Kinetin Inhibits Protein Oxidation and Glycoxidation *in Vitro*

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We tested the ability of N^6-furfuryladenine (kinetin) to protect against oxidative and glycoxidative protein damage generated *in vitro* by sugars and by an iron/ascorbate system. At 50 μM, kinetin was more efficient (82% inhibition) than adenine (49% inhibition) to inhibit the bovine serum albumin (BSA)-pentosidine formation in slow and fast glycation/glycoxidation models. Kinetin also inhibited the formation of BSA-carbonyls after oxidation significantly more than adenine did. However both compounds inhibited the advanced glycation end product (AGE) formation to the same extent (59–68% inhibition). At 200 μM, kinetin but not adenine, limited the aggregation of BSA during glycation. These data suggest that kinetin is a strong inhibitor of oxidative and glycoxidative protein-damage generated *in vitro.* © 2000 Academic Press

Key Words: adenine; glycoxidation; protein-oxidation; antioxidant; ageing; ROS.

N^6-furfuryladenine (kinetin) is a cytokinin and a secondary oxidation product of adenine (1). It has been shown to have various biological properties, such as stimulation of transcription, cell cycle control, calcium flux, and antioxidant activities (1). Kinetin also exerts anti-ageing effects in different systems including plants, fruitflies, and cultured human skin fibroblasts (1–4). Although the exact mechanisms of action of kinetin are not fully known, it has been suggested to act both as an inhibitor of the radical oxygen species (ROS) formation and as a scavenger of ROS (1). It prevents the oxidation of unsaturated fatty acids (5) and inhibits the *in vitro* formation of 8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA (6). Considering the anti-oxidative effects of kinetin on fatty acids and DNA, we examined whether kinetin also protects against protein oxidation and glycoxidation.

Oxidized proteins are important factors in the ageing process as glycation/glycoxidation products accumulate in cells and tissues during ageing (7–9). Glycation results from the linking of sugars or of intermediate metabolic products to free amino groups of amino acids or nucleotides whereas glycoxidation is due to a sequential glycation and oxidation reaction (7, 10, 11). The extracellular glycation/glycoxidation process is slow because it depends mainly on the glucose concentration, which is the least reactive sugar. However, the intracellular process is much faster because it is due to an increase of the cytosolic concentration of more reactive glycation agents such as pentoses (i.e., ribose, arabinose) (12) and α-oxoaldehydes (i.e., glyoxal, 3-deoxyglucosone) (13). The end-result of this post-translational protein modification process is the formation of the so-called advanced glycation/glycoxidation end products (AGE) such as pentosidine (7). In this study, we report that kinetin inhibits oxidation and glycation/glycoxidation of proteins as well as preventing structural changes induced by glucose, ribose, arabinose, and glyoxal.

MATERIALS AND METHODS

Chemicals. Kinetin (N^6-furfuryladenine) was kindly supplied by Senetek PLC (CA). Adenine was purchased from Serva (Heidelberg, Germany). If not specified otherwise, all other chemicals were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) and were of highest analytical grade available.

Glycation of bovine serum albumin in vitro. Fatty-acid-free Bovine Serum Albumin (BSA; 12 mg) was incubated under air with either glucose (300 mM) or glyoxal (5 mM), arabinose (100 mM), and ribose (50 mM), in 4 ml of PBS (pH 7.4) containing protease inhibitors (aprotinin 5 μg/ml; leupeptin 5 μg/ml; antipain 5 μg/ml; PMSF 100 μg/ml). Incubations were performed in capped test tubes at 37°C in the dark for 0, 2, 7, and 30 days in the absence or presence of kinetin or adenine at different final concentrations (50–200 μM). Each experiment was repeated three times with sampling in triplicate each time. At the end of the incubation, samples were dialyzed twice for 24 h against PBS (pH 7.4). Protein determination was performed by the Lowry method (Bio-Rad, Richmond, CA) and the samples were stored at -20°C until analysis.

Oxidation of BSA in vitro. Fatty-acid-free BSA (3 mg) was incubated under air with fresh stock mixture of 25 mM ascorbic acid/100 μM FeCl$_3$ in 1 ml of a buffer containing 25 mM Hepes, 100 mM KCl, 10 mM MgCl$_2$, pH 7.2. Incubations were performed in a water bath at 37°C for 2, 10, 30, 60, 120, and 300 min in the absence or presence of kinetin or adenine, added at different final concentrations (50–200 μM) prior to the addition of ascorbic acid and FeCl$_3$. Incubation was stopped by adding 2 mM of diethylenetriamine-pentaacetic acid (DTPA) on ice. Proteins were immediately precipitated by cold TCA (15% final concentration) and the pellet was resuspended in a potassium phosphate buffer before protein content determination. The experiment was repeated twice with sampling in triplicate each time.

