

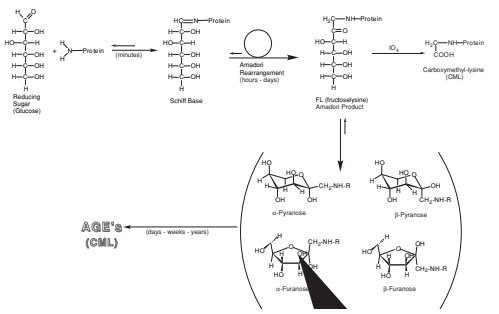
NON-ENZYMATIC GLYCATION OF PLASMA AND CELLULAR PROTEINS IN A SHORT TERM STZ-DIABETIC RAT MODEL

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INTRODUCTION

1. The chemical modification of proteins by non-enzymatic glycation and Advanced Glycation End-Product (AGE) formation is increased with diabetes but the role of these metabolic and physiological changes associated with diabetes is not well understood.
2. The Amadori product, a marker of non-enzymatic glycation, can be measured chemically and immunologically. We describe an HPLC assay and a novel anti-Amadori antibody that are used to quantitate the extent of protein glycation.
3. AGE's were measured with an anti-CML antibody, but there was no detectable increase immunologically up to 28 days following STZ injection.
4. Therefore, the aim of the study was to identify early changes in tissue proteins after the onset of diabetes by studying the time dependent increase in plasma and cellular protein glycation utilizing a short-term STZ-diabetic rat model.

Mechanism of Glycation Reactions (Glucose)



RESULTS (continued)

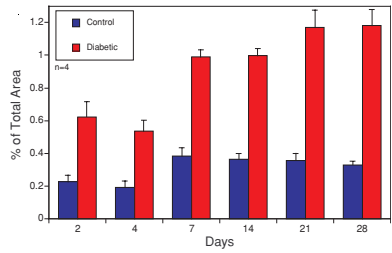


Figure 2: Time Course of Control and Diabetic Rat Plasma Protein Glycation

To develop a short-term model of non-enzymatic glycation, control and diabetic rat plasma protein glycation was determined at various time-points up to 28 days. Plasma from control and diabetic rats was analyzed by boronate affinity HPLC chromatography at 2, 4, 7, 14, 21 and 28 days following STZ injection. A significant difference in non-enzymatic glycation of plasma proteins exists as early as 2 days following STZ and seems to plateau as early as 7 days.

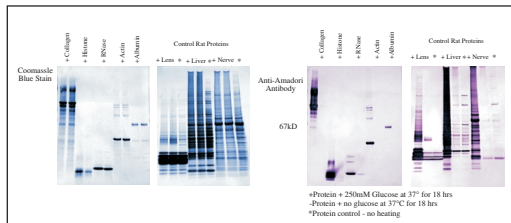


Figure 3: Anti-Amadori Antibody Reactivity with Glucose Modified Proteins

Non-enzymatic glycation is also measured immunologically with an antibody which recognizes the Amadori adduct on proteins incubated with glucose. The reactivity of the antibody was determined by testing the recognition of various glycated proteins using standard Western blot technology. The monoclonal anti-Amadori antibody, which was raised against an albumin peptide which contains the preferentially glycated lysine residue (525), detects glycation of collagen, histones, RNase, actin, albumin (left side) and also glycated tissue homogenates isolated from rat (right side).

