J. Biochem. 123, 353-357 (1998)

Overexpression of Aldehyde Reductase Protects PC12 Cells from the Cytotoxicity of Methylglyoxal or 3-Deoxyglucosone[1]

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Received for publication, October 20, 1997

The glycation reaction (Maillard reaction) plays a major role in diabetic complications, since some reaction intermediates are responsible for the modification and cross-linking of long-lived proteins, resulting, in turn, in a deterioration of normal cell function. The reaction intermediates include methylglyoxal (MG) and 3-deoxyglucosone (3-DG), both of which are cytotoxic dicarbonyl compounds and are elevated during hyperglycemia. Aldehyde reductase (ALR) catalyzes the reduction of both compounds. To examine the intracellular role of ALR in the diabetic complications of neural cells, its gene was overexpressed in rat pheochromocytoma PC12 cells, which normally express a low level of ALR. Western blot analysis showed that ALR protein in the ALR gene-transfected cells was more than twice as much as in the control cells. In the parental cells, cytotoxicity, including apoptotic cell death, which was determined by fluorescent microscopy using the fluorescent DNA binding dye Hoechst 33258, was observed at 100 μM MG. In the ALR gene-transfected cells, the cytotoxicity of both MG and 3-DG and apoptotic cell death were decreased. This suggests that intracellular ALR protects neural cells from the cytotoxicity of 3-DG or MG, and that neural cells, which normally express a low level of ALR, might be susceptible to diabetic complications caused by intermediate products of the Maillard reaction, such as 3-DG and MG.

Key words: aldehyde reductase, 3-deoxyglucosone, dicarbonyls, Maillard reaction, methylglyoxal.

Aldehyde reductase (ALR) is expressed in many organs and serves to reduce aldehyde carbonyl groups using NADPH as a hydrogen donor (*1, 2*). Recently, we identified aldehyde reductase as a major enzyme in rat liver which detoxifies 3-deoxyglucosone (3-DG) (*3*) and reported the inactivation of this enzyme as the result of a glycation reaction (*4*). In addition we demonstrated that 3-DG and methylglyoxal (MG) induce apoptotic cell death in macrophage-derived cell lines (*5*). Very recently we have also reported that 3-DG and methylglyoxal (MG) selectively induce heparin-binding epidermal growth factor-like growth factor (HB-EGF) in rat aortic smooth muscle cells and that this may be implicated in diabetic macroangiopathy (*6*).

Methylglyoxal, a reactive α,β-dicarbonyl metabolite and physiological substrate for the glyoxalase system (*7*), is formed by the nonenzymatic and enzymatic elimination of phosphate from dihydroxyacetone phosphate, glyceraldehyde-3-phosphate (*8, 9*), and by the oxidation of hydroxyacetone and aminoacetone (*10-12*). The serum concentration of MG increased by 5-6-fold in patients with insulin-dependent diabetes mellitus and by 2-3-fold in patients with non-insulin-dependent diabetic mellitus (*13*). 3-DG, another glucose-derived dicarbonyl compound, is a major and highly reactive intermediate in the glycation reaction and a potent cross-linker responsible for the polymerization of proteins and the formation of advanced glycation end-products (*14*). Plasma 3-DG levels are also increased under diabetic conditions (*15*).

Biochemical and clinical evidence suggests that the increased formation of MG or 3-DG in diabetes mellitus may be linked to the development of diabetic complications and that ALR plays a protective role in this process. However, the exact nature of its protective role remains unclear. In the present study we show that the overexpression of ALR exerts a protective effect against the cytotoxicity of MG or 3-DG in PC12 cells.

MATERIALS AND METHODS

Materials—MG was purchased from Sigma and further purified by distillation, and the solution concentration was determined as described previously (*6*). 3-DG was chemically synthesized and its structure and purity were confirmed using ^{1}H NMR (*3*). 2′7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes. Other chemicals were of the highest grade available.

Cell Culture—Rat pheochromocytoma PC12 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo). PC12 cells and the ALR gene-transfected cells were cultured in RPMI1640 medium, supplemented

[1] This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: MG, methylglyoxal; 3-DG, 3-deoxyglucosone; ALR, aldehyde reductase; DCFH-DA, 2′7′-dichlorofluorescein diacetate; ROS, reactive oxygen species.

with 10% fetal calf serum, 5% horse serum, and 0.1 mg/ml of kanamycin under a humid atmosphere of 95% air and 5% CO_2.

