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ALTERATION OF PROTEIN KINASE C ISOFORMS IN THE LIVER OF SEPTIC RAT

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ABSTRACT—The present study investigated the alteration of protein kinase C (PKC) isoforms in rat liver during the progression of sepsis. Cecal ligation and puncture (CLP) model of polymicrobial sepsis was used, with early and late sepsis referring to those animals sacrificed at 9 and 18 h, respectively, after CLP. The protein contents of various PKC isoforms were quantified by Western blot and densitometric analysis. PKCα activity was performed after immunoprecipitation and assayed based on the incorporation rate of ^{32}p from [γ-^{32}p] adenosine triphosphate (ATP) into histone. The distribution of PKCα was evaluated by immunohistochemical staining. The steady state expression of PKCα mRNA was estimated by reverse transcriptase-polymerase chain reaction (RT-PCR). The results indicated that 1) five isoforms (α, β, δ, ε, ζ) could be detected in normal rat liver. PKCα and β were predominantly present in the cytosolic fraction, while membrane-associated PKCδ was more prominent than that of cytosolic fraction; 2) the protein content of membrane-associated PKCα was significantly decreased at early ($P < 0.05$) and late ($P < 0.01$) sepsis; 3) there was no significant difference of protein contents of PKC-δ, -ε and -ζ between sham-operated and septic rat liver; 4) the activity of membrane-associated PKCα was significantly declined under detection level during sepsis; 5) at both early and late sepsis, the immunohistochemical staining of PKCα was significantly diminished, especially in the nucleus; 6) the RT-PCR product of PKCα mRNA of septic liver was significantly less than the sham-operated liver. These results suggest that inactivation and the suppression of PKC-α gene transcription might be involved in modulating hepatic failure during sepsis.

KEYWORDS—PKCα, hepatic failure, sepsis

INTRODUCTION

Protein kinase C (PKC) mediates phosphorylation of proteins and modulates the function of signal transduction pathways leading to gene expression (1), cell proliferation (2, 3), and cell survival (4, 5). In metabolic homeostasis, PKC plays an important role in the regulation of hepatic glucose metabolism (6). Our previous result has shown that PKC activity of rat liver is inactivated during the late the hypoglycemic phase of sepsis (7). Similar correlation has also been documented between PKC and numerous pathological situations, such as Alzheimer disease, pancreatic cancer, myocardium cardiomyopathy, or human proliferative glomerulonephritis. Currently, at least 12 mammalian PKC isoforms have been identified (8). Each individual PKC isoform possesses distinct mode of activation, kinetic property, substrate specificity, diverse patterns of tissue expression, and intracellular distribution (9, 10). PKC isoforms are mainly located in the cytoplasm. Once activated, they translocate from the cytoplasm to the plasma membrane and also to the nucleus, termed as membrane-associated form (11). The differential subcellular localization is associated with regulation of cell function *in situ* (12). Specific PKC isoform exerts spatially defined effects by virtue of their directed translocation to distinct intracellular sites (13).

In mammalian liver, Stravitz et al. (14) have immunochemically shown that there are at least 5 PKC isoforms, α, β, δ, ε, and ζ, expressed constitutively. Further functional studies revealed that PKC-α, δ, and ε isoforms are involved in cell growth events of human hepatoma Hep 3B cells (15). Inhibition of PKCβII was found to lead to cell cycle arrest in the G_2/M phase (16). The expressions of PKC-α, and -βII in hepatocytes were selectively increased in diabetes (17). Streptozotocin (STZ)-induced diabetes also induces the expression of a biologically inactive form of PKCα in liver by an undefined post-translational modification (18). PKCα and PKCδ are suggested to have different roles in liver regeneration and cell proliferation (19). Moreover, PKC-ζ is abundant in fetal liver and plays a role in cell proliferation (20). These data indicate that specific PKC isoform plays a different role in the hepatic survival and metabolism. Therefore, it is interesting and essential to investigate which isoform(s) is (are) major involved in modulating hepatic failure during sepsis, for both the pathogenetic research and clinical therapeutic intervention. In this study, we analyzed the expression and distribution of various PKC isoforms by immunochemical, immunohistochemical studies, and enzyme activity measurement to explore the molecular metabolic alternation leading to the liver dysfunction during sepsis.

