of Individuals With Type-2 Diabetes Mellitus, With Uncontrolled Cleavage of Insulin Receptors

Augusta E. Modestino^{a,b} , Elaine A. Skowronski^c , Chris Pruitt^d , Pam R. Taub^e , Karen Herbst^f , Geert W. Schmid-Schönbein^a , Michael J. Heller^{a,b} , and Paul J. Mills^d 

^aDepartment of Bioengineering, University of California San Diego, La Jolla, California, USA; ^bKnight Cancer Center, Center for Early Cancer Detection (CEDAR), Oregon Health Sciences University, Portland, Oregon, USA; ^cDepartment of Nanoengineering, University of California San Diego, La Jolla, California, USA; ^dDepartment of Family Medicine and Public Health, University of California San Diego, La Jolla, California, USA; ^eDepartment of Medicine, University of California San Diego, La Jolla, California, USA; ^fDepartment of Medicine, The University of Arizona Health Sciences, Tucson, Arizona, USA

ABSTRACT

Objective: To examine resting and postprandial peripheral protease activity in healthy controls and individuals with type 2 diabetes mellitus (T2DM) and pre-T2DM.

Methods: Individuals with T2DM or pre-T2DM and healthy controls (mean age 55.8 years) were studied before and for a span of 300 minutes following a single high-calorie McDonald's breakfast. Metalloproteases-2/-9 (MMP-2/-9), elastase, and trypsin activities were assessed in whole blood before and following the meal using a novel high-precision electrophoretic platform. Also assessed were circulating levels of inflammatory biomarkers and insulin receptor density on peripheral blood mononuclear cells (PBMCs) in relationship to protease activity.

Results: Premeal MMP-2/-9 and elastase activity levels in T2DM and in pre-T2DM participants were significantly elevated as compared to controls. The T2DM group showed a significant increase in elastase activity 15 minutes after the meal; elastase activity continued to increase to the 30-minute time point ($p < 0.01$). In control participants, MMP-2/-9, elastase, and trypsin were significantly increased at 15 minutes after the meal ($p < 0.05$) and returned to premeal values within a period of approximately 30 to 60 minutes post meal. PBMCs incubated for 1 hour with plasma from T2DM and pre-T2DM participants had significantly lower levels of insulin receptor density compared to those incubated with plasma from control participants ($p < 0.001$).

Conclusions: The results of this study suggest that individuals with T2DM and pre-T2DM have higher resting systemic protease activity than nonsymptomatic controls. A single high-calorie/high-carbohydrate meal results in further elevations of protease activity in the systemic circulation of T2DM and pre-T2DM, as well as in healthy controls. The protease activity in turn can lead to a downregulation of insulin receptor density, potentially supporting a state of insulin resistance.

ARTICLE HISTORY

Received 23 August 2018
Accepted 3 November 2018

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia and insulin resistance (1, 2). Despite the high prevalence of T2DM, a molecular mechanism for the development of insulin resistance has not yet been fully elucidated (3).

Unchecked degrading protease activity has been shown to contribute to the development of insulin resistance. In animal models of diabetes, hypertension, and metabolic syndrome, unchecked proteolytic activity cleaves the extracellular domain of the insulin receptor, leading to deficient insulin binding and signaling, that is, insulin resistance (3, 4). In the plasma of T2DM patients, elevated levels of “soluble” insulin receptor fragments have been reported (5),

and the proposed mechanism for insulin receptor cleavage is by proteases (6, 7).

As part of normal digestion, powerful pancreatic proteases reside inside the lumen of the small intestine where they degrade large volumes of macromolecules in food. Chronic overconsumption of a high-calorie diet is a major risk factor for the development of T2DM and is also associated with an increased permeability of the intestinal mucosa (8). While there is the possibility that digestive enzymes leak out of the small intestine into the central circulation, the link between nutrition and protease activity in blood remains relatively unexplored.

Table 1. McDonald's Breakfast Meal Composition (Estimates in Grams).

	Calories	Protein	Total fat	Saturated fat	Trans fat	Carbohydrates	Sodium
One Egg McMuffin	280	17	12	6	0	29	777
Two hash browns	300	2	18	3	0	32	640
One orange juice	150	2	0	0	0	34	0
One McCafé hot chocolate	380	11	15	9	0.1	53	170
Totals	1,110	32	45	18	0.1	148	1,587

Table 2. Baseline Characteristics of Study Participants (Mean \pm SEM).

Characteristics	Control	pre-T2DM	T2DM	Control Fasting
Sample size	7	10	10	3
Age (years)	53.0 \pm 5.6	57.4 \pm 4.0	57 \pm 4.4	44 \pm 2.6
Females (#)	5 (0.71)	6 (0.6)	6 (0.6)	1
Weight (kg)	78.5 \pm 6.8*	86.6 \pm 6.7	99.6 \pm 7.4	56.3 \pm 1.3**,#
Waist (cm)	98.9 \pm 6.0	102 \pm 5.5	112.2 \pm 5.2	81.9 \pm 3.2**,#
BMI (kg/m ²)	29.1 \pm 2.5	31.7 \pm 2.5	33.4 \pm 2.8	21.5 \pm 0.6**,#
Fasting glucose (mg/dL)	77.6 \pm 3.4**	114.1 \pm 1.8**	147.9 \pm 5.4	73.9 \pm 4.8**,#
SBP (mm Hg)	118.0 \pm 4.7*	129.0 \pm 1.25	133.0 \pm 2.9	108.0 \pm 0.9**
DBP (mm Hg)	69.4 \pm 1.5	71.3 \pm 4.6	75.7 \pm 3.0	73.3 \pm 1.1
	Race and ethnicity			
Non-Hispanic White	6 (0.91)	9 (0.9)	10 (1)	3 (1)
Hispanic	1 (0.09)	1 (0.1)	0 (0)	0 (0)

* $p < 0.05$ when compared to T2DM.** $p < 0.001$ when compared to T2DM.# $p < 0.05$ when compared to pre-T2DM.## $p < 0.001$ when compared to pre-T2DM.