Measurement of Intracellular ROS by Flow Cytometry—Levels of ROS were assessed using an oxidation-sensitive fluorescent probe DCFH-DA, as previously described (*16*). In the presence of a variety of ROS, such as intracellular peroxides, DCFH is oxidized to 2′7′-dichlorofluorescein, a highly fluorescent compound. PC12 cells which had been treated with MG were incubated with 5 mM DCFA-DA and their cellular fluorescence intensities were measured using a FACScan (Becton-Dickinson).

Staining of Nuclear DNA—PC12 cells which had been treated with MG or 3-DG were fixed for 30 min at room temperature with 3% paraformaldehyde in phosphate-buffered saline (PBS), washed with PBS, treated for 4 min with 1% Triton X-100/PBS, and given a final wash with PBS. The fixed and treated cells were incubated for 60 min at room temperature with 0.5 mg/ml of 4′,6-diamino-2-phenylindole hydrochloride in PBS, washed with PBS and then mounted. Cells were inspected using an Olympus microscope in the fluorescence mode. Cells which contained highly condensed chromatin and irregular DNA inclusions were defined as being apoptotic (*17*).

Gene Transfection—Rat ALR cDNA (*3*) was inserted into the pRc/CMV (Invitrogen). The PC12 cells used for the transfection of cDNA were plated in a 10-cm plastic culture dish coated with collagen to a density of 1×10^6/ml cells. After 24 h the medium was removed and the cells washed twice with cold PBS, pH 7.4, and the medium changed to serum-free DMEM. The ALR-pRc/CMV vector (20 μg) was mixed with Lipofectamine (Life Technologies) and 100 μl of this solution was added to PC12 cells. After a 5-h incubation, the medium was changed to the original as described above. Stable transfectants were screened with 0.5 mg/ml Neomycin. Out of 58 Neomysin-resistant clones, two clones with normal cell shapes and good cell growth were randomly chosen and designated as PC12-ALR-14 and PC12-ALR-15.

Immunoblotting and Activity of ALR—Cultured cells were harvested by pipetting and disrupted by sonication. Proteins were fractionated by 10% SDS-PAGE and then transferred to PVDF membranes (Immobilon, Millipore) using a semi-dry electroblotter (Trans-Blot SD, Bio-Rad). After blocking in 4% BSA for 30 min, the blots were incubated overnight at 4°C with a 1:500 dilution of anti-rat ALR antibody (*3*). After washing 3 times, the blots were incubated with 1:1,000 diluted peroxidase-conjugated goat anti-rabbit IgG. The blots were developed using the ECL chemiluminescence detection kit (Amersham) according to the manufacturer's instructions.

Enzyme activity of ALR was measured by the rate of decrease in the absorbance at 340 nm. The standard assay mixture contained 100 mM Na-phosphate, pH 7.0/0.1 mM NADPH/10 mM MG.

Northern Blotting—Total cellular RNA was prepared from the cultured cells using RNA Zol (Biotecx Laboratories). RNA (20 μg) was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred onto a Zeta-Probe membrane (Bio-Rad) by capillary action. After hybridization with a ^{32}P-labeled ALR probe (*3*) at 42°C in the presence of 50% formamide, the membrane was washed at 55°C in 2×SSC (1×SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.5) and 0.1% sodium dodecyl sulfate for 80 min. The Kodak XAR films were exposed for 1 day to an intensifying screen at −80°C.

Cell Viability Assay—The viability of cells in 96-well culture plates was determined 24 h after the addition of MG or 3-DG by the methylene blue method (*18*). Twenty microliters of a 25% glutaraldehyde solution was added to each well, followed by incubation for 15 min to fix the surviving cells. After washing with PBS, the fixed cells were stained for 15 min with 100 μl of a 0.05% methylene blue solution. After washing 3 to 4 times with PBS, 200 ml of 0.33 N HCl was added to each well in order to extract the methylene blue, whose level was determined by the measurement of the absorbance at 665 nm.