MATERIALS AND METHODS

Materials

Monoclonal antibodies against PKC-α, -β, δ, and -ε and positive control were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-PKC-ζ rabbit polyclonal antibody IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pre-stained protein molecular weight standard was obtained from Bio-Rad Laboratories (Hercules, CA). Biotin and streptoavidin-peroxidase were purchased from DAKO (Carpinteria, CA). Diaminobenzidine (DAB) was purchased from Vector Laboratories (Burlingame, CA). Other chemicals and reagents were of analytical grade.

Animal model

In conducting the research described in this study, the authors adhered to the guidelines of the National Institutes of Health for the use of the experimental animals and all experiments were approved by the Committee for the Use of Experi-

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mental Animal of Kaohsiung Medical University. Experiments were performed on male Sprague-Dawley rats weighing from 270–350 g. All animals were fasted overnight with free access to water prior to the experiment. Sepsis was induced by cecal ligation and puncture (CLP) according to the method of Wichterman et al. (21) with slight modification. Under halothane anesthesia, a laparotomy was performed and the cecum was ligated with a 3-0 silk ligature and punctured in two sites with an 18-gauge needle. The cecum was then returned to the peritoneal cavity, and the abdomen was closed in two layers. Control rats were sham operated (a laparotomy was performed, and the cecum was manipulated but neither ligated nor punctured). Some of the sham-operated rats were sampled at 9 h after sham operation and some of them were sampled at 18 h after sham operation. Finally, the data was combined because there is no significant difference between data sampled at 9 and 18 h after sham operation. All animals were resuscitated with 4mL/100 g body weight of normal saline at the completion of surgery and also at 7 h post-surgery. Animals were fasted but had free access to water after operative procedures. Early and late sepsis refers to those animals sacrificed at 9 and 18 h, respectively, after CLP. The livers were sampled from rats of each group under pentobarbital anesthesia. The tissues were then chilled immediately in ice-cold buffer A [(2 mM EDTA, 10 mM EGTA, 10 mM dithiothreitol, 0.15 M sucrose, 25 mM Tris-HC1, pH 7.4), and protease inhibitor (0.1 mM PMSF, 1 mM pepstatin A, 0.1 mM benzamide, 1 mM leupeptin, 1 mg/mL aprotinin, 1 mg/mL trypsin-inhibitor)] for further experiments.

Subcellular fraction preparation of rat liver homogenate

Different fraction of tissue proteins was extracted and partially purified by the method of Wise B.C. et al., (22) with modification. Approximately 2–3 mg of minced liver, trimmed free of visible fatty and vascular tissues, was homogenized in 2 mL of buffer A with a Tekmar tissumizer (Cincinnati, OH; Model SDT). The homogenate was centrifuged at 100,000 *g* for 60 min, and the resultant supernatant was filtered through glass wool and was used as a crude cytosolic PKC-contained extract. To prepare membrane-associated PKC-contained fraction, the above pellet was rehomogenized in 2 mL of buffer A containing 0.25% TritonX-100. The mixture was shaken for 2 h followed by centrifugation at 30, 000 *g* for 20 min. The resulting supernatant was filtered through glass wool and was used as crude membrane-associated PKC-contained extract.

Western blot analysis

The protein content was estimated using the Bio-Rad protein microassay procedure. Optimal amount (20 μg) of cytosolic extract and the same volume of membrane-associated extract were loaded and separated by 10% SDS-PAGE simultaneously. The gel was transferred to polyvinylidene fluoride (PVDF) membrane by a Bio-Rad transblot apparatus at constant 100 V for 1 h. The membranes were blocked with 5% nonfat dry milk/TBS-T (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h and then incubated with the monoclonal anti-PKC-α, -β, -δ, -ε Transduction Laboratories; Cat #P16520, P17720, P36520, P14820) or polyclonal anti-PKC-ζ (Santa Cruz; Cat #SC-216-G) antibody, respectively, in 5% nonfat dry milk/TBS-T for 1 h at room temperature. Membranes were washed completely with TBS-T buffer and incubated with anti-mouse IgG-HRP (Transduction Laboratories; Cat #M15345, for isoforms α, β, -δ, and -ε) or anti-rabbit IgG-HRP (Transduction Laboratories; Cat #R14745, for isoform ζ) for 1 h at room temperature. Target isoforms were developed using the ECL (Amersham Life Science; Piscataway, NJ) detection system and autoradiography. The bands were scanned and quantified.

