The Biological Significance of Glycyrrhizin- and Glycyrrhetic Acid Derivative-Induced Selective Phosphorylation of Histones H2A and H2B by A-kinase In Vitro

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SHAMSA, F., IWASA, T., SAITO, H., NAGATA, N. and OHTSUKI, K. *The Biological Significance of Glycyrrhizin- and Glycyrrhetic Acid Derivative-Induced Selective Phosphorylation of Histones H2A and H2B by A-kinase In Vitro*. Tohoku J. Exp. Med., 1994, **172** (2), 123–132 — Histones H2A and H2B were found to be glycyrrhizin (GL)-binding proteins, because (i) the two histones H2A-H2B pairs were isolated selectively from the crude histone preparations of calf thymus by means of GL-affinity column chromatography (HPLC); (ii) phosphorylation of these two histones by A-kinase was remarkably stimulated by native GL or oGA (a derivative of glycyrrhetic acid) at 20 μ M; and (iii) in the crude histone preparations of calf thymus, these two histones were selectively phosphorylated by A-kinase in the presence of both dsDNA and 20 μ M oGA or 20 μ M GL. The provided data suggest that the GL-induced selective phosphorylation of histones H2A and H2B by A-kinase may be implicated in the transcriptional activation involved in the biological activities of the drug. — glycyrrhizin; glycyrrhetic acid derivative; A-kinase; histone phosphorylation; anti-inflammatory effect

Glycyrrhizin (GL), a molecule of glycyrrhetic acid (GA) and two molecules of glucuronic acid, is one of the aqueous extracts from roots of the licorice, *Glycyrrhizin radix*, and is known for its anti-inflammatory property in Chinese

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Abbreviations: GL, glycyrrhizin; GA, glycyrrhetic acid; oGA, olean-11, 13 (18)-diene-3 β , 30-diol 3 β , 30-di-*o*-hemipthalate 2Na; A-kinase, cAMP-dependent protein kinase; CK-II, casein kinase II; dsDNA, double-stranded DNA; IFN γ , γ -type interferon; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

medicine (Finney and Somers 1959; Dargan and Subak-Sharpe 1985). It has an anti-viral activity in several RNA viruses (Pompei et al. 1979), including human immunodeficiency type I virus (HIV-I) (Ito et al. 1987), and an inducing activity of γ -type interferon (IFN γ) (Abe et al. 1982). Although recently it has also been reported that GL induces selectively extrathymic T cells in mouse liver at its clinical dose (Kimura et al. 1992), the biochemical mechanisms involved in these biological effects of GL remain to be elucidated. In the metabolism of cortisol in rat, GA inhibits both hepatic Δ^5 -reductase and 11β -hydroxysteroid dehydrogenase in a dose-dependent manner and this inhibition results in the decrease of conversion of cortisone, dihydrocortisol and tetrahydrocortisol from cortisol (Kojima et al. 1990).

Recently, we reported that (i) phosphorylation of lipocortin I by A-kinase is highly stimulated at the GL concentrations between 10 μ M and 20 μ M (Shamsa et al. 1991; Ohtsuki et al. 1992); (ii) one (olean-11,13 (18)-diene-3 β , 30-diol 3 β , 30-di-*o*-hemiphthalate 2Na, oGA) of the GA derivatives is the most effective compound for stimulation of the activity of A-kinase (Shamsa et al. 1991; Ohtsuki et al. 1992); and (iii) lipocortin I was identified as one of GL-binding proteins (Ishikawa et al. 1990; Ohtsuki et al. 1992). On the other hand, recent studies (Felsenfeld 1992; Grunstein 1992) on the physiological functions of histones revealed that these DNA-binding basic proteins are vital participants in gene regulation (Felsenfeld 1992). Therefore, the present study has been undertaken (i) to characterize histones as GL-binding polypeptides, which may function as mediators involved in the biological activities of GL; and (ii) to investigate mainly the biochemical effects of oGA on the phosphorylation of histones by A-kinase in vitro. Here, we describe (i) selective separation of histones (H2A and H2B) as a H2A-H2B pair from the crude histone preparations by means of GL-affinity column chromatography; (ii) the stimulatory and inhibitory effects of GL and oGA on the phosphorylation of histones by A-kinase; and (iii) the selective phosphorylation of histones H2A and H2B by A-kinase in the presence of both dsDNA and oGA or GL. From the results of these experiments, we can propose that the GL- and oGA-induced stimulation of the A-kinase catalyzed selective phosphorylation of histones H2A and H2B may be implicated in the transcriptional activation involved in the anti-inflammatory effect of the drug.

