Inhibitory Effect of Some Acetyl Esters and Acetamides on Glycation of the Histone H1

Georgi Stoynev[a,*], Ljuba Srebreva[a], Roumyana Gugova[b], Evgeny Golovinsky[b], and Ivan Ivanov[a]

[a] Department of Gene Regulations, Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria. Fax: +3592820158 6. E-mail: stoynev@obzor.bio21.bas.bg
[b] Department of Molecular Design and Biochemical Pharmacology, Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

* Author for correspondence and reprint requests

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Non-enzymatic glycosylation (glycation) is a spontaneous set of reactions between reducing sugars and free amino groups in proteins or other biomolecules leading to the formation of fluorescent and coloured compounds known as advanced glycation end products (AGEs). AGEs cause structural changes of key proteins in humans, and therefore they are related with a number of physiological processes and diseases such as aging, atherosclerosis, cataract, arthritis, Alzheimer's disease. Two main strategies have been employed to prevent the formation of AGEs: a) low carbohydrate diet and b) pharmacological intervention. The latter includes treatment with reactive compounds which might be either sugar competitors (type A), carbonyl traps (type B) or free radical trapping antioxidants (type C). Acetylsalicylic acid (ASA, aspirin) is a good example of sugar competitor capable of inhibiting glycation by acetylating ε-amino groups of lysine residues in proteins. Taking into consideration the inhibiting effect of ASA on glycation we designed to study the antiglycation activity of other acetyl group-containing compounds (acetamides and acetyl esters) using the lysine-rich protein histone H1 as a model. The glycation of the histone H1 was carried out by either fructose or a complex mixture of glycating agents obtained from *E. coli* and monitored by fluorescent spectroscopy, SDS-PAGE and measurement of the content of reactive carbonyl groups in the target protein. Our results showed that the inhibitory effect of phenyl acetate, acetanilide, 4-acetamidophenylacetic acid and isopropenyl acetate was comparable to that of ASA. Based on the obtained results we conclude that these compounds act as free radical scavengers protecting proteins from the damaging effect of reactive oxygen species produced during the formation of AGEs.

Key words: Glycation End Products, Glycation Inhibitors, Histone H1

Introduction

Non-enzymatic glycosylation (glycation) is a spontaneous and complex set of reactions between reducing sugars and free amino groups in proteins or other biomolecules leading to the formation of coloured, fluorescent and cross-linked compounds collectively called advanced glycation end products (AGEs) (Culbertson *et al.*, 2003). AGEs are mostly studied on long-lived proteins in living organisms including man (Nawroth *et al.*, 1999). Now it is known that glycation occurs also in prokaryotes. Recently, Mironova *et al.* (2001, 2003) have shown unequivocally that glycation affects both bacterial host and recombinant proteins in *Escherichia coli*.

In higher organisms AGEs cause pathological changes via the following general mechanisms: 1) AGEs alter signal transduction pathways interfering with normal matrix-matrix, matrix-cell and cell-cell interactions; 2) AGEs interfere with gene expression of soluble signals such as cytokines and hormones by interacting with AGE-specific cellular receptors; 3) AGEs bound to the endothelial AGE receptors mediate the generation of oxygen free radicals; 4) intracellular AGEs formed by glucose, fructose and various other glycating agents (intermediates of the carbohydrate metabolism) alter physicochemical properties of many intra- and extracellular proteins and even of the DNA (Brownlee, 1995). Due to this glycation is related to a number of diseases such as diabetes mellitus (Brownlee, 1995; Frye *et al.*, 1998), Alzheimer's disease (Munch *et al.*, 2002), atherosclerosis (Stitt *et al.*, 1997; Nagai *et al.*, 2002).

Two main strategies have been employed to counteract the detrimental effect of AGEs: a) low-

AGE diet and b) pharmacological interventions. The latter tends to inhibit the formation of AGEs or to destroy the AGEs-related crosslinks (Vlassara and Palace, 2003).