Immunoprecipitation of PKC-α

Samples were incubated with 5 μ g of monoclonal anti-PKCα (Transductional Lab.) by rotation for 2 h at 4° C. Then, protein G plus/protein A-agarose (CALBIOCHEM; Cat #IP05) was added to each sample, followed by agitation at 4°C for 4 h to precipitate the antigen-antibody complex. The suspension was subjected three times successively to a 5-min spin in a microcentrifuge followed by washing of the pellet. Immune complexes were recovered from the beads by suspension of the pellet in 100 μl of activity assay buffer.

Assay of PKC-α activity

PKC activity was assayed according to the method of Wise et al. with modification. The standard reaction mixture in a final volume of 0.25 mL contained 40 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM free Ca^{2+}, 0.25 mg histone, 0.2 m M [γ-^{32}p] ATP with a radioactivity of approximately 2.5×10^6 cpm. The reaction was performed with or without 70 mg of phosphatidylserine plus 40 mM of diacylglycerol. The reaction was initiated by the addition of partially purified PKC enzyme preparation and was allowed to proceed for 2 min at 37°C. At the end of incubation, the reaction was terminated by the addition of 1 mL of 15% trichloroacetic acid solution containing 1 mg of bovine serum albumin as a carrier protein. The mixture was centrifuged at 3,000 × *g* for 10 min. The resultant pellet was dissolved in 0.2 mL of 1 N NaOH, and the protein was reprecipitated. This procedure was repeated one more time. The final pellet was dissolved in 0.4 mL of 1 N NaOH, and the radioactivity was determined by a liquid scintillation counter. The enzymatic activity was then calculated based on the rate of incoporation of ^{32}p from [γ-^{32}p] ATP into histone.

Immunohistochemical staining

Rats for the study were perfused through the right ventricular with 200 mL 0.9% normal saline, followed by 200 mL 4% paraformaldehyde in the phosphate buffer (pH 7.4) before removal of the liver. Thirty minutes after perfusion, the liver was removed and immersed overnight in the same fixative. Then, the tissues were dehydrated in a series of graded alcohol steps and embedded in paraffin. The tissues were serially cut at 5 μm thickness. After deparaffinization, the sections were boiled in 10mmol/L sodium citrate (pH 6.0) for 5 min in a microwave oven and then cooled slowly to expose antigen epitope. To eliminate the endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in methanol for 10 min and then were incubated with blocking solution for 1 h at room temperature. The sections were incubated 2 h at 37°C in a humid chamber with PKC antibody. After washing, the sections were incubated with Biotin labeled secondary antibody for 20 min and then incubated with streptoavidin-peroxidase for another 20 min. The sections were finally developed with a DAB containing 0.01% hydrogen peroxidase for 5 min (Vector Laboratories).

TUNEL staining

The specimens were de-paraffinized and then subjected to proteinase K (20 μg/mL) digestion (25°C, 20 min). Three percent hydrogen peroxide was used to quench endogenous peroxidase activity. DNA strand breaks were identified by TUNEL assay using the KLENOW FragEL™ DNAfragmentation detection kit (Calbiochem, Cat #QIA21) following the manufacturer's protocol. The Signal of TdT-mediated dNTP nick end labeling (TUNEL) was then detected by streptavidin conjugated with HRP, a reporter enzyme that catalytically generates a brown-colored product from the chromogenic substrate diaminobenzidine (DAB). The cells were counter stained with methyl green. Negative controls were processed identically except that TdT was not added. The incidence of apoptosis was derived from the quotient of TUNEL positive cell mumber divided by the sum of total cell munbers in each section. All measurements were made by a single person.