MATERIALS AND METHODS

GL and oGA

Native GL and oGA (olean-11, 13(18)-diene-3 β , 30-diol 3 β , 30-di-*o*-hemiphthalate 2Na) (Fig. 1) were kindly supplied by Minophagen Pharmaceutical Co., Ltd. (Tokyo). A 10 mM preparation of these two compounds in 1% dimethylsulphonyl sulphate (DEMSO) was diluted with sterilized water. These drug preparations were freshly made for each experiment and used immediately.

(a) GL

(b) oGA

Fig. 1. The chemical structures of GL (a) and oGA(b).

Histones

Whole histone preparations (types II-A and III-S) of calf thymus were obtained from Sigma Chemical Corp. (St. Louis, MO, USA). Other crude histone fractions from rat liver were prepared by the modified method described originally by Strickland et al. (1980). Purified histones (H1, H2A, H2B, H3 and H4) of calf thymus were obtained from Boehringer-Mannheim Biochemicals (Mannheim, Germany).

GL-affinity column

A GL-affinity column was prepared as described originally by Nakamura et al. (1990) using Tresyl-5PW (packing gel for HPLC, Tosoh Mfg. Co., Ltd., Tokyo) and *N*-(Glycyrrhiziny)-30 α -Lysine.

Purification of A-kinase from Ehrlich ascites tumor cells

To purify A-kinase in the 0.35 M KCl extract of Ehrlich ascites tumor cells, the extract was applied on a column of DEAE-cellulose, equilibrated previously with 20 mM Tris-HCl (pH 7.6) containing 2 mM dithiothreitol, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.2 M NaCl and 10% glycerol. A-kinase was eluted step-wise from the column with 0.5 M NaCl. A-kinase in the DEAE-cellulose was further purified by gel filtration on Sephacryl S300 and phospho-cellulose column chromatography, successively. Finally, the partially purified A-kinase (phospho-cellulose fraction) was purified by Mono Q column chromatography (high-performance liquid chromatography, HPLC), as previously reported (Shamsa et al. 1991). The purified A-kinase was assayed in the presence of 2 μ M cAMP using whole histone (type II-A) of calf thymus or the indicated histones (calf thymus and rat liver) as a phosphate acceptor, as previously reported (Shamsa et al. 1991; Ohtsuki et al. 1992).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography

The 32 P-labeled polypeptides in the crude and purified histone fractions were analyzed by SDS-PAGE after incubation of the fractions with purified A-kinase and [γ - 32 P]ATP in the presence or absence of either GL or oGA, as previously reported (Ishikawa et al. 1990; Shamsa et al. 1991; Ohtsuki et al. 1992). After electrophoresis, the 32 P-labeled polypeptides on the gel were detected by exposure (approximately 6 hr at -80°C) to x-ray film (Fuji-aif RX, Tokyo).

