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Accelerated Formation of N^{ϵ}-(carboxymethyl) Lysine, an Advanced Glycation End Product, by Glyoxal and 3-Deoxyglucosone in Cultured Rat Sensory Neurons

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The formation of advanced glycation end products (AGEs) is associated with pathophysiological changes with aging and disease processes. In the neurodegeneration in Alzheimer's disease and other neurodegenerative diseases, AGEs are speculated to play a role in their pathogenesis. We provide the first evidence for the induction of AGEs in cultured neuronal cells. Glyoxal and 3-deoxyglucosone (3-DG), AGE precursors, induced N^{ϵ}-(carboxymethyl) lysine (CML), a well characterized and major AGE structure, in cultured rat sensory neurons in a time- and dose-dependent manner. CML formation was prevented by addition of aminoguanidine, an inhibitor of AGE formation. This culture system provides a useful model to analyze the role of the glycoxidation reaction in neuronal aging and neurodegenerative disorder.

The Maillard reaction is an nonenzymatic glycation and oxidation between carbohydrate-derived carbonyl compounds and protein amino groups. This reaction is initiated via formation Schiff base, which converts into a relatively stable form, Amadori product. It further undergoes a series of reaction to form advanced glycation end products (AGEs) which leads to insoluble, browning, fluorescence, cross-linking of the proteins and resistance to a protease (1, 2). AGEs include various structures, such as N^{ϵ}-(carboxymethyl)lysine (CML) (3) and pentosidine (4). In the early period of their investigation, the formation of AGEs was shown to be associated with the pathophysiological changes involved in aging and complications of diabetes (5). But recently, it was suggested that AGEs accumulated not only in aging or diabetic complications but also in many other disorders, namely, renal failure complications (6, 7, 8, 9), senile cataract (2, 10, 11), stroke (12), Alzheimer disease (13, 14, 15, 16, 17, 18), Pick's disease (19) and Parkinson's disease (20). AGEs bind to their specific receptor (receptor of AGEs; RAGE) (21, 22) and show various physiological and pathophysiological activities (23, 24, 25, 26). A significant role of AGEs has been suspected in neuronal aging and neuronal degeneration such as Alzheimer's disease, however, its underlying molecular mechanism has remained unknown because a useful model system to analyze it has not been available.

3-Deoxyglucosone (3-DG) is formed by rearrangement and decomposition of Amadori compounds (11, 27), and glyoxal is formed by autoxidation of carbohydrates and arachidonates (28). Both compounds accelerate AGE formation (29, 30), and induce cytotoxic effects with functional and morphological changes as well as apoptotic cell death in macrophage-derived cell lines (31).

In the present study, we demonstrate the experimental induction of CML, a major component of AGEs, by glyoxal and 3-DG in cultured adult rat dorsal root ganglion neurons. The effect of aminoguanidine, an inhibitor of AGE formation (12, 32, 33), was also investigated. This is the first report of the experimental induction of AGEs in neuronal cells.

MATERIALS AND METHODS

Explant culture of dorsal root ganglia. Dorsal root ganglia (DRGs) were obtained from Sprague Dawley (SD) rats (3- to 6-month-old) under deep anesthesia with diethylether. Ganglions were collected in the serum-free synthetic medium SFM-101 (Nissui Seiyaku Co.), and the loose connective tissue capsule was removed under a dissecting microscope using a sharp blade. We employed a collagen-embedded explant culture system, the details of which have been described previously (34, 35, 36). Three to four explants were embedded and cultured in a collagen gel in 16-mm plastic tissue culture dishes. Three hundred μl of a liquid type I collagen solution derived

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from porcine tendon (Wako Pure Chemical) was poured into a dish well and gelled at 37°C. Then the ganglion explants were put on the collagen gel, and another 500 μl of collagen solution was immediately poured onto the collagen gel and explants, then gelled at 37°C. The liquid collagen solution was composed of 0.21% type I collagen, 26 mM $NaHCO_3$, 20 mM HEPES and synthetic medium SFM-101. Collagen gel containing ganglions was overlaid with the defined synthetic culture medium supplemented with 50 units/ml penicillin and 50 μl streptomycin. The cultures were then incubated at 37°C in a 5% CO_2 in air-humidified atmosphere.