Based on the mode of action AGE inhibitors are classified (Khalifah *et al.*, 1999) as: 1) Carbonyl traps bearing nucleophilic function and reacting with aldoses and ketoses (inhibitors type B) or with reactive dicarbonyl intermediates (type D); 2) metal ion chelators (type C1); and 3) free radical trapping antioxidants which exhibit their activity by limiting the oxidative acceleration of glycation (type C2). Other types of AGE inhibitors (type A) act as sugar competitors by acylation of the reactive amino groups on proteins. Acetylsalicylic acid (ASA, aspirin) is the first example of sugar competitor capable of inhibiting the *in vitro* glycation of albumin by acetylating the ε-amino groups of lysine residues (Malik and Meek, 1994).

Using [^{14}C]-acetylsalicylic acid Rao and Cotlier (1988) proved that the inhibition of glycation of human and rat eye crystallins was due to the acetylation of their side chain amino groups. It was also shown that ASA blocks by acetylation the glycation of hemoglobin (Rendell *et al.*, 1986), collagen (Malik and Meek, 1996), bovine crystallins (Ajiboye and Harding, 1989), etc. Recently we have reported that ASA was very effective in inhibiting the glycation of the histone H1 by a mixture of glycated agents isolated from *E. coli* (Stoynev *et al.*, 2004). Taking into consideration these data it is tempting to assume that other acetylated organic compounds could also act as inhibitors of glycation. In this context the present work aims to study the ability of a series of acetylated aromatic and aliphatic compounds to inhibit the glycation of the highly basic protein histone H1 used as a model.

Materials and Methods

Isolation of the histone H1

Histone H1 was extracted from rat liver by direct homogenization of the tissue in 5% perchloric acid. After centrifugation at $1500 \times g$ for 10 min, the protein was precipitated by trichloroacetic acid (20% final content) for 1 h at 4 °C. The precipitate was washed twice with cold ethanol/diethyl ether, dried under vacuum and stored at −20 °C.

Animals

Procedures involving animals and their care were conducted in conformity with institutional guidelines in compliance with international laws and policies (approved by the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria).

Preparation of deproteinated bacterial lysates

LB medium (1 l) was inoculated with *E. coli* AB1157 cells and incubated for 16 h at 37 °C. Bacteria were harvested by centrifugation, resuspended in 200 ml TS buffer [10 mM tris(hydroxymethyl)aminomethane-HCl, pH 8.0, 0.15 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and disrupted by ultrasonication. The lysates were clarified by centrifugation at $3000 \times g$ for 20 min at 4 °C and the proteins were removed by bentonite (a native colloidal hydrated aluminium silicate). To this end the latter was added to 5% final content, lysates were stirred at room temperature for 1 h, centrifuged at $3000 \times g$ for 15 min and the clear supernatants were used in all further experiments.

Glycation of the histone H1 with bacterial lysates and fructose

Histone H1 dissolved in TS buffer (1 mg/ml) was placed in benzoylated dialysis tubings (2 kDa cut off) and dialyzed overnight at 37 °C against protein-free bacterial lysates.

In the case of fructose-induced glycation protein samples were incubated in the presence of 300 mM fructose, 1 mM PMSF and 1 mM NaN_3 at 37 °C for 30 d. This concentration was chosen based on the information derived from other *in vitro* experiments. The protein samples were then dialyzed against a vast excess of TS buffer for 24 h at 4 °C and stored at 4 °C.

Accumulation of AGEs in the histone H1 during storage was monitored by fluorescence measurements on a Shimadzu RF-5000 spectrophotometer at λ_{ex} = 365 nm and λ_{em} = 443 nm. The samples were also analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels according to Laemmli (1970).

