Ochnaflavone, Naturally Occurring Biflavonoid, Inhibits Phospholipase A₂ Dependent Phosphatidylethanolamine Degradation in a CCl₄-Induced Rat Liver Microsome

Tae Chul MOON,^a Hwa Shin HWANG,^a Zhejiu QUAN,^a Kun Ho SON,^b Cheorl-Ho KIM,^{*,c} Hyun Pyo KIM,^d Sam Sik KANG,^e Jong Keun SON,^a and Hyeun Wook CHANG^{*,a}

^a College of Pharmacy, Yeungnam University; Gyeongsan 712-749, Korea; ^b Department of Food and Nutrition, Andong National University; Andong 760-749, Korea; ^c Department of Biological Science, Sungkyunkwan University and National Research Laboratory for Glycobiology; Chunchun-Dong, Jangan-Gu, Suwon City, Kyunggi 440-746, Korea; ^d College of Pharmacy, Kangwon National University; Chunchen 200-701, Korea; and ^e Natural Products Research Institute, Seoul National University; Seoul 110-460, Korea. Received June 12, 2006; accepted September 19, 2006

This study investigated the protective effects of a group IIA secretory phospholipase A₂ (sPLA₂-IIA) inhibitor, ochnaflavone, on the progression of carbon tetrachloride (CCl₄)-induced acute liver injury in rat liver microsomes *in vitro*. When rat liver was incubated at 37°C in the presence of CCl₄, the level of phosphatidylethanolamine (PE) degradation increased markedly compared with the control. The rat 14 kDa platelet PLA₂ antibody, R377, suppressed the degradation of PE. Pretreating the microsome with ochnaflavone (2–16 μM) reduced the level of PE degradation in a dose dependent manner. In addition, *p*-bromophenacyl bromide (*p*-BPB), which is a PLA₂ inhibitor, also inhibited PE degradation. However, the inhibitory activity was weaker than that of ochnaflavone. Further investigation showed that ochnaflavone not only inhibited the purified rat platelet sPLA₂ activity in a dose dependent manner with an IC₅₀ value of 3.45 μM, when arachidonyl PE was used as a substrate, but also inhibited lipid peroxidation in a dose dependent manner with an IC₅₀ value of 7.16 μM. This result suggests that ochnaflavone prevents the progression of CCl₄-induced PE hydrolysis by inhibiting the endogenous sPLA₂ activity.

Key words ochnaflavone; biflavonoid; secretory phospholipase A₂; CCl₄; rat liver microsome; lipid peroxidation

The flower whole plants of *Lonicera japonica* have long been used as an antidote, diuretic, tonic, antipyresis and anti-inflammatory agent. The anti-inflammatory and analgesic activity of a water extract and its *n*-butanol (*n*-BuOH) fraction of *L. japonica* were previously reported.¹⁾

Ochnaflavone (Fig. 1), which was isolated from *L. japonica*, was found to be inhibitor of sPLA₂-IIA from rat platelets, and similar results were also obtained with morelloflavone.^{2,3)} Recently, it was reported that this compound inhibits LPS-induced iNOS expression, which is mediated by ERK1/2 via NF-κB regulation, in RAW264.7 cells.⁴⁾ In addition, biflavonoids such as ginkgetin and ochnaflavone (OC) were reported to be dual inhibitor of cyclooxygenase-2 and 5-lipoxygenase from the mast cells as well as inhibit the release of the arachidonic acid metabolites from the rat peritoneal macrophages stimulated by either phorbol ester or calcium ionophore, A₂₃₁₈₇.^{5–7)}

It has been suggested that CCl₄-induced stimulation of

peroxide breaks down the membrane lipids, which leads to PLA₂ activation.^{8–10)} Evidence of PLA₂ activation was also obtained in isolated hepatocytes treated with CCl₄ in *in vitro* and *in vivo* animal models.^{11,12)} Phospholipids hydrolysis by PLA₂ generates potentially bio-active lipids, such as free fatty acids including arachidonic acid and lysophospholipids that can promote inflammatory process. Arachidonic acid is converted to prostaglandins (PGs) and thromboxanes (TXs) via the cyclooxygenase (COX) pathway to leukotriens (LTs) via 5-lipoxygenase (LOX) pathway. These lipid derivatives have proinflammatory and vasoconstrictive actions and contribute to liver injury or post ischemic organ dysfunction. The potential importance of PLA₂ activity in CCl₄-induced cell injury and ischemic model is supported by the findings that PLA₂ inhibitors offer protection against phospholipids degradation in liver thereby reducing cell injury.^{13–15)}

Therefore, the aim of this study was to determine the protective role of sPLA₂ inhibitor, OC against CCl₄ induced PE degradation.

