Effect of Ginsenoside Rb₁ on Rat Liver Phosphoproteins Induced by Carbon Tetrachloride

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We investigated the effects of ginsenoside Rb₁ (G-Rb₁), a major saponin from Panax ginseng C. A. Meyer, on rat liver protein phosphorylation after intraperitoneal administration of CCl₄ alone or together with G-Rb₁. We found that 118, 63, and 34 kDa proteins were prominently phosphorylated in liver homogenates prepared from CCl₄-administered rats, while these protein-phosphorylations were inhibited in the homogenate prepared from the G-Rb₁ plus CCl₄-administration group. When inhibitors of protein kinases were exogenously added to the homogenates from either the CCl₄-administered group or the G-Rb₁ plus CCl₄-administered group, their phosphorylations were inhibited much more by W-7, an inhibitor of Ca²⁺/calmodulin-dependent protein kinase (CaM-PK), than by H-7, an inhibitor of protein kinase C (C-kinase). Interestingly, only 34 kDa was phosphorylated in homogenates prepared from the corn oil-, G-Rb₁-, and G-Rb₁ plus CCl₄-administered groups by the exogenous addition of sodium fluoride (NaF), an inhibitor of glycogen synthase. Additionally, G-Rb₁ inhibited the Ca²⁺-accumulation induced by CCl₄ both in liver homogenates and microsomes. The above results imply that G-Rb₁ inhibits the CCl₄-induced protein phosphorylations by modulating CaM-PK rather than C-kinase, and that 34 kDa protein may play a different biological role in cellular environment from 118 and 63 kDa proteins. Therefore, a study in which G-Rb₁ is employed as a modulator of critical CCl₄-induced phenomena ranging from the disturbance of Ca²⁺ concentration to protein phosphorylation may be successfully applicable to investigate the diverse physiological functions of liver.

Key words ginsenoside Rb₁; carbon tetrachloride; protein phosphorylation; rat liver

Protein phosphorylation is induced by many different protein kinases in cells and provides an important regulatory currency of cellular physiological functions.¹⁻³ Glycogen synthase is inactive in synthesizing glycogen when it is phosphorylated in liver.⁴ There have been many reports that the hepatotoxin, carbon tetrachloride (CCl₄) alters glycogen metabolism in liver,⁵⁻⁶ though the exact mechanism of CCl₄-induced decrease of liver glycogen is not fully explained. Recently, it has been reported that CCl₄-treatment increases intracellular Ca²⁺ concentration and induces calmodulin (CaM) alterations.⁷⁻⁸ Such reports fortify the prevailing presumption that the CCl₄-induced inhibition of glycogen synthesis is mediated by protein kinases' actions.

Ginsenoside Rb₁ (G-Rb₁) (Fig. 1), a kind of ginseng glycoside from the root of Panax ginseng C. A. Meyer (Araliaceae), is known to increase liver glycogen in abnormal conditions like diabetes.⁹⁻¹⁰ Therefore, we set out to investigate in this study whether treatment using CCl₄ increases phosphoproteins, whether G-Rb₁ has inhibitory effects on CCl₄-induced phosphorylation, and which, if any, kinases are responsible for the action of CCl₄ and, among them, which kinases are mainly affected by the treatment using G-Rb₁.

MATERIALS AND METHODS

Materials Figure 1 shows the structure of G-Rb₁ (M.W. 1108) that was tested. G-Rb₁ was purified as described by Paik et al.¹¹ from Ginseng Radix Rubra (red ginseng), which had been processed by drying and steaming fresh ginseng and was kindly supplied by the analysis center of the Korea Ginseng and Tobacco Research Institute (Taejon, Korea). [γ-³²P]ATP was purchased from Amersham Life Science Co. X-Ray film for autoradiography was from Fuji Co. The other chemical reagents were obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

Animals and Administration Sprague Dawley rats (male, 200 g) were grouped by fives and given normal laboratory diet and fresh tap water throughout the experiment. CCl₄ was diluted with corn oil and G-Rb₁ was dissolved in saline. The corn oil- or CCl₄-treated groups were injected once intraperitoneally (i.p.) with 0.35 ml per kg of body weight. The saline- or G-Rb₁-treated groups were injected i.p. with 10 mg per kg of body weight once a day for 3 d. To the G-Rb₁ plus CCl₄-

![Fig. 1. Structure of G-Rb₁](image-url)
treated group, after injecting 10 mg of G-Rb\textsubscript{1} per kg of body weight once a day for 3 d, CCl\textsubscript{4} was injected once with 0.35 ml per kg of body weight. After the respective treatments, all rats were fasted for 24 h and sacrificed to prepare liver homogenates and microsomes.

