D-Lysine Effectively Decreases the Non-Enzymic Glycation of Proteins in Vitro

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Excessive non-enzymic glycation of proteins alters their physicochemical properties, with possible pathological effects. We investigated the in vitro inhibition of protein glycation by D-lysine—an isomer not incorporated into mammalian proteins but possessing the same chemical characteristics as L-lysine. Glucose incorporation was studied as follows: (a) human albumin, IgG, collagen, and isolated glomerular basement membrane were incubated for 20 days with D-glucose (5.0, 10.0, and 20.0 mmol/L) in the presence of D-lysine at 1% of the sugar concentration; (b) albumin was incubated in similar glucose concentrations but with a constant amount (2.0 mmol/L) of D-lysine; (c) albumin and IgG were incubated for 10 days in buffer containing glucose (10 mmol/L) and increasing concentrations of D-lysine (0.25, 0.5, 1.0, 2.0, and 4.0 mmol/L); (d) inhibition specificity was tested by treating albumin as in c but with glycerol present rather than D-lysine. In addition, we measured ketoamine after incubating albumin (50 g/L) in 10 mmol/L glucose for 10 days in the presence of D-lysine (0.25, 0.5, 1.0, and 2.0 mmol/L). The results show that (a) the amount of glucose bound to the four proteins was significantly (P < 0.05) decreased in the presence of D-lysine at the higher concentrations of glucose; (b) the lower the glucose concentration, the higher was the inhibitory effect of D-lysine; (c) the inhibition of glucose incorporation into proteins correlated directly with the concentration of D-lysine; (d) no inhibition was observed with glycerol. Ketoamine decreased with increase in D-lysine (P < 0.01). The effective diminution to non-enzymic glycation by D-lysine highlights its potential use in vivo.

Additional Keyphrases: fructosamine · handling of diabetes

Many clinical and experimental observations have highlighted the important role played by the enhanced non-enzymic glycation of structural proteins in contributing to the pathogenesis of late diabetic complications (1). The initial step of non-enzymic glycation takes the form of a nucleophilic addition reaction between a molecule of glucose in the aldehydic form—0.001% of the total number of sugar molecules (2)—and a free amino group at the protein N-terminus or at a side-chain residue, mainly L-lysine. After an Amadori type of rearrangement, which leads from the initial unstable aldimine to the formation of the more stable ketoamine (3), further spontaneous slow reactions take place in long-lived proteins and lead to the appearance of highly stable, irreversible, and very reactive advanced glycation end-products (AGE) (4). These chemical entities, formed earlier and in increased concentrations in diabetic patients, are suspected to be responsible for causing such physicochemical changes that lead to increased protein-to-protein crosslinks in collagen (5), increased lens protein precipitability (6, 7), interference with the function of the myelin sheath protein (8), and other events of pathological significance. Formation of these end-products could also explain the increased trapping of plasma proteins by purified calf-skin collagen (9) and by isolated human glomerular basement membrane (GBM) (10) that have been non-enzymically glycated in vitro.

Optimization of diabetic metabolic control obviously would decrease the negative effects of excessive non-enzymic glycation as a damaging factor, but until this can be successfully achieved, chemico-pharmaceutical means to avoid protein over-glycation in conditions of chronic hyperglycemia must be sought. Aspirin reportedly decreases the non-enzymic glycation of albumin and other proteins (11–14), probably by acetylating the terminal or side-chain lysine groups that ordinarily react with glucose (15). More recently, aminoguanidine has been shown to be capable of decreasing the formation of damaging AGE in the aortic collagen of diabetic rats (16), probably by reacting irreversibly with the early Amadori products (ketoamine)—the first stable products of non-enzymic glycation.

D-Lysine, a naturally occurring amino acid, is not incorporated into mammalian proteins, but its chemical characteristics are the same as for its isomer. In therapy, its amino groups should compete with the free protein. In this event, the use of D-lysine could effectively be an interesting pharmacological method of controlling non-enzymic glycation in diabetes.

In this study we used multiple approaches to investigate the in vitro effect of D-lysine on the extent of non-enzymic glycation of selected circulating and structural proteins.

Materials and Methods

Proteins

Two circulating proteins, human albumin and human IgG (Sigma Chemical Co.), were studied as samples of short-half-life proteins and because of their high concentration in plasma. Human collagen from Sigma and GBM isolated and purified in our laboratory and treated for the experiment as described elsewhere (10) were also used in a part of the study, representing samples of long-lived structural proteins or protein structures that are directly involved in late complications of diabetes.