Determination of the carbonyl content in the histone H1

Reactive carbonyl groups in the histone H1 were determined as described by Reznick and Packer (1994) with some modifications. Treated protein samples were analyzed 12 d after storage at 4 °C. To 1 ml of the protein solution (3 mg/ml)

4 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl were added, and the samples were incubated for 1 h at room temperature. Trichloroacetic acid (TCA) was added to a final content of 20%, and the samples were stored overnight at 4 °C. The pellets were collected by centrifugation, washed consecutively with 1 ml 20% TCA, 3 times with 1 ml ethanol/ethyl acetate (1:1), air-dried and dissolved in 0.5 ml 6 M guanidine hydrochloride. The absorbance was measured at 360 nm and the content of carbonyl groups was expressed as nmols per mg of protein using the absorption coefficient ε = 22 000/M.

Inhibition of glycation

To study the inhibitory effect of acetylated compounds on glycation, clear bacterial lysates were supplemented with 20 mM inhibitor (including ASA as a control) and incubated as above.

Inhibitors used

The investigated substances phenyl acetate, acetanilide, 4-acetamidophenylacetic acid, methyl acetate, isopropenyl acetate and acetylsalicylic acid were chromatographically pure commercial products or chemicals prepared according to literature protocols (Kaufmann and Luterbacher, 1909; Lipton *et al.*, 1979; Robertson, 1932; Ferber and Bendix, 1939).

Results

Previous studies have shown firstly that, besides in eukaryotes, glycation takes place also in bacteria (Mironova *et al.*, 2001, 2003), and secondly, that the histone H1 (due to its high lysine content) is a suitable substrate for the detection of glycating agents in bacterial lysates (Stoynev *et al.*, 2004). To avoid degradation of the histone H1 by hydrolytic enzymes from the lysates, two precautions were adopted in our experiments: (i) Bacterial proteins were removed from the *E. coli* lysates by bentonite, and (ii) the target protein was exposed to glycation by placing the histone H1 in dialysis bags. Besides bacterial lysates, in some experiments fructose was also used as an alternative glycating agent.

In this study four aromatic (ASA, phenyl acetate, acetanilide and 4-acetamidophenylacetic acid) and two aliphatic (methyl acetate and isopropenyl acetate) acetylated compounds were tested for their ability to inhibit glycation *in vitro* (see Fig. 1).

Taking into consideration that glycation is a slow running process, protein samples were incubated in the lysate. Then they were stored for 30 days at 4 °C (see Materials and Methods) and the accumulation of AGEs was monitored by AGE-specific fluorescence (443 nm, after excitation at 365 nm). The results presented in Fig. 2A show

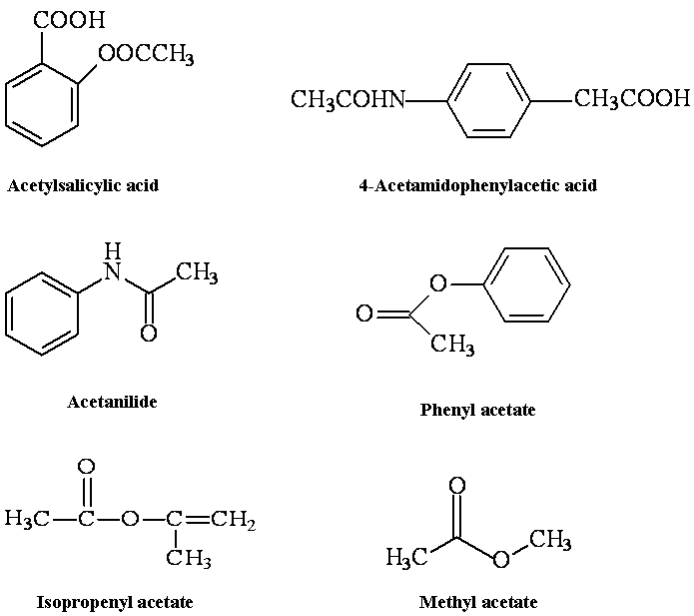

Fig. 1. Chemical structure of the inhibitors.