**Preparation of Liver Homogenates and Microsomes**

After removing the livers from rats, 5 ml of ice-cold homogenate buffer A (0.25 M sucrose, 5 mM Tris, 5 mM MgCl\textsubscript{2}, pH 7.4) containing 1 mM mercaptoethanol was added per gram of liver. Liver tissues were cut into small pieces and homogenated in a glass potter homogenizer with a tight-fitting teflon pestle (600–1000 rpm, 5 times). Homogenates were filtered using a cotton cheesecloth to remove tissue debris. Part of the filtrate was used as a homogenate fraction and the other was centrifuged at 700 \times g for 10 min. The upper layer was recentrifuged for 10 min at 7000 \times g in buffer A containing 0.1 mM EDTA (buffer B). The supernatant was collected and centrifuged once more for 60 min at 105000 \times g. The pellet was collected and suspended in buffer B and used as a microsomal fraction. All the procedures above were carried out in a cold room at 4°C. The homogenates and microsomes were stored at −70°C immediately after their preparation and thawed at 4°C when assayed.

**Protein Phosphorylations**

After preincubating the liver homogenates containing 100 µg of protein for 3 min at 25°C with either 1-(5-isooquinolylsulfanyl)-2-methyl-piperazine (H-7, 6 µM),\textsuperscript{12} N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7, 25 µM),\textsuperscript{13} sodium fluoride (NaF, 50 mM) or nothing, the phosphorylation reaction was initiated by adding 2 µCi of [γ-\textsuperscript{32}P]ATP. The above reaction buffer did not contain Ca\textsuperscript{2+} because 3.35 ± 0.39 mmol/g-tissue of Ca\textsuperscript{2+} already existed in the liver homogenate from the CCl\textsubscript{4} treated-group and, in the amount of 1.67 ± 0.32 mmol/g-tissue in the control group. The reaction was stopped after 5 min by adding an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (0.125 M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8). The reaction tubes were boiled for 5 min to completely denature the proteins. Twenty µg of protein were taken from each reaction tube and subjected to SDS-PAGE (10%, 1.5 mm gel) according to the method of Laemmli.\textsuperscript{14} Separated proteins were stained, destained, dried and autoradiographed onto X-ray film (Fuji Medical X-ray film) at −70°C for 15 d.

**Determination of Ca\textsuperscript{2+}**

The levels of Ca\textsuperscript{2+} in liver homogenates and microsomes were determined by the method of Moore.\textsuperscript{15} Homogenates and microsomes were dried overnight at 95°C, then ashed for 72 h at 600°C. The ashed samples were collected in 0.1 N HCl solution containing 0.1% LaCl\textsubscript{3}. One ml of supernatant was assayed with an atomic absorption spectrophotometer (Varian, Spect AA-30).

**Other Methods**

Liver glycogen was determined by the method of Roe and Daily.\textsuperscript{16} Protein concentration was determined by the method of Lowry et al.\textsuperscript{17}

**RESULTS**

**Suppression of G-Rb\textsubscript{1} on the CCL\textsubscript{4}-Induced Liver Glyco-**

Fig. 2. Protein Phosphorylations Induced by in Vivo Treatment of Either CCL\textsubscript{4} or G-Rb\textsubscript{1} in Rat Liver

Animal treatments and protein phosphorylations were performed as described in Materials and Methods. Lane 1: corn oil-treated group used as control of CCL\textsubscript{4}-treated group. Lane 2: saline-treated group used as control of G-Rb\textsubscript{1}-treated group. Lane 3: G-Rb\textsubscript{1}-treated group. Lane 4: CCL\textsubscript{4}-treated group. Lane 5: G-Rb\textsubscript{1} plus CCL\textsubscript{4}-treated group.