RT-PCR of PKC-α mRNA

Total RNA was isolated from liver tissue according to a method described by Chomczynski P. & Sacchi N (23). First-strand cDNA was synthesized using oligo (dT) primers and reverse transcriptase using an RT-PCR kit (Titan ™ Boehringer Mannheim). The sense primer used was 5′-TGAACCCTCAGTGGAATGAGT-3′ and the antisense primer was 5′-GGCTGCTTCCTGTCTTCTGAA-3′. The cDNA of β-actin was also amplified as an internal standard. The primers used were 5′ primer: 5′-CTACAATGAGCTGCGTGTGG-3′. 3′ primer: 5′-TAGCTCTTCTC-CAGGGAGGA-3′. The amplification profile involved denaturation at 95°C for 1 min, primer annealing at 59°C for 45 s, and extension at 72°C for 4 min. This cycle was repeated 35 times using a Perkin-Elmer thermocycle (model 2400) with 1U of Taq DNA polymerase.

Statistical analysis

The statistical analysis of the data was performed using one-way analysis of variance followed by Newman-Keuls test. A 95% confidence limit was accepted as statistically significant.

RESULTS

Expression of PKC isoforms in normal rat liver

The expression of PKC isoforms in normal rat liver was investigated by Western bolt and immunochemical staining by antibodies recognizing PKC-α, -β, -δ, -ε, -ζ. The identification of each isozyme was confirmed by characterizing immunoreactive proteins purchased from Transduction Laboratories. As shown in Figure 1, all five detected PKC isoforms were visualized in cytosolic and membrane-associated fractions of the liver extract.

Alterations in expression of PKC isoforms of rat liver during CLP-induced sepsis

After quantification of the band of protein by densitometer, the content of membrane-associated PKC-α was decreased by 25% ($P < 0.05$) and 35% ($P < 0.01$) during early and late sepsis, respectively. However, the content of cytosolic PKC-α was not altered significantly between the early or late stage of

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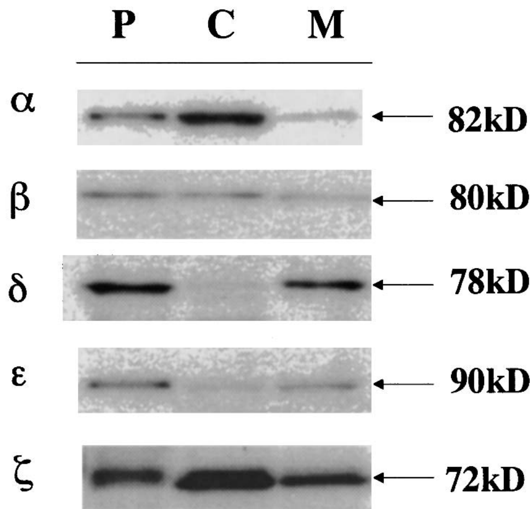

P: positive control, C: cytosolic, M: membrane-associated

FIG. 1. **Protein kinase C isoform distribution in rat liver.** Protein extracts from rat liver were prepared as described in Materials and Method. Liver cytosolic and membrane-associated and nuclear protein (20 μ g protein/lane) extract were separated in an 10% SDS-polyacrylamide gel, transferred to PVDF membrane, and subjected to Western immunoblotting. PKC isoforms were detected by enhanced chemiluminescence (ECL, 5–20 min exposure). For all isoforms, rat brain was extracted as a positive control. A molecular mass maker for each isozyme is shown at the left. Specificity of immunoreactive bands was demonstrated for each antibody by preincubating the respective primary antibody with the PKC isoform-specific peptide against which it was raised. P: positive control, C: Cytosolic, M: membrane-associated.

sepsis (Fig. 2). As shown in Figure 3, the content of cytosolic PKC-β was increased by 46% ($P < 0.05$) during late sepsis, while no significant difference of membrane-associated PKCβ was found between sham and septic rats. No significant difference was observed PKCδ (Fig. 4), PKC-ε (Fig. 5), and PKC-ζ (Fig. 6), both in cytosolic and membrane-associated fractions, between sham and septic rat liver.