RESULTS

Flow Cytometric Analysis of Intracellular ROS—Changes in intracellular ROS levels in PC12 cells after treatment with MG was investigated using DCFH-DA (Fig. 1). Treatment of PC12 cells with MG shifted the peak, thus indicating that the cells treated with MG had undergone more oxidative stress than the control cells.

Detection of Apoptosis of PC12 Cells—Because internucleosomal cleavage of the DNA is one of the biochemical phenomena associated with apoptotic cell death, we investigated whether these dicarbonyl compounds affect the morphology of cells by fluorescence microscopy using a fluorescent DNA-binding dye, 4′,6-diamino-2-phenylindole hydrochloride (Fig. 2). Highly condensed chromatin and irregular DNA inclusions were observed by treatment of the cells with 500 mM MG at 6 h (Fig. 2B). These morphological changes are characteristic of apoptotic cell death, and this finding suggests that MG shows cytotoxicity including apoptosis toward PC12 cells.

ALR Activity and Immunoblotting of ALR Transfected Cells—ALR activities of control (wild), mock, PC12-ALR-14, and PC12-ALR-15 cells were 18.0, 15.4, 22.2, and 16.5 units/mg cellular protein, respectively. However, ALR belongs to the aldo-keto reductase superfamily, and MG or 3-DG can be also substrates for other reductases, which makes it difficult to distinguish each activity. There-

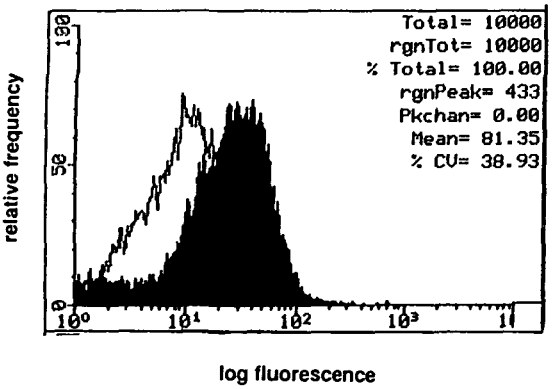

Fig. 1. **Flow cytometric analysis of intracellular peroxides.** The production of peroxides by MG-treated PC12 cells was evaluated by flow cytometry. Cells treated with MG (0.5 mM) for 4 h showed markedly increased ROS contents. White, control; black, treated with MG.

fore Western blotting using a specific antibody was employed (*3*). Western blotting analysis shows that PC12-ALR-14 cells have more abundant levels of ALR protein (38 kDa) than mock, control (wild), and PC12-ALR-15 cells. PC12-ALR-15 cells, which are Neomycin-resistant cells with normal cell shapes and good cell growth, have smaller amounts of ALR protein than control cells. This reason for this low level of ALR is still unclear, but gene transfection might have a negative effect on ALR protein synthesis.

Northern Blotting—Figure 4 shows Northern blot analysis of ALR mRNA from control PC12 cells and PC12-ALR-14 cells. PC12-ALR-14 cells expressed more mRNA of ALR than control PC12 cells both in normal conditions and after treatment with MG. This result is consistent with the result of the immunoblotting.

Effects of ALR Overexpression on the Cytotoxicity by MG in PC12 Cells—We examined the cytotoxicity of MG to PC12 cells as a function of their ALR content (Fig. 5). The PC12-ALR-14 cells, which highly overexpressed ALR, were more resistant to MG than control, mock or the PC12-ALR-15 cells. These findings suggest that overexpression of ALR has a protective effect against the cytotoxicity of MG.

