COMMENTARY

The fructosamine 3-kinase knockout mouse: a tool for testing the glycation hypothesis of intracellular protein damage in diabetes and aging

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Protein glycation and the formation of AGEs (advanced glycation end-products) and cross-links have been hypothesized to play a role in the pathogenesis of age- and diabetes-related complications. The discovery that FN3K (fructosamine 3-kinase) results in protein deglycation upon phosphorylation of glucose-derived Amadori products suggests that intracellular glycation could be deleterious under certain circumstances. In order to approach the question of the biological relevance of intracellular glycation, in this issue of the *Biochemical Journal*, Veiga-da-Cunha and colleagues generated an FN3K-knockout mouse. The

mice grow normally and are apparently healthy, and levels of protein-bound and free fructoselysine are elevated in several tissues of importance to diabetic complications. This commentary discusses the clinical and evolutionary significance of FN3K, and proposes experimental approaches for revealing the existence of a biological phenotype.

Key words: fructose, galactosaemia, glucose, glycated haemoglobin, HbA_{1c}, Maillard reaction, starvation.

D-Glucose is the biological ‘parent’ of a series of reactive carbonyl compounds that can non-enzymatically react with nucleophiles in proteins, peptides, amino acids, glycolipids or DNA. The reaction, first described by L. C. Maillard in 1912 [1], can be initiated by most reducing sugars, whereby the sequence proceeds via an Amadori product (Figure 1), the formation rate of which is inversely related to the anomerization rate of the sugar, i.e. glycoaldehyde > glyceraldehyde > threose/erythrose > ribose > glucose. Indeed, it has been noted that evolution might have chosen glucose as its main carrier of energy from cell to cell, precisely because the rate of damage inflicted by glucose is the slowest of all reducing sugars [2]. Once the glucose-derived Amadori product (also called fructosamine) has formed, it can either slowly revert back into glucose and its epimer mannose, or it can undergo glycoxidation and form AGEs (advanced glycation end-products), such as CML (carboxymethyl-lysine) and pentosidine. It can also react further without fragmentation with arginine or lysine residues and form protein cross-links, such as glucospane and crossline, as well as all sorts of modifications that have been implicated in molecular damage in diabetes and aging [3,4].

In 1990, Szwergold and colleagues [5] discovered the enzymatic conversion of fructose into fructose 3-phosphate in the lens and erythrocyte leading to the formation of deoxyglucosone, implying the existence of a 3-phosphokinase enzyme. The enzyme was first cloned by Van Schaftingen and colleagues and was found to phosphorylate protein-bound fructosamines with much higher affinity than free fructose itself [6]. Because the phosphorylated fructosamine spontaneously detached from the protein, FN3K (fructosamine 3-kinase) emerged as a deglycation and protein-repair enzyme (Figure 1), thus joining the growing list of protein-repair enzymes, such as methionine sulfoxide reductase, PCMT-1 (L-isoaspartyl methyltransferase), *cis*-proline isomerase and TDOR (thiol-disulfide oxidoreductase).

Molecular studies revealed that FN3K is a 34 kDa enzyme that can phosphorylate fructose, fructoselysine and protein-bound

Amadori products, with relative K_m values of > 50 mM for fructose but only 10 μ M for fructoselysine respectively [6]. Thus the enzyme has a much higher affinity for Amadori products than for fructose. The mRNA was detected in several rodent and human tissues; in particular, brain, bone marrow, kidney and spleen, and at lower levels in the heart, liver and skeletal muscle. FN3K was found to phosphorylate fructosamines at specific lysine residues, as well as other low-molecular-mass glycation substrates. However, it was inactive against the N-terminal valine that forms HbA_{1c} (stable minor haemoglobin variant containing glycation N-terminal β -chains). Importantly, FN3K is constitutively expressed, and no significant correlation between FN3K activity and the levels of HbA_{1c}, total GlcHb (glycated haemoglobin) and haemoglobin fructoselysine residues, either in the normoglycaemic or diabetic group, was found. Its gene, located on chromosome 17, contains six exons and is thought to have arisen through duplication of an ancestral gene, the FN3K-RP (FN3K-related protein) that is found as far back as the fish, sea squirt and thermophilic bacteria [7]. FN3K-RP is conserved in most vertebrates and can perform similar reactions as FN3K, except that its substrate specificity is limited to ribolusamines.

In order to probe the biological impact of FN3K, in this issue of the *Biochemical Journal* Veiga-da-Cunha and colleagues [8] have generated an FN3K-deficient mouse by replacing exons 1 and 2 with a heterologous gene consisting of β -galactosidase and a neomycin-resistance gene. The homozygous mice are viable and fertile. FN3K activity was undetectable in erythrocytes, brain and kidney, and presumably most other tissues. Curiously, β -galactosidase expression was not detectable in any tissue, suggesting that the exon 1–2 region of the FN3K gene contains important regulatory elements. KO (knockout) mice showed no differences in body mass, life span, plasma fructosamines, creatinine or tissue histology at multiple ages and were, by all accounts, indistinguishable from wild-type mice.