Protocols

We investigated the inhibitory effect of D-lysine on the in vitro non-enzymic glycation of the above proteins by measuring:

(a) the direct incorporation of glucose after the incubation of (i) D-lysine at concentrations sufficient to maintain a constant molar ratio between the D-amino acid and the sugar, (ii) D-lysine at a constant concentration but with increasing concentrations of glucose, to vary the molar ratios between the D-amino acid and the sugar, (iii) D-lysine at increasing concentrations but with a constant glucose concentration, to study the dose-related inhibitory activity of D-amino acid, (iv) D-glycerol at increasing concentrations.
but with a constant glucose concentration, to exclude a nonspecific inhibitory effect of the \( \text{d-lysine} \) acid; and

(b) the ketoamine formation in the presence of a constant glucose concentration and increasing \( \text{d-lysine} \) concentrations.

Control glycation values were obtained by performing experiments without \( \text{d-lysine} \). In all the experiments, temperature (37 °C) and the pH of the phosphate buffer (7.3) were constant. Sodium azide (2 mmol/L) was added as an antibacterial agent.

Procedures

(a) Glucose incorporation. i) Human albumin, IgG (2 g/L), collagen, and GBM (0.5 g/L) were incubated for 20 days in phosphate buffer containing glucose at concentrations of 5, 10, and 20 mmol/L. These sugar concentrations were selected to reproduce a situation resembling good, poor, and bad metabolic control, respectively. \( \text{d-Lysine} \) (Sigma) was present at \( \frac{1}{30} \) the sugar concentration.

ii) Human albumin (2 g/L) was incubated for 20 days in phosphate buffer containing the same glucose concentrations as described above and \( \text{d-lysine} \) at a constant concentration of 2.0 mmol/L.

iii) Human albumin and IgG (2 g/L) were incubated for 10 days with glucose (10 mmol/L) in the presence of increasing concentrations (0.25, 0.5, 1.0, 2.0, and 4.0 mmol/L) of \( \text{d-lysine} \). The effect of these increasing \( \text{d-lysine} \) concentrations was also measured with human albumin at a physiological concentration (50 g/L), to compare it with the degree of inhibition of ketoamine formation.

iv) Human albumin (2 g/L) was incubated for 10 days with glucose (10 mmol/L) in the presence of increasing concentrations (0.25, 0.5, 1.0, 2.0, and 4.0 mmol/L) of \( \text{d-glycerol} \) instead of \( \text{d-lysine} \).

In all these experiments, the amount of glucose bound to the proteins was measured by adding tracer amounts of \(^{14} \text{C}-\)labeled glucose (Amersham International plc, Amersham, U.K.; specific activity 270 mCi/mol) to the sugar solutions so that the ratio of radioactive glucose to nonradioactive glucose remained constant, 0.04 mCi per mole of unlabeled sugar. At the end of the incubation period, the excess unreacted glucose, \( \text{d-lysine} \), and any glycosyl-\( \text{d-lysine} \) formed were removed by protein precipitation with a 0.6 mol/L solution of trichloroacetic acid (final concentration), followed by repeated washes of the pellet with 0.12 mol/L trichloroacetic acid. In the case of GBM, the excess solutes were removed by repeated washes of the suspension in phosphate buffer. The protein and membrane pellets were then solubilized in 0.4 mL of 0.2 mol/L NaOH dissolved in 10 mL of "Aqualuma" (Lumac b.v., Landgraaf, The Netherlands), and their radioactivities were measured in a Beckman beta-counter.

(b) Ketoamine (fructosamine) formation. Human albumin (50 g/L) was incubated for 10 days in 10 mmol/L glucose in the presence of increasing concentrations of \( \text{d-lysine} \) (0.25, 0.5, 1.0, and 2.0 mmol/L). Excess unreacted glucose, \( \text{d-lysine} \), and the glycosyl-\( \text{d-lysine} \) formed were then removed by 24-h dialysis at 4 °C against sugar-free phosphate buffer. The dialysates were then subjected to the "fructosamine" test (Roche, Basel, Switzerland).

Statistical evaluation of the results. Student's two-tailed \( t \)-test for paired data ("ST") and the linear regression coefficient \( r \) ("LR") were used in evaluating the results.

Results

Glucose Incorporation

(a) The amount of glucose (expressed as nanomoles of sugar per milligram of protein or GBM) bound to human albumin, IgG, collagen, and GBM was lessened significantly in the presence of \( \text{d-lysine} \) when the results obtained either at all glucose concentrations or at the two highest concentrations were considered (\( P < 0.05 \), in both cases; "ST" test). By contrast, the inhibition measured at the physiological glucose concentration, 5 mmol/L, did not reach statistical significance. Moreover, the inhibitory effect (i.e., the percent difference) of \( \text{d-lysine} \) increased with increasing molar concentration of both sugar and amino acid (Table 1).

(b) At a constant concentration of \( \text{d-lysine} \), 2 mmol/L, the inhibitory effect on human albumin glycation was increased at 5 mmol of glucose per liter, still slightly increased at 10 mmol/L, and unchanged at 20 mmol/L when compared with the data obtained at a constant concentration ratio of glucose to amino acid (Table 2).

