Expression of Protein Kinase C Alpha in Biopsies and Surgical Specimens of Human Hepatocellular Carcinoma

Jen-Hsiang Tsai[1], Min-Ting Tsai[2, 3], Wei-Wen Su[4], Yao-Li Chen[5], Trang-Tiau Wu[6], Yih-Shou Hsieh[2], Chih-Yang Huang[2], Kun-Tu Yeh[3], and Jer-Yuh Liu[2]

[1]*Department of Nursing*
Fooyin University
Kaohsiung 831, Taiwan
[2]*Institute of Biochemistry and Biotechnology, Medical College*
Chung Shan Medical University
Taichung 402, Taiwan
[3]*Department of Pathology, Changhua Christian Hospital*
Changhua 500, Taiwan
[4]*Division of Hepatogastroenterology, Department of Internal Medicine*
[5]*Department of General Surgery*
Changhua Christian Hospital
Changhua 500, Taiwan
and
[6]*Department of Surgery*
School of Medicine, Medical College
Chung Shan Medical University Hospital
Taichung 402, Taiwan
Republic of China

Abstract

Variations in the activity of protein kinase C (PKC) have been observed in different types of tumors. Although these inconsistent findings may be attributed to the alterations of individual PKC isoforms, the effects of general anesthetic may not be neglected. In this study, biopsies and surgical specimens were obtained from patients with HCC, and the levels of PKCα were analyzed by immunohistochemistry. PKCα expression in biopsies was mainly revealed on the cell membrane of hepatocytes whereas that in the surgical specimens was in the cytosol and on the membrane. In both types of specimens, the PKCα level in HCC was significantly higher than that in the adjacent non-tumorous tissue. Moreover, the level of PKCα in biopsies was significantly higher than that in surgical specimens of the corresponding tissue type. These findings suggested that general anesthetics may significantly affect the expression of PKCα, and the effects of anesthetics should not be neglected in observations which were made only based on surgical specimens.

Key Words: anesthetic, hepatocellular carcinoma, protein kinase C alpha

Introduction

Protein kinase C (PKC) is a family of phospholipid-dependent protein kinases involving in the regulation of a wide range of cellular functions (14, 16). Moreover, changes in the activity of this enzyme have been observed in different types of tumors. However, the activity of PKC has been determined to be elevated in

Corresponding author: Dr. Jer-Yuh Liu, Institute of Biochemistry and Biotechnology, Medical College, Chung Shan Medical University, Taichung 402, Taiwan, R.O.C. Tel: 886-4-24730022 ext. 11673, Fax: 886-4-23248195, E-mail: jyl@csmu.edu.tw.
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human breast tumor (18), pituitary tumor (1), and malignant gliomas (4) and reduced in colonic carcinoma (12, 13) and hepatocellular carcinoma (HCC) (25). Although these inconsistent findings may be attributed to the alterations of individual PKC isoforms in the cancer tissue, whether the anesthetic drugs used during the surgical operation modulate the expression of PKC isoforms remains unclear.

PKC has been considered to be a target for general anesthetic action (10). Although n-alknols and volatile anesthetics may inhibit the activity of purified brain PKC (23), halothane and propofol may stimulate PKC activity (6) by stabilizing the active confirmation of PKC through a possible binding site on their target (7, 24). In addition, halothane may also down-regulate the activity of membrane-associated PKC (8) and exposure of phenobarbital to male rat liver may decrease approximately 63% of the levels of cytosolic PKCα (28). It has been concluded that administration of general anesthetics may have potency to induce activation-dependent down-regulation of PKC (9). In this study, we analyzed PKCα in biopsies and surgical specimens from patients with HCC by immunohistochemistry. The results indicated that general anesthetics may affect the expression of PKCα, and the effects of anesthetics should not be neglected in observations which were made only based on surgical specimens.

