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Gymnemic acid interacts with mammalian glycerol-3-phosphate dehydrogenase

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Abstract In a previous study, we found interaction of gymnemic acid (GA) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis. We now examined interaction of GA with glycolytic and related enzymes. We found that (1) GA induced a band smearing of glycerol-3-phosphate dehydrogenase (G3PDH) as well as that of GAPDH in SDS-PAGE, (2) GA diminished the G3PDH band detected by an antibody to phosphoserine, and (3) GA inhibited the G3PDH activity. The GA-induced smearing of the G3PDH band was diminished by prior incubation of GA with γ-cyclodextrin. GA gave no effects on the electrophoretic and phosphoserine bands of other glycolytic enzymes. NAD and NADH diminished the GA-induced smearing of the G3PDH and GAPDH bands in different concentration-dependent manner. Pretreatment of G3PDH with heated SDS-containing buffer or pretreatment with hydroxylamine diminished the GA-induced smearing of G3PDH. Deacylation of GA by alkaline hydrolysis diminished the smearing of G3PDH band, thereby indicating that the acyl moieties of GA were necessary for the GA-induced smearing of G3PDH. These results indicated the interaction of GA with G3PDH, an enzyme involved in glycerol metabolism. These studies suggest that GA may have some pharmacological activities including antidiabetic activity and lipid lowering effects via interaction with GAPDH and G3PDH.

Keywords Gymnemic acid · Glycerol-3-phosphate dehydrogenase · Glyceraldehyde-3-phosphate dehydrogenase · Phosphoserine · γ-Cyclodextrin · Hydroxylamine

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Introduction

Gymnemic acid (GA), a mixture of triterpene glucuronides, is contained in the leaves of the Indian plant *Gymnema sylvestre*. GA has various physiological effects. GA suppresses taste sensitivity to sweetness [1, 2], inhibits intestinal glucose absorption [3], and elicits the antihyperglycemic effect [4, 5]. There are, however, few reports about molecular interaction of GA with proteins.

We previously found interaction of GA with mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC: 1.2.1.12) [6, 7]. GAPDH is a key enzyme in glycolysis, but cumulative results demonstrate that mammalian GAPDH displays a number of diverse activities unrelated to its glycolytic function, including its role in membrane fusion, phosphotransferase activity and apoptosis [8]. We found that GA inhibits rabbit GAPDH and induces a smearing of its band in SDS-PAGE [7]. The results also suggest that GA induces dephosphorylation of GAPDH at Ser residues.

Gymnema sylvestre is used for the control of diabetes mellitus in several parts of India. There are reports that *G. sylvestre* leaf extracts reduce hyperglycemia and change enzyme activities in experimentally induced diabetic animals [9], and these glucose-decreasing effects may be mediated by increases in insulin secretion [5]. We now examined the interaction of GA with glycolytic and related enzymes with an indication of band smearing in SDS-PAGE. GA induced a band smearing of glycerol-3-phosphate dehydrogenase (G3PDH, EC: 1.1.1.8) in SDS-PAGE. G3PDH catalyzes the reversible biological reduction of glycerone phosphate using NADH as a reducing equivalent to form glycerol 3-phosphate, and is involved in the hepatic metabolism of glycerol. GA diminished the G3PDH band detected by an antibody to phosphoserine, and GA inhibited the G3PDH activity. The results indicated interaction of GA

with glycerol-3-phosphate dehydrogenase (G3PDH) as well as GAPDH, and suggest the possibility that GA may have some physiological effects on glucose, glycerol, and lipid metabolism via interaction with GAPDH and G3PDH.