Based on this existing literature, we hypothesized that a high-calorie/high-carbohydrate meal would induce an increase in protease activity in the systemic circulation due to increased intestinal mucosal permeability. A key requirement to test this hypothesis was to measure protease activity in whole blood (9). Previous efforts to study proteases in T2DM have yielded conflicting results and focused on proteases in isolated plasma and with limitations in assay sensitivity (9). To overcome these limitations, we used a new electrophoretic technique that uses charge-changing fluorescent peptides capable of detecting the activities of nanomolar concentrations of active proteases in a single drop of fresh whole blood without the need for sample preparation (10–12). Furthermore, in addition to protease activity, we examined whether an elevated proteolytic activity would trigger proteolytic cleavage of the extracellular domain of the insulin receptor on peripheral blood monocytes (PBMCs).

To elucidate potential relationships between a high-calorie/high-carbohydrate meal and circulating protease activity and its potential proteolytic consequences, we therefore measured the activities of the pancreatic proteases trypsin and elastase and the metalloproteases MMP-2 and -9, and the PBMC insulin receptor density in whole blood of T2DM, pre-T2DM, and healthy controls at rest and following a single high-calorie/high-carbohydrate meal.

Material and methods

Ten normal control (fasting glucose < 100 mg/dL), 10 pre-T2DM (fasting glucose 100 mg/dL to 125 mg/dL), and 10 T2DM (fasting glucose > 126 mg/dL) volunteers participated in the study. Other than T2DM and pre-T2DM, all participants were otherwise asymptomatic. Study participants

fasted for a minimum of 12 hours prior to study. Anthropomorphic measurements and blood pressure were collected. The study was approved by the Institutional Review Board of the Human Research Protections Program at the University of California, San Diego. All participants provided written informed consent.

Meal tolerance test

An intravenous catheter was placed in the antecubital vein of the nondominant arm. Blood was collected into tubes containing either lithium heparin or ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson). An initial blood sample was collected to serve as the baseline. A high-calorie/high-carbohydrate McDonald's breakfast meal consisting of the following was then served to the participants: one hot chocolate, one orange juice, two hash browns, and one Egg McMuffin (see Table 1 for estimated totals in grams for calories, proteins, fats, carbohydrates [carbs], and sodium). Subsequent blood samples were collected postprandially at 15, 30, 45, 60, 120, 180, 240, and 300 minutes. For all participants, the protocol was started between the hours of 8:00 and 9:00 a.m. Participants were told to come in a fasting state, only having ingested water the morning of testing, and to continue as usual any prescribed medications they were taking.

Protease activity measurements: charge-changing fluorescent peptide substrates

Samples were collected into lithium heparin tubes and immediately stored at 4 °C. Protease activity was determined within 2 hours. Activities of MMP-2 and -9, elastase, and trypsin were measured using charge-changing protease

substrates and an electrophoretic method (10–12). This method uses synthetic fluorescent peptides that are specifically cleaved by a protease. The peptides are originally negatively or neutrally charged, but upon cleavage by the protease of interest two fragments are produced: a negative one and a fluorescently labeled positive one. This allows for electrophoretic separation and detection in the whole blood without sample preparation. The substrate Ac-NGDPVGLTAGAGK-NH₂ was used for MMP-2 and MMP-9; the substrate Ac-NDGDAGRAGAGK-NH₂ was used for trypsin; and the substrate Ac-DAGSVAGAGK-NH₂ was used for elastase. Substrates were tagged with a fluorophore (Bodipy-FL-SE, Invitrogen, Carlsbad, CA) via the peptide's diaminoethyl-amine group. Stock solutions of the substrates with concentrations of 1.2 mg/mL were prepared using 1 × phosphate-buffered saline (1 × PBS; pH 7.8).

Whole blood (4.0 μL) was mixed with the substrate (4.0 μL) to a final concentration of the substrate in the reaction of 0.6 mg/mL. As negative control, 1 × PBS was combined with the substrate. Positive controls consisted of a combination of known concentrations of enzyme (1000, 100, and 10 nM) and the fluorescent substrates. Reactions were allowed to proceed for 30 minutes and then 6-μL aliquots of the reaction were loaded in different lanes of 20% polyacrylamide gels (Novex, Invitrogen, Carlsbad, CA) submerged in trisborate running buffer (0.5 × TBE; 44.5 mM, 1 mM EDTA, pH 8.0). The loaded gels were electrophoresed at 500 V for 12 minutes and then imaged at excitation and emission wavelengths of 302 nm and 500–580 nm, respectively (BioDoc-It M-26 transilluminator, UVP, Upland, CA). Gels were scanned (Storm 840 Workstation; Molecular Dynamics, Sunnyvale, CA) and loaded with the ImageQuant v5.2 software using fluorescence mode, high sensitivity, 100 μm pixel size, 1000 V photomultiplier tube with a 450 nm excitation filter, and a 520-nm long-pass emission filter. The fluorescence intensity of the image was determined digitally (National Institutes of Health [NIH] Image J 1.440) and calibrated with intensities by known concentrations of protease activities in each gel.

