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Original article

In vitro glycation of brain aminophospholipids by acetoacetate and its inhibition by urea

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Abstract

Amino groups of amino acids, nucleic acids and lipids can react non-enzymatically with reducing sugars to form unstable Schiff bases that can then undergo the Amadori rearrangement to form irreversible advanced glycation end products (AGEs). Ketoacidosis is a life-threatening complication in patients with untreated diabetes mellitus and it is characterized by increased circulating ketone body concentrations.

Recently, the *in vitro* glycation of hemoglobin by β-hydroxybutyrate and acetone was described by our laboratory.

This study was designed to evaluate the *in vitro* effect of acetoacetate on brain aminophospholipids at similar concentrations to that observed in ketoacidosis (16.13 mM total ketone bodies). The effect of acetoacetate was compared to that of glucose and the other ketone bodies; β-hydroxybutyrate and acetone. The antiglycating activity of urea and glycylglycine was also investigated.

The incubation of aminophospholipids with acetoacetate results in the formation of a new compound with an absorption peak at 280 nm. When this reaction product was analyzed by thin layer chromatography using an elusion system of methanol:chloroform:acetic acid:water (8:1:1:0.4), the R_f value obtained (0.24–0.26) was similar to that of the compound formed by aminophospholipids with glucose. In contrast, this reaction product was not detected in those samples containing β-hydroxybutyrate and acetone. The formation of this new compound was inhibited by urea more effectively than glycylglycine.

In conclusion, this study provides the evidence that brain aminophospholipids react with acetoacetate forming AGEs and that this glycating effect of acetoacetate was remarkably decreased by urea, suggesting a protective physiological role for urea in the body as it was previously stated.

Finally, this information adds knowledge about the contribution of ketoacidosis in the pathophysiology of diabetic complications, especially in type 1 diabetic patients.

Keywords: Advanced glycation end products; Ketoacidosis; Ketone bodies; Urea; Aminophospholipids; Glycylglycine

1. Introduction

The process of nonenzymatic reaction of glucose with proteins results in the formation of highly reactive intermediate and terminal adducts called advanced glycation end products (AGEs) [1].

The aldehyde group or keto groups of reducing sugars are able to react with amino groups of amino acids, nucleic acids and lipids such as phosphatidylethanolamine to form Schiff bases, which can then rearrange to the more stable Amadori-type early glycation end products [2–5]. The AGE levels increase markedly in diabetic patients as a result of sustained hyperglycemia [5–7] leading to tissue damage through a variety of mechanisms, including alteration of tissue structure and function [2,5], stimulation of cellular responses through AGE-specific receptors [8–10] or the generation of reactive oxygen intermediates [11,12].

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Ketoacidosis is the most common acute complication of type 1 diabetes mellitus, however, patients with type 2 diabetes are also at risk during the catabolic stress of acute illness [6]. This condition is characterized by hyperglycemia, metabolic acidosis, and high levels of ketone bodies. This last symptom is due to the lack of insulin or insulin resistance with concomitant elevation of glucagon, catecholamines, cortisol, and growth hormone [13,14].

Because ketone bodies are carbonyl compounds and they are increased in uncontrolled diabetic patients, especially in type 1 diabetics, it makes sense to consider that they can contribute to the AGEs formation.

Recently, we reported the *in vitro* glycation of hemoglobin by β-hydroxybutyrate and acetone [15]. On the other hand, AGE inhibitors are substances containing guanidinium or amino groups that can act as nucleophilic agents by trapping reactive carbonyl intermediates in AGEs' formation. Some of these AGE inhibitors are aminoguanidine [16], pyridoxamine [17], L-arginine [18], polyamines [19], and recently urea has been suggested as a possible natural protector of advanced glycation end-products' formation [20].

This study was designed to evaluate the *in vitro* effect of acetoacetate on brain aminophospholipids at similar concentrations to that observed in ketoacidosis (16.13 mM total ketone bodies). The effect of acetoacetate was compared to that of glucose and the other ketone bodies; β-hydroxybutyrate and acetone. The antiglycating activity of urea and glycylglycine was also investigated.

2. Materials and methods

D-(+)-glucose, urea, β-hydroxybutyrate, ninhydrin and lithium acetoacetate were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Acetone, sodium phosphate salts, chloroform, sulfuric ether, sulfuric acid, sodium molybdate and hydrazine sulfate were purchased from J.T. Baker S.A. de C.V. (Xalostoc, Mexico). Silica gel plates were purchased from Merck (Cat. 5721 DC-Fertigplatten Kieselgel 60, E. Merck, Darmstadt, Germany). Glycylglycine was purchased from Nutritional Biochemicals Corporation (Cleveland, OH, USA).

2.1. Lipid extraction

Aminophospholipids were extracted from bovine brain white matter by two-step selective method. First, cholesterol was removed from the sample with acetone and then the aminophospholipids were extracted with sulfuric ether [21]. The solvent was evaporated and the extracted fraction was placed in covered vials and refrigerated.

The presence of aminophospholipids was verified by thin layer chromatography using a mixture of chloroform:methanol:acetic acid:water (65:25:8:2) [21]. The plates were revealed with 0.2% ninhydrin solution prepared in acetone.

2.2. Preparation of solutions

The following solutions were prepared: 0.26 g/L and 0.53 g/L β-hydroxybutyrate; 0.13 g/L lithium acetoacetate; 1.2 g/L and 2.4 g/L urea.

2.3. Sample incubation

As shown in Table 1, same amount of aminophospholipids (70 mg) were incubated with glucose (0.08 mM); acetoacetate (5.3×10^{-3} mM); β-hydroxybutyrate (0.02 mM) and acetone (5.2×10^{-4} mM) in 8 ml of 0.1 M phosphate buffer solution (pH = 7.4). Also the three ketone bodies were incubated in the presence of glycylglycine (0.16 mM) and urea

Table 1
Concentration of substrate added to each vial under the same experimental conditions[a]

Vial	Aminophospholipids (mg)	Ketone bodies (mM)[b]		Glucose (mM)[b]	Antiglycating agent (mM)	
1	70		–	–		–
2	70		–	0.08		–
3	70	β-hydroxybutyrate	0.02	–		–
4	70	Acetone	5.2×10^{-4}	–		–
5	70	Acetoacetate	5.3×10^{-3}	–		–
6	70	β-hydroxybutyrate	0.02	–	Gly-gly	0.16
7	70	Acetone	5.2×10^{-4}	–	Gly-gly	0.16
8	70	Acetoacetate	5.3×10^{-3}	–	Gly-gly	0.16
9	70	β-hydroxybutyrate	0.02	–	Urea	0.16
10	70	Acetone	5.2×10^{-4}	–	Urea	0.16
11	70	Acetoacetate	5.3×10^{-3}	–	Urea	0.16
12	70	β-hydroxybutyrate	0.02	0.08		–
13	70	Acetone	5.2×10^{-4}	0.08		–
14	70	Acetoacetate	5.3×10^{-3}	0.08		–
		β-hydroxybutyrate	0.02			
		Acetone	5.2×10^{-4}			
15	70	Acetoacetate	5.3×10^{-3}	–		–
		β-hydroxybutyrate	0.02			
		Acetone	5.2×10^{-4}			
16	70	Acetoacetate	5.3×10^{-3}	0.08		–

[a] pH = 7.4; T = 37 °C (light protected).
[b] Physiological equivalent concentrations [22]: glucose 55 mM; β-hydroxybutyrate 12.5 mM; acetoacetate 3.31 mM; acetone 0.32 mM.