Glycolaldehyde Induces Cytotoxicity and Increases Glutathione and Multidrug-Resistance-Associated Protein Levels in Schwann Cells

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Schwann cell injury is observed in diabetic neuropathy. It is speculated that glycolaldehyde (GA), a precursor of advanced glycation end products (AGEs), contributes to the pathogenesis and development of diabetic neuropathy. Here, we demonstrated for the first time that GA at near-physiological concentration decreased the viability of rat Schwann cells. In contrast, methylglyoxal, glyoxal, and 3-deoxyglucosone, all of which are AGE precursors, had no effects on cell viability. It is well known that methylglyoxal causes oxidative damage. In the present study, however, GA failed to induce reactive oxygen species production in Schwann cells. The addition of glutathione (GSH) or N-acetyl-l-cysteine protected Schwann cells from the loss of viability induced by GA. Moreover, GA increased intracellular GSH level and γ-glutamylcysteine synthetase mRNA level. Flow cytometric analysis revealed that GA increased multidrug-resistance-associated protein 1 (MRP1) level as well. Moreover, we demonstrated that the knockdown of MRP1 with small interfering RNA (siRNA) enhanced the loss of cell viability induced by GA. Taken together, these findings suggest that MRP1, together with GSH, plays an important role in the GA-induced toxicity in Schwann cells.

Key words glycolaldehyde; Schwann cell; glutathione; multidrug-resistance-associated protein 1; cytotoxicity

Glycolaldehyde (GA), a highly reactive α-hydroxyaldehyde, is formed from glycated proteins and plays an important role in the production of advanced glycation end products (AGEs) that lead to diabetic complications.1) GA is also formed by the reaction of l-serine with the myeloperoxidase system.2) During the production of AGEs from glycated proteins, dicarbonyl compounds, such as methylglyoxal, glyoxal, and 3-deoxyglucosone, are formed as intermediates, as well as GA.3) The levels of those dicarbonyl compounds are elevated in diabetic patients.4–7) Plasma methylglyoxal level is 2 µM in diabetic patients compared to approximately 0.6 µM in nondiabetic subjects.4,6) On the other hand, plasma GA concentrations in healthy or diabetic patients have not been quantified so far. Nonetheless, its physiological concentration is estimated to range from 0.1 to 1 mm.8–11) Although the physiological purpose of the intensive production of GA is unclear, it is postulated that the elevated GA is involved in the development of diabetic complications.

Oxidative stress resulting from the accumulation of reactive oxygen species (ROS) has been well characterized in diabetic complications. Studies of various cultured cell types have demonstrated that dicarbonyl compounds induce oxidative stress. For example, methylglyoxal increases ROS production in mouse Schwann cells, bovine retinal pericytes, and human aortic endothelial cells.12–14) Intracellular glutathione (GSH) is depleted in Schwann cells incubated with methylglyoxal.15) It has also been revealed that N-acetyl-l-cysteine, which serves as an antioxidant and a precursor for GSH synthesis, restores the methylglyoxal-induced GSH depletion in Schwann cells. It is reported that GA increases intracellular superoxide production in MCF7 human breast cancer cells.16) However, it remains unknown whether GA affects cells other than breast cancer cells. Moreover, the effects of GA on GSH in cells and tissues have not been reported so far.

GSH not only acts as an antioxidant but also plays an important role in xenobiotic elimination reactions. The multidrug-resistance-associated protein (MRP) transporter, which is able to export GSH, various GSH derivatives, and many xenobiotics, contributes to the regulation of cellular GSH levels and the thiol redox state.17) MRP1, which is expressed ubiquitously in humans,18,19) is important for the management of cell injury. However, it is unclear whether MRP1 is affected by GA and dicarbonyl compounds, such as methylglyoxal.

In the peripheral nervous system of diabetic patients, the major pathology includes fiber loss, axonal degeneration, demyelination, and microangiopathic changes.20) Various features of degenerating Schwann cells are observed in human and animal diabetic peripheral nerves.20) In this study, we examined whether GA exerts any influence on Schwann cells. The effects of GA on cell viability as well as GSH and MRP1 levels were explored using cultured Schwann cells.