Insulin receptor labeling

Freshly isolated PBMC cells from a healthy volunteer were incubated with plasma from control fasting, control, pre-T2DM, and T2DM participants collected at baseline and at different time points after the meal or with spiked proteases (trypsin, elastase, and MMP-2). A PBMC suspension (50 μL) and plasma (150 μL) were incubated for 1 hour at 37 °C. The cells were spread as a blood smear, fixed (10% formalin, neutral buffered), and labeled with a primary antibody against the extracellular domain of the insulin receptor (R-alpha-710 rabbit polyclonal antibody, Santa Cruz Biotech) followed by the biotin/avidin and Vector TMB peroxidase enzyme substrate. The cell images were collected using a bright-field microscope (40× and 80× objective) and digitally analyzed (NIH Image J 1.440) to determine light absorption as a measure of surface density for the extracellular domain of the insulin receptor.

Glucose, inflammatory markers, and soluble adhesion molecules

Glucose was determined using a standard glucose analyzer by the UCSD Medical Center Clinical Chemistry Laboratory. Circulating levels of C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular cell adhesion molecule-1 (sICAM-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1) were determined by commercial enzyme-linked immunosorbent assay (ELISA) (MSD, Rockville, MD). Intra- and inter-assay coefficients were less than 7%.

Statistical analyses

Measurements are presented as mean ± standard error (SEM). Potential group differences were examined by analysis of variance (ANOVA) and, in response to the meal, repeated-measures ANOVA. Post hoc paired *t*-tests were used where significant group by time interactions were observed. Statistical significance was set at $p \leq 0.05$. Correlations were examined using Pearson coefficients.

Results

Study participants

The 30 participants in the study included T2DM ($n = 10$); pre-T2DM ($n = 10$); controls ($n = 7$); and controls who fasted during the experiment (fasting controls, $n = 3$) (Table 2). Weight was significantly higher in T2DM when compared to controls (Table 2, $p < 0.05$) and to fasting controls (Table 2, $p < 0.001$). Pre-T2DM weight was significantly higher than fasting controls (Table 2, $p < 0.05$). Waist circumference and body mass index (BMI) were significantly higher in T2DM and pre-T2DM when compared to fasting controls (Table 2, $p < 0.05$), but not compared to control participants. By definition, fasting glucose levels were significantly higher in T2DM than in all other participants, and in pre-T2DM as compared to healthy controls (Table 2, $p < 0.001$). Systolic blood pressure (SBP) was significantly higher in T2DM compared to controls (Table 2, $p < 0.05$) and fasting controls (Table 2, $p < 0.001$).

Baseline circulatory protease activity

We observed higher baseline MMP-2/-9 and elastase activities in T2DM and in pre-T2DM as compared to controls (Figure 1A–B, $p < 0.05$) and fasting controls (Figure 1A–B, $p < 0.001$). Baseline trypsin activity was not statistically significant among the groups (Figure 1C).

Postprandial increases in circulatory protease activity

In control participants, MMP-2/-9, elastase, and trypsin increased significantly 15 minutes after the meal (Figure 2A–C, $p < 0.05$) and returned back to premeal values within a period of approximately 30 to 60 minutes post meal. Fasting controls showed no significant change in any protease activity.

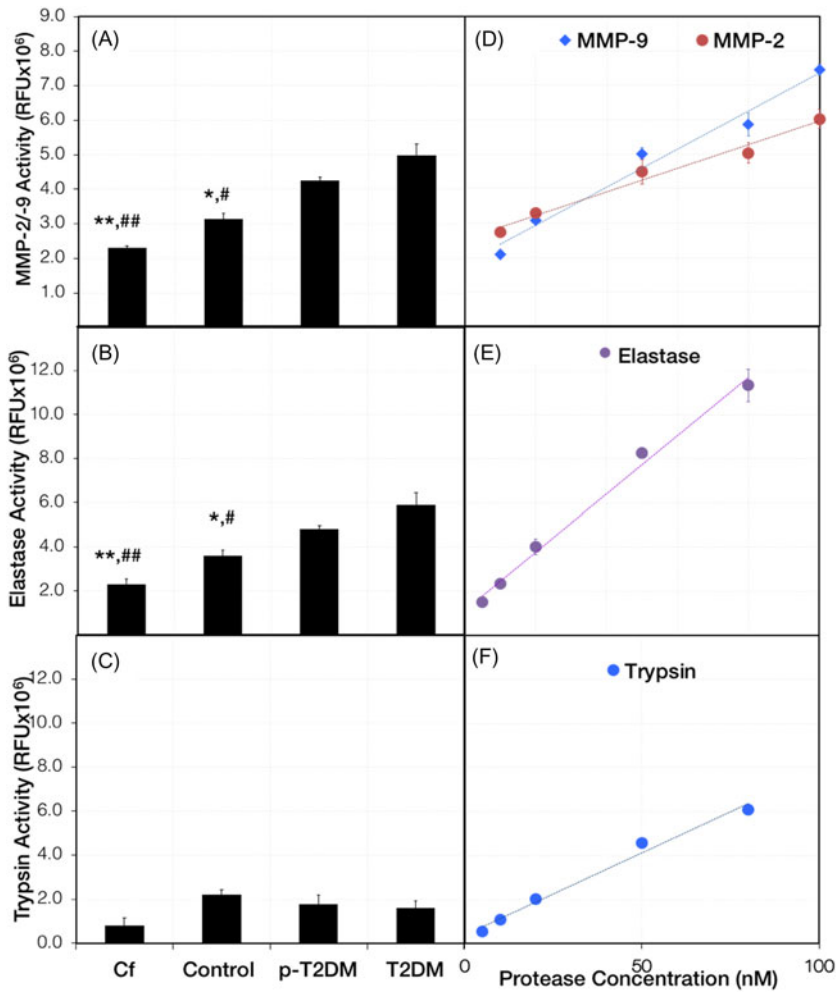


Figure 1. Baseline protease activity. Mean baseline activity for (A) MMP-2/-9, (B) elastase, and (C) trypsin (* $p < 0.05$, ** $p < 0.001$ versus T2DM and # $p < 0.05$, ## $p < 0.001$ versus pre-T2DM). Calibration curves in $1 \times$ PBS spiked with different concentrations of (D) MMP-2/MMP-9, (E) elastase, and (F) trypsin (Cf = control fasting; p-T2DM = pre-T2DM).