Changes of PKC-α activities in rat liver during CLP-induced sepsis

Owing to the significant change observed in the quantitative study, we concentrated the focus on the PKC-α in the following investigations. Being similar with the result of protein

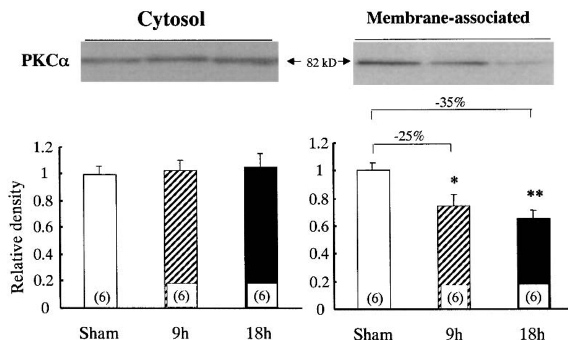

FIG. 2. **The cytosolic and membrane-associated PKCα protein expressions in rat liver derived from sham-operated rats or at 9 and 18 h after CLP.** The molecular weight of PKCα is 82KD. The data shown indicates mean ± SD of six samples in each group. *Indicates $P < 0.05$, **indicates $P < 0.01$.

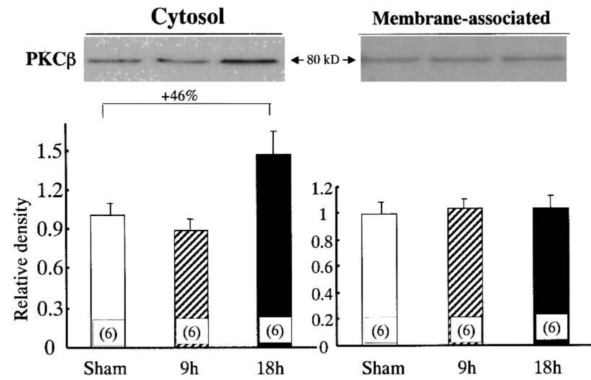

FIG. 3. **The cytosolic and membrane-associated PKCβ protein expressions in rat liver derived from sham-operated rats or at 9 and 18 h after CLP.** The molecular weight of PKCβ is 80KD. The data shown indicates mean ± SD of six samples in each group. *Indicates $P < 0.05$.

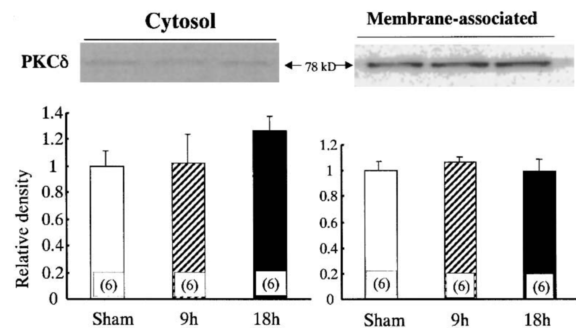

FIG. 4. **The cytosolic and membrane-associated PKCδ protein expressions in rat liver derived from sham-operated rats or at 9 and 18 h after CLP.** The molecular weight of PKCδ is 78KD. The data shown indicates mean ± SD of six samples in each group.

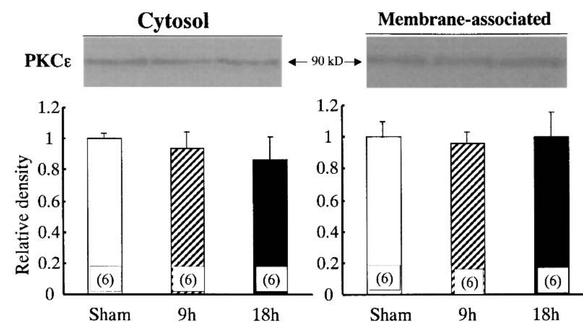

FIG. 5. **The cytosolic and membrane-associated PKCε protein expressions in rat liver derived from sham-operated rats or at 9 and 18 h after CLP.** The molecular weight of PKCε is 90KD. The data shown indicates mean ± SD of six samples in each group.

content, the *in vitro* activities of cytosolic PKC-α showed no significant difference between sham- operated and septic rat livers. As expected, the membrane-associated PKC-α activities were undetectable by the current method in both early and late septic rat liver (Table 1).

