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Article

Gymnemic acid II inhibits heme binding to glyceraldehyde-3-phosphate dehydrogenase

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Gymnemic acid (GA) is a saponin of triterpene glycoside isolated from the plant *Gymnema sylvestre*. GA is not a pure entity but is comprised of several types of homologs. GA suppresses taste sensitivity to sweetness and inhibits intestinal glucose absorption. We previously found that GA inhibits rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. GAPDH is a key enzyme in glycolysis, and binds with heme. We now found that purified GA II inhibited heme-binding to murine GAPDH. Heme binding did not affect the GA II binding to GAPDH. The inhibition of heme-binding to GAPDH was not observed with the aglycone part, gymnemagenin and was reduced by deacylation of GA II. Thus, the acetyl and/or 2-methylbutyroyl moieties and glycone glucuronic acid of GA II were necessary for the inhibitory effect of GA II on the formation of the GAPDH-heme complex.

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Introduction

Gymnemic acid (GA) is a saponin of triterpene glycoside isolated from the plant *Gymnema sylvestre*. *Gymnema sylvestre* is used to manage diabetes mellitus in several parts of India. *Gymnema sylvestre* extract is known to have antimicrobial and antihypercholesteolemic effects, hepatoprotective properties, and especially effects on obesity and diabetes mellitus [1]. These pharmacological activities are mainly due to triterpenoid saponins present in the leaf extracts, and GA is the major active compound [2]. GA has various physiological effects. GA suppresses taste sensitivity to sweetness [3,4], inhibits intestinal glucose absorption [5], and elicits antihyperglycemic effects through its ability to decrease blood glucose levels and enhance insulin sensitivity in patients with type 2 diabetes [6,7]. GA binds to several proteins, including glucose transporter [8], taste receptors T1R2/T1R3 [9], and liver X-receptor which regulates lipid metabolism

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in the liver [10].

GA is not a pure entity but is comprised of several types of homologs [11]. The aglycone part of GA is gymnemagenin, which is linked with glycone glucuronic acid and diversified esters (tigloyl, methylbutyroyl, etc.). Eighteen different types of gymnemic acids (GAs) were found in the leaves of *G. sylvestre* [2]. *Gymnema sylvestre* extracts contain triterpene saponins belonging to the oleanane and dammarane classes [1]. GAs, a complex mixture of oleanane saponins, are generally considered the active constituents [12]. Some triterpene compounds from *G. sylvestre* inhibit intracellular enzymes such as aldose reductase, protein tyrosine phosphatase 1B, glycogen phosphorylase and 11β-hydroxysteroid dehydrogenase type 1 [13].

We previously found an interaction of GA with rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC: 1.2.1.12) [14-16]. GA inhibits GAPDH activity and induces a smearing of its band in SDS-PAGE. We also found that purified gymnemic acid II (GA II) inhibits glycerol-3-phosphate dehydrogenase (G3PDH, EC: 1.1.1.8) [17]. GAPDH is a key enzyme in glycolysis, and G3PDH catalyzes the reversible biological reduction of glycerone phosphate to form glycerol 3-phosphate, and it is involved in the hepatic metabolism of glycerol. These studies suggest that GA may have some pharmacological activities, including antidiabetic activity and lipid-lowering effects, via their interactions with GAPDH and G3PDH. Gymnemic acids I-IV were the components considered to be of prime importance and were first reported from the leaves of *G. sylvestre* [18]. GA II has been found to completely suppress the sweet sensation [19] and exhibit inhibitory activity against glucose uptake into rat small intestinal fragments [5].

GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate into glycerate 1,3-bisphosphate. Besides its house-keeping role in the glycolytic pathway, eukaryotic GAPDH participates in an array of cellular function, including transcription, oxidative stress, apoptosis, and autophagy [20,21]. Recent studies reported that GAPDH binds with heme. Intracellular heme trafficking enables hemeproteins to mature and function outside the mitochondria. GAPDH behaves like a heme-chaperone that allocates labile heme [22] and delivers heme to inducible NO synthase [23] and soluble guanylyl cyclase [24]. GAPDH is involved in the maturation of myoglobin and hemoglobin [25]. We now found that GA II inhibited heme-binding to murine GAPDH.