Total AGE measurement. AGE assays were performed by competitive ELISA as previously described (14) except that 1,2-phenylene-diamine-dihydrochloride substrate solution (OPD tablets, Dako A/S, Denmark) was used as peroxidase substrate. Absorbance was measured at dual wavelengths (490/655 nm) on a microplate reader (Bio-Rad, Richmond, CA). AGE content was quantified according to the calibration curve (0–20000 A.U. AGE/ml).

Pentosidine quantification by HPLC. Pentosidine content in the samples was measured using a modification of the method from Odetti *et al.* (15). Briefly, 50 μg of acid-hydrolyzed protein was injected into an HPLC fluorescence system (GILSON). Separations were made on a Vydac C18 reversed-phase column (4.6 × 250 mm) equipped with a precolumn. The HPLC was programmed with a linear gradient from 0 to 35 min of 1.2–17% acetonitrile (Baker, Holland) in water containing 0.1% heptafluorobutyric acid as a counterion. The effluent was monitored with a Fluorescence Spectrophotometer (Hitachi, F-1000) set at excitation/emission wavelengths of 335/385 nm, respectively. Pentosidine eluted at ~37 min. The protein-linked pentosidine was quantified according to a calibration curve. Data were expressed as pmol pentosidine/nmol BSA.

Protein carbonyl determination. Protein carbonyl content was measured by a reaction with dinitrophenylhydrazine (DNPH) (16). 750 μg of freeze-dried oxidized BSA was gently resuspended in 200 μl of 6% SDS and incubated at room temperature for 25 min. Then, either 10 mM DNPH diluted in trifluoroacetic acid (1.3 M) or trifluoroacetic acid alone was added and the incubation followed for 40 min with gentle shaking every 10 min. Proteins were precipitated by 15% (w/v) TCA on ice for 15 min. Samples were spun at 10,000g, and the protein pellets were washed twice with 20% TCA. The final protein pellet was resuspended in 0.2 M sodium phosphate buffer and the absorbance was measured at 360 nm using 17.530/M/cm as the molar extinction coefficient of DNP. The recovery of protein was determined from the A$_{280}$ of the non-derivatized samples. The protein-carbonyl content was expressed as mol carbonyl/mol BSA.

Protein structural changes. Protein-crosslinks and fragmentation can be generated during incubation of BSA with sugars (17, 18). We have determined the effects of different concentrations of adenine and kinetin on these potential structural changes by incubation of either kinetin or adenine with BSA, glyoxal, arabinose, and ribose as described above, for 1 month. After extensive dialysis and protein content determination, the samples were submitted to an 8% SDS–PAGE subsequently stained by coomassie blue dye. The percentage of aggregates and native BSA have been quantified in each sample by computer-assisted densitometry (Molecular Analyst software, Bio-Rad, Richmond, CA). The experiment has been repeated twice.

Statistical analysis. Results were compared by analysis of variance followed by the Student's-*t*-test for unpaired data. Data were expressed as mean ± S.D., a value of $P < 0.05$ was considered as statistically significant.

RESULTS

Effects on the formation of AGE and pentosidine. We have assessed the effects of kinetin and adenine on the AGE and pentosidine formation by using two different glycation/glycoxidation models: (i) a slow process (Figs. 1A and 1B) by incubating BSA with glucose, and (ii) a fast process (Figs. 1C and 1D) by incubating BSA with highly reactive compounds (arabinose, ribose, glyoxal). Whereas, a plateau of AGE (Fig. 1C) and pentosidine (Fig. 1D) formation is reached in less than a month in the fast process, their formation was still in progress in the slow process. (Figs. 1A and 1B). After 1 month, the level of pentosidine was about 8 times higher in the fast process (Fig. 1D) than in the slow process (Fig. 1B), but the AGE concentrations were identical (Figs. 1A and 1C). Both kinetin and adenine inhibited the AGE (Figs. 1A and 1C) and pentosidine (Figs. 1B and 1D) formation in both processes. The inhibitory effect of both compounds on the formation of AGE was similar (59 ± 33% for 50 μM of adenine, 68 ± 5% for 50 μM of kinetin) in both processes after 1 month. However, kinetin was a stronger inhibitor (95 ± 1% inhibition) than adenine (61 ± 4% inhibition) after 1 week in the fast process. After 1 month, 50 μM of kinetin inhibited the pentosidine formation in both processes significantly more (82 ± 11%) than adenine did (49 ± 20%) (Figs. 1B and 1D).