Immunohistochemical study of PKC-α in rat liver during CLP-induced sepsis

To understand the definite cellular redistribution of PKC-α in liver cells during sepsis, we performed the immunohistochemical study. As shown in Figure 7A, immunochemical activity was homogenous stained in cytoplasm, with condensed

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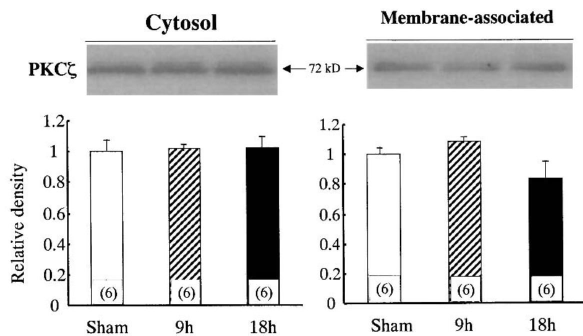

FIG. 6. **The cytosolic and membrane-associated PKCζ protein expressions in rat liver derived from sham-operated rats or at 9 and 18 h after CLP.** The molecular weight of PKCζ is 72KD. The data shown indicates mean ± SD of six samples in each group.

TABLE 1. **Activities of cytosolic and membrane-associated PKCα of the liver from sham and CLP-induced septic rats**

	Activity (p moles/mg.min)		
Fraction	Sham	9 h after CLP	18 h after CLP
Cytosol	32.36 ± 4.33 (3)	39.14 ± 4.18 (3)	38.75 ± 5.66 (3)
Membrane-associated	28.24 ± 4.24 (3)	U.D. (3)	U.D. (3)

Activities are expressed as p moles/mg of protein/min (m ± SEM).
Numbers in parenthesis indicates number of rats.
U.D.: under detection.

stain in the nucleus, in the specimen of sham rat. During the early stage of sepsis, the staining of nuclei was fading away while the cytosolic staining was not significantly changed (Fig. 7B). As the sepsis progressed, the characteristics of condensed staining in nuclei nearly disappeared from the specimen of late sepsis, leading to an ill-defined picture through the whole field (Fig. 7C).

The increase of apoptotic incidence in rat liver during CLP-induced sepsis

The incidence of hepatic apoptosis per 1,000 cells in the liver of sham-operated rat (2.96% ± 0.97%) was significantly lower than that in early (10.45% ± 1.24%) ($P < 0.01$) and late phases of sepsis (13.87% ± 1.76%) ($P < 0.01$) (Fig. 8). The number of rats in each group was six.

The RT-PCR product of PKCα-mRNA in rat liver during sepsis

Figure 9 showed the changes of the RT-PCR product of the steady-state level of PKC-α mRNA in rat liver during sepsis. The reverse transcription and amplification of total RNA isolated from liver tissues resulted in a single band of 325 bp using PKC-α specific primer and a single band of 450 bp using β -actin specific primer. Analysis of the densitometric signals revealed that the RT-PCR product of PKC-α mRNA was significantly decreased by 59% ($P < 0.01$) and 69% ($P < 0.01$) during early and late sepsis, respectively.