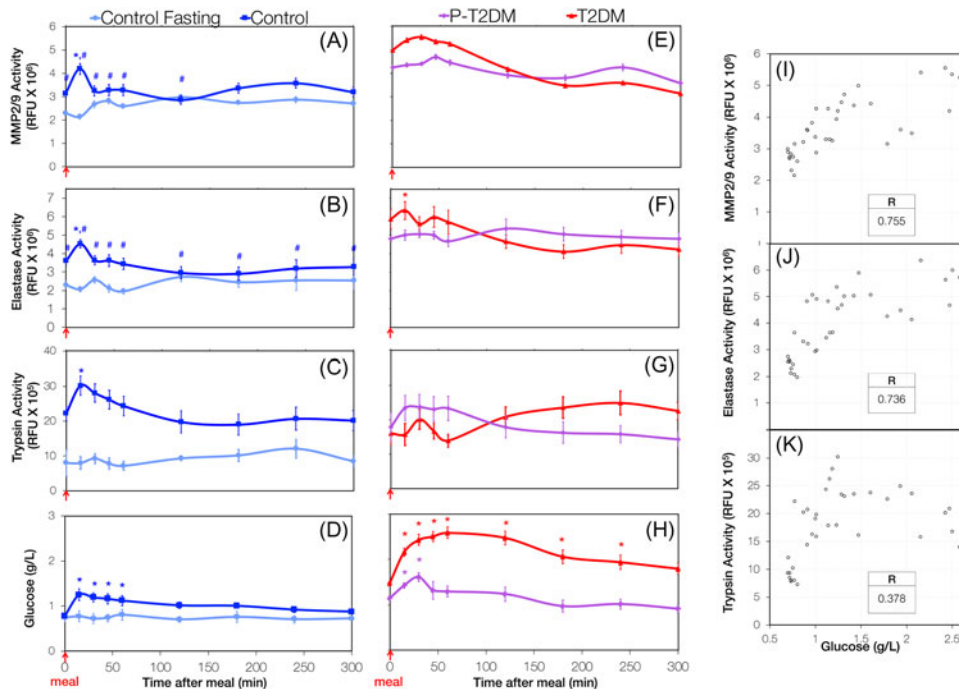


Figure 2. Postprandial protease activity and glucose levels. MMP-2/-9 activity, elastase, trypsin, and glucose in control, and control fasting (A–D) and in pre-T2DM and T2DM participants (E–H) (* $p < 0.05$ versus time 0 within group, and # $p < 0.05$ control versus T2DM). Linear correlations of blood glucose levels and (I) MMP-2/-9 activity ($p < 0.001$), (J) elastase activity ($p < 0.001$), and (K) and trypsin activity ($p < 0.001$) (p-T2DM = pre-T2DM).

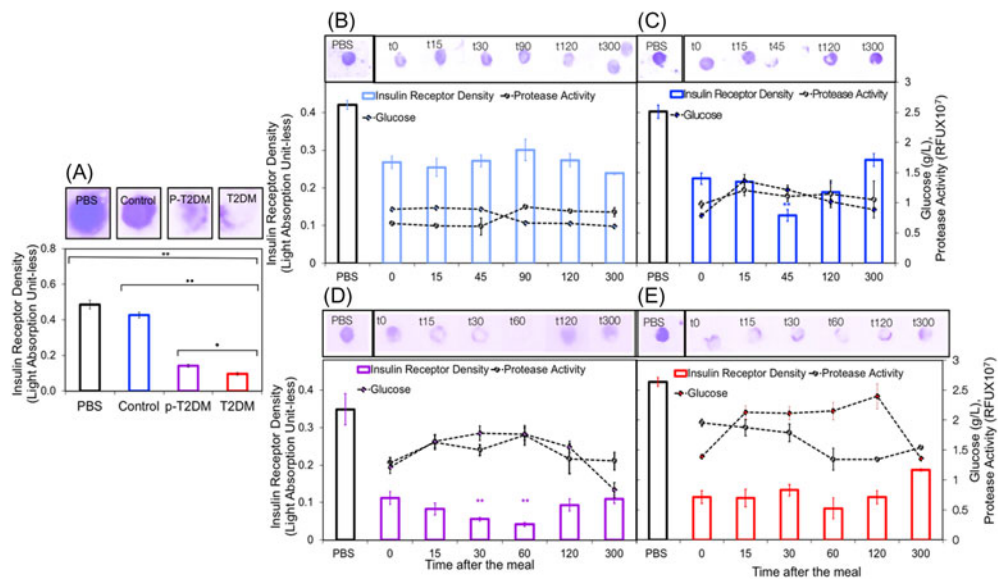


Figure 3. Insulin receptor density in PBMC. (A) Ectodomain insulin receptor density in PBMC incubated with PBS and baseline plasma from control, pre-T2DM, and T2DM (**, ** $p < 0.01$). Incubation with $1 \times$ PBS versus control, pre-T2DM, and T2DM plasma (** $p < 0.01$ for control plasma versus incubation with pre-T2DM, and T2DM plasma and * $p < 0.05$ for incubation with pre-T2DM plasma when compared to cells incubated with T2DM plasma). Density of insulin receptor label after incubation with plasma samples at different times after a meal and with $1 \times$ PBS as control: (B) Shows incubation with plasma from control fasting ($n = 2$), (C) with plasma from control ($n = 3$) (** $p < 0.01$ versus time 0), (D) with plasma from pre-T2DM ($n = 3$) (** $p < 0.01$ versus time 0) and (E) plasma from T2DM ($n = 3$).