Administration of carbonyl stress compounds, and blocking by aminoguanidine. Glyoxal (at 0.001, 0.01, 0.1, 1 and 10 mM) or 3-DG (at 0.001, 0.01, 0.1 and 1 mM) was added to the overlay medium on the first day of culture, or vehicle only. The medium change was performed every day. The cultures were incubated up to 6 days. For the blocking experiments, aminoguanidine (Sigma) was prepared as 500 mM stock solution in 1 N HCl and diluted in medium to give final concentrations of 0.1, 1 and 10 mM with glyoxal or 3-DG at 0.1 mM. The medium was changed every day, and cultures were incubated up to 3 days.

Histology and immunohistochemistry. Cultured dorsal root ganglions picked up from the collagen gel were fixed in 10% buffered-formalin, then embedded in paraffin. Sections were cut at 4 μm thickness. Hematoxylin-eosin (H-E) staining was performed by the usual method. For indirect immunostaining, sections were deparaffined and rehydrated, and heated in a microwave oven. Then sections were incubated in 0.3% H_2O_2 in methanol for 30 minutes, blocked with diluted normal horse serum, and then incubated with monoclonal anti-AGE IgG (0.5 μg/ml, 6D12, Wako Pure Chemical) in humid chambers for 1 h at room temperature. The binding specificity of anti-AGE IgG to CML-modified protein was described previously (37). The detection was performed using an ABC staining kit (Vectastain) according to the manufacturer's protocol. For a competition experiment, the antibody preincubated with excess bovine serum albumin (BSA) modified with AGEs (AGEs-BSA) was used (14). AGEs-BSA was prepared by incubating BSA with 0.1 M glucose at 37°C for 60 or 10 days, respectively, in 0.1 M phosphate buffer (pH 7.4). For semiquantitive analysis, more than 300 sensory neurons per one stained section were evaluated in observer-blind fashion.

Western blot analysis of CML accumulation. The samples were homogenized in the buffer (pH 7.6, 50 mM Tris, 5 mM EDTA, 25 mM NaCl, 2 mM PMSF, 1 μg/ml lenpeptin, 1 μg/ml pepstatin). The samples containing 10 μg protein were incubated with the same volume of SDS buffer (4% SDS, 2% β-mercaptoethanol in water) for 30 minutes at 60°C. They were separated by 12.5% SDS-polyacrylamide gel (Bio-Rad Laboratories) electrophoresis and transferred to polyvinylidene fluoride membranes (Amersham) electrophoretically. The blots were blocked in 5% nonfat dry milk, 0.1% Tween-20, and TBS (10 mM Tris, 150 mM NaCl, pH 8.0) at room temperature and incubated overnight at 4°C with anti-AGE monoclonal antibody (0.25 μg/ml, Wako Pure Chemical). Next, they were rinsed three times and incubated for 1 hour with horseradish peroxidase conjugated sheep anti-mouse IgG (1:2000) in blocking buffer, then rinsed three times. Immunoreactive proteins were visualized using enhanced chemluminescence (Amersham). The blots were visualized by the exposure of the membranes to high-performance chemiluminescence film (Amersham).

Assays for DNA nicking (TUNEL procedure). For the detection of DNA nicks, an in situ apoptosis detection kit (Oncor) was used according to the manufacturer's protocol. After deparaffinization and rehydration, the protein digesting enzyme was applied to the specimens. After washings, specimens were quenched in 2-3% hydrogen peroxide and rinsed with PBS. Equilibration buffer was applied followed by 10-15 second incubation. Working Strength TdT Enzyme (38 μl of reaction buffer and 16 μl of TdT enzyme) was applied. After 1 hour incubation at 37°C, specimens were dipped into Working Strength Stop/Wash Buffer (1 ml of stop/wash buffer and 34 ml of distilled water) for 30 minutes at 37°C followed by washings with PBS. Anti-digoxigenin-peroxidase was applied, then incubated for 30 minutes at room temperature followed by washings with PBS. Specimens were stained by DAB (diaminobenzidene) substrate working solution (117 μl of DAB dilution buffer and 13 μl of DAB substrate) and washed. Specimens were further stained by methylgreen and washed. After washing by 100% butanol and xylene, specimens were mounted and observed by microscope.