Effects of ALR Overexpression on the Cytotoxicity by 3-

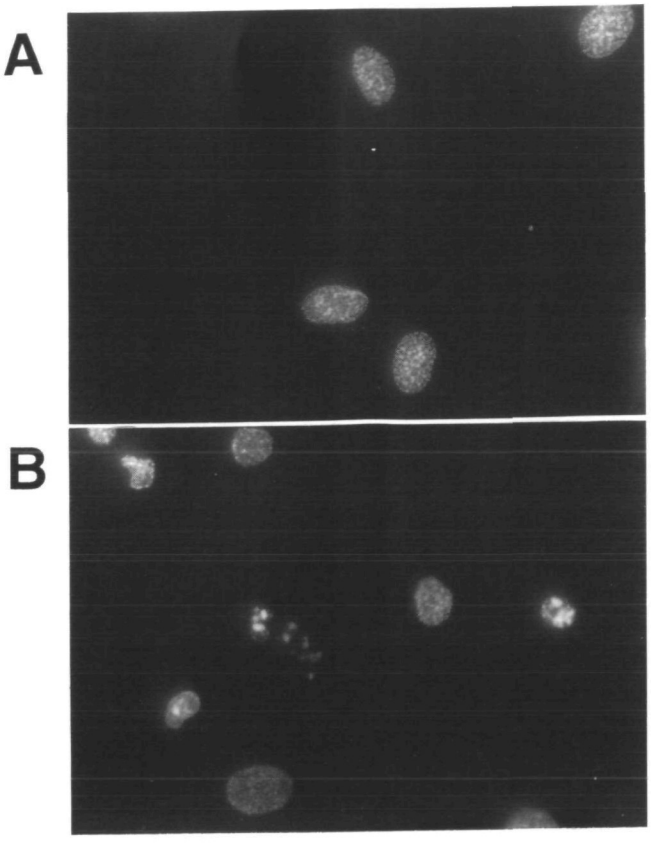

Fig. 2. **Detection of apoptosis of PC12 cells.** Fragmentation of nuclear chromatin in PC12 cells treated with MG. A, control; B, treated with MG (0.5 mM) for 6 h.

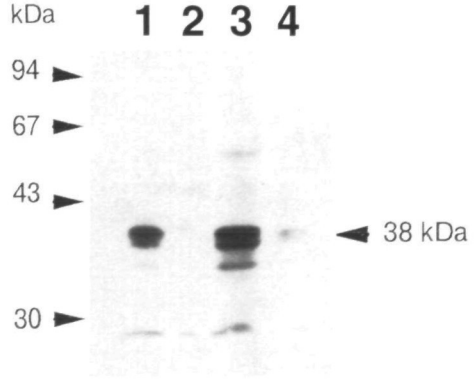

Fig. 3. **Immunoblotting of ALR transfected cells.** Lane 1, control PC12 cells; lane 2, mock; lane 3, PC12-ALR-14; lane 4, PC12-ALR-15. PC12-ALR-14 cells show overexpression of ALR protein (38 kDa).

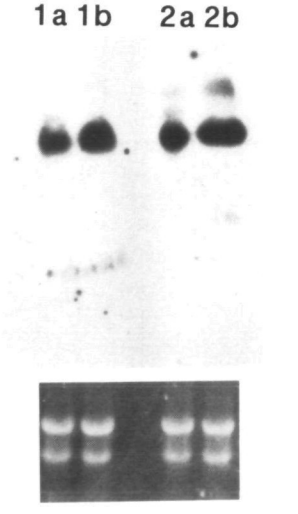

Fig. 4. **Northern blotting of ALR transfected cells.** Lane 1a, control PC12 cells; lane 1b, control PC12 cells treated with MG (0.5 mM) for 6 h; lane 2a, PC12-ALR-14 cells; lane 2b, PC12-ALR-14 cells treated with MG (0.5 mM) for 6 h. Ribosomal RNA is indicated in the lower panel.

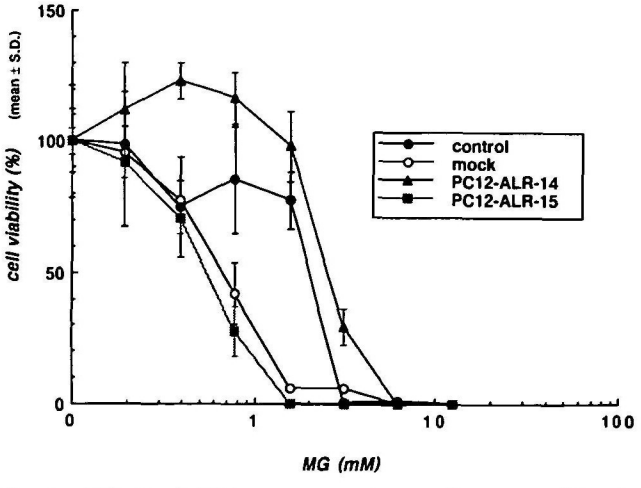

Fig. 5. **Effects of ALR overexpression on the cytotoxicity by MG in PC12 cells.** The effect of ALR overexpression on the viability of PC12 was examined after treatment with MG. ALR overexpression increased cell viability (PC12-ALR-14).