RESULTS AND DISCUSSION

The stimulatory effects of GL and oGA on the phosphorylation of histones by A-kinase in vitro

As previously reported (Shamsa et al. 1991; Ohtsuki et al. 1992), A-kinase highly phosphorylated histones (H2A, H2B, H3 and H4) when whole histone (type II-A) of calf thymus was incubated with purified A-kinase and [γ - 32 P]ATP in the absence of GL or oGA (lane 2, Fig. 2). Under the experimental conditions, the kinase did not phosphorylate histone H1. Phosphorylation of histones H2A, H2B and H3 by the kinase was highly stimulated by GL and oGA. The highest stimulation [approximately 4-fold as compared with the absence of the drugs] was obtained at 20 μ M oGA (lane 4, Fig. 2). 40 μ M oGA inhibited selectively phosphorylation of histones H2A and H2B, but histone H3 and other polypeptides (6.8 kDa, 8 kDa and 10 kDa polypeptides) were still phosphorylated (lane 5); at 100 μ M oGA (lane 7), over 95% of the A-kinase catalyzed phosphorylation of these polypeptides was inhibited (Fig. 2). Similar dose effects of the drugs were obtained when other crude histone preparations of rat liver were used as phosphate acceptors (results not shown). These observations suggest that (i) phosphorylation of histones by A-kinase is significantly stimulated by GL or oGA at between 10 μ M and 20 μ M; and (ii) oGA at concentrations above 40 μ M causes

Fig. 2. The effect of GL and oGA on the phosphorylation of whole histone (type II-A) by A-kinase in vitro. To detect histones highly phosphorylated by A-kinase in response to different doses of GL or oGA, whole histone preparation (type II-A) of calf thymus was incubated for 15 min at 30°C with A-kinase and [γ - 32 P]ATP in the presence of various concentrations of either GL or oGA. After incubation, the reaction mixtures were analyzed directly by SDS-PAGE followed by autoradiography. Lane 1, A-kinase alone; lane 2, whole histone preparation (type II-A) after incubation with [γ - 32 P]ATP and A-kinase in the absence of GL or oGA; and whole histone after incubation with A-kinase and 20 μ M [γ - 32 P]ATP in the presence of oGA [10 μ M (lane 3), 20 μ M (lane 4), 40 μ M (lane 5), 60 μ M (lane 6) and 100 μ M (lane 7)] or 20 μ M GL (lane 8).

selective inhibition of the A-kinase catalyzed phosphorylation of histones H2A and H2B, whereas histone H3 and other polypeptides (6.8 kDa, 8 kDa and 10 kDa polypeptides) in the crude histone preparations of calf thymus remain to be detectable as phosphorylated polypeptides. This differential phosphorylation of histones H2A and H2B versus histone H3 and other polypeptides (6.8 kDa and 8 kDa polypeptides) by A-kinase may be due to differences in binding affinity with GL and oGA during activation and inhibition of their phosphorylation by the kinase in vitro. In addition, the 8 kDa and 10 kDa polypeptides were identified as degraded fragments of histone H2A and histone H2B, respectively, by determination of their partial primary structures (data not shown). However, it was not successful to determine the partial primary structure of the 6.8 kDa polypeptide since the protein yield from the gel was extremely low. Ubiquitin (8.4 kDa), a basic nuclear protein, was also contained in the crude histone preparation, but it was distinguished from the histones since it did not function as a phosphate acceptor for A-kinase.

Separation of the histones H2A-H2B pairs from the crude histone preparations of calf thymus by GL-affinity column chromatography

To further investigate the binding affinity of histones H2A and H2B with GL, they were partially purified from whole histone preparation (type II-A) of calf thymus by GL-affinity column chromatography (HPLC). Following elution with a KCl gradient, five fractions (peaks I through V, Fig. 3A) were recognized. SDS-PAGE revealed that the peak V fractions, eluted between 0.6 M and 0.7 M KCl (Fig. 3A), containing histones H2A and H2B and a trace amount of 10 kDa polypeptide (lane 6, Fig. 3B), were eluted between 0.5 M and 0.6 M KCl (peak IV, Fig. 3A), whereas histone H3 (lane 4, Fig. 3B) was eluted between 0.45 M and 0.50 M KCl (peak III, Fig. 3A). The 6.8 kDa and 8 kDa polypeptides were detected in the peak II fraction (Fig. 3A), as phosphorylated polypeptides, on separate incubation of these two fractions with A-kinase and 20 μ M [γ - 32 P]ATP in the presence of 60 μ M oGA, as shown in the result (lane 6) in Fig. 2. No phosphorylated polypeptides were detected in the peak I (breakthrough fraction). The elution profiles (Fig. 3A) of the histones in the crude histone preparation (type II-A) of calf thymus by GL-affinity column chromatography were confirmed using purified histone preparations (H1, H2A, H2B, H3 and H4) under the same experimental conditions. These results suggest that (i) histones H2A and H2B have a similar binding affinity with GL; and (ii) these two histones have a higher affinity to the drug rather than the other histones (H1, H3 and H4).