Materials and Methods

Specimen Collection

Biopsies and surgical specimens of liver tissue were obtained from five patients with HCC from Division of Hepatogastroenterology, Department of Internal Medicine, Changhua Christian Hospital. An informed consent was given to each patient and/or guardian before collection of specimens. Liver biopsies were obtained by an experienced gastroenterologist under the monitoring of ultrasounds. Surgical specimens of human liver cancer and adjacent non-tumorous hepatic tissue were obtained by mastectomies from the same patients who received liver puncture biopsy. The specimens were immediately fixed in 4% formalin after collection.

Preparation of Polyclonal Anti-PKCα Antibodies

PKCα peptide (657-672) was obtained from Dr. Ta-Hsiu Liao, Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei. After coupling to carbonic anhydrase (Sigma, St. Louis, MO, USA) using glutaradehyde (Sigma), the PKCα peptide preparation was immunized to male New Zealand white rabbits (26). Serum samples were then obtained from the rabbits since day 42 after immunization. Antibodies were purified by affinity chromatography on a column bearing the PKCα peptide.

Immunohistochemistry

The formalin-fixed specimens were dehydrated through graded alcohol and embedded in paraffin. Sections (3 μm) were prepared from the paraffin-embedded specimens and then deparaffinized in xylene and rehydrated through an alcohol series. The sections were then incubated with 3% H_2O_2 for 5 min. After washing with phosphate buffer saline (PBS), the sections were heated to boiling in an EDTA buffer containing 1 mM EDTA and 0.1% NP-40 (pH 8.0) for 5 min in a microwave oven. After cooling for 20 min, the sections were washed triplicate in PBS for 5 min and incubated with 3% normal bovine serum in PBS for 25 min. The sections were washed with PBS again and then incubated with the purified polyclonal antibodies to PKCα (10 ng/ml PBS plus 0.2% BSA) at room temperature for 1 h. After washing triplicate in PBS for 5 min, the sections were incubated with peroxidase-labeled goat anti-rabbit IgG (Sigma) at room temperature for 30 min. The sections were then washed with PBS and PKCα antigen staining was visualized by adding 3,3′-diaminobenzidine substrate (Sigma). The reaction was terminated by rinsing the sections in distilled water. The sections were counterstained with Gill's hematoxylin V (Mute pure Chemicals Ltd., Tokyo, Japan), dehydrated through graded alcohol, and cleared with xylene before mounting with Malinol (Mute Pure Chemicals Ltd.). Immunoreactivity of PKCα in the sections was examined using the BX40 system microscope (Olympus, Tokyo, Japan) with a CCD DPII Camera (Olympus, Tokyo, Japan). Images were analyzed by the Image-Pro(r) Plus software (Media Cybernetics, Silver Spring, MD, USA). Two sections were prepared from each specimen, and measurements were obtained from the four quadrants in each section. Corresponding non-specific binding of section performed by parallel incubation with the antibody preneutralized with antigenic peptide was revealed no immnoreactivity (data not shown).

Statistical Analysis

Levels of PKCα were represented by the percentages of the measurements to the median of measurements in the adjacent non-tumorous hepatic tissue obtained by liver puncture and expressed as mean ± standard error of the mean. Differences between groups were analyzed by the unpaired Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Table 1. Clinical characteristics of patients with hepatocellular carcinoma

Patient	Sex	Age (years)	HBs-Ag	Anti-HCV	TNM staging	Histopathology of non-tumorous tissue	Histopathological grading of cancer tissue	Anesthetics used	Anesthetic time (min)
1	F	67	–	+	T1	Normal	Moderately differentiated	Pentothal (250 mg)	135
2	M	67	+	–	T1	Chronic active hepatitis	Well differentiated	Propofol (200 mg)	255
3	M	64	–	–	T2	Normal	Well differentiated	Pentothal (250 mg)	150
4	M	74	–	–	T2	Cirrhosis	Moderately differentiated	Pentothal (250 mg)	100
5	F	55	–	+	T3	Chronic active hepatitis	Moderately differentiated	Pentothal (250 mg)	115