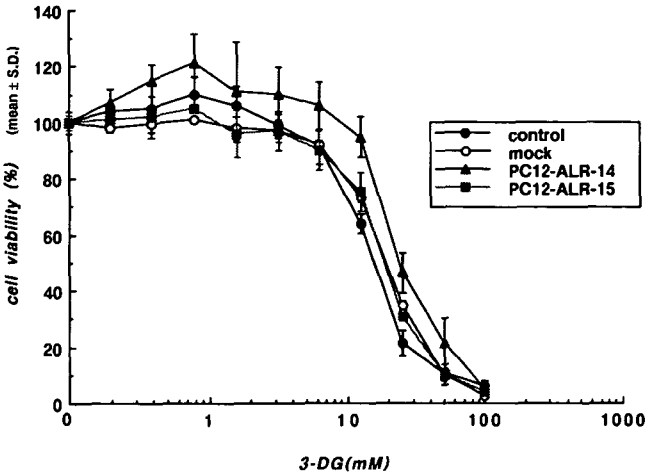

Fig. 6. **Effects of ALR overexpression on the cytotoxicity by 3-DG in PC12 cells.** The effect of ALR overexpression on the viability of PC12 was observed after treatment with 3-DG. ALR overexpression increased cell viability (PC12-ALR-14).

DG in PC12 Cells—We also examined the cytotoxicity of 3- DG to PC12 cells which contained varying levels of ALR (Fig. 6). While the difference is not as clear as in the case of MG, the PC12-ALR-14 cells, which highly overexpressed ALR, also show higher resistance to 3-DG than the control, mock, or the PC12-ALR-15 cells. These findings are consistent with the overexpression of ALR having a protective effect against the cytotoxicity of 3-DG.

DISCUSSION

Several 2-oxoaldehyde compounds are elevated under diabetic conditions (*13, 15*). These compounds are capable of inducing cellular damage, as well as accelerating the glycation process. Recently we reported that MG and 3-DG accumulate ROS and induce apoptotic cell death in macrophage-derived cell lines (*5*). These compounds can be detoxified by ALR, which is distributed over a variety of organs. However, ALR levels in nervous tissues are relatively low (*3*), suggesting that the nerve tissue could be prone to the cytotoxic effects of dicarbonyl compounds, and that this may be a link to diabetic complications. The data collected herein show a protective effect by ALR in PC12 cells, which originated from rat pheochromocytoma, and which contain a relatively low level of ALR. MG induced the accumulation of ROS and as a result, cell death including apoptosis occurred in PC12 cells, similar to those in macrophage-derived cell lines as reported previously by our group (*5*). While the concentrations of MG used in this study might appear to be high, we have already reported that the incorporation of MG into cells is only a fraction of the total MG in the culture medium. For example, in the case of rat smooth muscle cells (*6*), only 1.8% is incorporated. Therefore, the concentration of MG employed in this study results in physiologically relevant levels inside the cells.

In the ALR gene-transfected cells, the cytotoxicity of MG and 3-DG was decreased and apoptotic cell death was also decreased. These data suggest that intracellular ALR protects neural cells from the cytotoxicity of 3-DG or MG, and that neural cells, which normally express low levels of ALR, would be predicted to be susceptible to diabetic complications evoked by intermediate products of the Maillard reaction, such as 3-DG and MG. Since in diabetic conditions ALR is inactivated by the glycation reaction, as we previously reported (*4*), this may accelerate cell death, including apoptosis, and may be an important issue with respect to diabetic complications in neural tissues.

We would like to thank Dr. Motoko Takahashi for advice on the construction of the ALR-pRc/CMV vector and Dr. Paul J. Thonalley for advice on the purification of MG. We also thank Dr. Milton S. Feather for editing this manuscript. K. Suzuki is deeply grateful to Miss Kimiko Sakane and Miss Naomi Okamoto for secretarial assistance and encouragement.

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