(c) At a constant concentration of 10 mmol/L glucose (Figure 1), the inhibitory action of \( \text{d-lysine} \) on human albumin (A) and IgG (B) glycation was directly correlated to its concentration in the buffer (A: \( r = -0.922 \), \( P < 0.01 \); B: \( r = -0.833 \), \( P < 0.02 \), "LR" test). Figure 2 shows results obtained in the experiment where human albumin was used at a physiological concentration, 50 g/L. The results are expressed according to the ratio:

\[
\frac{\text{nmol d-glucose/mg protein} + \text{d-lysine}}{\text{nmol d-glucose/mg protein} - \text{d-lysine}}
\]

After precipitation of albumin from the initial solution with trichloroacetic acid, glycation inhibition (i.e., a ratio <1)

| Table 1. Effect of \( \text{d-Lysine} \), Present at 0.1 the Glucose Concentration, on the Glucose Incorporation by Human Albumin, IgG, Collagen, and GBM* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| \( \text{d-Glucose concn. mmol/L} \) | \( \text{Albumin} \) | \( \text{IgG} \) | \( \text{Collagen} \) | \( \text{GBM} \) | \( \text{GBM} \) |
| \( \text{d-Lysine} \) | \( \text{d-Lysine} \) | \( \text{d-Lysine} \) | \( \text{d-Lysine} \) | \( \text{d-Lysine} \) | \( \text{d-Lysine} \) | \( \text{d-Lysine} \) |
| \( \text{+d-Lysine} \) | \( \text{+d-Lysine} \) | \( \text{+d-Lysine} \) | \( \text{+d-Lysine} \) | \( \text{+d-Lysine} \) | \( \text{+d-Lysine} \) | \( \text{+d-Lysine} \) |
| \( \% \text{ diff.} \) | \( \% \text{ diff.} \) | \( \% \text{ diff.} \) | \( \% \text{ diff.} \) | \( \% \text{ diff.} \) | \( \% \text{ diff.} \) | \( \% \text{ diff.} \) |
| \( 5 \) | \( 4.45 \pm 0.65 \) | \( 1.14 \pm 0.17 \) | \( 0.24 \pm 0.05 \) | \( 0.91 \pm 0.06 \) | \( 0.91 \pm 0.06 \) | \( 0.91 \pm 0.06 \) |
| \( 10 \) | \( 8.37 \pm 0.99 \) | \( 2.30 \pm 0.41 \) | \( 0.41 \pm 0.02 \) | \( 1.71 \pm 0.09 \) | \( 1.71 \pm 0.09 \) | \( 1.71 \pm 0.09 \) |
| \( 20 \) | \( 17.46 \pm 1.84 \) | \( 3.92 \pm 0.46 \) | \( 0.94 \pm 0.08 \) | \( 3.43 \pm 0.06 \) | \( 3.43 \pm 0.06 \) | \( 3.43 \pm 0.06 \) |

*Values given are nmol d-glucose/mg protein ± SD (n = 3).
was observed only in the presence of 0.25 mmol/L D-lysine (Figure 2a). The dilution of the albumin solution 1:10 (Figure 2b) and 1:20 (Figure 2c) with distilled water before precipitation with trichloroacetic acid also revealed a D-lysine inhibitory effect at 0.5 mmol/L, while at the same time reducing the ratio measured at the higher amino acid concentrations.

(d) No inhibitory effect on albumin glycation was observed when glycerol was substituted for D-lysine (Figure 1C; r = 0.44, not significant by the "LS" test).

Ketoamine (Fructosamine) Formation

The results of the Roche "fructosamine" test showed a decline from 2.41 mmol of fructosamine per liter without D-lysine to 1.60 mmol/L in the presence of 2.0 mmol/L D-lysine. As expected, the ratio between the amount of ketoamine formed with and without D-lysine decreased proportionately with increasing concentrations of the amino acid (r = −0.945, P < 0.001, "LR" test) (Figure 2d).

Discussion

Currently, pharmacological or chemical intervention, in addition to attempts to achieve and maintain a good metabolic control, may be of the greatest help in controlling the extent of non-enzymic glycation of protein in diabetes mellitus.

Two inhibitory agents have been proposed to date: aspirin and aminoguanidine. However, with regard to a possible therapeutic use of these compounds, it must be stressed that it is not yet sufficiently clear what would be the effect of (perhaps) irreversibly bound foreign chemical groups in place of glucose in very long-lived protein structures. Thus, we could speculate that such groups might be chemically more reactive than the stable glucose-protein adducts, the formation of which they have helped to prevent. A second important point to consider is that it would be necessary to administer the inhibiting compounds as soon as possible after the diagnosis of diabetes and regularly thereafter during the patient's lifetime. This is necessary because it has been suggested that, once the process of excessive non-enzymic glycation has begun, subsequent correction of hyperglycemia would not prevent its far-reaching and damaging effects (I). Important characteristics of an ideal inhibitor of glycation must therefore be easy absorption and excretion, low or no toxicity, and absence of serious side effects. Chronic therapy with aspirin would be contraindicated, owing to its effects on the gastric mucosa. Aminoguanidine seems to have little or no toxicity (16), but this needs to be investigated further.