MATERIALS AND METHODS

Materials GA dimer (G6805), N-acetyl-l-cysteine (NAC), GSH, oxidized glutathione (GSSG), and 1,3-buthionine-(S,R)- sulfoximine (BSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Methylglyoxal (40% aqueous solution) was from Nacalai Tesque Inc. (Kyoto, Japan). Glyoxal (40% aqueous solution) was from Kanto Chemical Co., Inc. (Tokyo, Japan). 3-Deoxyglucosone was from Dojindo Laboratories (Kumamoto, Japan). Polyethylene glycol-copper zinc superoxide dismutase (PEG-SOD, from bovine erythrocytes) and PEG-catalase (from bovine liver) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Polyclonal gamma-collider-zinc superoxide dismutase (PEG-SOD, from bovine erythrocytes) and PEG-catalase (from bovine liver) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-human MRP1-phycocerythrin monoclonal antibody was from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Hydroethidine was from Dojindo Laboratories (Kumamoto, Japan). Anti-human MRP1-phycocerythrin monoclonal antibody was from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Hydrothidine was from Inviogen (Eugene, OR, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline (DPBS), and phosphate-buffered saline (PBS) at pH 7.4 were from Gibco BRL (Grand Island, NY, U.S.A.). All other chemicals used were of reagent grade.

Schwann Cell Culture and Treatment with GA Rat Schwann cells were purchased from Dainippon Sumi-
Cells were grown to 80–90% confluence in DMEM containing 10% fetal bovine serum (FBS), l-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Then, the cells were passaged by trypsinization. Before treating the cells with GA, the culture medium was replaced with DMEM containing 2% FBS because serum may include antioxidants, chelates of transition metal ions, and high-density lipoproteins. GA (100–500 µM) was subsequently added to the medium.

Cell Viability

Cell viability was assessed by using Cell Titer 96 AQueous One Solution Cell Proliferation Assay ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay) from Promega (Madison, WI, U.S.A.). Briefly, Schwann cells on 96-well plates were treated with GA (100, 250, 500 µM) for 24 h. After the treatment with GA, the medium containing detached cells was removed. Cells remaining on the 96-well plates were washed with DMEM (FBS-free) and incubated with fresh DMEM (100 µl) and MTS assay solution (10 µl) at 37°C for 60 min. The produced MTS formazan was measured at 490 nm with a Bio-Rad Model 680 microplate reader (Hercules, CA, U.S.A.).

Detection of Intracellular Superoxide

Intracellular superoxide levels were estimated by using hydroethidine, a superoxide-sensitive fluorescent probe, and by measuring the activity of aconitase, a superoxide-sensitive enzyme. GA (100–500 µM) was subsequently added to the medium.

Determination of Nrf2 Nuclear Translocation

A nuclear extract of Schwann cells was prepared using the Active Motif Nuclear Extraction Kit (Tokyo, Japan) according to the manufacturer’s protocol. The amount of active Nrf2 in the nuclear extract was determined by measuring 20 µg protein samples with a TransAM Nrf2 DNA Binding ELISA Kit (Active Motif, CA, U.S.A.).

Measurement of γ-Glutamylcysteine Synthetase (γ-GCS) and Nuclear Factor E2-Related Factor 2 (Nrf2) mRNA Levels

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to measure γ-GCS mRNA and Nrf2 mRNA levels. Total RNA from treated cells was extracted with RNAspin Mini (GE Healthcare) according to the manufacturer’s protocol. mRNAs were reverse-transcribed into cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, U.S.A.). Quantitative RT-PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems). Primers for rat γ-GCS (Rn00689046_g1) and rat Nrf2 (Rn00477784_m1) were purchased from Applied Biosystems. mRNA levels were acquired from the value of the threshold cycle (Ct) of γ-GCS or Nrf2, normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative mRNA levels were compared and expressed as percentage of control levels.
Motif). The assay was performed according to the manufacturer’s protocol.