Materials and methods

Materials

Rabbit muscle GAPDH, G3PDH, triosephosphate isomerase, and L-lactate dehydrogenase were from Roche, and 6-phosphofructokinase from *Bacillus stearothermophilus*, yeast glucose-6-phosphate isomerase and 3-phosphoglycerate kinase, rabbit muscle pyruvate kinase, and monoclonal antibody to phosphoserine (clone PSR-45) were obtained from Sigma. *Gymnema sylvestre* extract was donated by Dai-Nippon Meiji Sugar Co., Ltd. (Tokyo, Japan). GA was purified from the extract as described previously [7]. GA is the saponins with a triterpenoid structure, and more than 10 kinds of GA and related compounds were isolated [10]. Owing to the difficulty and tediousness of the isolation of each GA, we used here the GA preparation obtained above without isolation of each GA. Gymnemagenin was obtained from MARUZEN PHARMACEUTICALS CO., LTD. (Onomichi, Japan). Diaion HP20 was obtained from Mitsubishi Chemical Corporation (Tokyo, Japan). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Binding reaction, electrophoresis, and Western blotting

Enzymes (125 μg/ml) were incubated with 2 mM GA at 20°C for 8 h, unless otherwise specified, and SDS-PAGE sample buffer was added. The proteins were heated and separated by SDS-PAGE. Gels were subsequently either stained for protein or electroblotted. Western blotting analysis was performed as described previously [7]. Horseradish peroxidase linked secondary antibody was used in combination with an ECL detection system to visualize immunoreactive bands.

Determination of G3PDH activity

The activity of G3PDH was measured spectrophotometrically as described [11]. Briefly, a sample was added to a cuvette containing 0.3 M triethanolamine-HCl (pH 7.6), 0.1 M NaCl, 0.1 mg/ml of bovine serum albumin and 0.2 mM NADH. The reaction was initiated by the addition of dihydroxyacetone phosphate (DAP) and absorbance at 340 nm was measured for 2 min at 25°C.

Chemical treatment of G3PDH with hydroxylamine

G3PDH was treated with 1.8 M hydroxylamine (pH 9.0) for 3 h at 45°C as described [12]. The reaction was quenched by addition of trifluoroacetic acid, and the reaction products were analyzed by SDS-PAGE.

Hydrolysis with β-glucuronidase and deacylation of GA

GA (3.3 mM) was incubated with β-glucuronidase (170 units/ml) from *Escherichia coli* at 37°C for 6 h. Hydrolysis of GA was confirmed by determination of released β-glucuronic acid and reverse-phase HPLC analysis after alkaline hydrolysis to gymnemagenin.

To obtain deacylgymnemic acid GA in EtOH (2.5 mg/ml) was treated with 0.9% KOH at 100°C for 2 h. The obtained deacylgymnemic acid was analyzed using reverse-phase HPLC.

Results

Effect of GA on electrophoretic bands of glycolytic and related enzymes

We previously observed that incubation of GAPDH with GA induces a smearing of the GAPDH band in SDS-PAGE [7]. We now examined the effect of GA on electrophoretic bands of glycolytic and related enzymes (Fig. 1). Among the enzymes examined, incubation of G3PDH with GA induced a significant smearing of the G3PDH protein band at the position of 37 kDa in SDS-PAGE (Fig. 1a) and no other bands were observed. GA treatment did not induce a smearing of other enzymes in glycolysis such as 6-phosphofructokinase and triosephosphate isomerase. Incubation with GA in the presence of γ-cyclodextrin abolished the GA-induced smearing of the G3PDH band (Fig. 1b). GA specifically binds to γ-cyclodextrin [13]. When G3PDH was denatured in SDS-containing sample buffer, and then incubated with GA, smearing of the G3PDH band was not observed. These observations indicated that the band smearing of G3PDH was not due to mere presence of GA in SDS-PAGE, but rather due to the interaction of GA with G3PDH. The GA-induced smearing of the G3PDH band was observed in a time-dependent manner from 10 min to 24 h of incubation with 2 mM GA, and in a dose-dependent manner from 0.1 to 2 mM GA as shown in Fig. 1c and d. Similar concentration range of GA is reported to stimulate insulin secretion from pancreatic β-cells [14], and to inhibit maltose absorption in rat intestine [15]. On the basis of the results, subsequent experiments were carried out with incubation of G3PDH with 2 mM GA for 8 h.