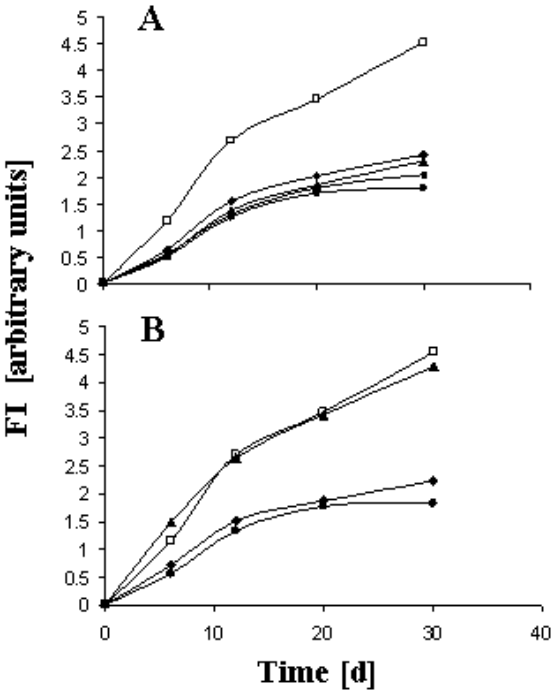

Fig. 2 Inhibition of AGEs production in the histone H1 after incubation with *E. coli* lysate. (A) In the presence of aromatic compounds: 4-acetamidophenylacetic acid (⧫); acetanilide (▲); phenyl acetate (■); acetylsalicylic acid (●); control sample, histone H1 incubated without inhibitor (□). (B) In the presence of aliphatic compounds: methyl acetate (▲); isopropenyl acetate (⧫); acetylsalicylic acid (●); control sample, histone H1 incubated without inhibitor (□). All inhibitors tested were used at 20 mM. AGE-specific fluorescence was measured at λ_{ex} = 365 nm and λ_{em} = 443 nm. Mean values were calculated from three independent experiments.

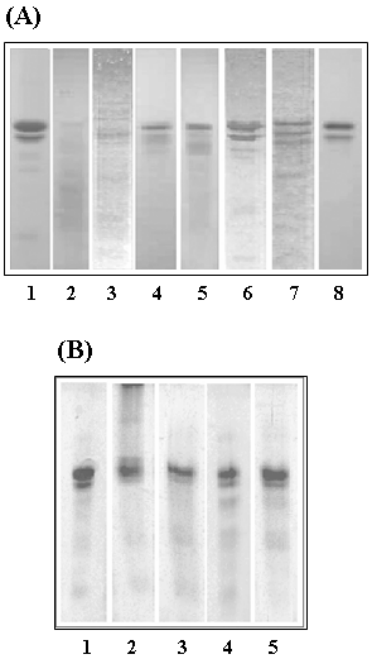

Fig. 3. SDS-PAGE analysis of the histone H1 in the presence of the inhibitors. Histone H1 was treated with (A) *E. coli* lysate or (B) fructose as described in Materials and Methods and stored at 4 °C. (A) Samples analyzed 6 days after incubation: 1, negative control sample (histone H1 incubated in TS buffer); 2, positive control sample (histone H1 treated with bacterial lysate); histone H1 in the presence of the inhibitors: 3, methyl acetate; 4, isopropenyl acetate; 5, 4-acetamidophenylacetic acid; 6, acetanilide; 7, phenyl acetate; 8, acetylsalicylic acid. (B) Samples analyzed 30 days after incubation: 1, negative control sample (histone H1 incubated in TS buffer); 2, positive control sample (histone H1 treated with fructose); histone H1 in the presence of the inhibitors: 3, 4-acetamidophenylacetic acid; 4, isopropenyl acetate; 5, acetylsalicylic acid. Each lane contained 20 μg protein stained with Coomassie Brilliant Blue.

that the content of AGEs in the histone H1 gradually increased on incubation and that all aromatic compounds led to a considerable decrease in the glycation-related fluorescence. Similar results were obtained also with one of the aliphatic compounds (isopropenyl acetate) whereas no such effect was observed with the other one (methyl acetate) (Fig. 2B).