D-Lysine is one of the D-amino acids that occur naturally in bacterial products such as antibiotics, and in bacterial cell and spore walls. Although not incorporated into mammalian proteins, it has the same chemical characteristics as its more "physiological" L-isomer. It follows that the non-enzymic reaction between the aldehydic form of glucose and the free, N-terminus or side chain amino groups (such as

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**Table 2. Comparison of the Effect of D-Lysine, Present at 0.1 the Glucose Concentration (A) or at a Constant 2.0 mmol/L Concentration (B), on the Nonenzymic Glycation of Human Albumin**

<table>
<thead>
<tr>
<th>D-Glucose concn, mmol/L</th>
<th>(A)</th>
<th>(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.45 ± 0.65</td>
<td>4.50 ± 0.53</td>
</tr>
<tr>
<td>10</td>
<td>8.37 ± 0.99</td>
<td>6.60 ± 0.52</td>
</tr>
<tr>
<td>20</td>
<td>17.46 ± 1.84</td>
<td>13.04 ± 0.69</td>
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</tbody>
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Results are given as nmol glucose/mg albumin ± SD. Experiments A and B were performed at different times (n = 3).

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**Fig. 1.** Effect of increasing concentrations of D-lysine on the binding of glucose to human albumin (A) and IgG (B), and of increasing concentrations of D-glycerol on the binding of glucose to human albumin (C). Results expressed in nanomoles of glucose per milligram of protein.

**Fig. 2.** Effect of increasing concentrations of D-lysine on the binding of glucose and ketoamine formation on human albumin at a physiological concentration (50 g/L).

Results expressed as (nmol D-glucose/mg albumin + D-lysine per mmol D-glucose/mg albumin − D-lysine) after (a) precipitation of the initial albumin solution with trichloroacetic acid; (b) precipitation with trichloroacetic acid after 1:10 dilution was distilled water; (c) precipitation with trichloroacetic acid after 1:20 dilution with distilled water, and as [nmol/L fructosamine + D-lysine per mmol/L trichloroacetic acid − D-lysine] after (d) 24-h dialysis at 4 °C vs sugar-free buffer.
those belonging to L-lysine) could, in theory, be controlled by D-lysine. By reacting directly with glucose in the plasma, the D-amino acid could impede the sugar from binding to the free amino groups of circulating and structural proteins.

The results obtained in this in vitro study show that, under various experimental conditions, D-lysine has a significant inhibitory activity. Thus the amount of bound glucose detected on plasma proteins (such as human albumin and IgG), structural proteins such as human collagen, or structures such as GBM was significantly decreased when incubated together with the D-amino acid. Furthermore, the higher the glucose (and, consequently, the D-lysine) concentration, the greater was the corresponding inhibitory effect of D-lysine. At a constant protein concentration, this finding can be easily explained in terms of proportionately larger numbers of reacting glucose and D-lysine molecules per unit volume. Similarly, the presence of a proportionately greater number of D-lysine molecules per unit volume can explain why, at increasing concentrations of glucose vs a standard concentration of the D-amino acid, the lower the concentration of the former, the higher was the inhibitory effect of the latter.

In addition, the inhibitory effect of D-lysine was linearly correlated with its concentration in the medium, and its action appeared to be specific, because glycerol—a compound with no free amino groups or carbonyls to react with glucose or proteins, respectively—had no equivalent inhibitory activity.

The glucose bound in the presence of a physiological concentration of human albumin was also decreased by the D-amino acid. However, where a higher concentration of the latter was used, its inhibitory effect was not observed, probably because it was masked by the large mass of the precipitating albumin trapping some of the labeled products formed between glucose and D-lysine in solution.

The inhibitory efficiency of D-lysine was further confirmed by the measurement of the amount of ketoamine (fructosamine) formed during the glycation reaction. Thus, in the presence of the amino acid, ketoamine concentrations declined linearly as the D-lysine concentration increased.

In conclusion, this study has shown that, in vitro, D-lysine can specifically inhibit the non-enzymatic attachment of glucose to soluble and structural proteins. In vivo studies in normal animals and in animals with artificially induced diabetes are now required. This will help to define the physiological, pharmacological, chemical, and toxicity indices that might allow D-lysine to be used safely and efficiently in humans in an attempt to minimize the non-enzyme glycation of proteins, the hallmark of diabetic hyperglycemia.

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References