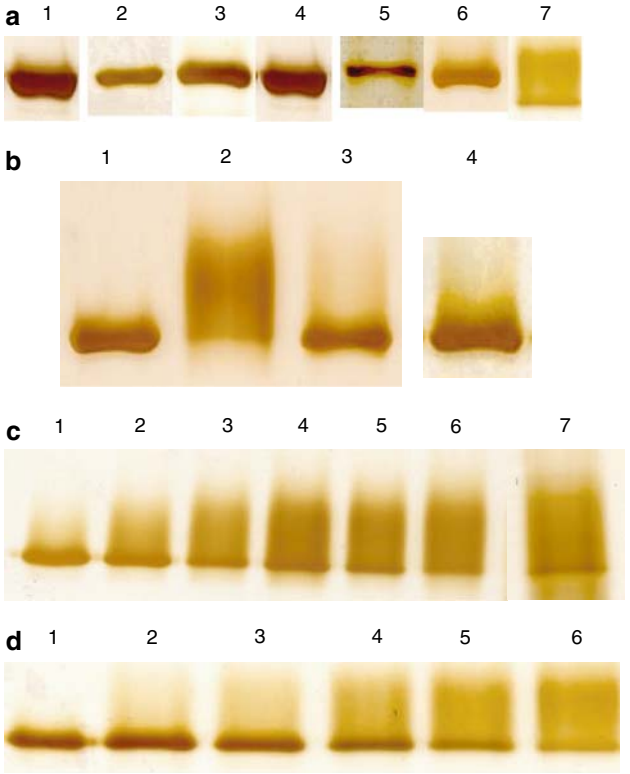

Fig. 1 The effect of GA on electrophoretic mobility of glycolytic enzymes and G3PDH. Enzymes (125 μg/ml) were incubated with GA, and 0.625 μg of protein was subjected to SDS-PAGE. The gel was stained with silver. (**a**) Enzymes were incubated with 2 mM GA for 8 h. Lane 1; phosphoglucose isomerase, lane 2; 6-phosphofructokinase, lane 3; triosephosphate isomerase, lane 4; 3-phosphoglycerate kinase, lane 5; pyruvate kinase, lane 6; lactate dehydrogenase, lane 7; G3PDH. (**b**) G3PDH was incubated without (lane 1) and with (lanes 2, 3 and 4) 2 mM GA for 8 h in the absence (lanes 1, 2 and 4) and presence (lane 3) of 10 mM γ-cyclodextrin. In lane 4, the enzyme was treated in SDS-containing sample buffer at 95°C for 3 min, then incubated with GA for 8 h, and was subjected to SDS-PAGE. (**c**) G3PDH was incubated with 2 mM GA for 10 min (lane 1), 1 h (lane 2), 3 h (lane 3), 6 h (lane 4), 12 h (lane 5), 18 h (lane 6), and 24 h (lane 7). (**d**) G3PDH was incubated with 0 (lane 1), 0.1 (lane 2), 0.2 (lane 3), 0.5 (lane 4), 1 (lane 5), and 2 (lane 6) mM GA for 8 h

Effect of GA on phosphorylation of glycolytic enzymes and G3PDH

We previously found that GA treatment diminished the GAPDH band detected by an antibody to phosphoserine, suggesting that GA induces dephosphorylation of GAPDH [7]. Phosphoserine bands were detected in commercially obtained G3PDH and glycolytic enzymes, such as phosphoglucose isomerase (PGI), 6-phosphofructokinase (PFK), triosephosphate isomerase (TPI), pyruvate kinase (PK), and lactate dehydrogenase (LDH) (Fig. 2). No phosphoserine band was detected in 3-phophoglycerate kinase. Incubation with GA significantly diminished the phosphoserine band of G3PDH, but had no significant effect for the other enzymes. Incubation with GA in the