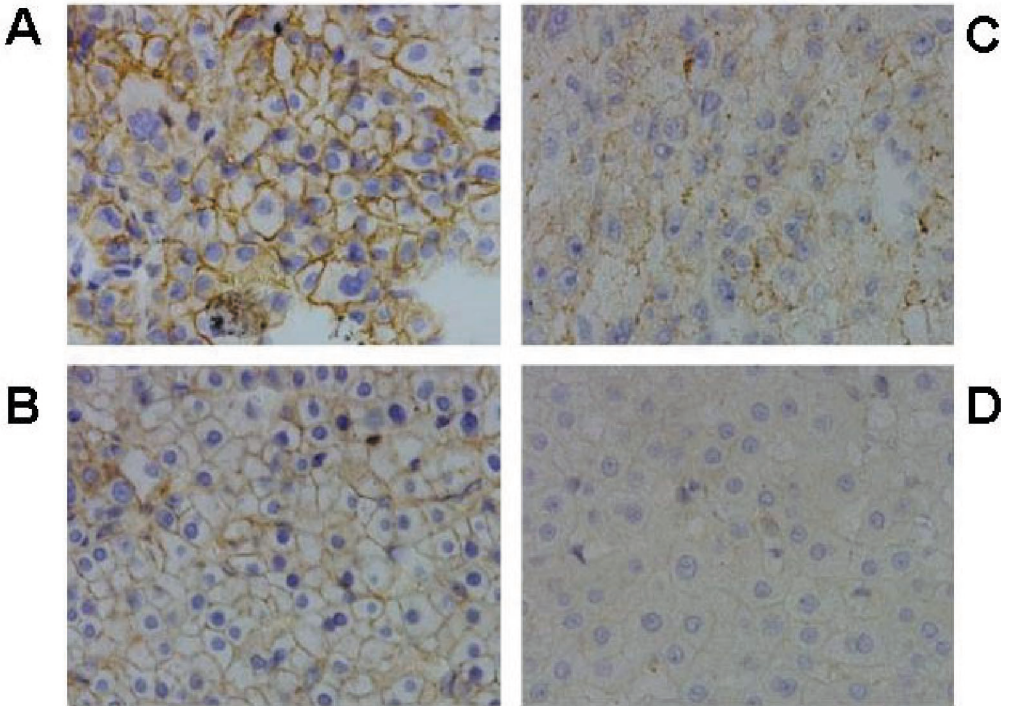

Fig. 1. Sections of human hepatocellular carcinoma (HCC) and adjacent non-tumorous hepatic tissue after incubating with purified polyclonal antibodies to PKCα, and then with peroxidase-labeled goat anti-rabbit IgG before visualization by adding 3,3′-diaminobenzidine substrate. A. Biopsy specimen of HCC. B. Biopsy specimen of adjacent non-tumorous hepatic tissue. C. Surgical specimen of HCC. D. Surgical specimen of adjacent non-tumorous hepatic tissue (magnification, ×400).

Results

The five patients included three males and two females with a mean age of 65.4 ± 3.4 years. One patient was found to be positive for hepatitis B virus surface antigens, and two with hepatitis C virus antibodies. TNM staging identified two T1, two T2, and one T3 lesions. Histopathologic analysis of the tumors revealed two with well-differentiated cells and three with moderate-differentiated cells. Pentothal was administered to four patients and propofol to the remaining one as an anesthetic during the surgery and the anesthetic time ranged from 100 to 255 min (Table 1).