The stimulatory effect of oGA on the selective phosphorylation of histones H2A and H2B by A-kinase in the presence of dsDNA

To determine the dose effect of oGA on the phosphorylation of histones H2A, H2B and H3 by A-kinase, these three purified histones were incubated separately

Fig. 3. [A] Separation of histones H2A and H2B from crude whole histone preparation of calf thymus by GL-affinity column chromatography (HPLC). Whole histone preparation (type II-A) was applied on a GL-affinity column (HPLC), equilibrated previously with 20 mM potassium phosphate buffer (pH 7.0) containing 0.2 M KCl. Elution was carried out with a linear gradient between 0.2 M and 0.8 M KCl, and fractions (1.0 ml) were collected. Absorbance at 280 nm (—). [B] Polypeptides of the indicated peak fractions (I through V in [A]) were analyzed by SDS-PAGE, and were stained with Coomassie brilliant blue R-250. Lane 1, whole histone preparation (type II-A); lane 2, peak I (breakthrough fraction); lane 3, peak II; lane 4, peak III; lane 5, peak IV; lane 6, peak V; and purified calf thymus histones H2B (lane 7) and H2A (lane 8).

with A-kinase and 20 μM [$\gamma\text{-}^{32}\text{P}$]ATP in the presence of various concentrations of oGA. It was found that (i) phosphorylation of histones H2A and H2B was stimulated about 4-fold by 20 μM oGA; and (ii) a similar stimulation was observed with histone H3 in the presence of 30 μM oGA was (Fig. 4B), but a higher concentration (about 200 μM) of oGA was required to inhibit completely phosphorylation of histone H3 by the kinase. Interestingly, histones H2A and H2B were phosphorylated selectively by A-kinase when whole histone (type II-A) of calf thymus was incubated with the kinase and 20 μM [$\gamma\text{-}^{32}\text{P}$]ATP in the presence of both dsDNA (30 μg) and 20 μM oGA (lane 5, Fig. 5). Similar results were obtained when histone mixtures (H1, H2A, H2B, H3 and H4, 2 μg each) were

Fig. 4. [A] The dose effects of oGA on the phosphorylation of histones H2A and H2B by A-kinase in vitro. To determine the dose effect of oGA on the phosphorylation of histones H2A and H2B by A-kinase, the purified histones (5 μ g each) were incubated separately for 15 min at 30°C with the kinase (0.1 μ g) and 20 μ M [γ - 32 P]ATP in the presence of various concentrations of oGA, and the directly analyzed by SDS-PAGE followed by autoradiography. Purified histone H2A (a), histone H2B (b) and histone H3 (c) were incubated separately with A-kinase and 20 μ M [γ - 32 P]ATP in the presence (lanes 3 through 10) or absence (lane 2) of oGA [5 μ M oGA (lane 3); 10 μ M (lane 4); 20 μ M (lane 5); 30 μ M (lane 6); 50 μ M oGA (lane 7); 100 μ M oGA (lane 8); and 200 μ M oGA (lane 9)]; and A-kinase alone (lane 1). [B] Comparison of the stimulatory effects of oGA on the phosphorylation of histones H2A, H2B and H3 by A-kinase. Each spot on the film (a, b and c, Fig. 3A) was scanned with a densitometer. 100% represents the degree of phosphorylation of histones [H2A (○), H2B (●) and H3 (■)] by A-kinase in the absence of oGA.

incubated with the kinase in the presence of dsDNA (30 μ g) and 20 μ M oGA or 20 μ M GL (data not shown). However, the A-kinase catalyzed phosphorylation of these histones was inhibited significantly by 30 μ g of dsDNA (lane 7, Fig. 5). These results suggest that histones H2A and H2B bound to dsDNA are highly phosphorylated by GL- or oGA-stimulated A-kinase. As expected, the DNA binding ability of histones decreased in proportion to the rate of their phosphorylation by A-kinase (data not shown). However, it failed to detect the stimulatory effect of some steroid hormones (cortisone, androgen and estrogen), which have a chemical structure similar to GL, on the A-kinase catalyzed phosphorylation of histones H2A and H2B in vitro.