Although biological parameters were apparently unaffected by the KO, elevated fructosamine levels were detected in a number

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Figure 1 The sequence of protein glycation and phosphorylation by FN3K, followed by spontaneous deglycation of Amadori products (fructoselysine)

of tissues. GlcHb measured by boronate affinity chromatography was increased 2–5-fold. The increase was linked to glycation at lysine residues and not the N-terminal valine of the β chain, since the latter is not accessible to FN3K [9]. In other tissues, glycation was approx. 20% higher in enzymatic protein digests from the brain, kidney and liver of FN3K-KO mice. Fructose- ϵ -lysine in its free form was increased approx. 10-fold in erythrocytes, brain and heart, and approx. 4-fold in liver and skeletal muscle, but not significantly in serum (fed state) and kidney, compared with the wild-type mouse. In the fasting state, the FN3K-KO mouse excreted 2.5 times more fructoselysine than the wild-type mouse, strongly suggesting that body protein breakdown contributes to circulating levels of fructoselysine.

The creation of a mouse deficient in FN3K that lives and reproduces normally in spite of elevated cellular levels of glycated proteins and free fructoselysine raises a number of important questions of evolutionary, biological and clinical significance. First, it should be noted that many mouse KOs display no biological phenotype, indicating that considerable redundancy exists in biology, and that biological stresses are often needed to reveal a phenotype. In the case of FN3K, the most obvious stresses are hyperglycaemia as in diabetes, galactosaemia and a high-fructose diet. All conditions have been associated with various pathologies that include nephropathy, retinopathy and neuropathy, cataracts and increased protein glycation [10–13]. If these stresses were to be applied to the FN3K-KO diabetic mouse, kinetic experiments would be necessary, since single end-points after prolonged diabetes may not show differences with the wild-type diabetic mouse.

Useful complication end-points should include retinal parameters, vascular permeability, basement membrane thickening, nerve conduction velocity, leucocyte adhesion, macrophage function, albuminuria, wound healing, lens opacification and susceptibility to infections, to name but a few. For an investigation of cardiovascular phenotypes, crosses between the FN3K-KO and apoE (apolipoprotein E)-null diabetic mouse could reveal useful insights. Similarly, gestational diabetes might be particularly dele-

terious in offspring from FN3K-deficient mice, and various embryopathy end-points are available for such studies [14].

Assuming a biological phenotype with severe complications emerges from the combination of diabetes and FN3K deficiency, the approach towards finding the glycated culprit is expected to be very difficult. A promising approach would be to focus on cells that are highly permeable to glucose, such as endothelial cells, and preferentially proteins with a slow turnover rate. Interestingly, an siRNA (small interference RNA) knockdown in cultured fibroblasts was associated with growth inhibition [15]. Assuming FN3K indeed works as a deglycation enzyme, extensive proteomics experiments will be needed, whereby the detection of glycated proteins with anti-1-deoxyglucitolyl antibodies [16] in two-dimensional gels combined with LC (liquid chromatography)/MS/MS is expected to be helpful.

The above proposition automatically leads to the quantitative question. How much of a decrease in cellular protein activity is required for a phenotype to emerge? The answer is straightforward: a great many animals and humans that are heterozygous for a non-redundant gene display a 50% reduction in protein level, but no biological phenotype. As for glycation, the quintessential example is haemoglobin, which even in poorly controlled diabetes reaches a level of glycation that seldom (if ever) exceeds 20% [17]. The major component of haemoglobin, HbA_{1c} , shows a lower rate of dissociation of oxygen than HbA_0 [18], but this increased affinity is thought to be insufficient to affect oxygen delivery in diabetes [17]. Applying a 20% glycation ceiling to intracellular proteins with non-redundant functions, would such a decrease in activity be sufficient to impart a metabolic disturbance, even if combined with a similar decrease in other molecules?

The availability of the FN3K mouse will help address the questions raised above, and hopefully why evolution has retained this enzyme. Intracellular glycation somehow must be a greater threat to survival in early life than extracellular glycation, since no enzyme has yet been found that can reverse collagen-linked Amadori products. Apparently, although the enormous increase in

glucose-derived cross-linking of the ECM (extracellular matrix) during aging and in diabetes [19] could have detrimental effects on vascular compliance, glucose-derived matrix protein cross-linking during the growing years did not exert sufficient pressure on survival for such a deglycation gene to evolve.

Finally, if the FN3K-KO mouse proves to be totally resilient to biological stresses, one will have to consider that the enzyme, like aldose reductase, might be detrimental rather than beneficial under hyperglycaemic conditions by virtue of forming high levels of the toxic 3-deoxyglucosone [20]. If so, the fructose-fed diabetic KO mouse would develop complications at a lower rate than the wild-type.

In summary, Veiga-da-Cunha and colleagues [8] have achieved a major milestone. Obviously, the various experiments proposed above represent only the tip of the investigation of an 'iceberg'. The most gratifying outcome of FN3K-KO will be to find a biological phenotype. Whatever the latter may be, it will increase our insight into the pathogenesis of diabetic complications.

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