Modulation of *in vivo* 3-deoxyglucosone levels

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Abstract

Fructoselysine 3-phosphate is synthesized *in vivo* by the recently discovered fructoseamine-3-kinase (F3K) from fructoselysine and ATP and decomposes to lysine, Pi, and 3-deoxyglucosone (3DG). This pathway appears to dominate 3DG production *in vivo*, making it possible to modulate 3DG levels by stimulating or inhibiting the reaction. Present inhibitors are non-reacting substrate analogues with relatively high Ki values and can inhibit F3K sufficiently *in vivo* to reduce 3DG in diabetic rat plasma by approx. 50%. Stimulation of the F3K pathway by feeding glycated casein causes an increase of 10–20-fold in plasma levels of 3DG and 3-fold in kidney tubules. Consequences of this increase were studied in two systems: the Eker rat, a model of susceptible kidney tubules; and birth rates in two rat strains. In both cases substantial pathological effects were observed. In the Eker rats, an approx. 3-fold increase in kidney lesions was observed (P < 0.00001). In both Fischer 344 and Sprague–Dawley rats, birth rates were reduced by 56% (P < 0.0001) and 12% (P < 0.015) respectively. These results suggest that inhibition of F3K is a promising new therapeutic target for diabetic complications, as well as other 3DG-dependent pathologies.

Introduction

Glycation of lysine by glucose was first described by Maillard [1]. In this reaction the ε-amino group of lysine condenses with the carbonyl on glucose to form a Schiff’s base, which rearranges to a carbonyl at C2 of glucose to produce the modified amino acid, fructoselysine (FL). This reaction has been studied for many years, since it is the primary route of lysine loss in heated milk products [2]. FL was thought not to be metabolized in mammals until the recent discovery of a phosphorylated derivative of FL, FL 3-phosphate (FL3P), showed otherwise [3]. The enzyme responsible was isolated and sequenced from human red cells by Delpierre et al. [4]. It has been found in rats, mice, chickens, pigs and rabbits. The reaction phosphorylates the three position of FL, or other fructoseamines, using ATP as a phosphate donor. The enzyme’s likely function is to act as a lysine recovery pathway, by recovery of lysine from FL3P on or off protein.

FL3P spontaneously decomposes with a lifetime of approx. 6 h under standard physiological conditions to yield lysine, inorganic phosphate (Pi), and the reactive di-carbonyl 3-deoxyglucosone (3DG), an early component in the formation of advanced glycation end-products (AGEs) [5]. Non-enzymatic glycation, during which reducing sugars covalently attach to free amino groups and ultimately proceed to form AGES, occurs during normal aging and at an accelerated rate in diabetes mellitus [6]. This process changes irreversibly the structural and functional properties of proteins, lipid components and nucleic acids [6]. Its acceleration has been postulated to contribute to the development of diabetic complications including nephropathy, retinopathy and neuropathy [7]. AGE inhibitors prevent and/or reverse endothelial cell proliferation [8], and inhibition of AGE formation reduces the extent of nephropathy in diabetic rats [9].

3DG plays a key role in this process [10–12]. Plasma levels of 3DG in a diabetic population suggested a causal connection to microangiopathy, retinopathy and neuropathy [13]. Whether this is due to the direct action of 3DG on proteins or its more indirect role in raising AGE levels is unknown. In any case, higher plasma levels of 3DG are significantly associated with pathology. Until the discovery of fructoseamine-3-kinase (F3K), *in vivo* 3DG was believed to result entirely from non-enzymatic processes involving FL-containing protein [14,15]. Measurements in tissue extracts, *in vitro* solutions and estimates of 3DG production *in vivo* indicate that levels of F3K-produced 3DG are 20-fold higher than those of non-enzymatically produced 3DG. Thus, by stimulating or inhibiting F3K, we can modulate *in vivo* 3DG levels. In this paper, we review the pathological consequences of stimulating the F3K pathway through dietary manipulation and the changes in 3DG levels *in vivo* due to inhibition of the reaction.