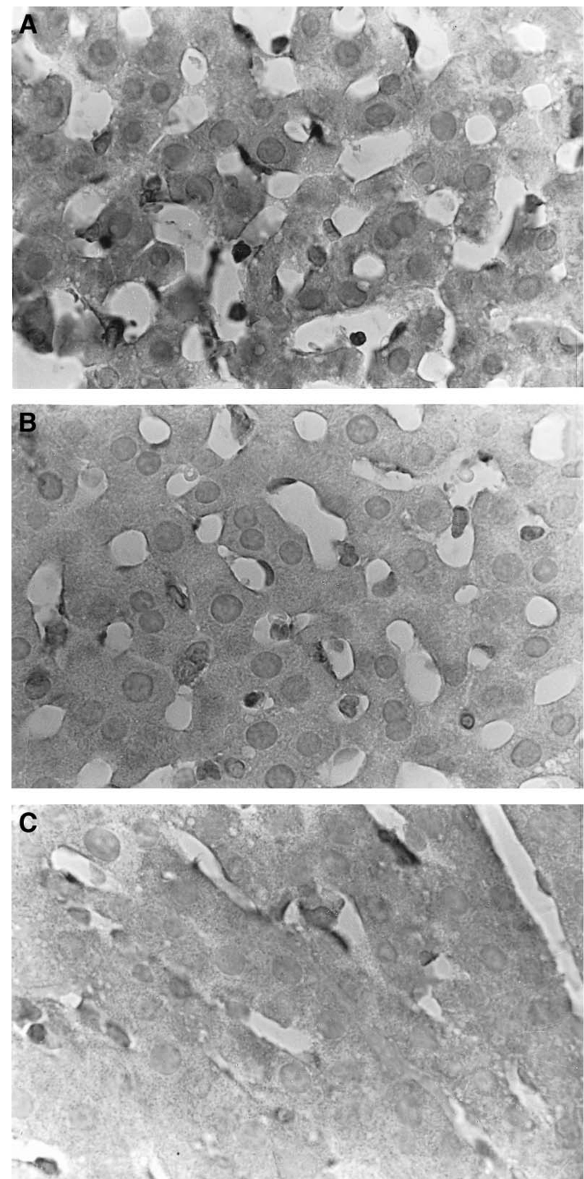

FIG. 7. **Immunohistochemical staining of the PKCα-isoform of liver tissues in sham-operated or septic rats.** (A) liver tissue of sham operated rat, (B) 9 h after CLP, and (C) 18 h after CLP (original magnification ×200).

DISCUSSION

It is well known that inactivation of PKC causes cellular dysfunction resulting in various organ failures. With the advance in biochemical characterization, at least 12 isoforms have been reported (8). Each isoform was regulated by its own way and exerted its physiological function independently. In a pathological situation, specific isoform was revealed to be responsible for specific diseases. In our previous study, we have shown that the hepatic PKC was inactivated during late sepsis and suggested to be the factor leading to the hypometabolic phase during severe infection (7). However, which isoform is the major one is still obscure.

In the present study, we demonstrated that 5 PKC isoforms, -α, -β, -δ, -ε, and -ζ are expressed in rat liver. During sepsis, only isoforms -α, and -β altered in specific distribution. The protein content of membrane-associated PKC-α was significantly decreased, which was further confirmed by decline of enzyme activity. Although PKC-α protein content was only decreased by 25–35% (Fig. 2), there was a near complete loss of kinase activity. Since post-translational modification may occur during septic condition, the PKC-α activity might not