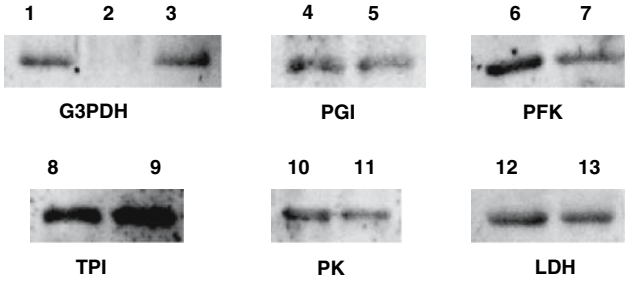

Fig. 2 The effect of GA on phosphorylation at serine residues of G3PDH and glycolytic enzymes (Western blotting). Enzymes were incubated without (lanes 1, 4, 6, 8, 10 and 12) and with (lanes 2, 3, 5, 7, 9, 11 and 13) 2 mM GA for 8 h in the absence (lanes 1, 2, 4–13) and presence (lane 3) of 10 mM γ-cyclodextrin. Lanes 1–3; G3PDH, lanes 4 and 5; phosphoglucose isomerase (PGI), lanes 6 and 7; 6-phosphofructokinase (PFK), lanes 8 and 9; triosephosphate isomerase (TPI), lanes 10 and 11; pyruvate kinase (PK), lanes 12 and 13; lactate dehydrogenase (LDH)

presence of γ-cyclodextrin abolished the effect of GA on G3PDH phosphorylation.

Phosphotyrosine band was also detected in GAPDH using monoclonal antibody to phosphotyrosine, but was not detected in G3PDH. Little effect of GA was observed for phosphotyrosine band of GAPDH (data not shown).

Inhibition of G3PDH activity by GA

The above results indicated interaction of GA with GAPDH and G3PDH. G3PDH activity was inhibited by GA (Fig. 3). The linear plot of Dixon indicated that the GA inhibition was non-competitive with respect to dihydroxyacetone phosphate (DAP) and the K_i value obtained was 0.2 mM.

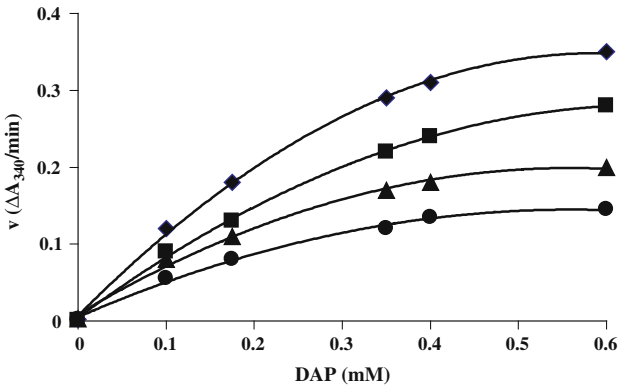

Fig. 3 Inhibition of G3PDH activity by GA. The initial velocity measurements were carried out at GA concentrations of 0 (◆), 0.05 (■), 0.1 (▲), and 0.15 (●) mM. All points were done in triplicate. SEs were <3% and were covered by the symbols

Effect of NAD and NADH on GA-induced smearing of GAPDH and G3PDH bands

Both GAPDH and G3PDH are NAD-linked dehydrogenases and catalyze the reactions reversibly. NAD and NADH gave different effects on the GA-induced smearing of GAPDH and G3PDH bands (Fig. 4). NAD diminished the GA-induced smearing of GAPDH band at a concentration of 3 mM, while 30 mM NADH gave little effect on the GA-induced smearing of GAPDH band. In contrast, NADH was more effective than NAD for the decrease of the smearing of G3PDH band. NADH diminished the GA-induced smearing of G3PDH band at a concentration of 3 mM, but 3 mM NAD gave little effect on its smearing.