The T2DM group showed an initial 15-minute postmeal increase in elastase activity, which continued to remain elevated to the 60-minute time point (Figure 2F, $p < 0.01$).

Among all study participants, we observed a positive correlation between blood glucose during the meal and MMP-2/-9 activity (Figure 2I, $p < 0.001$), elastase activity (Figure 2J, $p < 0.001$), and trypsin activity (Figure 2K, $p < 0.001$).

Insulin receptor density reduction in PBMC after incubation with plasma

PBMCs incubated for 1 hour with baseline plasma from T2DM and pre-T2DM had significantly lower levels of insulin receptor density compared to those incubated with plasma from control participants (Figure 3A, $p < 0.001$). PBMCs incubated with T2DM plasma also showed significantly lower levels of insulin receptor density when compared to cells incubated with pre-T2DM plasma (Figure 3A, $p < 0.05$).

Insulin receptor density on PBMCs incubated with plasma collected at different time points from fasting control participants remained the same throughout the 5-hour observation period (Figure 3B). In nonfasting controls, PBMC insulin receptor density decreased significantly when incubated with plasma collected 45 minutes after the meal (Figure 3C, $p < 0.01$). Insulin receptor density decreased significantly when incubated with pre-T2DM plasma collected at 30 and 60 minutes after the meal (Figure 3D, $p < 0.01$). For T2DM, the insulin receptor density in the PBMCs showed no statistically significant changes before and after the meal (Figure 3E).

There was a significant decrease in insulin receptor density in cells incubated with plasma from all groups compared to cells incubated with $1 \times$ PBS (Figure 3A, $p < 0.001$). Incubation of PBMCs with 5 nM of purified trypsin, elastase,

and MMP-2 spiked into $1 \times$ PBS produced a significant decrease in insulin receptor density on the cell membrane ($p < 0.05$).

Glucose, inflammatory markers, and soluble adhesion molecules

Postprandial glucose levels increased significantly in all participants, except fasting controls ($p < 0.001$) (Figures 2 and 4).

Levels of inflammatory markers and soluble adhesion molecules rose in the circulation of control participants after the meal: CRP values increased significantly at 45 and 60 minutes (Figure 4A, $p < 0.05$) while the SAA values rose significantly at 45, 60, and 120 minutes after the meal (Figure 4B, $p < 0.05$). The sICAM-1 values increased significantly at 45 and 120 minutes (Figure 4C, $p < 0.05$), while the sVCAM-1 values rose at 45, 60, and 120 minutes after the meal (Figure 4, $p < 0.05$). No such changes were observed for T2DM or pre-T2DM participants (Figure 4 F–J).

Among all participants, at baseline we observed negative correlations between blood glucose and plasma CRP levels (Figure 4K, $p < 0.001$), SAA (Figure 4L, $p < 0.001$), sICAM-1 (Figure 4M, $p < 0.001$), and sVCAM-1 (Figure 4N, $p < 0.001$).

Discussion

In the animal literature, unchecked proteolytic activity has been shown to cleave the extracellular domain of the insulin receptor, leading to deficient insulin binding and signaling (3, 4). While there have been limited human data examining this issue, elevated levels of “soluble” insulin receptor fragments have been observed in the plasma of T2DM patients (5). The findings from this study support the hypothesis that a potential mechanism leading to insulin resistance and