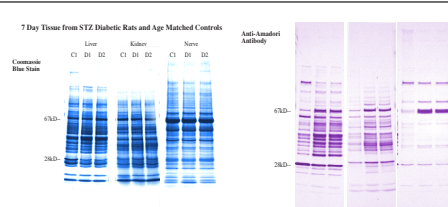
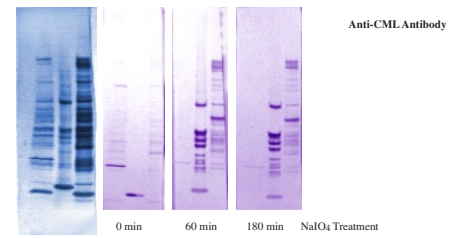


Figure 4: Detection of Non-Enzymatic Glycation of Control and 7-Day Diabetic Rat Tissue Proteins Using an Anti-Amadori Antibody

The extent of glycation in control and diabetic rat tissue was determined in soluble proteins from liver, kidney and nerve homogenates by SDS-PAGE Western blots using the anti-Amadori antibody. One control (C1) and two diabetic (D1, D2) animals are shown for each tissue. A general increase in glycation occurs in most protein bands as evident by the increased staining by the antibody. The protein band at 67kD, which increases dramatically in antibody reactivity in the nerve, is albumin. All lanes contain equal protein loads as evident in the Coomassie Blue stained gels. Similar results are seen 21 days following STZ injection but with a greater difference in antibody reactivity between control and diabetic animals. Control proteins in liver tissue display a higher level of antibody reactivity than do control tissues in kidney or nerve. One such protein band, carbonic anhydrase at 28 kD, has been shown to decrease in the STZ rat model. Figure 6 will show that this reactivity is specific for the Amadori adduct.

RESULTS (continued)



Effect of Periodate Treatment on Anti-Amadori Antibody

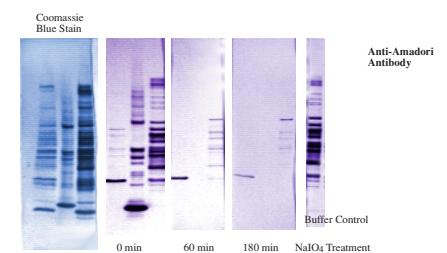


Figure 6: Periodate Oxidation of the Amadori Adduct on Protein Yields CML

Specific anti-Amadori antibody reactivity is further confirmed by sodium periodate treatment used to convert protein bound Amadori adducts to carboxymethyllysine (CML) by oxidative cleavage of vicinal hydroxyl groups leaving a carboxymethyl group attached to the protein. A polyclonal anti-CML antibody was prepared from glyoxylic acid modified KLH which produces only one AGE, CML-KLH. A mixture of proteins (lane 2, and seen also in figure 3 left panel) was incubated with glucose for 18 hours in PO buffer, pH 7.4, at 37°C. Lanes 1 and 3 represent intracellular liver proteins from a control rat and a diabetic rat, respectively. Loss of anti-Amadori antibody reactivity in the top panel is expected if the antibody is specific for the Amadori adduct. The detection of increased CML in the bottom panel shows that the periodate reaction was successful in converting the Amadori adduct to a CML product.

METHODS

Animal Model:

Weight and age-matched male Sprague Dawley rats were placed into a control (n=5) or diabetic (n=7) group (streptozotocin [STZ] 30 mg/kg i.p. with resultant blood glucose > 250 mg/dl). Rats were euthanized by CO₂ and blood was collected by heart puncture at the indicated time points. Liver, kidney and nerve tissue was dissected, placed into PBS containing protease inhibitors (PMSF, DFP), rinsed, and frozen in liquid N₂ until analysis.

Boronate HPLC:

Plasma (150 µl) was diluted with 75 µl buffer (50 mM magnesium chloride, 250 mM ammonium acetate, pH 8.05) and 50 µl (2 mg protein) was injected onto a Glyco-Gel phenylboronate chromatography column (5 mm x 60 mm) at a flow rate of 2 ml/min. Non-glycated protein was eluted in the void while the bound glycated protein fraction was eluted with 0.1 M acetic acid. Data reported as percent glycation is the ratio of the bound glycated peak area to total protein area.

SDS-PAGE/Western blot:

Tissues were homogenized in PBS buffer using a Branson Sonifier 450 at 30% pulse for 15-20 pulses. Homogenates were centrifuged (5000xg) and the supernatant was removed for analysis. Protein content was determined by BCA and 7.5 µg were loaded onto a 4%-20% PAGE gel run under reducing conditions. Following separation, proteins were transferred to a PVDF membrane using a Hoeffer Mini-Gel transfer chamber for 1200 volt-hours.