Materials and methods

Animals

Sprague–Dawley or Fisher 344 rats were purchased from Charles River Labs (Wilmington, MA, U.S.A.). Rats carrying the Tsc2 gene (Eker rats) in a Fisher 344 background were kindly supplied by Dr Raymond Yeung (University of Washington, Seattle, WA, U.S.A.) and bred with wild-type
Fisher 344 to produce F1 crosses. Pups were genotyped from tail clippings by Dr Yeung.

**Synthesis of 3-O-methylsorbitol-lysine**

3-O-Methylglucose (25 g, 129 mmol) and α-Cbz (benzyl-oxyacarbonyl)-lysine (12 g, 43 mmol) were dissolved in 200 ml of water/methanol (2:1, v/v). Sodium cyanoborohydride (10 g, 162 mmol) was added and the reaction was stirred at room temperature. Reaction of α-Cbz-lysine was monitored by TLC on a silica gel employing 1-butanol/acetic acid/water (5/2/3, by vol.) using ninhydrin for visualization. The reaction was complete when no α-Cbz-lysine remained. The solution was neutralized with 3 M acetic acid and then applied to a column (5 cm × 50 cm) of Dowex-50 (H+) and the column was washed well with water to remove excess 3-O-methylglucose. The target compound was eluted with 5% ammonium hydroxide. After evaporation the residue was dissolved in 50 ml of water/methanol (2:1, v/v) and 10% Pd/C (0.5 g) was added. The mixture was shaken under 20 lbf/in² of hydrogen for 1 h. The charcoal was filtered off and the filtrate evaporated to a white powder. The material was converted to its phenylisothiocyanate derivative. Elemental analysis: calculated for C₁₃H₂₈N₂O₇ · CH₃OH; C, 42.86; H, 9.18; N, 7.14. Found: C, 42.94; H, 8.50; N, 6.95.

**Enzyme isolation**

Samples (100 g) of chicken kidney were homogenized in 300 ml of 10 mM Tricine, pH 7.4, and centrifuged at 40 000 g for 0.5 h; the supernatant was then centrifuged at 100 000 g for 0.5 h, the precipitate was collected and dissolved in the minimum volume of 10 mM Tricine, pH 7.0, and dialysed against the same buffer containing 1 mM dithiothreitol, 0.02 mM EDTA, 1 mg/l antipain and 1 mg/l leupeptin. The reaction was complete when no α-Cbz-lysine remained. The solution was neutralized with 3 M acetic acid and then applied to a column (5 cm × 50 cm) of Dowex-50 (H+) and the column was washed well with water to remove excess 3-O-methylglucose. The target compound was eluted with 5% ammonium hydroxide. After evaporation the residue was dissolved in 50 ml of water/methanol (2:1, v/v) and 10% Pd/C (0.5 g) was added. The mixture was shaken under 20 lbf/in² of hydrogen for 1 h. The charcoal was filtered off and the filtrate evaporated to a white powder (10.7 g, 77% yield based on α-Cbz-lysine) that was homogeneous when analysed by reversed-phase HPLC as the phenylisothiocyanate derivative. Elemental analysis: calculated for C₁₀H₁₉N₂O₆ · CH₃OH · 2 H₂O; C, 42.86; H, 9.18; N, 7.14. Found: C, 42.94; H, 8.50; N, 6.95.

**Results**

The consequences of stimulating the F3K pathway were investigated in two model systems. Stimulation of the F3K pathway is relatively easy; simply feeding a diet rich in FL residues created by heating glucose and casein together dramatically increases 3DG levels. Preliminary experiments showed that 5% (w/w) of this mildly glycated casein in a standard diet stimulated 3DG levels 10–20-fold in the plasma and approx. 3 fold in the kidney compared with the standard diet stimulated 3DG levels 10–20-fold in the plasma and approx. 3 fold in the kidney compared with the control diet containing identical constituents, except that the glucose and casein were heated at 60°C for 70 h, together in a paste for the glycated diet and separately for the control diet. In the glycated diet, the lysine residues of the casein are converted into FL by this procedure. As the casein is digested, these become available as free FL, which is phosphorylated intracellularly by F3K.
Table 1 | Tumor lesions per kidney section in Eker rats on glycated diet ($P = 0.00001$)