Effects on the formation of protein-carbonyls. To evaluate whether kinetin and adenine could limit the protein oxidation process, we have incubated BSA with both compounds in different concentrations along with ascorbic acid and FeCl$_3$ up to 5 h. After 2 h, the carbonyl formation reached a plateau (Table 1). Both adenine and kinetin at different concentrations (50–200 μM) protected BSA against oxidation. However, kinetin was more effective than adenine in inhibiting this oxidative process. For instance, 200 μM of kinetin reduced the protein-carbonyl content by 51 ± 12% during the last 270 min of oxidation whereas adenine reduced it by 29 ± 8% only (Table 1).

Effect on the structural changes induced by incubation of BSA with glyoxal, arabinose, and ribose. The sugar-induced modifications of BSA and the related effects of adenine and kinetin were determined by SDS–PAGE (Fig. 2). Incubation of BSA for 1 month with glyoxal, arabinose, and ribose led to the appearance of protein aggregates at the top of the gel (25 ± 3%) in comparison with the non-glycated BSA (4 ± 2%). At 200 μM, kinetin maintained the integrity of the native BSA and inhibited the formation of aggregates by 45% during the glycation process. At the same concentration, adenine also maintained the integrity of BSA but was not so effective in limiting the formation of aggregates (14% of inhibition) during glycation.