In the biopsy specimens, PKCα expression was mainly revealed on the membrane of the hepatocytes (Fig. 1A and 1B). The level of PKCα in HCC (166.5 ± 8.1%) was significantly higher than that in the non-tumorous hepatic tissue (91.7 ± 3.6%) ($P < 0.001$)

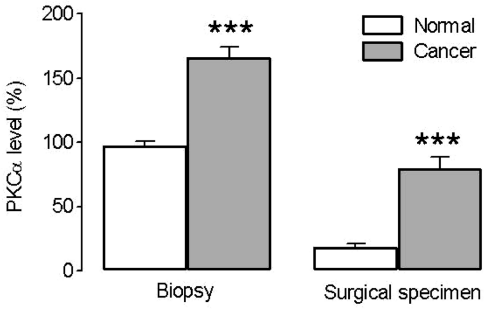

Fig. 2. Comparison of the levels of PKCα in biopsy and surgical specimens of human hepatocellular carcinoma (HCC) and adjacent non-tumorous hepatic tissue. The levels were represented by the percentages of the measurements to the median of measurements in the adjacent non-tumorous hepatic tissue obtained by liver puncture and expressed as mean ± standard error of the mean. HCC *vs.* adjacent non-tumorous tissue: ***$P < 0.001$.

(Fig. 2). In the surgical specimens, PKCα expression was found in the cytosol and on the membrane of hepatocytes (Fig. 1C and 1D), and the level of PKCα in HCC (79.8 ± 8.6%) was also significantly higher than that in the non-tumorous tissue (18.7 ± 2.1%) ($P < 0.001$) (Fig. 2). Moreover, the level of PKCα in biopsies was significantly higher than that in surgical specimens of the corresponding tissue type ($P < 0.001$) (Fig. 2).

Discussion

Although the results in this study were based on the findings in five patients, we prepared two sections from each specimen and four measurements were obtained from each section. This design makes the number of measurements in each group amount to 40, which is sufficient for the comparisons of the different groups.

Barbiturates are a group of intravenous anesthetics. Phenobarbital may inhibit PKC of rat brain by competitively displacing diacylglycerol. This drug appears to occupy the triple hydrogen bonding site which bonds diacylglycerol or phorbol esters to the enzyme (19). Thiamylal, thiopentone, pentobarbitone, mepivacaine or bupivacaine may inhibit PKC purified from rat brain, and are considered to be inhibition of the activation process rather than to direct interaction with the active site of the enzyme (15). Exposure of rat colon to pentobarbital may produce up to 90% inhibition of calcium-dependent PKC activity, whereas calcium-independent activity may stimulate up to 35%. It is suggested that a prior anesthetic use may complicate observations in PKC-mediated effects on epithelial barrier function using epithelial tissue models (22). This study was the first time to compare levels of a PKC isoform in human HCC specimens obtained by liver puncture and surgical resection. The levels of PKCα in cancer or adjacent non-tumorous tissues were significantly higher in liver biopsies than the corresponding levels obtained from the hepatectomy specimens under general anesthesia with pentothal (4 patients) or propofol (1 patient). This change in the PKCα level may be due to ischemia, since the amount of PKC and the phorbol-binding capacity of the enzyme are decreased during ischemia (3).

In the hepatic vein, serum concentration of lipid-peroxidation reactants may increase before and after transient dearterialization of the liver in human subjects with unresectable hepatocellular carcinoma (27). The increase in the level of lipid peroxidation may also occur during ischemia in liver (20) and in hippocampus or cerebral cortex (5). This phenomenon may be correlated with the early PKC translocation to membrane and a sharp immediate increase of a 50-kDa isoform (PKM) and confirms that lipid peroxidation may induce PKC translocation and activation (16). During ischemia, the decrease in the content of phospholipids, phatidylcholine, and phosphatidylethanolamine in rat liver and increase in the content of DAG (19) signify the elevation in inositol phospholipid hydrolysis (21). In this study, the decrease in PKCα level in hepatectomy specimens obtained under general anesthesia with pentothal or propofol might also be due to ischemia, since the amount of PKC and the phorbol-binding capacity of the enzyme decrease during ischemia (19).