<table>
<thead>
<tr>
<th>Control diet</th>
<th>Glycated diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n = 9$</td>
<td>$n = 8$</td>
</tr>
<tr>
<td>Mean</td>
<td>0.82</td>
</tr>
<tr>
<td>Variance</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Pathology. Two cohorts of rats carrying the Eker mutation were placed *ad lib* for 12 weeks on the control diet and the glycated diet respectively, and subsequently killed. Their kidneys were fixed and sectioned for histopathology. The sections were analysed blindly by Dr Klein-Santo of the Fox Chase Cancer Center (Philadelphia, PA, U.S.A.) who scored each section for lesions [18]. Comparison of the two cohorts is presented in Table 1. The glycated diet increased the number of kidney lesions approx. 3-fold. Lesion counts in both diets showed both statistical distributions to be Poisson, with the mean for the glycated diet nearly 3 times larger than for the control diet ($P < 0.00001$).

Recent work has also implicated 3DG in the aetiology of diabetic embryopathy, suggesting that elevation of 3DG may increase developmental abnormalities [21]. To test this, relative birth rates of cohorts with *ad lib* access to the control diet and the glycated diet were compared. Two cohorts of Fischer 344 rats consisting of six breeding pairs each were placed on each diet for 10 months and the number of live pups produced recorded. Over this period the cohort on the control diet gave birth to 295 pups compared with 131 for the cohort on the glycated diet. The time course is shown in Figure 1. Litter sizes were not significantly different in the two cohorts (6.4 compared with 5.7 respectively), but the number of litters in the cohort on the glycated diet was significantly less (23 compared with 46). Assuming Poisson statistics for the litters, $P < 0.0001$. The experiment was repeated with two cohorts of six pairs of Sprague–Dawley rats. After 9 months, the cohort on the glycated diet gave birth to 456 pups compared with 516 for the cohort on the control diet. Again, the litter sizes were similar, but the litter number was significantly less for the cohort on the glycated diet (47 compared with 59; $P = 0.015$).

Inhibitors that were FL analogues, such as morpholino fructose, were successful in blocking the formation of FL3P *in vitro*. Unfortunately, these compounds are also substrates for F3K, and the resulting products also decompose to 3DG, making them unsuitable for use *in vivo*. Inhibitors based on sorbitol, on the other hand, show competitive inhibition of the F3K reaction but also prevent the formation of 3DG, because there is no spontaneous decomposition of the product to 3DG. For compounds blocked at C3, also there is no product formed. The $K_i$ values of these compounds are approx. 500 µM. Although high for an *in vivo* drug, their apparent low toxicity make it possible to use them, at least in animals, to lower 3DG levels.

These inhibitors were tested in diabetic rats. Of the four cohorts in the experiment two were made diabetic by intraperitoneal injection of streptozotocin (60 mg/kg). After 3 days any animal with blood glucose less than 300 mg/dl was removed from the experiment, leaving six rats in each diabetic cohort. One control and one diabetic cohort were injected intraperitoneally daily with 3-O-methylsorbitol-lysine (50 mg/kg) or saline for a period of 6 days. The animals were then killed and blood was obtained by cardiac puncture. As can be seen in Table 2, both control and diabetic animals showed an approx. 50% reduction in 3DG levels. This is the first report of reduction in 3DG levels in the plasma of diabetic animals by therapeutic intervention, although 3DG levels in erythrocytes have been reduced using aldose reductase inhibitors [22].

**Discussion**

Using the F3K pathway, 3DG levels *in vivo* can be manipulated in a controlled way. Our preliminary experiments demonstrate that is possible to reduce 3DG levels *in vivo* although our present inhibitors are not ideal, since they...
have high $K_i$ values and dosages in the mg/kg range are needed to inhibit F3K [23]. Because of this, we are unable to conclusively confirm 3DG’s role in diabetic and other pathologies by lowering its level for extended periods. Nevertheless, the experiments have proven that at least 50% of 3DG in vivo comes from the F3K reaction. The apparently linear relationship observed between FL and 3DG levels in the kidney tubules of rats suggests that inhibiting F3K more effectively could reduce 3DG levels further.