Materials and methods

Materials

GA II was purified from *G. sylvestre* extract as described previously [17]. Gymnemagenin and hemin chloride were purchased from Nacalai Tesque. Deacylgymnemic acid II was obtained by treating GA II with 1% KOH at 100°C for 1 h [16]. The oligonucleotides used for site-directed mutagenesis (CAGCAATGCA TCCAGCACCACCAACTGC and GCAGTTGGTGGTGCTGGATGCATTGCTG) were purchased from Invitrogen.

Protein expression and purification

Coding sequences of wild-type murine GAPDH isoform 2 and C150S mutant, made using site-directed mutagenesis, were subcloned in-frame at the NdeI site of the pET28a vector. Plasmid sequences were confirmed by DNA sequencing. The resulting GAPDH constructs containing a His_6 tag attached to their N-termini were expressed in *Escherichia coli* strain BL21(DE3) and purified according to standard procedures using Ni-NTA affinity chromatography.

UV-visible spectroscopy

UV-visible spectra of GAPDH, hemin and GAPDH-hemin complexes were measured at room temperature on a Shimadzu UV-1800 using freshly prepared samples of GAPDH and hemin. Hemin stock solutions (2.5 mM) were prepared by dissolving solid hemin in 0.01 M NaOH.

Binding of GA II and SDS–PAGE

GAPDH treated and untreated with hemin was incubated with 0.5 mM GA II in 0.1 M Tris-HCl buffer (pH 7.4) at 20°C for 8 h, and then SDS–PAGE sample buffer was added. The protein was heated and separated by SDS–PAGE. The gel was stained with silver.

Results and discussion

GA inhibits rabbit GAPDH and its inhibition is non-competitive with respect to glyceraldehyde 3-phosphate and NAD [15]. It is thought that the GAPDH reaction proceeds through a covalent intermediate between the substrate and the active site cysteine. Cys150 of the murine GAPDH, corresponding to Cys149 in rabbit GAPDH and Cys152 in human GAPDH, is the active site cysteine and is reported to be completely conserved in all mammalian GAPDH sequences [26]. A specific *S*-nitrosylation of human GAPDH at Cys152 causes inhibition of cellular heme insertion into

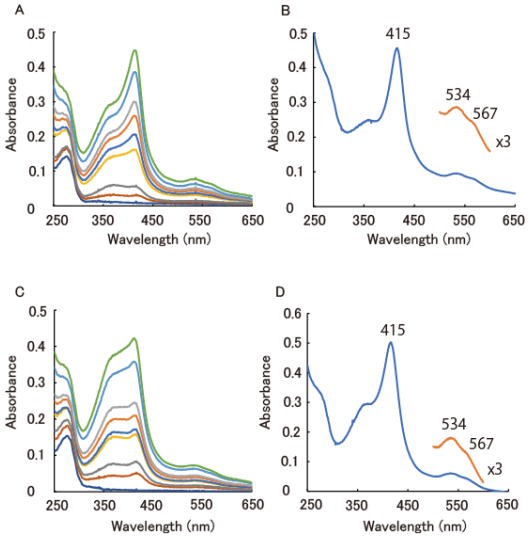

Fig. 1. Binding of wild-type (A, B) and C150S mutant (C, D) GAPDH for hemin. (A, C) Spectrophotometric titrations were performed by stepwise addition of hemin (0, 0.1, 0.2, 0.5, 0.6, 0.7, 0.8, 1.0 and 1.1 molar equivalent to 24 µM GAPDH tetramer) in 100 mM Tris-HCl buffer, pH 7.4, and incubation at 0°C for 5 min. (B, D) GAPDH-heme complex was prepared by incubating hemin and GAPDH tetramer in a 1.1:1 molar ratio in 100 mM Tris-HCl, pH 7.4 at 0°C overnight. After centrifugation (20,000 x g, 10 min at 4°C), spectra of the supernatants were measured. The insets show enlarged absorption spectra in the 500 – 600 nm range.

inducible NO synthase [23], and binding to the E3 ubiquitin ligase Siah1 [27]. Oxidative stresses induce aggregation of rabbit and human GAPDH and cell death via disulfide bonds of the active site cysteine [26]. We now examined the effects of GA II on heme binding of murine GAPDH using purified wild-type and C150S mutant GAPDH.