Statistical analysis. The statistical analysis employed in this report is Mann-Whitney's U-test.

RESULTS

CML Induction by Glyoxal and 3-DG

CML formation in the sensory neurons was accelerated in the presence of glyoxal and 3-DG in the culture

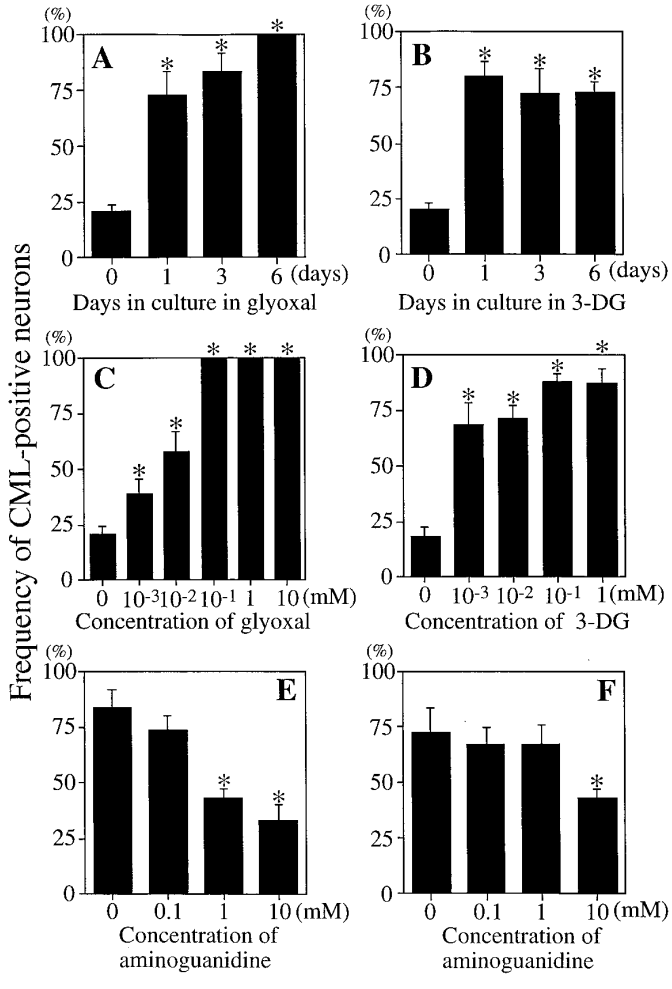

FIG. 1. Time course of CML induction. **A:** Glyoxal 0.1 mM; **B:** 3-DG 0.1 mM; **C:** Induction of CML by glyoxal (6 days in culture); **D:** Induction of CML by 3-DG (1 day in culture); **E:** Aminoguanidine blocked CML induction by glyoxal 0.1 mM, 3 days in culture; **F:** Aminoguanidine blocked CML induction by 3-DG 0.1 mM, 3 days in culture. Each value shows the percentage of stained neurons to the number of total neurons (mean ± SD, n=4). Asterisk indicates $P<0.05$.