**Gen Depletion** Levels of glycogen were very low in the CCL\textsubscript{4}-treated group relative to the corn oil-treated group and was nearly normalized in the G-Rb\textsubscript{1} plus CCL\textsubscript{4}-treated group. The slightly decreased glycogen in the saline-treated group may be explained as 155 mM Cl− ions are present in the saline solution (0.9% NaCl) which was used for dissolving G-Rb\textsubscript{1}, and this concentration is higher than the physiological concentration of Cl− (30–50 mM) sufficiently resulting in the inhibition of glycogen synthase phosphatase.\textsuperscript{18}

**G-Rb\textsubscript{1}'s Inhibition of CCL\textsubscript{4}-Induced Protein Phosphorylations**

As the hepatotoxicity of CCL\textsubscript{4} is mediated by increased intracellular Ca\textsuperscript{2+}, which leads to an abnormal protein phosphorylation pattern, we compared the patterns between the CCL\textsubscript{4}-treated group and the other groups (Fig. 2).118, 63, and 34 kDa proteins were most prominently phosphorylated in the homogenate from the CCL\textsubscript{4}-treated group, but their phosphorylations were inhibited in the G-Rb\textsubscript{1} plus CCL\textsubscript{4}-treated group. Both in the group given G-Rb\textsubscript{1} only and the group given corn oil, the control of the CCL\textsubscript{4}-treated group, these proteins were not phosphorylated. However, the saline-treated group, taken as the control of the G-Rb\textsubscript{1}-treated group, showed 118 and 34 kDa phosphorylations. We interpret this unpredictably, as the Ca\textsuperscript{2+} in the saline-treated group was 50–60 mol (Table 1) higher than the group with G-Rb\textsubscript{1}-treatment or corn oil-treatment, which did not show phosphorylations of 118 kDa and 34 kDa. Moreover, Ca\textsuperscript{2+} in total from the CCL\textsubscript{4}-treated group, measured in homogenate, was higher than that of the corn oil-treated group (Table 1), and the protein phosphorylations from the CCL\textsubscript{4}-treated group was dependent upon calmodulin-dependent protein kinase (CaM-PK, Figs. 3, 4). These findings mean that CCL\textsubscript{4}-induced pro-
Table 1. Levels of Ca$^{2+}$ in Liver Homogenates and Microsomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Corn oil</th>
<th>Saline</th>
<th>Rb$_1$</th>
<th>CCl$_4$</th>
<th>G-Rb$_1$ + CCl$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenates (mmol/liver-g)</td>
<td>1.67 ± 0.32</td>
<td>1.73 ± 0.38</td>
<td>1.68 ± 0.16</td>
<td>3.35 ± 0.39***</td>
<td>2.58 ± 0.08**</td>
</tr>
<tr>
<td>Microsomes (ng/protein-mg)</td>
<td>32.10 ± 0.17</td>
<td>35.94 ± 0.89</td>
<td>31.83 ± 0.35</td>
<td>124.70 ± 4.13***</td>
<td>108.60 ± 4.58**</td>
</tr>
</tbody>
</table>

The level of Ca$^{2+}$ was determined as described in Materials and Methods. Corn oil was taken as the control of the CCl$_4$-treated group, saline was taken as the control of the G-Rb$_1$-treated group. Data are given as the mean ± S.D. (n = 3). *** Significant at p < 0.01, ** significant at p < 0.05.

Fig. 3. Inhibitory Effects of CaM-PK Inhibitor on Protein Phosphorylation Induced by CCl$_4$

Protein phosphorylations were performed as described in Materials and Methods. W-7 was added exogenously to the homogenates prepared from the CCl$_4$-treated group or the G-Rb$_1$ plus CCl$_4$-treated group. Lane 1: CCl$_4$-treated group. Lane 2: CCl$_4$-treated group with W-7 (25 µM). Lane 3: G-Rb$_1$ plus CCl$_4$-treated group with W-7 (25 µM).