MATERIALS AND METHODS

Materials OC was isolated from the flower and leaves of *L. japonica*. The isolated compound was chemically and structurally identified according to the previous reports.¹⁶⁾ OC used in this study showed a single spot on TLC and was prepared by dissolving in dimethyl sulfoxide (DMSO) and final concentrations of DMSO were adjusted to 0.1% (v/v) in culture media. Control with DMSO alone was run in all cases.

Preparation of Liver Microsomes Livers obtained from rat were removed rapidly, and 10% (w/v) liver homogenated

Fig. 1. Chemical Structure of the Ochnaflavone

* To whom correspondence should be addressed. e-mail: hwchang@yu.ac.kr; chkimbio@mail.dongguk.ac.kr

in ice-cold buffer were prepared using Potter-Elelenmeyer teflon homogenizer. The homogenates were centrifuged at $9000\times g$ for 10 min at 4°C , recentrifuged at $12000\times g$ for 20 min to remove any contamination of mitochondria and then centrifuged at $105000\times g$ for 60 min to yield the microsomal fraction and the supernatant fraction was re-centrifuged $13000\times g$ for 10 min at 4°C . Then the supernatant fraction was ultracentrifuged for 1 h and the pellet fraction was suspended with 1 mM Tris-HCl (pH 7.4) include 0.15 M NaCl.

Lipid Analysis One milligram of rats liver microsome was indicated amounts of rat platelet PLA₂ antibody R377 (a gift from Dr. Kudo Ichiro Showa University, Tokyo, Japan) and rat IgG or OC were pretreated 30 min on ice and additional 90 min incubated at 37°C in the presence of 8 mM CCl₄/0.3 mM NADPH with shaking. The reaction was stopped by the addition of 1 ml of chloroform and 1 ml of methanol. Total lipids in the reaction mixtures were extracted twice by the method of Bligh and Dyer.¹⁷⁾ The combined chloroform phase was evaporated to dryness under reduced pressure, dissolved in a small volume of chloroform-methanol (2:1, v/v), and subjected to thin layer chromatography. Individual phospholipids were separated by two-dimensional silica gel thin layer chromatography (No. 5721; Merck, Darmstadt, Germany, 20×20 cm, silica gel 60, without a fluorescent indicator) with an initial solvent system of chloroform-methanol-acetic acid (65:25:10, v/v/v) and a second system of chloroform-methanol-88% aqueous formic acid (65:25:10, v/v/v). The TLC plates were exposed with iodine vapor to visualize phospholipid areas. After evaporation of the iodide, the area corresponding to each phospholipid was scraped from the plate and the power was used for determination of lipid phosphorus was determined according to the method described previously.¹⁶⁾

Assay of Phospholipase A₂ Activity The standard reaction mixture (200 μl) contained 100 mM Tris-HCl (pH 9.0), 6 mM CaCl₂, 1% BSA, 2.5 μM radiolabeled 1-acyl-2-[1-¹⁴C] arachidonoyl-*sn*-glycerol-3-phosphorylethanolamine (48 mCi/m mole, NEN), and OC. The reaction was started by addition of 20 ng of purified rat platelet PLA₂ and carried out at 37°C for 20 min, and [¹⁴C] arachidonic acid released was extracted by the method described previously,¹⁷⁾ and counted in a liquid scintillation counter. Under these conditions, reaction mixture without the OC revealed 10% hydrolysis of the substrate. Inhibition was expressed as a percent regarding the enzyme activity in the absence of OC as 0% inhibition. OC was solved in dimethylsulfoxide (DMSO) and added to the enzyme assay tubes at 2% of the final volume. Control experiments showed that DMSO at concentrations up to 2% had no effect on enzymatic activity. All data are the mean of duplicate determinations. The 14 kDa rat platelet sPLA₂ was purified as described previously.¹⁸⁾

Determination of Malonaldehyde Lipid peroxidation was estimated by accumulation of MDA using the TBA-test.¹⁹⁾ One milligram of rats liver microsome was incubated at 37°C for 90 min in the presence of 8 mM CCl₄/0.3 mM NADPH with shaking. The reaction was stopped by the addition of 0.4 ml of 10% sodium dodecyl sulfate (SDS) at room temperature for 30 min. The reaction mixture was mixed with 2 ml of 0.1 N HCl and 1 ml of 1% thiobarbituric and heated for 50 min at 95°C . To avoid interference from turbidity, the

Fig. 2. Effect of Rat 14kDa Platelet sPLA₂ Antibody, R377 and Rat IgG on PE Degradation

One milligram of the rat liver microsomes was first preincubated for 1 h with the indicated amount of R377 and IgG on ice. Additional incubation was then carried out in the presence 8 mM CCl₄/0.3 mM NADPH. Lipid analysis was performed according to Materials and Methods in detail. *** $p < 0.001$ was considered significantly different from the CCl₄-treated mice using a one-way ANOVA test.

coloured complex was extracted into 5 ml of the *n*-butanol phase measured.