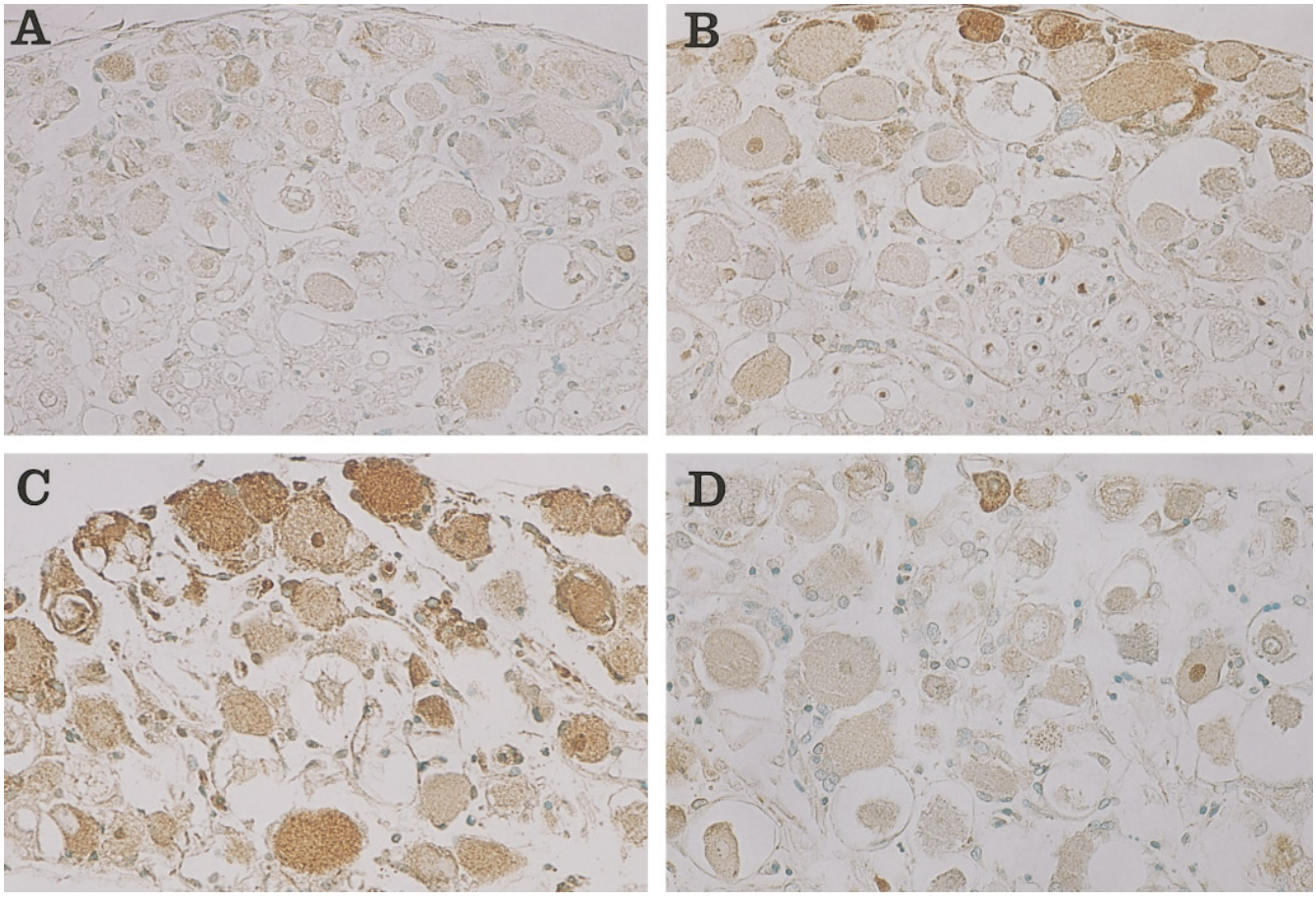

FIG. 2. **A, B** and **C** show CML induction by glyoxal. **A:** Control; **B:** glyoxal 0.01 mM; **C:** glyoxal 0.1 mM. **D** shows that aminoguanidine (10 mM) blocked the induction of CML by glyoxal (0.1 mM) at 3 days in culture.

media in their time- and dose-dependent manner (Fig. 1A-D, Fig. 2A-C). Both glyoxal and 3-DG accelerated CML formation in neurons within one day in culture. Glyoxal gradually increased the CML accumulation level, and 3-DG rapidly increased CML, reaching the plateau level in three days and one day in culture, respectively (Fig 1 A, B). Half maximum concentrations for CML induction were 0.01 mM for glyoxal and less than 0.001 mM for 3-DG, respectively. CML immunostaining was observed in the cytoplasm of the sensory neurons, both in the large and small neurons, and was also present in the nuclei in the subpopulation of neurons. CML immunostaining was more prominently observed in the surface area of DRGs. Preincubation of anti-AGE antibodies with AGE-BSA virtually reduced the immunostaining on cultured sensory neurons (data not shown). In the sensory neurons, western blot analysis revealed that CML formation was accelerated in the presence of glyoxal in a dose-dependent manner. The broad and intensive band was induced by 10 mM glyoxal, moderate dense band by glyoxal 0.1 mM, and minimum band in the control (Fig. 3, left column).