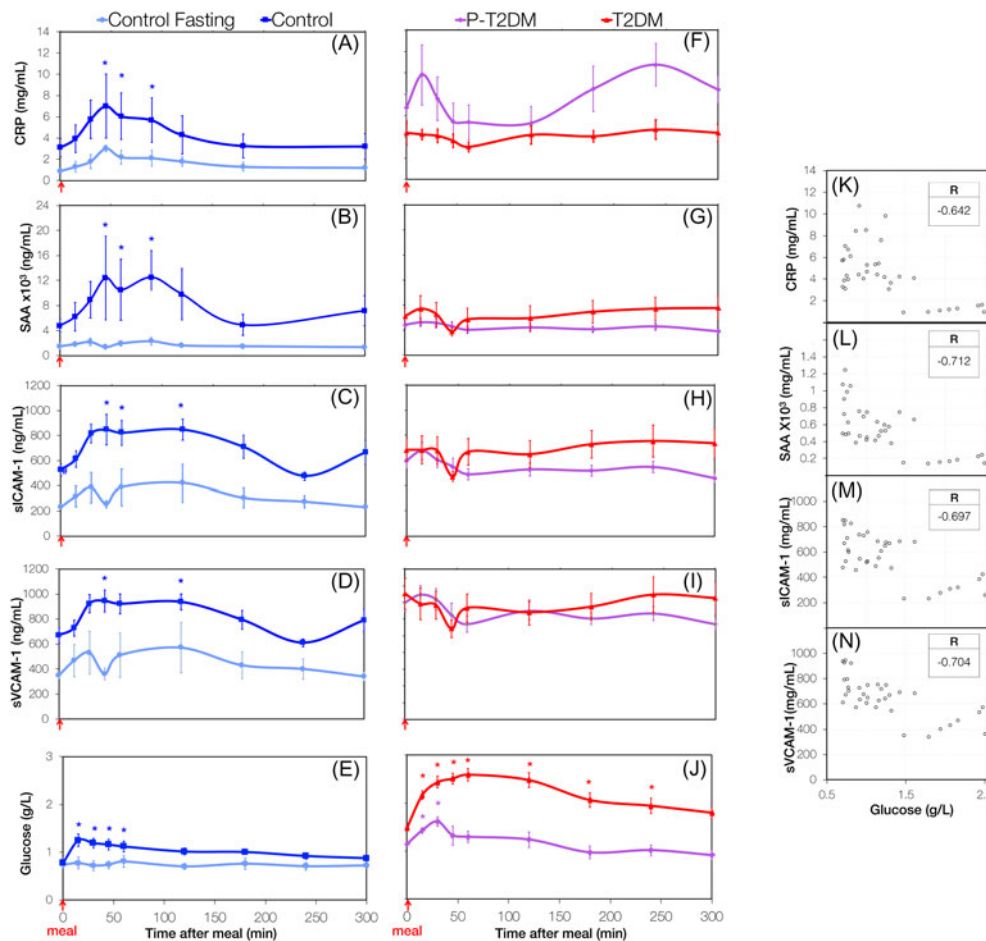


Figure 4. Postprandial levels of CRP, SAA, sICAM-1, and sVCAM-1 in control and control fasting (A–D) and in pre-T2DM and T2DM (F–J) for 5 hours ($*p < 0.05$ when compared to time 0 for control participants). Linear correlations of blood glucose levels and (k) CRP ($p < 0.001$), (l) SAA ($p < 0.001$), (m) sICAM-1 ($p < 0.001$), and (n) sVCAM-1 ($p < 0.001$).

T2DM may be the presence of digestive proteases from the lumen of the intestine in the peripheral circulation, due to an elevated mucosal permeability in the small intestine. We observed an elevation in elastase and MMP-2/-9 activity in the circulation of pre-T2DM and T2DM as compared to controls. In addition, and consistent with the animal literature (3–5), PBMCs incubated with plasma exhibiting this proteolytic activity showed a decrease in the density of the insulin receptor ectodomain. These findings serve to document that proteases cleave artificial substrates for enzyme activity detection when added to them, and that proteases present in plasma can cause a significant decrease in the membrane density of the insulin receptor ectodomain.

Extracellular cleavage of the insulin receptor was not limited to individuals with overt signs of T2DM. Even in non-symptomatic control participants, we detected a postprandial transient elevation of protease activity in the circulation, which was associated with a reduced insulin receptor density on cells incubated with their plasma collected in a postprandial period. Overall, the evidence links an elevation in plasma protease activity, membrane depletion of insulin receptor, and an increase in blood glucose levels and inflammatory markers in response to consumption of a high-calorie/high-carbohydrate meal. While few studies have directly examined carbohydrate load on proteases, a study of lean

non-T2DM men found that, as compared to a high-fat meal, an isoenergetic low-fat (higher carbohydrate) meal led to higher MMP-2 and MMP-9 levels, which were associated with higher glucose levels (13). The authors speculated that hyperglycemia may induce MMP-2 and MMP-9 expression from key cellular sources of MMPs, namely, endothelial cells and macrophages. The study did not assess elastase or trypsin.

Previous efforts to study proteases in T2DM have yielded conflicting results and have focused mainly on plasma MMP-2 and MMP-9 levels. Some reports indicate no correlation between plasma levels of MMP-2 and -9 and T2DM (14), while others show elevated levels of MMP-2 and -9 in T2DM (15–17), or lower levels of MMP-2 and -9 after an oral glucose tolerance test for T2DM (18). These discrepancies may be due to the limitations in previous assay techniques. For example, immunoassays, such as ELISAs, use antibodies that do not distinguish between the active and inactive forms of proteases; moreover, they require ample preprocessing of the samples. The novel electrophoretic assay used in this report bypasses these limitations by (1) measuring protease activity in whole blood and not just in plasma or serum, (2) detecting protease activity derived from nanomolar (as compared to micromolar) levels of proteases, (3) determining only the activity of proteases (and

not only protein levels), and (4) working with a small blood sample volume (3.5 μ L) (10–12).

The baseline elevation of MMP-2/-9 and elastase activity in T2DM may be responsible for the reduction of insulin receptor density and their resulting poor blood glucose control. Proteases, such as elastase, MMP-2, and MMP-9, not only are capable of reshaping the extracellular matrix (19) and activating other proteases, but also are capable of cleaving extracellular receptors (20, 21). Here we present evidence that freshly isolated PBMCs incubated with plasma from T2DM or with purified proteases (MMP-2, elastase, and trypsin) exhibit significant depletion of their insulin receptor density. These results are consistent with the evidence for soluble insulin receptors in the circulation of T2DM patients and *in vitro* cleavage of the insulin receptor by a protease (5, 6). In addition to insulin receptor cleavage, receptor internalization may also occur under these circumstances. The finding that cells incubated with proteases spiked into $1 \times$ PBS also showed depletion of the insulin receptor supports the hypothesis that cleavage of the insulin receptor is a significant process. The exact fate of receptors in these patient groups subject to ectodomain cleavage and the rate of reconstitution into the membrane from endoplasmic pools of newly synthesized receptors remain to be determined in the future.