In our previous studies, the activity of the membrane-bound PKC was found to be significantly decreased in HCC, whereas no significant difference in the activity was observed in the cytostolic fractions of HCC and adjacent non-tumorous hepatic tissue (2). By Western blotting, the level of membrane-bound PKCα in HCC was demonstrated to be significantly lower than that in the adjacent non-tumorous tissue (25). By immunocytochemistry, we also observed that the activation of PKC in Hep3B cells by a low PMA concentration may cause translocation of PKC isoforms from cytosol to membrane or nucleus (11). It has been reported that halothane may decrease the membrane-associated PKC activity (8). In this study, PKCα expression was mainly revealed on the cell membrane in biopsies, in the cytosol and on the membrane of hepatocytes in surgical specimens. These changes indicated that barbiturates might also have similar effects in the translocation of PKC and the reduction in the PKCα level of in the surgical specimens might be mainly due to stimulation and down-regulation of PKC. Based on the results of this study, we concluded that general anesthetics may significantly affect the expression of PKCα, and the effects of anesthetics should not be neglected in

observations which were made only based on surgical specimens.

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References

1. Alvaro, V., Touratine, P.H., Vozari, R.R., Bai-Grenier, F., Birman, P. and Joubert, D. Protein kinase C activity and expression in normal and adenomatous human pituitaries. *Int. J. Cancer* 50: 724-730, 1992.
2. Chang, K.J., Lin, J.K., Lee, P.H., Hsieh, Y.S., Cheng, C.K. and Liu, J.Y. The altered activity of membrane-bound protein kinase C in human liver cancer. *Cancer Lett.* 105: 211-215, 1996.
3. Chauhan, V.P. and Brockerhoff, H. Phenobarbital competes with diacylglycerol for protein kinase C. *Life Sci.* 40: 89-93, 1987.
4. Couldwell, W.T., Antel, J.P. and Yong, V.W. Protein kinase C activity correlates with the growth rate of malignant gliomas: Part II. Effects of glioma mitogens and modulators of protein kinase C. *Neurosurgery* 31: 717-724, 1992.
5. Domanska-Janik, K. and Zablocka, B. Protein kinase C as an early and sensitive marker of ischemia-induced progressive neuronal damage in gerbil hippocampus. *Mol. Chem. Neuropathol*. 20: 111-123, 1993.
6. Hemmings, H.C. Jr., Adamo, A.I. and Hoffman, M.M. Biochemical characterization of the stimulatory effects of halothane and propofol on purified brain protein kinase C. *Anesth. Analg*. 81: 1216-1222, 1995.
7. Hemmings, H.C. Jr. and Adamo, A.I. Activation of endogenous protein kinase C by halothane in synaptosomes. *Anesthesiology* 84: 652-662, 1996.
8. Hemmings, H.C. Jr. and Adamo A.I. Effect of halothane on conventional protein kinase C translocation and down-regulation in rat cerebrocortical synaptosomes. *Br. J. Anaesth*. 78: 189-196, 1997.
9. Hemmings, H.C. Jr. and Adamo, A.I. Effects of halothane and propofol on purified brain protein kinase C activation. *Anesthesiology* 81: 147-155, 1994.
10. Hemmings, H.C. Jr. General anesthetic effects on protein kinase C. *Toxicol. Lett*. 100-101: 89-95, 1998.
11. Hsu, S.L., Chou, Y.H., Yin, S.C. and Liu, J.Y. Differential effects of phorbol ester on growth and protein kinase C isoenzyme regulation in human hepatoma Hep3B cells. *Biochem. J*. 333: 57-64, 1998.