AGE products
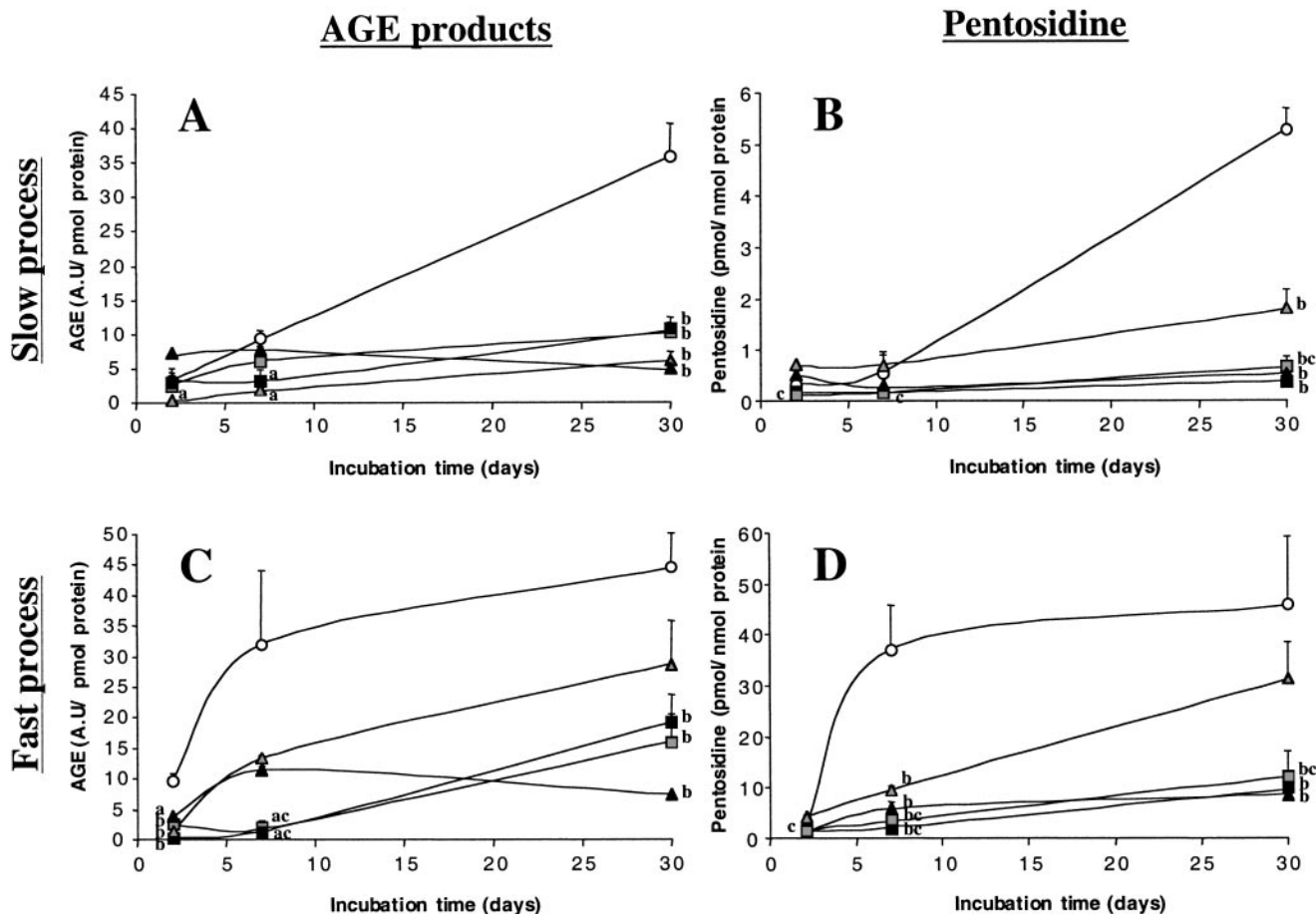
Pentosidine

FIG. 1. Effect of adenine and kinetin on the formation of AGE and pentosidine. BSA was incubated with either glucose (A, B) or ribose, arabinose, and glyoxal (C, D) alone (○) or in the presence of 50 μM (△) or 200 μM (▲) of adenine or 50 μM (□) or 200 μM (■) of kinetin. The yields of AGE (A, C) and pentosidine (B, D) in the incubation mixtures were determined as indicated. The levels of AGE and pentosidine in the control BSA incubated for 1 month without glycation agent were respectively 1.36 $\pm$ 0.6 A.U./pmol of protein and 0.168 $\pm$ 0.098 pmol/nmol of protein. [a] $P < 0.05$ vs. control without adenine and kinetin, [b] $P < 0.01$ vs. control without adenine and kinetin, [c] $P < 0.05$ vs. sample incubated with the same concentration of adenine.

DISCUSSION

To evaluate the effects of kinetin on the glycation/glycoxidation process, we have developed two models *in vitro:* a slow process due to the low reactivity of glucose and a fast process due to the high reactivity of pentoses and glyoxal. The first one is representative of the extracellular glycation process and the second is a phenomenon that could occur in the cytosol. We have shown that kinetin inhibited both the slow and fast processes of glycation/glycoxidation by up to 81 and 68%, respectively, as measured by the levels of AGE products and pentosidine. We have also shown that kinetin inhibited the aggregation of BSA occurring under glycation *in vitro* (17). Finally, kinetin also inhibited protein oxidation in response to ascorbate/OH-mediated oxidative stress as measured by the formation of protein carbonyls. In comparison, although adenine also inhibited the AGE formation to

the same extent, its effect on the formation of pentosidine, carbonyl, and crosslinks was significantly lower than kinetin.

The known endogenous and pharmacological AGE-inhibitors act either as quenchers of sugars and carbonyls (hydrazines), as metal ion chelators (phytate, penicillamine) or as antioxidants (SOD, catalase, vitamins C, and E) (17, 19). It is unlikely that kinetin could quench sugars or carbonyl compounds and limit their reactivity towards the amino groups of proteins, because kinetin does not possess amino groups susceptible to glycation (1, 20). Since the formation of crosslinking glycoxidation products, such as pentosidine, is facilitated under oxidation, kinetin could inhibit its formation by decreasing the ROS formation or by scavenging the ROS formed *in vitro* by autoxidation of sugars (18). This hypothesis is reinforced by our observations that kinetin inhibits protein oxidation as well as protein aggregation *in vitro,* both processes being

TABLE 1

Inhibitory Effects of Adenine and Kinetin on the Formation of Protein Carbonyls in BSA

Oxidation time (min)	Compound				
	None	Adenine (μM)		Kinetin (μM)	
		50	200	50	200
0	0.28 ± 0.07	—	—	—	—
2	0.46 ± 0.11	0.49 ± 0.01	0.42 ± 0.06	0.45 ± 0.26	0.43 ± 0.13
10	0.94 ± 0.08	0.57 ± 0.03^c	0.55 ± 0.03^c	0.48 ± 0.002^{cd}	0.43 ± 0.18
30	0.92 ± 0.04	0.74 ± 0.12	0.73 ± 0.01^c	0.53 ± 0.23	0.53 ± 0.01^{cd}
60	1.03 ± 0.12	0.77 ± 0.03^b	0.65 ± 0.12^b	0.61 ± 0.03^{cd}	0.51 ± 0.02^{cd}
120	1.26 ± 0.07	0.95 ± 0.12^a	0.8 ± 0.04^c	0.77 ± 0.14^a	0.4 ± 0.05^{cd}
300	1.09 ± 0.09	0.89 ± 0.16	0.84 ± 0.09^a	0.75 ± 0.03^c	0.64 ± 0.1^{bd}