It seems likely, therefore, that the GL-induced stimulation of the A-kinase

Fig. 5. Selective phosphorylation of histones H2A and H2B by A-kinase in the presence of both dsDNA and oGA. To determine the effect of dsDNA (calf thymus) on the phosphorylation of whole histone preparation (type II-A, 5 μ g) by A-kinase, the histone preparation was incubated for 20 min at 30°C with A-kinase (0.1 μ g) and 20 μ M [γ - 32 P]ATP in the presence or absence of both dsDNA and oGA. Lane 1, whole histone preparation (type II-A) in the absence of oGA; lane 2, whole histone in the presence of 5 μ M oGA; lane 3, 20 μ M oGA; lane 4, 5 μ M oGA and 30 μ g of dsDNA; lane 5, 20 μ M oGA and 30 μ g of dsDNA; lane 6, 5 μ g of dsDNA; and lane 7, 30 μ g of dsDNA.

catalyzed selective phosphorylation of the two histones H2A-H2B pairs may be involved in the transcriptional activation of certain genes, because (i) the histones H2A-H2B pairs may initiate transcription by their temporary ejection from nucleosomes in promoter regions following their specific phosphorylation (Felsenfeld 1992; Grunstein 1992); and (ii) administration of mouse with relatively low doses of GL results in the induction of IFN γ gene product (Abe et al. 1982) and the induction of extrathymic T cells in liver (Kimura et al. 1992). Although the biological significance of the GL-induced stimulation of the A-kinase catalyzed phosphorylation of the two histones H2A and H2B pairs in vitro is unclear at present, a complicated regulatory mechanism at the transcriptional level may be involved in the anti-inflammatory effects of GL and glucocorticoids, which have a similar chemical structure to GL. This speculation is supported by recent reports (Northrop et al. 1992; Nishio et al. 1993) concerning anti-inflammatory mechanisms, which show that a variety of serum proteins, named acute-phase proteins, such as haptoglobin, hemopexin and fibrinogen are induced by anti-inflammatory drugs and inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF α). These cytokines ultimately augment the synthesis of acute-phase proteins at the transcriptional level since most their genes harbor a cytokine responsive elements (CREs) in the 5'-flanking regions of these genes and a specific binding of DNA-binding transcriptional factors, such as glucocorticoid hormone receptor (GHR), to the specific elements involved in the initiation of these selected genes (Evans 1988; Burnstein and Cidlowski 1989; Kapiloff et al. 1991) is regulated by serine/threonine protein kinases (Denton et al. 1992). In addition, the anti-inflammatory effect of GL is also mediated by other inhibitions induced by the drug, because the doses above

10 μ M of GL inhibit (i) the A-kinase catalyzed phosphorylation of lipocortin I (an inhibitor of phospholipase A2) in vitro (Ohtsuki et al. 1992), (ii) the CK-II catalyzed phosphorylation of a 100 kDa GL-binding protein, which is crossreacted with anti-glucocorticoid hormone receptor, in vitro (Ohtsuki et al. 1994), and (iii) the activities of both hepatic Δ^5 -reductase and 11β -hydroxysteroid dehydrogenase in rat (Kojima et al. 1990). However, to understand the biochemical mechanisms involved in the GL-induced biological effects, such as gene activation and cell activation, it is necessary to investigate in detail the transcriptional regulation of selective genes by specific phosphorylation of the histones H2A-H2B pairs on chromatin by GL-activated A-kinase.

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