Measurement of MRP1 Protein Levels After treating Schwann cells with GA, the cells were fixed with 4% p-formaldehyde and incubated with anti-human MRP1-phycoerythrin monoclonal antibody in Hanks’ balanced salt solution containing saponin (0.1%) and sodium azide (0.05%) for 20 min. Following the incubation, the cells were washed with DPBS, collected with a cell scraper, resuspended in DPBS, and analyzed with a flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.). Fluorescence was detected with fluorescence channel 2 (Fl2).

Knockdown of MRP1 with Small Interfering RNA (siRNA) Oligonucleotides directed against rat MRP1 and control siRNA (Ambion, Austin, TX, U.S.A.) were transfected into Schwann cells using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s protocol. Briefly, both MRPI siRNA and control siRNA were diluted in Opti-MEM medium and then, diluted Lipofectamine RNAiMax was added. The transfection mixture was incubated at room temperature for 20 min. When Schwann cells reached 30–50% semiconfluence, the culture medium was replaced with DMEM (without FBS) and the transfection mixture was added to each well. The final concentration of siRNA was 50 nM. After Schwann cells transfected with MRPI siRNA and control siRNA were treated with GA, the effect of MRPI knockdown on cell viability was assessed by the MTS assay as described above.

Other Procedures Intracellular GSH levels were measured by spectrophotometric methods, as described previously.26 Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

Statistical Analysis All experiments were performed independently at least three times. Data were combined and expressed as means±S.D. Statistical significance between two groups was evaluated using the Student’s t-test after analysis of variance or the Scheffe test after the Kruskal–Wallis test. A p value of <0.05 was considered to be significant.

RESULTS

GA-Induced Toxicity in Schwann Cells Schwann cells were treated with GA (100, 250 or 500 µM) for various times (4, 8, 16, or 24 h) and cell viability was measured by the MTS assay (Fig. 1A). GA at 100 µM had no effect on cell viability up to 24 h. The viability of Schwann cells was decreased time-dependently by treatment with 250 and 500 µM GA. After 24 h incubation at those concentrations of GA, viability was decreased by 20% and 60%, respectively, compared to the untreated cells. When Schwann cells were treated with methylglyoxal, glyoxal, or 3-deoxyglucosone at 500 µM for 24 h, the decrease in cell viability was not observed (Fig. 1B). The results indicate that Schwann cells are susceptible to GA and GA is cytotoxic.

In MCF7 human breast cancer cells, GA induces oxidative stress by increasing intracellular superoxide levels.6 To
examine whether GA is capable of promoting ROS formation in Schwann cells, we measured intracellular superoxide levels by using hydroethidine, a superoxide-sensitive fluorescent probe.\(^{22}\) GA at 500 µM failed to promote the increase in hydroethidine fluorescence intensity (Fig. 1C). Moreover, there were no changes in the activity of aconitase, which is rapidly inactivated by superoxide,\(^{23,24}\) in Schwann cells treated with 1 mM GA (Fig. 1D). Menadione (positive control), a generator of intracellular superoxide, induced an increase in hydroethidine fluorescence intensity and a decrease in aconitase activity (Figs. 1C, D). From the results, it seems that ROS, such as superoxide, are not a major contributor to the GA-induced loss of cell viability.

**Effect of Antioxidant Enzymes and GSH on GA-Induced Toxicity in Schwann Cells** Then, we examined the effects of exogenously added antioxidant enzymes and GSH on GA-induced cytotoxicity. The two antioxidant enzymes, cell-permeable PEG-SOD and PEG-catalase, did not suppress the loss of viability of Schwann cells treated with 500 µM GA for 24 h (Fig. 2A). PEG-catalase suppressed the loss of viability of Schwann cells treated with 10 µM menadione for 24 h (control, 100±1%; menadione alone, 21±9%; menadione and PEG-SOD, 31±9%; menadione and PEG-catalase, 84±6%).