[a] $P < 0.05$ vs. control without adenine and kinetin.
[b] $P < 0.01$ vs. control without adenine and kinetin.
[c] $P < 0.001$ vs. control without adenine and kinetin.
[d] $P < 0.05$ vs. sample incubated with the same concentration of adenine.

linked to an oxidative phenomenon (9). Indeed, kinetin has been reported to act as an antioxidant catalysing $O_2^{\cdot-}$ dismutation by SOD mimetic activity (21) and protects against ascorbate mediated oxidative stress (22). Previously, we have shown that kinetin is able to inhibit the Fenton-reaction mediated formation of 8-oxo-dG in the DNA (6).

Adenine was also shown to inhibit the glycation process as well as kinetin. One could consider adenine as a quencher of sugar or carbonyl derivatives since it contains one well-exposed amino group. However, the data supporting this are conflicting. It has been reported that adenine is not able to react with intermediates of glycation (23), yet it has been described as the most reactive base towards other aldehydes formed from lipid peroxidation (24, 25). Moreover, the glycoxi-

FIG. 2. SDS–polyacrylamide gel electrophoresis profile of BSA treated with ribose, arabinose, and glyoxal for 1 month in the presence and absence of kinetin and adenine. Each lane represents 10 μg of protein loaded. Arrows indicate the area of aggregation.

dation of another purine (guanine) has been shown to be highly favoured in the presence of amino acid, peptide, or protein (23, 26). Since adenine also inhibited to some extent the carbonyl formation in BSA under oxidation, it suggests that it also has some antioxidant properties. It has been previously reported that adenine can limit the formation of ROS generated through the Haber Weiss cycle by iron ion systems (27) and can also be preferentially oxidized (28–30).

Although the abilities of kinetin and adenine to reduce protein glycation/glycoxidation (AGE formation) were similar, kinetin was more effective compared to adenine in slowing down oxidation-mediated protein damages generated *in vitro,* such as proteinglycoxidation (pentosidine formation), protein oxidation (carbonyl formation), and protein aggregation. Therefore, it appears that the N^6-furfuryl substitution is important for the protective functions of kinetin. Paradoxically, this substitution is due to ROS damage and seems responsible for the antioxidant proprieties of kinetin thereby making kinetin more easily oxidized than adenine (20).

Previously, we have reported that kinetin limits the accumulation of autofluorescent material in human fibroblasts during ageing in culture (4). Autofluorescent materials such as ceroid and lipofuscin accumulate in the lysosomal compartment during ageing in both mitotic and post-mitotic cells following an imbalance between ROS generation and the cellular antioxidative defence capacity (31, 32). This pro-oxidative status allows the production of oxidized proteins and lipids and glycation/glycoxidation products of proteins such as pentosidine, all prone to crosslinks, aggregation, and formation of age pigments-like fluorophores (32–34). Therefore, the anti-ceroid effect of kinetin observed in human cells could be due to its antioxidant effect in inhibiting protein oxidation and protein oxidative by-
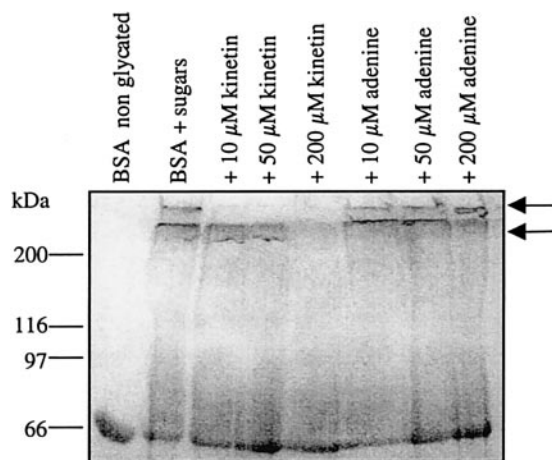
processes that we have reported in this study. In experimental animal models, dietary supplementation with the antioxidant vitamin β-tocopherol resulted in a decrease of lipofuscin accumulation in brain (35) suggesting that antioxidative defence systems prevent this aggregation occurring during ageing.

In conclusion, our results suggest that kinetin acts as an anti protein glycoxidation agent by inhibiting the oxidative process. Together with the previous reports showing that kinetin mimics the SOD activity (21), activates both SOD and catalase expression (2, 36), and quenches ROS (6), our results strengthen the view that kinetin is a powerful antioxidant with significant biological properties and useful potential for the prevention of oxidative damage.

ACKNOWLEDGMENTS

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