A major source for the proteolytic activity in the circulation of T2DM may be the intestine, where proteases derived from the pancreas need to be active as a requirement for daily digestion. The evidence that a single high-calorie/high-carbohydrate meal produces an immediate rise in protease activity after the meal in control participants, whereas this did not change significantly in fasting controls, suggests that the increase of pancreatic proteases in the circulation may be linked to digestion. In control participants, the meal also caused an initial increase in soluble adhesion molecules and inflammatory markers and an initial decrease in the insulin receptor density in cells incubated with plasma collected 45 minutes after the meal. In contrast to control participants, T2DM had a significant initial increase after the meal in elastase activity only and no significant depletion in insulin receptor density. The elevation in elastase activity in T2DM could be caused not only by an increase in pancreatic elastase, which can be derived from the intestine and the pancreas itself, but also from neutrophil elastase, which increases in states of inflammation (22). T2DM participants with chronically elevated intestinal permeability (8, 23) may also have chronically elevated proteolytic activity in the circulation, making daily proteolytic activity and insulin receptor density fluctuations less visible than in healthy individuals. In pre-T2DM we also found a decrease in the insulin receptor density of PBMC incubated with plasma taken 30 and 60 minutes after the meal. Though potential mechanisms require further analysis, membrane-bound proteases may cleave the extracellular portion of the adhesion molecules, releasing the extracellular fragments into the plasma as peptide (24, 25). One might expect elevated proteolytic activity in T2DM to yield higher levels of soluble adhesion molecules, but this was not observed. Instead,

reduced values in soluble adhesion molecules in T2DM with chronic elevation of proteolytic activity may be caused by further cleavage of the soluble adhesion molecules, degrading their antibody binding sites and preventing their detection by ELISA.

The relatively high trypsin activity levels in some control participants may be due to their relatively high BMI and waist circumference, as we observed a correlation to trypsin activity values in the circulation of controls. This may explain why control fasting participants, who on average had the lowest BMI and waist circumference, had the lowest trypsin activity. In addition, trypsin activity levels were low and close to the detection limit of our electrophoretic assay, making it difficult to identify significant differences in activities between groups.

Limitations of this exploratory study include the modest number of participants. In addition, we had no detailed information on medications, sleep, or physical activity levels. A larger trial is required utilizing this new protease detection technology. Future studies would also benefit by including measurements of intestine permeability, for example, by measuring large size polysaccharides, or indigestible probes such as ^{51}Cr -EDTA in the plasma.

Conclusion

Despite the high prevalence of T2DM, the molecular mechanisms for the development of insulin resistance have not yet been fully elucidated. The results of this study provide evidence for the hypothesis that the digestive system, and in particular pancreatic proteases, may be associated with the development of insulin resistance and T2DM. In response to a typical high-calorie/high-carbohydrate meal, pancreatic proteases that are normally compartmentalized inside the intestinal lumen may reach the circulation due to an increase in intestinal permeability. Once in the systemic circulation, these pancreatic proteases can degrade biomolecules. Proteases may be responsible for the reduction of the insulin receptor on cells and impairing glucose control. Many membrane receptors have extracellular domains that may be subject to cleavage by proteases, which could be the reason for the diversity of cell dysfunctions that accompany T2DM. Blockage of proteolytic activity in circulation may be a therapeutic target for T2DM, and as a prior proof-of-principle of this concept, studies indicate that the broad metalloprotease inhibitor doxycycline improved the overall health of T2DM (7).

Acknowledgments

This project was supported by a Pilot Grant from the UC San Diego Altman Clinical and Translational Research Institute (ACTRI) Translational Research Award, and partially supported by NIH grant UL1TR000100. We thank the ACTRI Center for Clinical Research for assistance with this study, and Augustine Oberieze for collection of the peripheral blood monocytes.

Conflicts of interest

P.R.T. has no disclosures related to this manuscript. She is a consultant for Sanofi/Regeneron, Novo-Nordisk, Boehringer-Ingelheim, Janssen, Pfizer and Amgen. She is a stock holder of Cardero Therapeutics. G.W.S.-S. owns shares in Leading Bioscience Inc., a company developing treatments for shock. P.J.M. has no disclosures related to this manuscript.

ORCID

Augusta E. Modestino  <http://orcid.org/0000-0001-8132-5381>
 Elaine A. Skowronski  <http://orcid.org/0000-0002-7982-5974>
 Chris Pruitt  <http://orcid.org/0000-0002-1638-2467>
 Pam R. Taub  <http://orcid.org/0000-0002-0684-0655>
 Karen Herbst  <http://orcid.org/0000-0002-9079-9754>
 Geert W. Schmid-Schönbein  <http://orcid.org/0000-0002-1803-1521>
 Michael J. Heller  <http://orcid.org/0000-0001-6436-0337>
 Paul J. Mills  <http://orcid.org/0000-0003-4263-7686>