Protein phosphorylations are inhibited by pretreatment of G-Rb$_1$. As a next step, we investigated which kinases were responsible for the phosphorylation of those above proteins.

Effects of Protein Kinase Inhibitors on Protein Phosphorylation When liver homogenates from the CCl$_4$-treated group were incubated with W-7 (K$_i$ = 25 µM), an inhibitor of CaM-PK, or H-7 (K$_i$ = 6 µM), an inhibitor of protein kinase C (C-kinase) in vitro, the phosphorylation of 118 kDa was more strongly inhibited by W-7 (Fig. 3, lane 2) than by H-7 (Fig. 4, lane 2). The inhibition of 118 kDa by W-7 or H-7 was most prominently observed in the G-Rb$_1$ plus CCl$_4$-treated group (Fig. 3, lane 3 and Fig. 4, lane 4) among the experimental groups. Even though 118 kDa tends to be phosphorylated by both kinases, 118 kDa was much more influenced by CaM-PK than by C-kinase. The inhibition of 63 kDa-phosphorylation was equal following treatment with either W-7 (Fig. 3, lane 2) or H-7 (Fig. 4, lane 2). The phosphorylation of 34 kDa in the homogenate from the CCl$_4$-treated group was inhibited only by W-7 (Fig. 3, lane 2) and was not affected by H-7 (Fig. 4, lane 2). It seems that G-Rb$_1$ inhibits protein phosphorylation by mainly inhibiting CaM-PK.

Effect of NaF, a Phosphoprotein Phosphatase Inhibitor CCl$_4$-Treatment caused the depletion of liver glycogen in our experiment (Table 2). As the CCl$_4$-induced liver glycogen decrease is contributed to the phosphorylation of glycogen synthase, resulting from the inhibition of glycogen synthase phosphatase, we predicted that the same phosphorylation bands would be obtained by the exogenous addition of NaF, a glycogen synthase phosphatase inhibitor, in the homogenates from either the G-Rb$_1$- or G-Rb$_2$ plus CCl$_4$-treated groups in which glycogen was not decreased. 34 kDa-phosphorylation (Fig. 2, lane 4), which was observed in the CCl$_4$-treated group, was also observed by the exogenous addition of 50 mM NaF in the corn oil, G-Rb$_1$, and G-Rb$_1$ plus CCl$_4$-treated groups (Fig. 5, lanes 2, 3 and 4). However, 63 kDa and 118 kDa were hardly phosphorylated in the G-Rb$_1$- and G-Rb$_2$ plus CCl$_4$-treated groups, even with the exogenous addition of NaF (Fig. 5, lanes 3 and 4). It seems that 34 kDa is actively given the actions of both CaM-PK and phosphoprotein phosphatase, and we can not fully explain right now whether the inhibition of 34 kDa phosphorylation is contributed to either the inhibition of CaM-PK, the activation of phosphoprotein phosphatase, or both.

Fig. 4. Inhibitory Effects of C-Kinase Inhibitor on Protein Phosphorylation Induced by CCl$_4$

Protein phosphorylations were performed as described in Materials and Methods. H-7 was exogenously added in the homogenates prepared from the CCl$_4$-treated group or the G-Rb$_1$ plus CCl$_4$-treated group. Lane 1: CCl$_4$-treated group. Lane 2: CCl$_4$-treated group with H-7 (6 µM). Lane 3: G-Rb$_1$-treated group. Lane 4: G-Rb$_1$ plus CCl$_4$-treated group with H-7 (6 µM).
Table 2. Levels of Glycogen in Liver Homogenates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Corn oil</th>
<th>Saline</th>
<th>Rb&lt;sub&gt;1&lt;/sub&gt;</th>
<th>CCl&lt;sub&gt;4&lt;/sub&gt;</th>
<th>G-Rb&lt;sub&gt;1&lt;/sub&gt; + CCl&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>Glycogen (mg/liver-g)</td>
<td>30.3 ± 0.37</td>
<td>29.8 ± 0.80</td>
<td>31.9 ± 0.53**</td>
<td>24.7 ± 0.05***</td>
<td>31.0 ± 0.12***</td>
</tr>
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</table>

The level of glycogen was determined as described in Materials and Methods. Corn oil was taken as the control of the CCl<sub>4</sub>-treated group; saline was taken as the control of the G-Rb<sub>1</sub>-treated group. Data are given as the mean ± S.D. (n = 3). ** Significant at p < 0.01, *** significant at p < 0.05.