We examined the interaction of free hemin with murine GAPDH by UV-visible spectroscopy. The ferric GAPDH-heme complex presents a Soret band with a peak wavelength maximum at 415 nm, indicative of axial coordination to the heme [28]. Titration of wild-type GAPDH with increasing concentrations of hemin led to the formation of the GAPDH-heme complex (Fig. 1A). After

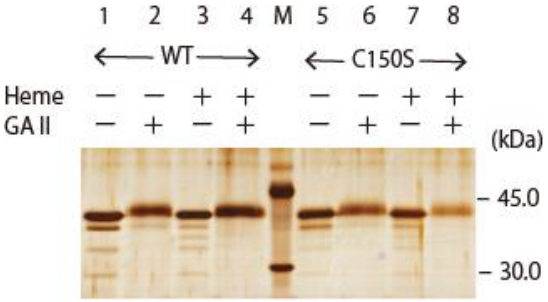

Fig. 2. The effect of C150S mutation and heme binding of murine GAPDH on GA II binding. The wild-type (lanes 1 and 2) and C150S (lanes 5 and 6) GAPDH and the wild-type (lanes 3 and 4) and C150S (lanes 7 and 8) GAPDH-heme complex were incubated without (lanes 1, 3, 5 and 7) and with (lanes 2, 4, 6 and 8) 0.5 mM GA II at 20°C for 8 h and then subjected to SDS–PAGE. The gel was stained with silver. Lane M: protein markers.

incubating hemin and GAPDH tetramer in a 1.1:1 molar ratio overnight and centrifugation to remove unbound hemin, spectroscopic features showed the formation of the ferric GAPDH-heme complex presenting a Soret band with a peak wavelength at 415 nm (Fig. 1B). Titration of C150S mutant GAPDH with increasing concentrations of hemin showed the spectra that differed from those of wild-type GAPDH (Fig. 1C), but after incubation overnight and centrifugation, the spectrum did not differ markedly from those of wild-type GAPDH (Fig. 1D). These results suggested that the C150S mutant GAPDH-heme complex presenting a Soret band was formed more slowly than that of wild-type GAPDH, and indicated that the active site Cys residue was not directly coordinated to the heme. The result shown in Fig. 1D are consistent with the results with rabbit GAPDH, where a Cys-null mutant of GAPDH (all four Cys residues replaced with Ser residues) showed no differences in the spectra of the GAPDH-heme complex to that of wild-type GAPDH [28].

We previously found an interaction of GA with rabbit GAPDH [15]. GA binding to GAPDH induces a smearing of its band in SDS-PAGE. Purified GA II induced a band smearing

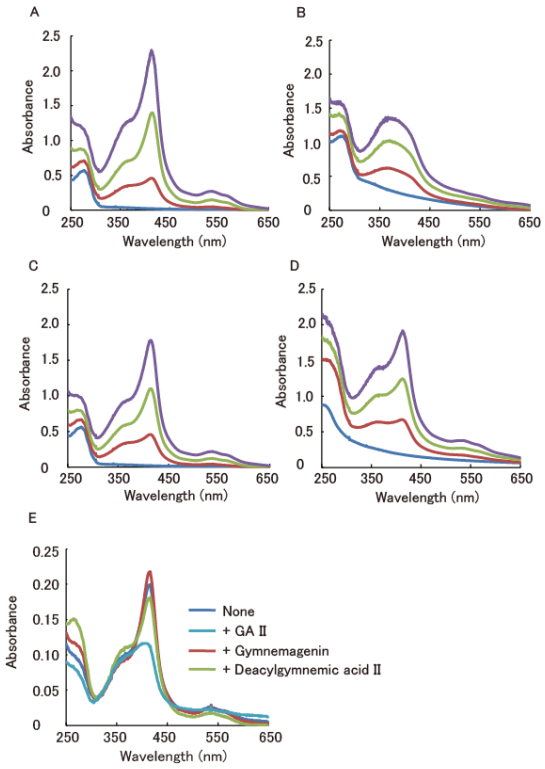

Fig. 3. The effect of GA II, gymnemagenin and deacylgymnemic acid II on heme binding to murine GAPDH. (A-D) Spectrophotometric titrations were performed by addition of hemin (0, 0.7, 1.4 and 2.1 molar equivalent to GAPDH tetramer) in 100 mM Tris-HCl buffer, pH 7.4, and incubation without (A) and with 1.0 mM GA II (B), 2.6 mM gymnemagenin (C) and 1.6 mM deacylgymnemic acid II (D) at 0°C for 30 min. (E) The wild-type GAPDH was incubated with hemin in a 2.1:1 molar ratio of hemin and GAPDH tetramer in 100 mM Tris-HCl, pH 7.4 without and with 1.0 mM GA II, 2.6 mM gymnemagenin and 1.6 mM deacylgymnemic acid II at 0°C overnight. After centrifugation (20,000 x g, 10 min at 4°C), spectra of the supernatants were measured.