Effect of chemical treatment on GA-induced band smearing of G3PDH

The above results indicated that GA bound to G3PDH, and induced a smearing of its protein band.

The proteins were eluted from the gel of the lower and upper parts of the smearing G3PDH band. The eluted proteins were precipitated with 80% acetone, treated in SDS-containing sample buffer at 95°C for 3 min, and then subjected to SDS-PAGE. The protein eluted from the lower part did not show a smearing of the band, but that from the upper part did show a smearing again (Fig. 5a). The results further indicated that the effect of GA on G3PDH was stable to precipitation with 80% acetone and then heat treatment in SDS-containing sample buffer.

The GA-induced smearing was diminished by chemical treatment with hydroxylamine (Fig. 5b). When GA-treated G3PDH was treated with hydroxylamine (lane 4), or when hydroxylamine-treated G3PDH was incubated with GA (lane 5), no smearing of the G3PDH band was observed.

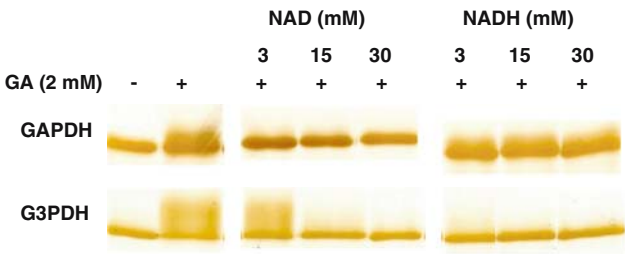

Fig. 4 The effect of NAD and NADH on GA-induced smearing of GAPDH and G3PDH. GAPDH and G3PDH (125 μg/ml) were incubated with 2 mM GA for 8 h in the presence and absence of NAD and NADH

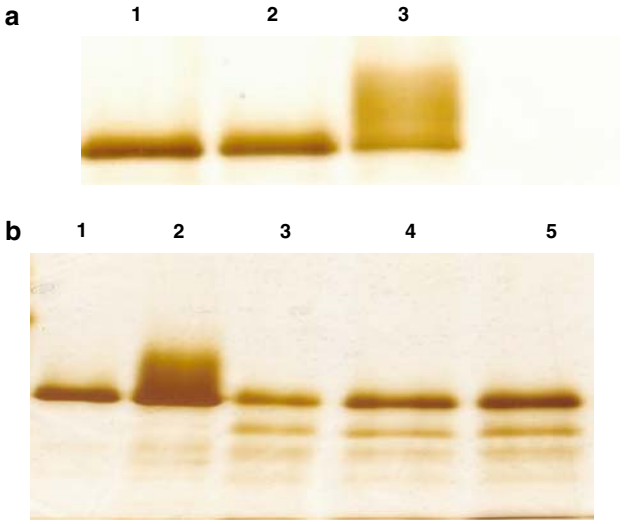

Fig. 5 Smearing of extracted and re-electrophoresed G3PDH band and effects of treatment with hydroxylamine. (**a**) G3PDH was treated with and without GA, and subjected to SDS-PAGE. The proteins were eluted from the gel of untreated G3PDH band and eluted from the lower and upper parts of the smearing G3PDH band. The eluted proteins were precipitated with 80% acetone, and treated in SDS-containing sample buffer at 95°C for 3 min. The protein sample eluted from the gel of G3PDH band untreated with GA (lane 1) and samples eluted from the lower (lane 2) and upper (lane 3) parts of the smearing G3PDH band were re-electrophoresed. (**b**) G3PDH was incubated without (lanes 1 and 3) and with (lanes 2 and 4) GA, and then treated with hydroxylamine (lanes 3 and 4). In lanes 5, G3PDH was treated with hydroxylamine, and then incubated with GA

Effect of deacylation and elimination of glucuronate moiety of GA

Gymnemic acid is a mixture of glucuronides of gymnemagenin, being differently acylated [16]. The smearing of GAPDH band is not observed with gymnemagenin [7], the aglycone of GA, indicating that glucuronate and/or acyl moieties are necessary for the effect of GA. Deacylation of GA by alkaline hydrolysis diminished the smearing of GAPDH and G3PDH bands (Fig. 6, lanes 3). A smearing of GAPDH and G3PDH bands was observed with β-glucuronidase-treated GA, but the smearing of G3PDH band was reduced as compared with the smearing induced by untreated GA (Fig. 6, lanes 4).