G-Rb<sub>1</sub>'s Inhibition of Ca<sup>2+</sup>-Accumulation in Liver Homogenates and Microsomes in Vivo In liver homogenates and microsomes, Ca<sup>2+</sup> was increased significantly in the CCl<sub>4</sub>-treated group compared with the control group, and was decreased significantly in the G-Rb<sub>1</sub> plus CCl<sub>4</sub>-treated group compared with CCl<sub>4</sub>-treated group (Table 1). These results indicate that G-Rb<sub>1</sub> inhibits the CCl<sub>4</sub>-induced hepatopathy in vivo resulting from the sustained levels of Ca<sup>2+</sup>, 7,8,20.

DISCUSSION

It is known that the necrogenic effect of CCl<sub>4</sub> on liver is actively participated by Ca<sup>2+</sup> and CaM. There have been some reports revealing the contributions from each one; one says that thioridazine, an anti-CaM drug, decreases the liver Ca<sup>2+</sup> content in CCl<sub>4</sub>-poisoned animals, 21 and the other says that C-kinase alpha-type was activated in hepatocytes at 24 h after CCl<sub>4</sub>-administration. 20 In our experiment, the action of CCl<sub>4</sub> was mediated more strongly by CaM-PK than by C-kinase when employing a cognate inhibitor of each kinase with a specific concentration, K<sub>i</sub> in order to maximally exclude the crosslinking between kinases. The CCl<sub>4</sub>-induced increase in Ca<sup>2+</sup> concentration, both in the homogenates and in the microsomes, was significantly inhibited by pre-treatment with G-Rb<sub>1</sub> (Table 1), corresponding well with the fact that Ca<sup>2+</sup>-accumulation is a typical phenomenon of CCl<sub>4</sub>-induced hepatopathy. 22 Also, serum glutamate oxaloacetate transaminase (GOT) increased in the CCl<sub>4</sub>-treated group, but returned to normal levels in the G-Rb<sub>1</sub>-pretreated group (data not shown).

Since a CCl<sub>4</sub>-induced liver Ca<sup>2+</sup> increase activates CaM-PK, 12 it is accordingly suggested that G-Rb<sub>1</sub> is able to be involved in alterations of calmodulin. In the protein phosphorylation reaction using a homogenate from CCl<sub>4</sub>-treated group, the CCl<sub>4</sub>-induced phosphorylation of 118 and 34 kDa seems to be under the influence of CaM-PK among phosphorylating bands according to the following evidence. 118 and 34 kDa were strongly phosphorylated and were extensively inhibited by the inhibitor of CaM-PK in the CCl<sub>4</sub>-treated group. Also, the CCl<sub>4</sub>-induced 118 and 34 kDa phosphorylations were distinctly inhibited by pre-treatment with G-Rb<sub>1</sub>, and these inhibitions were mediated by G-Rb<sub>1</sub>'s acting on CaM-PK. These proteins were also phosphorylated in the saline-treated group, the control for the G-Rb<sub>1</sub>-treated group, more than in the G-Rb<sub>1</sub>-treated group (Fig. 2), and the levels of total Ca<sup>2+</sup> (Table 1) and glycogen (Table 2) in the saline-treated group were above and below that of the G-Rb<sub>1</sub>-treated group, respectively. Because the increased Ca<sup>2+</sup> in total, by 50 μmol, in the saline-treated group could activate CaM-PK, leading to the phosphorylation of glycogen synthase, and since the 155 mM Cl<sup>-</sup> ions, present in saline solution, were higher than the physiological concentration (30—50 mM) inhibiting glycogen synthase phosphatase, likely as the F<sup>-</sup> ion of NaF, 18,19) we can successfully dissolve the discrepancy between the saline-treated group and G-Rb<sub>1</sub>-treated group. Particularly, 34 kDa was only phosphorylated in the saline-treated group and a similar phosphorylating pattern was present when using the homogenate from the G-Rb<sub>1</sub> plus CCl<sub>4</sub>-treated group with the exogenous addition of NaF (Fig. 5).