12. Kopp, R., Noelke, B., Sauter, G., Schileberg, F.W., Paumgartner, G. and Pfeiffer, A. Altered protein kinase C activity in human colonic adenoma and carcinomas. *Cancer Res.* 51: 205-210, 1991.
13. Kusunoki, M., Sakanoue, Y., Yanagi, F., Tamamura, T. and Utsunomiya, J. Protein kinase C activity in human colonic adenoma and colorectal carcinomas. *Cancer* 69: 24-30, 1992.
14. Liu, W.S. and Heckman, C.A. The sevenfold way of PKC regulation. *Cell Signal*. 8: 529-542, 1998.
15. Mikawa, K., Maekawa, N., Hoshina, H., Tanaka, O., Shirakawa, J., Goto, R., Obara, H. and Kusunoki, M. Inhibitory effect of barbiturates and local anaesthetics on protein kinase C activation. *J. Int. Med. Res.* 18: 153-160, 1990.
16. Nakajima, T. and Yukawa, O. Radiation-induced translocation of protein kinase C through membrane lipid peroxidation in primary cultured rat hepatocytes. *Int. J. Radiat. Biol*. 70: 473-480, 1996.
17. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334: 661-665, 1988.
18. O'Brian, C.A., Vogel, V.G., Singletary, S.E. and Ward, N.E. Elevated protein kinase expression in human breast tumor biopsies relative to normal breast tissue. *Cancer Res.* 49: 3215-3217, 1989.
19. Piccoletti, R., Bendinelli, P., Arienti, D. and Bernelli-Zazzera, A. State and activity of protein kinase C in postischemic reperfused liver. *Exp. Mol. Pathol.* 56: 219-228, 1992.
20. Saito, M. Polychlorinated biphenyls-induced lipid peroxidation as measured by thiobarbituric acid-reactive substances in liver subcellular fractions of rats. *Biochim. Biophys. Acta* 1046: 301-308, 1990.
21. Seren, M.S., Aldinio, C., Zanoni, R., Leon, A. and Nicoletti, F. Stimulation of inositol phospholipid hydrolysis by excitatory amino acids is enhanced in brain slices from vulnerable regions after transient global ischemia. *J. Neurochem*. 53:1700-1705, 1989.
22. Simons, R.M., Laughlin, K.V., Kampherstein, J.A., Desai, D.C., Shurina, R.D. and Mullin, J.M. Pentobarbital affects transepithelial electrophysiological parameters regulated by protein kinase C in rat distal colon. *Dig. Dis. Sci.* 43: 632-640, 1998.
23. Slater, S.J., Cox, K.J., Lombardi, J.V., Ho, C., Kelly, M.B., Rubin, E. and Stubbs, C.D. Inhibition of protein kinase C by alcohols and anaesthetics. *Nature* 364: 82-84, 1993.
24. Slater, S.J., Kelly, M.B., Larkin, J.D., Ho, C., Mazurek, A., Taddeo, F.J., Yeager, M.D. and Stubbs, C.D. Interaction of alcohols and anesthetics with protein kinase Cα *J. Biol. Chem*. 272: 6167-6173, 1997.
25. Tsai, J.H., Hsieh, Y.S., Kuo, S.J., Chen, S.T., Yu, S.Y., Huang, C.Y., Chang, A.C., Wang, Y.W., Tsai, M.T. and Liu, J.Y. Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma. *Cancer Lett*. 161: 171-175, 2000.
26. Wetsel, W.C., Khan, W.A., Merchenthaler, I., Rivera, H., Halpern, A.E., Phung, H.M., Negro-Vilar, A. and Hannun, Y.A. Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. *J. Cell Biol.* 117:121-133, 1992.
27. Yamanoi, A., Nagasue, N., Kohno, H., Kimoto, T. and Nakamura, T. Clinical and enzymatic investigation of induction of oxygen free radicals by ischemia and reperfusion in human hepatocellular carcinoma and adjacent liver. *HPB Surg.* 8: 193-199, 1995.
28. Zangar, R.C., Buhler, D.R. and Springer, D.L. Neonatal exposure to xenobiotics alters adult hepatic protein kinase C alpha levels and testosterone metabolism: differential effects by diethylstilbestrol and phenobarbital. *J. Toxicol. Environ. Health* 45: 47-58, 1995.