An alternative approach is to stimulate the flux in the F3K pathway, thereby increasing 3DG levels. Several points should be made regarding this class of experiments. First, the consequences of the stimulation are observed against a control diet made up of the same constituents but heated separately to prevent the formation of glycated residues. Since the heating was very mild (60°C for 70 h) and the same for both diets, we have been unable to conceive of a mechanism other than the elevation of 3DG as the cause of the pathological sequelae. Secondly, the intracellular levels of 3DG almost certainly will be distributed differently from those found in hyperglycaemia because they will depend on how the glycated amino acid, FL, is distributed after casein digestion. In hyperglycaemia, either the lysine is deglycated by F3K while on the protein, or the protein is recycled within the cell. We do not know how much of the resultant FL is released into the plasma and available systemically. Finally, 3DG produced by the F3K pathway will be different than 3DG directly fed to an animal for several reasons: (1) dietary 3DG can react in the gut with other amines and be detoxified; (2) 3DG created intracellularly is able to interact directly with protein and DNA without needing to be transported; and (3) the conformations of newly synthesized 3DG will be different from the equilibrium distribution of conformers. This latter is important, as the reactive form of a carbonyl is the unhydrated open chain form. This is always low at equilibrium, whereas it could be high immediately following FL3P decomposition.

It is clear from Table 1 that Eker rats are sensitive to the glycated diet, increasing their number of kidney lesions almost 3-fold. Comparing this with the approx. 3-fold increase in 3DG observed in the tubules of normal rats on the same diet suggests that the dose–response relationship is in the correct range for 3DG to be a significant agent in stimulating kidney lesion formation in these susceptible rats. It is impossible to know whether the mechanism is direct adduct formation on DNA, excess production of various growth factors by 3DG [24], or some other effect. Further work is needed to confirm these observations and identify the causal pathway.

Since 3DG is known to cause embryopathy the birth rate reductions for animals on the glycated diet are very compelling. Because there are many factors which affect an animal’s birth rate (season, lighting, handling, etc.), they were located in the same room, handled and cared for by the same staff in an attempt to minimize all influences other than the diet. Although elevation of 3DG appears responsible for the birth rate decline, the precise mechanism is unknown. It may be a problem in the pup development, but sperm viability in the fathers or other effects of 3DG in the mothers cannot be excluded.

In summary, our experiments strongly suggest that 3DG produced as a consequence of the formation of FL3P by the F3K pathway is a significant contributor to a wide range of pathological sequelae. Observations of increased pathology following stimulation of the pathway by feeding a glycated diet are powerful, but not conclusive indicators of this. To demonstrate conclusive causality we must be able to inhibit the pathway successfully in vivo. Thus far, we have been able to reduce 3DG levels by 50%, although higher reduction may be possible with our present inhibitors, since there were no signs of toxicity at the dosages used. Our results show this reaction to be a new potential therapeutic target for diabetic complications as well as other 3DG-dependent pathologies.

We acknowledge the invaluable contributions of R. Yeung, A. Klein-Santo and A. Taylor to this work. Much of the early work on this problem was done in collaboration with B. Szwergold and A. Petersen.

References

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Table 2 | Reduction of in vivo 3DG by 3-0-methylsorbitol-lysine

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Control</th>
<th>Control</th>
<th>Plasma/day 8</th>
<th>Plasma/day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample-Time</td>
<td>Urine*/day 1</td>
<td>Urine*/day 8</td>
<td>Plasma/day 8</td>
<td>Plasma/day 8</td>
</tr>
<tr>
<td>Saline ($n=6$)</td>
<td>6.55 ± 2.43</td>
<td>5.56 ± 0.84</td>
<td>0.23 ± 0.07 μM</td>
<td>0.94 ± 0.28 μM</td>
</tr>
<tr>
<td>Inhibitor ($n=6$)</td>
<td>6.35 ± 1.41 μmol/g</td>
<td>4.05 ± 0.24 μmol/g</td>
<td>0.13 ± 0.02 μM</td>
<td>0.44 ± 0.10 μM</td>
</tr>
<tr>
<td>% Reduction</td>
<td>0.3%</td>
<td>27.0%</td>
<td>43.0%</td>
<td>53.0%</td>
</tr>
<tr>
<td>$P$ value ($t$ test)</td>
<td>0.43</td>
<td>0.0007</td>
<td>0.0024</td>
<td>0.0006</td>
</tr>
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</table>

* Urine values are normalized to creatinine.
16 Reference deleted


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