The addition of NAC, a GSH precursor, suppressed the loss of viability of Schwann cells induced by GA (Fig. 2B). Interestingly, the addition of GSH (free), which does not permeate the cell membrane, also completely suppressed the loss of viability of GA-treated cells, whereas GSSG addition failed to do so. BSO is an inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme that catalyzes the first step in GSH synthesis.\(^{27}\) When Schwann cells were pretreated with BSO, cellular GSH levels were decreased by 90% (data not shown). The pretreatment of Schwann cells with BSO did not enhance the loss of cell viability induced by GA, adversely BSO had a protective effect (Fig. 2C). From these results, it is expected that GSH exerts a protective action outside the plasma membrane.

**Effect of GA on Intracellular GSH, γ-GCS mRNA, and MRP1 Protein Levels in Schwann Cells** Next, we examined the effects of GA on intracellular GSH levels. As shown in Fig. 3A, treatment of Schwann cells with GA induced a dramatic increase in intracellular GSH levels; the GSH levels of cells treated with 250 and 500 µM GA were increased by approximately 4.5- and 3.5-fold, respectively, compared to that of the untreated cells. GA at 100 µM had no influence on the intracellular GSH level. Fig. 3B demonstrates that GA induced an increase in γ-GCS mRNA level in Schwann cells; the γ-GCS mRNA level was increased by 11.3-fold by treatment with 500 µM GA. In contrast, 100 and 250 µM GA did not significantly increase the γ-GCS mRNA level.

Intracellular GSH levels are regulated by adjusting their rates of synthesis and export from cells.\(^{17}\) MRP1 is expressed in all mammalian cells and acts as a major transporter of GSH, glutathione S-conjugates, and GSH derivatives. Thus, we examined the effects of GA on MRP1 protein levels. MRP1 protein levels in Schwann cells were estimated by flow
cytometry using phycoerythrin-conjugated anti-MRP1 monoclonal antibody. Figures 3C and D show that MRP1 protein levels were increased in GA-treated cells; MRP1 protein level was increased by 3.5-fold by treatment with 500µM GA. In contrast, GA at 100 and 250µM did not significantly increase the MRP1 protein level. These results were similar to that of γ-GCS mRNA.

Then, we further studied how GA at 500µM increased the levels of γ-GCS and MRP1. One study has shown that the transcription factor Nrf2 plays a pivotal role in inducing the expression of genes encoding detoxifying/defensive proteins, including γ-GCS and MRP1, by binding to the antioxidant response element (ARE). Figure 4A shows that 500µM GA induced an increase in Nrf2 mRNA level. Then, the effect of GA on Nrf2 activation and nuclear translocation in Schwann cells was tested by measuring its binding to an ARE-specific double-stranded oligonucleotide. Figure 4B demonstrates that 500µM GA increased the nuclear level of active Nrf2.

Finally, we examined whether MRP1 levels could alter the toxicity of GA, by using MRP1 knockdown in Schwann cells. MRP1 mRNA expression levels in cells transfected with MRP1 siRNA were reduced by 80% relative to those in control siRNA-transfected cells (data not shown). Both control and MRP1 knockdown cells were treated with GA (250 or 500µM) for 24h. As shown in Fig. 4C, MRP1 knockdown enhanced the loss of cell viability induced by 500µM GA. Nevertheless, MRP1 knockdown alone, i.e., without GA treatment, had no effect on cell viability.

**DISCUSSION**

GA is generated during hyperglycemia and inflammation.1,2 Although plasma GA level has not been quantified, its physiological concentration is estimated to range from 0.1 to 1mM8–11 and several in vitro studies have employed that range.9–11 In this study, we demonstrated for the first time that GA at near-physiological concentration exerted biological effects on Schwann cells by effectively decreasing cell viability (Fig. 1A). In contrast, methlyglyoxal, glyoxal, and 3-deoxyglucosone, all of which are AGE precursors, had no effects on cell viability (Fig. 1B). Because the loss of Schwann cells plays a pivotal role in diabetic nerve dysfunction,6,12 our data suggest the probable involvement of GA in the pathogenesis of diabetic neuropathy.