Statistical Analysis All values were represented as arithmetic mean $\pm$ S.D. One-way ANOVA was used to determine the statistical significance.

RESULTS AND DISCUSSION

PE degradation proceeded in a time-dependent manner when the rat liver microsomes were incubated *in vitro* at 37°C in the presence of CCl₄. Approximately 20% of total ethanolamine containing glycerophospholipids had been converted to lysoPE after 90 min, whereas levels of other major phospholipids, such as phosphatidylcholine (PC), phosphatidylserin (PS), and spingomyelin (SM) did not change significantly (data not shown).

It should be noted that the observed increase in the levels of PE degradation is consistent with a previous study that reported increases in both PE and PS levels when incubated the liver homogenates obtained from CCl₄-injected rats at 37°C .⁹⁾ Currently, it is unclear why the degradation of PS was not detected in this system. The PE degradation of CCl₄-stimulated rat liver microsomes was examined in order to determine if the endogenous PLA₂ activity mediated this degradation. As shown in Fig. 2, the sPLA₂ antibody, R377,¹³⁾ which was raised against rat platelet 14 kDa sPLA₂ was found to suppress PE degradation, but IgG which was used as control antibody did not prevent PE degradation. This result shows that the degradation of endogenous PE observed in CCl₄-treated rat liver microsome may be mainly due to the activation of enzyme closely related sPLA₂-IIA. The kinetics of PE degradation was examined in rat liver microsomes stimulated with CCl₄ in order to determine if OC inhibits PE degradation. OC inhibited PE degradation in a dose-dependent manner, with an IC₅₀ value of 5.42 μM (Fig. 3A). The inhibitory effects of OC on sPLA₂, which had been purified from rat platelets, were examined to determine if this effect is mediated *via* the direct PLA₂ inhibitory activity. The results showed that OC inhibited the purified rat platelet sPLA₂ activity in a dose dependent manner, with an IC₅₀ value of 3.45 μM (Fig. 3B). This IC₅₀ value is consistent with that reported elsewhere.³⁾ PE degradation was confirmed to occur *via* the activation of PLA₂ activity by observing the effects of *p*-bromophenacyl bromide (*p*-BPB), which is one of well known PLA₂ inhibitor. *p*-BPB inhibited PE degradation

Fig. 3. Effect of OC on PE Degradation (A) and the Inhibitory Activity of OC on Purified Rat Platelet 14 kDa sPLA₂ (B)

One milligram of the rat liver microsomes was first preincubated for 1 h with the indicated concentration of OC on ice. Additional incubation was then carried out in the presence 8 mM CCl₄/0.3 mM NADPH. Lipid analysis and PLA₂ activity were performed according to Materials and Methods in detail. **p*<0.05, ****p*<0.001 were considered significantly different from the CCl₄-treated mice using a one-way ANOVA test.

Fig. 4. Effect of *p*-BPB on PE Degradation (A) and on Purified Rat Platelet 14 kDa sPLA₂ (B)

One milligram of the rat liver microsomes was first preincubated with indicated concentration of *p*-BPB on ice for 1 h and additional incubation was then carried out in the presence of 8 mM CCl₄/0.3 mM NADPH. Lipid analysis and PLA₂ activity were performed according to Materials and Methods. **p*<0.05, ***p*<0.01, ****p*<0.001 were considered significantly different from the CCl₄-treated mice using a one-way ANOVA test.

as well as the purified rat platelet sPLA₂ in a dose dependent manner, with an IC₅₀ value of 230 μM, 990 μM, respectively (Figs. 4A, B). However, the inhibitory activity of *p*-BPB was weaker than that of OC. In addition, OC also inhibited lipid peroxidation in a dose dependent manner, with IC₅₀ value, 7.16 μM, under similar experimental conditions (Fig. 5). However, the relationship between the endogenous sPLA₂ inhibitory activity and the inhibition of lipid peroxidation by OC is still unclear. These results demonstrate that OC can prevent the progression of CCl₄-induced PE hydrolysis by

Fig. 5. Effect of OC on the Lipid Peroxidation

One milligram of the rat liver microsomes was first preincubated for 1 h with the indicated concentration of OC. Additional incubation was then carried out in the presence 8 mM CCl₄/0.3 mM NADPH. Malonaldehyde was measured according to Materials and Methods. **p*<0.05, ****p*<0.001 were considered significantly different from the CCl₄-treated mice using a one-way ANOVA test.

inhibiting the endogenous sPLA₂ activity. Therefore, this compound might provide a basis for novel anti-inflammatory drugs.