In parallel experiments histone H1 was glycated with fructose at 300 mM to study the anti-AGE effect of isopropenyl acetate and 4-acetamidophenylacetic acid (representing the aliphatic and aromatic acetylated compounds, respectively) in an alternative glycating system. The obtained results demonstrated that the formation of fluorescent AGEs in the latter case was inhibited to an equal extent by both substances (data not shown).

To investigate the ability of all tested compounds to prevent structural changes (fragmentation and crosslinking) in the histone H1, protein samples were analyzed in parallel by PAGE. Fig. 3A, lane 2 shows that 6 days after treatment with bacterial lysates the histone H1 underwent extensive degradation and/or crosslinking, whereas the control (untreated sample) remained stable (lane 1). This figure shows also that isopropenyl acetate, 4-acetamidophenylacetic acid, acetanilide and phenyl acetate (lanes 4 –7) but not methyl acetate (lane 3) had a protective effect on the target (histone H1).

Unlike bacterial lysates, fructose led to a remarkable change in the electrophoretic mobility

of the histone H1 and generated high-molecular weight aggregates remaining on the start point of the separating gel (Fig. 3B, lane 2). When 4-acetamidophenylacetic acid and isopropenyl acetate were used as inhibitors, the protein polymerization reaction was completely suppressed (Fig. 3B, lanes 3 and 4). Judging by the electrophoretic pattern (heterogeneity) and mobility of the histone H1, we concluded that in both glycating systems (bacterial lysates and fructose) ASA was the most potent inhibitor of glycation (see Fig. 3A, lane 8 and Fig. 3B, lane 5).

To assess the antioxidant activity of isopropenyl acetate and 4-acetamidophenylacetic acid we evaluated their effect on the basis of accumulation of carbonyl compounds in the histone H1 during incubation in either bacterial lysates or fructose solutions. Our results demonstrated that the exposure of the histone H1 to bacterial lysates resulted in a remarkable increase in the content of carbonyl compounds in the protein (approaching 13 nmol/mg) as compared to the level of such compounds in the control sample (3.6 nmol/mg) (Fig. 4A, lanes 1 and 5). The accumulation of carbonyl compounds in the histone H1 was drastically inhibited by ASA (lane 2) and to a lesser extent by isopropenyl acetate and 4-acetamidophenylacetic acid (lane 3 and lane 4). Fig. 4B, lane 1 shows also that fructose is less active as glycating agent in comparison with the bacterial lysates since the content of carbonyl groups in the histone H1 was 9.3 nmol/mg protein. The presence of the two tested compounds in the incubation mixture lead to diminution of these groups (lanes 3 and 4).