It was reported that GA induced oxidative stress: intravenous injection of GA caused oxidative damage in rat kidney, heart, and liver.8,29,30 In breast cancer cells, GA increased intracellular superoxide production and MnTMPyP, a superoxide dismutase/catalase mimetic, failed to protect cells from the GA-induced apoptotic cell death.16,31 Studies have indicated that GA is oxidized by ROS, such as superoxide, during oxidative stress in isolated hepatocytes to form glyoxal and glyoxal subsequently induces cytotoxicity.32,33 In the present study, however, we found that glyoxal was less toxic to Schwann cells than GA (Fig. 1B). The effect of GA on ROS production, which was determined by measuring the fluorescence intensity of hydroethidine and aconitase activity, was negligible in Schwann cells (Figs. 1C,D). Moreover, PEG-SOD and PEG-catalasase did not suppress the GA-induced loss of viability of Schwann cells (Fig. 2A). Therefore, our results suggest that oxidative stress is not a major contributor to the GA-induced toxicity in Schwann cells. One interesting finding is that exogenous GSH as well as NAC suppressed the GA-induced loss of cell viability (Fig. 2B). Because exogenous GSH does not permeate the cell membrane easily, it is expected that the added GSH will exert a protective effect outside the cell membrane. NAC may also exert the same effect as GSH. We examined whether GSH or NAC directly reacts with GA in the extracellular milieu. When GSH or NAC was incubated with GA, no change in sulfhydryl content (data not shown) was noted, suggesting that sulfhydryl compounds did not react directly with GA. It is possible that exogenous GSH or NAC protects plasma membrane function, such as transport function.

We also found that GA increased intracellular GSH levels in Schwann cells (Fig. 3A). In contrast, methlyglyoxal, another AGE precursor, causes GSH depletion in Schwann cells, leading to oxidative stress.15 In addition, it has been revealed that methlyglyoxal induces apoptosis in Schwann cells through oxidative-stress-mediated p38 MAPK and JNK activation.12,15 Because GA had no effect on ROS production (Figs. 1C,D), we suggest that the mechanism of GA cytotoxicity is different from that of methlyglyoxal cytotoxicity.

Then, we looked into why GA could increase intracellular GSH levels. GA at 500µM induced an increase in γ-GCS...
mRNA level in Schwann cells (Fig. 3B), indicating that GA increases GSH levels through transcription regulation. Interestingly, MRPI protein levels in Schwann cells were increased by 500 μM GA (Figs. 3C, D). MRPI, which is a likely candidate for the major GSH transporter, contributes to the regulation of intracellular GSH levels and the thiol redox state, and thus to the many key signaling and biochemical pathways. From these results, we speculate that GSH is excreted from the cells through MRPI and protects the cells from the GA-induced toxicity. This speculation is supported by the result that GA-induced toxicity is enhanced in MRPI knockdown cells (Fig. 4C). Moreover, Nrf2, which regulates the expression of γ-GCS and MRPI,28 was activated after 500 μM GA treatment, probably by increasing mRNA expression (Figs. 4A, B). Meanwhile, the Education and Research Grant from Hokkaido Pharmaceutical University.

MRP1, together with GSH, plays an important role in the GA-production. GA increased intracellular GSH and γ-GCS activity of Schwann cells but had a negligible effect on ROS induced toxicity pathway. Further investigations are needed to examine the roles of GSH and MRP1 in the GA-induced toxicity pathway.

In conclusion, we showed that GA induced the loss of viability of Schwann cells but had a negligible effect on ROS production. GA increased intracellular GSH and γ-GCS mRNA levels. Flow cytometric analysis revealed that GA increased MRPI protein levels. The present results suggest that MRPI, together with GSH, plays an important role in the GA-induced toxicity in Schwann cells.

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REFERENCES