Acknowledgment This work was supported by Yeungnam University Research Grant (YN-106120).

REFERENCES

- 1) Lee S. J., Shin E. J., Son K. H., Chang H. W., Kang S. S., Kim H. P., *Arch. Pharm. Res.*, **18**, 133—135 (1995).
- 2) Chang H. W., Baek S. H., Chung K. W., Son K. H., Kim H. P., Kang S. S., *Biochem. Biophys. Res. Commun.*, **205**, 843—849 (1994).
- 3) Gil B., Sanz M. J., Terencio M. C., Gunasegaran R., Paya M., Alcaraz M. J., *Biochem. Pharmacol.*, **53**, 733—740 (1997).
- 4) Suh S. J., Chung T. W., Son M. J., Kim S. H., Moon T. C., Son K. H., Kim H. P., Chang H. W., Kim C. H., *Arch. Biochem. Biophys.*, **447**, 136—146 (2005).
- 5) Son J. K., Son M. J., Lee E., Moon T. C., Son K. H., Kim C. H., Kim H. P., Kang S. S., Chang H. W., *Biol. Pharm. Bull.*, **28**, 2181—2184 (2005).
- 6) Lee S. J., Choi J. H., Son K. H., Chang H. W., Kang S. S., Kim H. P., *Life Sci.*, **57**, 551—558 (1995).
- 7) Son M. K., Moon T. C., Lee E. K., Son K. H., Kim H. P., Kang S. S., Son J. K., Lee S. H., Chang H. W., *Arch. Pharm. Res.*, **29**, 282—286 (2006).
- 8) Glende E. A., Jr., Recknagel R. O., *Toxicol. Appl. Pharmacol.*, **113**, 159—162 (1992).
- 9) Chiarotto E., Biasi F., Marmo G., Poli G., Dianzani M. U., "Eicosanoid, Lipid Peroxidation and Cancer," ed. by Nigam S., Springer Verlag, Berlin, Heidelberg, 1988, pp. 233—241.
- 10) Kikuchi Y. R., Yanoshita R., Kudo I., Arai H., Takamura T., Nomoto K., Inoue K., *J. Biochem. (Tokyo)*, **114**, 33—38 (1993).
- 11) Glende E. A., Jr., Pushpendran C. K., *Biochem. Pharmacol.*, **35**, 3301—3307 (1986).
- 12) Ryu A., Itabe H., Mutoh M., Kudo I., Arai H., Inoue K., *J. Health Sci.*, **46**, 275—281 (2000).
- 13) Estevez A. Y., Phillis J. W., *Brain Res.*, **752**, 203—208 (1997).
- 14) Ogata K., Jin M. B., Taniguchi M., Suzuki T., Shimamura T., Kitagawa N., Magata S., Fukai M., Ishikawa H., Ono T., Furukawa H., Fujita M., Toda S., *Transplantation*, **71**, 1040—1046 (2001).
- 15) Ogata K., Jin M. B., Suzuki T., Taniguchi M., Shimamura T., Fukai M., Furukawa H., Todo S., *Transplant. Proc.*, **33**, 860—861 (2001).
- 16) Son K. H., Park J. O., Chung K. C., Chang H. W., Kim H. P., Kim J. S., Kang S. S., *Arch. Pharm. Res.*, **15**, 365—370 (1992).
- 17) Bligh E. G., Dyer W. J., *Can. J. Biochem. Physiol.*, **37**, 911—917 (1959).
- 18) Gerlach E., Deuticke B., *Biochem. Z.*, **337**, 477—479 (1963).
- 19) Fujimoto Y., Mino T., Fujita T., *Res. Commun. Chem. Pathol. Pharmacol.*, **35**, 173—176 (1982).
- 20) Chang H. W., Kudo I., Tomita M., Inoue K., *J. Biochem. (Tokyo)*, **102**, 147—154 (1987).
- 21) Horigome K., Hayakawa M., Inoue K., Nojima S., *J. Biochem. (Tokyo)*, **101**, 625—631 (1987).