CML Induction Blocking Effect of Aminoguanidine

The CML formation in the sensory neurons accelerated by the addition of glyoxal and 3-DG was effectively prevented by the presence of aminoguanidine (Fig. 1E, F, Fig. 2D). Minimal blocking effect was observed at 1 mM aminoguanidine, and more than 60% of CML accumulation was blocked at 10 mM of aminoguanidine. This inhibitory effect of aminoguanidine for CML formation was also confirmed by western blot analysis (Fig. 3, right column).

Apoptosis

No fragmentation and condensation of were observed in the nucleus of DRG neurons by H-E staining, and no apoptotic figure was observed by TUNEL procedure (data not shown).

DISCUSSION

In this study, we demonstrated that carbonyl compounds, glyoxal and 3-DG, are capable of enhancing

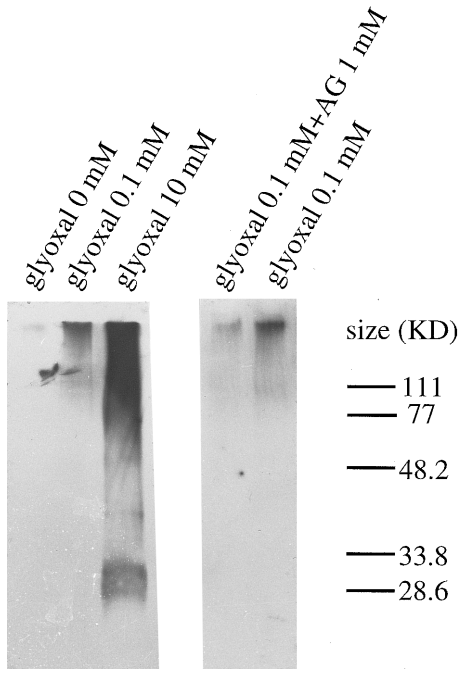

FIG. 3. The results of western blot analysis.

CML formation in the adult rat sensory neurons in explant culture. Aminoguanidine inhibited CML formation in a dose-response manner, indicating that CML in sensory neurons is accelerated by these carbonyl compounds. 3-DG has been reported to accelerate AGE formation in macrophage-derived cell lines (31), but this is the first demonstration of experimental AGE formation in neurons.

AGEs have been thought to require a long-time incubation for their formation (2), particularly in aging process as well as in various pathophysiological conditions. Our results, however, documented that once the carbonyl compounds are present in tissues, AGEs could be formed in a short period.

On the other hand, these carbonyl compounds also exhibit cytotoxic effects on certain cell lines, probably through Schiff base formation and cross-linking with cell surface proteins, leading to the intracellular signal transduction. Okado et al (31) showed that methylglyoxal and 3-DG induced apoptotic cell death in macrophage-derived cell lines in one day, with accompanying increased intracellular oxidant levels. It is not well understood whether carbonyl stress directly increases the intracellular oxidant level and induces a cytotoxic effect, or whether AGEs induced by carbonyl stress exert cytotoxic effects on these cells. Our study has shown carbonyl stress-induced AGE formation in sensory neurons was as rapidly as carbonyl stress-induced apoptosis in macrophage-derived cell lines in several hours. However, we could not demonstrate fragmentation of the nucleus in this neuronal system. Post-mitotic neurons, such as adult sensory neurons would not show a similar apoptotic figure as demonstrated in the mitotic cells like lymphocytes or macrophage-derived cell lines. Since there is no evidence that AGEs directly induce cytotoxic effects on neurons, we may assume that the AGEs have markers for intracellular oxidant stress in neurons.

Recently, there is an increasing body of evidence that AGE accumulation occurs in aged neurons as well as degenerating neurons in Alzheimer's disease (13, 14, 15, 16, 17, 18), Pick's disease (19) and Parkinson's disease (20), suggesting that AGE formation in these neurons is directly or indirectly related to the selective neuronal cell death in these neurodegenerative diseases. The essential proteins forming neurofibrillary tangles in Alzheimer's disease, Pick bodies in Pick's disease and Lewy bodies in Parkinson's disease are likely glycated (16, 17, 19, 20).

The system presented here for carbonyl compound-induced intraneuronal formation of CML is the first system of experimental induction of AGEs in neuronal cells, and it provides a useful model to analyze the underlying mechanism of neuronal degeneration.

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