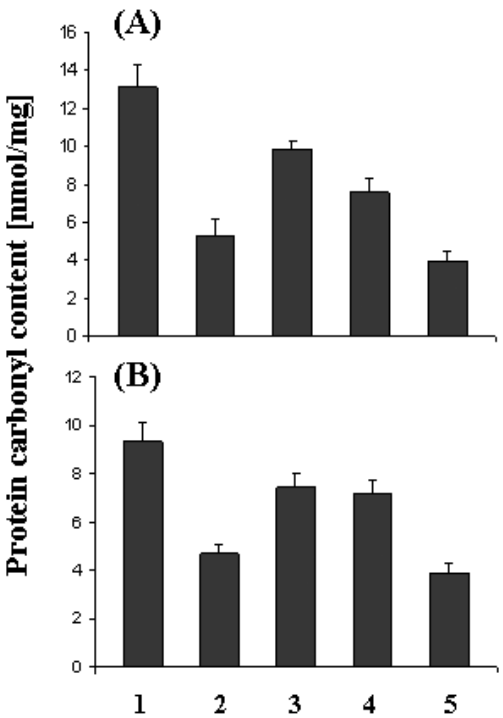

Fig. 4. Inhibition of increase of protein carbonyl content of the histone H1 after glycation with (A) bacterial lysate and (B) fructose; 1, positive control; histone H1 in the presence of: 2, acetylsalicylic acid; 3, 4-acetamidophenylacetic acid; 4, isopropenyl acetate; 5, negative control (untreated protein). The concentration of the inhibitors was 20 mM. Each column represents the mean ± SD of three replicate experiments.

Discussion

Both *in vitro* and *in vivo* experiments have shown that the protecting effect of ASA against glycation is due to a non-enzymatic transacetylation affecting the reactive amino groups in proteins (Rao and Cotlier, 1988; Crompton *et al.*, 1985; Rao *et al.*, 1985). Rendell *et al.* (1986) studied the inhibitory effect of ASA on albumin glycation and concluded that the sites of glycation coincide with those of transacetylation. To confirm transacetylation Rao and Cotlier (1988) used ^{14}C-labelled ASA to treat human lens crystallins *in vitro* and undoubtedly showed that the ^{14}C-radioactive atom is covalently bound to the proteins. In similar experiments Cherian and Abraham (1993) have found that the ^{14}C-radioactive atoms of ASA are preferentially (four times more) incorporated into the rat gamma-crystallin molecules compared to the alpha- and beta-crystallins. On the other hand, Yue *et al.* (1984, 1985) reported that ASA had no protective effect on glycation of rat tail tendon collagen. Based on their observation that crosslinking of the protein was inhibited, the authors suggested that ASA might not be acting by the mechanism of transacetylation. Furthermore, Fu *et al.* (1994) postulated an alternative mechanism for the protection by ASA assuming a free radical scavenging action.

The results in the present paper clearly show that phenyl acetate, acetanilide, 4-acetamidophenylacetic acid and isopropenyl acetate are capable of protecting the histone H1 from glycation and therefore from degradation and crosslinking caused by both bacterial lysates and fructose (Figs. 2 and 3). Taking into consideration that their effect

is similar to that of ASA, we are tempted to assume that the inhibitory effect of all these (acetyl group-containing) compounds is due to transacetylation, *i. e.* to acetylation of the side chain amino groups in proteins, which are also potential targets of glycation.

The reason to use fructose in some of our experiments in parallel with bacterial lysates was due to the fact that this sugar (used as a reference) is 10-fold more reactive than glucose (McPherson *et al.*, 1988) and also because it causes crosslinking of proteins by forming 3-deoxyglucosone (Shin *et al.*, 1988). The fact that isopropenyl acetate and 4-acetamidophenylacetic acid prevent structural changes, formation of AGE-related crosslinks (Fig. 3B) and accumulation of carbonyl groups (Fig. 4) in the histone H1 is a reason to assume that these compounds might act as inhibitors of formation of oxygen reactive species or neutralizers of their damaging effect.

Antioxidant and/or antiradical activities have been attributed to many phenol derivatives. Thus, acetaminophen, salicylic acid and 5-ASA may act as free radical scavengers because of the presence of phenol moieties in their molecules (Dinis *et al.*, 1994). Therefore the functionality as a free radical scavenger should not be excluded for phenyl acetate, acetanilide and 4-acetamidophenylacetic acid. On the other hand, there is also evidence that acetamides do not affect the activity of cell enzymes like histone acetyltransferase and histone deacetylase which have a crucial role in the regulation of transcription and replication (Kring and Böger, 1994).

Based on the results obtained in this paper we are tempted to classify the group of compounds studied above as inhibitors of both AGE-related fluorescence and crosslinking in proteins. Their activity is comparable to that of ASA and they could find application as alternative (to ASA) future drugs for preventing protein glycation and related pathological complications.

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