Discussion

GA has various physiological effects. GA is a multidirectional antihyperglycemic agent which has antisweet taste, blood-glucose lowering, glucose uptake inhibitory, and gut glycosidase inhibitory actions [17]. GA also improves lipid metabolism: Luo et al. reported in an animal model of multifactor syndrome, which exhibits a progressive polyphagia, dislipidaemia, and hyperglycemia, GA suppresses

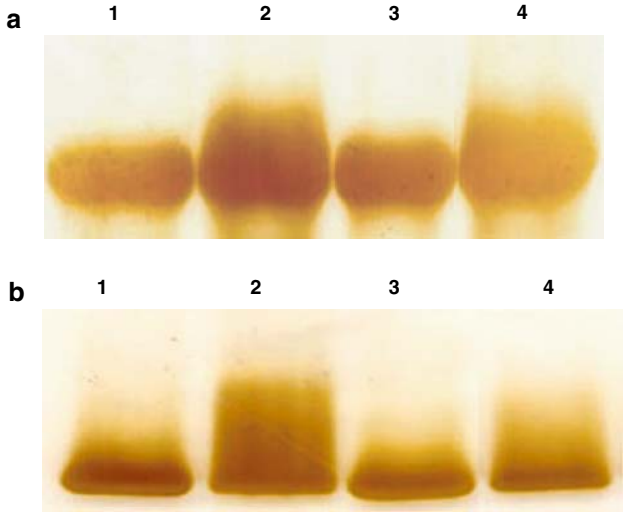

Fig. 6 Effects of deacylation and hydrolysis with β-glucuronidase of GA on smearing of GAPDH and G3PDH bands. GA was deacylated by alkaline hydrolysis or treated with β-glucuronidase. GAPDH (**a**) and G3PDH (**b**) were incubated without (lanes 1) and with GA (lanes 2), deacylated GA (lanes 3), and GA treated with β-glucuronidase (lanes 4)

serum triglyceride and total cholesterol, and thus GA inhibits the hyperlipidemia [18]. Although GA has various effects, molecules that interact with GA have not been fully understood. We previously found that GA interacted with GAPDH, a key enzyme in glycolysis [7]. We now examined interaction of GA with enzymes involved in glucose and glycerol metabolism, and we found that GA induced a smearing of the G3PDH band in SDS-PAGE (Fig. 1). G3PDH is involved in the glycerol metabolism. Glycerol, a product of adipose tissue lipolysis, is an important substrate for glucose synthesis. Thus, G3PDH occupies a key position in metabolism linking glycolysis to phospholipid and triglyceride pathways. The results of this study indicate that GA interacts with GAPDH and G3PDH. It is interesting that the GA-induced band smearing was not observed for triosephosphate isomerase (Fig. 1). Triosephosphate isomerase catalyzes the reversible reaction between glyceraldehyde 3-phosphate and glycerone phosphate, which are substrates of GAPDH and G3PDH, respectively.

Phosphoserine bands were detected in commercially obtained G3PDH and other glycolytic enzymes. GA treatment diminished the phosphoserine band of G3PDH, but had no significant effect for the other enzymes (Fig. 2). The results suggested that GA induced dephosphorylation of G3PDH. We previously observed GA-induced dephosphorylation of GAPDH [7]. Some works demonstrated acyl phosphatase to be an alternative activity of GAPDH itself [19], but the appearance of acyl phosphatase activity was not detected in GA-treated GAPDH. Thus, the phosphatase activity observed in GA-treated GAPDH (and G3PDH) is likely to be different from the acyl phosphatase activity.