References

- Muoio DM, Newgard CB. Molecular and metabolic mechanism of insulin resistance and beta-cell failure in type 2 diabetes. *Mol Cell Biol.* 2006;9:193–205.
- Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol.* 2011;11(2):98–107. doi:10.1038/nri2925.
- DeLano FA, Zhang H, Tran EE, Zhang C, Schmid-Schönbein GW. A new hypothesis for insulin resistance in hypertension due to receptor cleavage. *Expert Rev Endocrinol Metab.* 2010;5(1):149–158.
- DeLano FA, Schmid-Schönbein GW. Pancreatic digestive enzyme blockade in the small intestine prevents insulin resistance in hemorrhagic shock. *Shock.* 2014;41:56–62.
- Umehara S, Nishioka M, Obata T, Ebina Y, Shiota H, Hashida S. A novel ultra-sensitive enzyme immunoassay for soluble human insulin receptor ectodomain and its measurement in urine from healthy subjects and patients with diabetes mellitus. *Clin Biochem.* 2009;42(13-14):1468–1475. doi:10.1016/j.clinbiochem.2009.06.014.
- Tomoyuki YT, Amo K, Ishikura S, Nagaya H, Uchiyama K, Hashida S, Ebina Y. Development of in vitro model of insulin receptor cleavage induced by high glucose in HepG2 cells. *Biochem Biophysical Resh Commun.* 2014;445:236–243. doi:10.1016/j.bbrc.2014.01.187.
- Frankwich K, Tibble C, Torres-Gonzalez M, Bonner M, Lefkowitz R, Tyndall M, Schmid-Schönbein GW, Villarreal F, Heller MJ, Herbst K. Proof of concept: matrix metalloproteinase inhibitor decreases inflammation and improves muscle insulin sensitivity in people with type 2 diabetes. *J Inflamm.* 2012;9:1–10.
- Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabet.* 2008;57(6):1470–1480. doi:10.2337/db07-1403.
- Sampson M, Davies I, Gavrilovic J, Sussams B, Brown J, Astley S, Hughes DA. Plasma matrix metalloproteinases, low density lipoprotein oxidisability and soluble adhesion molecules after a glucose load in type 2 diabetes. *Cardiovasc Diabetol.* 2004;3:1–7. doi:10.1186/1475-2840-3-7.
- Lefkowitz RB, Marciniak J, Hu C, Schmid-Schönbein GW, Heller MJ. An electrophoretic method for the detection of chymotrypsin and trypsin activity directly in whole blood. *Electrophoresis.* 2010;31(2):403–410. doi:10.1002/elps.200900424.
- Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. *Anal Chem.* 2010;82(19):8251–8258. doi:10.1021/ac101462c.
- Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for trypsin activity using polyanionic focusing gel electrophoresis. *Electrophoresis.* 2010;31(14):2442–2451. doi:10.1002/elps.201000011.
- Kennedy A, Spiers JP, Crowley V, Williams E, Lithander FE. Postprandial adiponectin and gelatinase response to a high-fat versus an isoenergetic low-fat meal in lean, healthy men. *Nutrition.* 2015;31(6):863–870. doi:10.1016/j.nut.2015.01.009.
- Papazafiropoulou A, Perrea D, Moysakis I, Kokkinos A, Katsilambros N, Tentolouris N. Plasma levels of MMP-2, MMP-9 and TIMP-1 are not associated with arterial stiffness in subjects with type 2 diabetes mellitus. *J Diabetes Complicat.* 2010;24(1):20–27. doi:10.1016/j.jdiacomp.2008.10.004.
- Signorellu S, Malaponte G, Libra M, Di Pinto L, Celotta G, Bevalacqua V, Petrina M, Nicotra GS, Indelicato M, Navolanica PM, et al. Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease. *Vasc Med.* 2005;10:1–6. doi:10.1191/1358863x05vm582oa.
- Thraillkill K, Bunn C, Fowlkes JL. Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy. *Endocr.* 2009;35(1):1–10. doi:10.1007/s12020-008-9114-6.
- Noji Y, Kajinami K, Kawashiri MA, Todo Y, Horita T, Nohara A, Higashikata T, Inazu A, Koizumi J, Takegoshi T, Mabuchi H. Circulating matrix metalloproteinases and their inhibitors in premature coronary atherosclerosis. *Clin Chem Lab Med.* 2001;39(5):380–384. doi:10.1515/CCLM.2001.060.
- Lewandowski KC, Banach E, Bieńkiewicz M, Lewiński A. Matrix metalloproteinases in type 2 diabetes and non-diabetic controls: effects of short-term and chronic hyperglycaemia. *Arch Med Sci.* 2011;7(2):294–303. doi:10.5114/aoms.2011.22081.
- Mazzone T, Chait A, Plutzky J. Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. *Lancet.* 2008;9626:24–30.
- Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, Brain JD, Accurso FJ, Henson PM. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest.* 2002;109(5):661–670. doi:10.1172/JCI200213572.
- Rodrigues SF, Tran ED, Fortes ZB, Schmid-Schönbein GW. Matrix metalloproteinases cleave the beta2-adrenergic receptor in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol.* 2010;299(1):H25–H35. doi:10.1152/ajpheart.00620.2009.
- Talukdar S, Oh DY, Bandyopadhyay G, Li D, Xu J, McNelis J, Lu M, Li P, Yan Q, Zhu Y, et al. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nat Med.* 2012;18(9):1407–1412. doi:10.1038/nm.2885.
- Horton F, Wright J, Smith L, Hinton PJ, Robertson MD. Increased intestinal permeability to oral chromium (51Cr)-EDTA in human Type 2 diabetes. *Diabetic Med.* 2014;31:1464–5491.
- Ceriello A, Falletti E, Motz E, Taboga C, Tonutti L, Ezsol Z, Gonano F, Bartoli E. Hyperglycemia-induced circulating ICAM-1 increase in diabetes mellitus: the possible role of oxidative stress. *Horm Metab Res.* 1998;30(3):146–149. doi:10.1055/s-2007-978854.
- Mazor R, Schmid-Schönbein GW. Proteolytic receptor cleavage in the pathogenesis of blood rheology and co-morbidities in metabolic syndrome. Early forms of autodigestion. *BIR.* 2015;52(5–6):337–352. doi:10.3233/BIR-15045.