According to the reported molecular weight of Ca<sup>2+</sup>-ATPase, a plasma membrane ATP-dependent Ca<sup>2+</sup> transporter (M.W. 118 kDa) 23 or endoplasmic reticular Ca<sup>2+</sup>-ATPase (M.W. 118, 63 kDa), 24,25 the 118 and 63 kDa (Fig. 2) observed in our experiments are very similar to the previously reported Ca<sup>2+</sup>-ATPase, but it is not evident at the present whether G-Rb<sub>1</sub> regulates Ca<sup>2+</sup>-concentration by regulating Ca<sup>2+</sup>-ATPase in vivo.

Because NaF is a general inhibitor of protein phosphatases, we employed it expecting the exposure of a nascent state of 34 kDa only given upon the action of protein kinases and stripped off the action of phosphatases. As expected, 34 kDa was only phosphorylated in Fig. 5. Effects of NaF on Protein Phosphorylation

Protein phosphorylations were performed as described in Materials and Methods. NaF was added exogenously to the homogenates prepared from the corn oil-treated group taken as the control of the CCl<sub>4</sub>-treated group, the G-Rb<sub>1</sub>-treated group or the G-Rb<sub>1</sub> plus CCl<sub>4</sub>-treated group. Lane 1: corn oil-treated group. Lane 2: corn oil-treated group with NaF (50 mM). Lane 3: G-Rb<sub>1</sub>-treated group with NaF (50 mM). Lane 4: G-Rb<sub>1</sub> plus CCl<sub>4</sub>-treated group with NaF (50 mM).
the homogenates from the corn-oil-, G-Rb₁⁻, and G-Rb₁, plus CCl₄-treated groups by the exogenous addition of NaF. At the same time, the glycogen level of the CCl₄-treated group was largely decreased from the control, the corn oil-treated group (p<0.01), whereas that of the G-Rb₁ plus CCl₄-treated group was significantly increased (p<0.01). Although it is too early to conclude that 34 kDa is a glycogen synthase, 34 kDa seems unquestionably to be deeply involved in the regulation of glycogen synthesis.

On the other hand, there have been reports which stated that the primary cause of the hepatotoxicity of CCl₄ is a highly reactive free radical-CCl₃, which resulted from the conversion of microsomal cytochrome P-450's action, and that this radical causes a malfunction of the microsomal ATP-dependent Ca²⁺-pump.²⁶) Recently, it has also been suggested that CCl₄ acts as a suicide inhibitor of certain types of cytochrome P-450 (P450) isozymes.²⁷) However, a distinct shape was observed in our experiment, not yet published. In our experiment, P450 content and NADPH-dependent cytochrome P450 reductase (NADPH-P450-reductase) activity in microsomes from the CCl₄-treated group were 0.88±0.02 nmol/mg-protein and 51.33±2.49 nmol/mg-protein/min, respectively and these are lower than those of the corn oil-treated group, the control for the CCl₄-treated group, which were 1.27±0.01 nmol/mg-protein and 65.45±2.16 nmol/mg-protein/min. Those parameters in the G-Rb₁⁻-treated group were not much different from the control, which were 1.17±0.01 nmol/mg-protein and 64.59±0.33 nmol/mg-protein/min, implying that G-Rb₁⁻ itself does not massively disturb the metabolic environment of P450. However, much more work is to be done in order to establish whether or not G-Rb₁⁻ makes changes in the P450 system and whether those changes are affected by CCl₄ in vivo.

From the above results, it is clear that G-Rb₁⁻ is involved in the inhibition of Ca²⁺ accumulation and glycogen reduction induced by the in vivo treatment of CCl₄. However, whether the action of G-Rb₁⁻ on the CCl₄-induced phosphorylation of 34 kDa is mediated by phosphoprotein phosphatase or CaM-PK is not elucidated in this work, therefore, future work remains to be done on that subject.

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REFERENCES