Both GAPDH and G3PDH utilize NAD and NADH as a ligand. Incubation with higher concentrations of NAD and NADH diminished the GA-induced smearing of GAPDH and G3PDH bands (Fig. 4). The results indicated that GA did not bind to NAD- and NADH-bound forms of GAPDH and G3PDH. Lower concentrations of NAD and NADH gave different effects on the GA-induced smearing of GAPDH and G3PDH bands. NAD was more effective on GAPDH band than NADH, while NADH was more effective on G3PDH band. The difference of the effects of NAD and NADH may be due to different affinity of GAPDH and G3PDH for NAD and NADH. The reported K_m values of rabbit muscle GAPDH are 0.06 mM for NAD and 0.012 mM for NADH [20], and those of G3PDH are 0.01 mM for NAD and NADH [21], but the present results may suggest that GAPDH has higher affinity for NAD than NADH, while G3PDH has higher affinity for NADH than NAD under the conditions.

The above effects of NAD and NADH and the results that pretreatment of the enzymes in SDS-containing sample buffer diminished the GA-induced smearing of GAPDH and G3PDH bands indicated that the binding of GA was sensitive to conformational change of the enzymes, and required the native structure. Pretreatment of G3PDH with hydroxylamine also diminished the GA-induced smearing of G3PDH (Fig. 5b, lane 5). Hydroxylamine, a nucleophilic compound, attacks Asn–Gly bonds of proteins, and produces the hydroxylamine adducts [22].

The proteins eluted from the upper and lower parts of the smearing G3PDH band again migrated to the positions corresponding to the upper and lower parts of the smearing G3PDH, respectively (Fig. 5a). The results indicated that the smearing was not due to the equilibrium state during electrophoresis. The GA-induced smearing was not observed by treatment of GA-treated G3PDH with hydroxylamine (Fig. 5b, lane 4). Hydroxylamine partially cleaves Asn–Gly bonds of proteins, and cleavage of G3PDH at single ^{292}Asn–^{293}Gly peptide bond produces 30,991- and 6,510-Da polypeptides, but it should be noted that no smearing of the uncleaved G3PDH band was observed. Thus, the effect of GA was sensitive to hydroxylamine treatment. The results suggested a possibility that (i) GAPDH and G3PDH migrated in electrophoresis as GA-bound forms, (ii) GA-bound GAPDH and G3PDH showed smearing of their bands, and (iii) the binding of GA to GAPDH and G3PDH was cleaved by hydroxylamine treatment. GA was not detected by HPLC analysis from the GA-treated G3PDH sample eluted from the gel (data not shown). GA in solution is present in the aggregate form [13]. The results indicated that this amount of GA (>10 GA molecules/37.5 kDa G3PDH molecule) was not bound to G3PDH, if any. Recently, Yang et al. indicated that palmitoyl-CoA specifically thioesterifies

Cys-244 in GAPDH inhibiting the enzyme activity [23]. Studies using mass spectrometry are now in progress to examine the possibility that GAPDH and G3PDH migrate in electrophoresis as GA-bound forms.

The present results that the acyl moieties of GA were necessary for the GA-induced smearing of GAPDH and G3PDH (Fig. 6) suggested that the acyl moieties were necessary for the binding of GA to GAPDH and G3PDH. The results were consistent with importance of the acyl moieties for some physiological effects of GA [16]. Some acyl groups seem to play an important role on antihyperglycemic action in GA derivatives [5].

In conclusion, the present results indicated that GA bound to GAPDH and G3PDH. The results suggest the possibility that GA may have some physiological effects on glucose, glycerol, and lipid metabolism via interaction with GAPDH and G3PDH. Physiological role of the binding of GA to GAPDH and G3PDH remains to be elucidated.

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