of murine GAPDH in SDS-PAGE (Fig. 2, lane 2). Heme binding did not affect the GAPDH band in SDS-PAGE (lane 3) and GA II-induced smearing of the GAPDH band (lane 4), indicating that there is no effect of GAPDH-heme complex formation on the GA II binding. Analysis of the C150S GAPDH showed no difference in the GA II-induced band smearing (lanes 6 and 8) compared to that of wild-type

GAPDH, indicating that the active site Cys residue of GAPDH is not necessary for the binding of GA II.

GA II is a triterpenoid glycoside with gymnemagenin as aglycone, which is linked with glucuronic acid and acylated with acetic acid and 2-methylbutyric acid. Titration of murine GAPDH with increasing concentration of hemin in the presence of GA II did not lead to the formation of the GAPDH-heme complex, with a wavelength peak at 415 nm (Fig. 3B), which was observed without GA II (Fig. 3A). Titration with gymnemagenin (Fig. 3C) showed no difference in the spectrum compared to that without GA II shown in Fig. 3A, whereas titration with deacylated GA II led to the smaller spectral changes of the GAPDH-heme complex (Fig. 3D) than that with GA II. These results indicated that GA II inhibited heme-binding to murine GAPDH (Fig. 3E). The inhibition of heme-binding to GAPDH was not observed with gymnemagenin and was reduced by deacylation of GA II. Thus, the acetyl and/or 2-methylbutyroyl moieties and glycone glucuronic acid of GA II were necessary for the inhibitory effect of GA II on the formation of the GAPDH-heme complex. The reduced inhibition of heme-binding to GAPDH by deacylation of GA II suggested that deacylated GA II reduced affinity of heme to GAPDH and that deacylation of GA II reduced affinity of GA II to GAPDH.

GAPDH is a ubiquitous enzyme (constituting ~10-20% of the total cellular protein content) that catalyzes the key step of glycolysis [29]. The active site Cys150 of the murine GAPDH supplies a sulfhydryl group for nucleophilic attack on glyceraldehyde 3-phosphate. Besides the traditional role in a glucose metabolism, GAPDH also delivers heme to some key hemeproteins and operates as a chaperone to control the reactivity and toxicity of heme prior to its downstream delivery [22]. GAPDH is responsible for heme transfer to inducible nitric oxide synthase. GAPDH is a NO-sensitive heme binding protein, and the GAPDH-heme binding is diminished upon S-nitrosylation of the active site Cys152 of human GAPDH. Thus, Cys152 of GAPDH is not directly coordinated to the heme [28], but S-nitrosylation of Cys152 of GAPDH regulates the binding of heme [23]. Our current study showed for the first time that a low molecular compound, GA II inhibited the binding of heme to GAPDH. The results that deacylation of GA II reduced the inhibition and gymnemagenin did not show the inhibition indicated the specificity of the GA II inhibition. It was reported that treatment with a deacyl GA resulted in a decrease in insulin resistance accompanied with a decrease in systolic blood pressure and improved glucose and lipid profile without decreasing body weight in a rat model of metabolic syndrome [30], but acyl groups of GA were important for antihyperglycemic action in GA derivatives [7]. There are no reports on the effects of GA II and gymnemagenin on the GAPDH activity yet, but gymnemagenin does not induce a smearing of the GAPDH protein band in SDS-PAGE [15].

Conclusion

GA II inhibited heme-binding to murine GAPDH. Heme binding did not affect the GA II binding to GAPDH. The inhibition of heme-binding to GAPDH was not observed with the aglycone part, gymnemagenin and was reduced by deacylation of GA II. Thus, the acetyl and/or 2-methylbutyroyl moieties and glycone glucuronic acid of GA II were necessary for the inhibitory effect of GA II on the formation of the GAPDH-heme complex. Our findings provide a new insight into the role of interaction of GA with GAPDH.

Conflict of interest

The authors declare that there are no conflicts of interest.

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