Detection of Amadori modified protein: The membrane was incubated in a 1:1250 dilution of monoclonal IgM antibody raised against glucose-derived Amadori-modified lysine residues (1-deoxyfructosyl lysine). The membrane was subsequently incubated with a goat anti-mouse alkaline phosphatase conjugated secondary antibody at a concentration of 1:5000. Detection was accomplished using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl Phosphate (BCIP) substrate.

Detection of CML modified protein: The membrane was incubated in a 1:1250 dilution of polyclonal IgG antibody raised against CML-modified Keyhole Limpet Hemocyanin (KLH). The membrane was subsequently incubated with a goat anti-rabbit alkaline phosphatase conjugated secondary antibody at a concentration of 1:5000. Detection was accomplished using NBT/BCIP substrate.

RESULTS

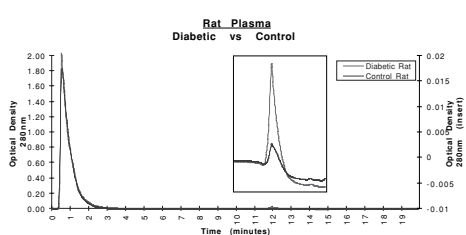


Figure 1: Boronate Affinity HPLC Chromatogram of Control and Diabetic Rat Plasma

Boronate affinity chromatography is used to determine the extent of glycated protein (bound fraction) relative to total protein (bound + flow-through fraction) analyzed. The fraction of glycated plasma proteins is minor relative to total protein content as shown by the difference in areas of the bound glycated peak (11.9 minutes) compared to the non-bound non-glycated peak (1.1 minute). A 3-fold increase in the extent of glycation of diabetic over control rat plasma proteins occurs in just four days following STZ injection and is shown as the difference in peak areas (inset).

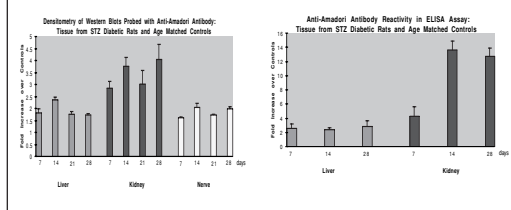


Figure 5: Time Course of Glycation in Control and Diabetic Rat Tissue

The extent of glycation in control and diabetic rat tissues was quantitated with the anti-Amadori antibody using both Western and ELISA blot techniques. The increase over control was approximately 2-fold for liver and nerve proteins and 3.5-fold for kidney proteins using the Western blot technique. The ELISA analysis of the liver proteins correlated well with the Western data showing a 2 fold increase over control which plateaus at 7 days. This was also seen in the plasma boronate data in figure 2. The larger increase in glycation seen in the diabetic kidney between 7 and 28 days using ELISA assays may be due to low control background in these experiments. Nerve proteins (neither control nor diabetic) did not react in the ELISA assay.

CONCLUSIONS

1. Non-enzymatic glycation of intracellular proteins occurs very rapidly in STZ-induced diabetic rats. Two days following STZ injection blood glucose concentration and protein glycation levels are elevated compared to control rats not injected with STZ. Increased protein glycation measured in plasma (Boronate HPLC) was consistent with increases measured immunologically (Amadori antibody) in tissues. Glycation was observed to increase in the liver and nerve and the greatest increase was seen in the kidney.
2. The formation of AGE products, measured immunologically as CML, was not detectable in tissues of rats after 28 days of hyperglycemia. The accumulation of AGE's may take several months before levels are sufficient for detection, even though changes in glycation with diabetes are seen at very early in time.
3. This data suggest that early physiological changes that occur in diabetics may be associated with increases in the Amadori product since AGE formation is insignificant, and ultimately lead to the development of diabetic complications.

REFERENCES

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