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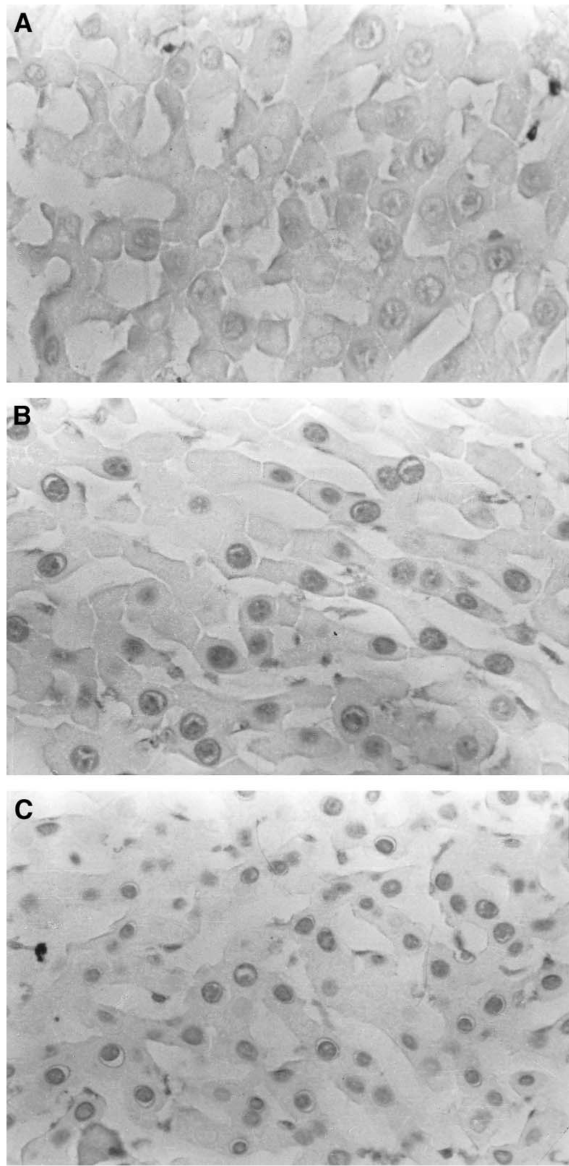

FIG. 8. **TUNEL stain positive cells in rat liver of sham-operated (A), 9 h (B) and 18 h (C) after CLP.** Strongly labeled nuclei are observed in the cells of septic rat liver tissue.

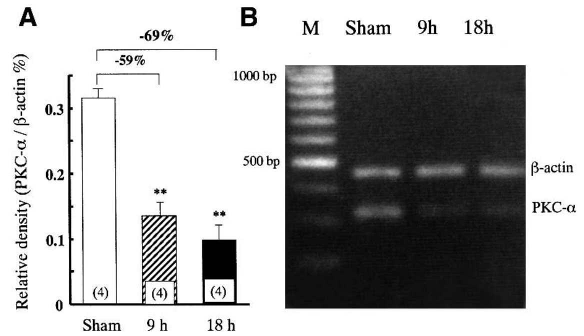

FIG. 9. **RT-PCR product of PKCα mRNA in rat liver of sham-operated, 9 and 18 h after CLP.** (A) Alteration in relative density of PKCα mRNA. (B) RT-PCR product of PKCα mRNA in liver derived from sham-operated rats or septic rats. The RT-PCR product of PKCα mRNA was 325 bp. The β-actin (450 bp) was used as a housekeeper. DNA crude preparations were prepared and separated by 2% agarose gel. M: 100 bp DNA ladder. Data are representative of four independents.

absolutely correlate with protein content. However, we can't exclude the possibility that the activity assay might not be sensitive enough to detect the very low protein content of membrane-associated PKC-α, which is significantly less than that of cytosolic fraction, especially during sepsis. Our previous report (7) showed that, during early sepsis, both membrane-associated and cytosolic PKC activity remained relatively unaltered. However, the membrane-associated PKC-α activity and protein expression were decreased ($P < 0.05$) during early sepsis in the present result. The PKC activity showed in the previous report represented the total activity of all PKC isoforms (at least all calcium-dependent isoforms). Whereas, the PKC-α activity showed in the current data is the activity of PKC-α only, because immunoprecipitation of PKC-α isoform was performed before activity assay. A decrease of specific isoform could be present, while the total activity unchanged. The present observation indicated that PKC-α might be crucial in hepatic failure during sepsis.

PKC modulates the function of a variety of signal transduction pathways leading to gene expression and each isoform may perform distinct functions via its translocation to discrete regions within the cell (13). Recent studies indicate that PKC itself may translocate to the nucleus and directly phosphorylate nuclear regulatory proteins (24, 25, 26). Our data of both Western blot study and activity assay showed that the membrane fraction decreased progressively and activity became undetectable during the progression of sepsis. In addition, the result of immunohistochemical staining showed that the decrease of PKCα during sepsis seems more prominent in nucleus than in cytosol or plasma membrane. Recently, we further found that the nuclear PKCα was significantly decreased at early and late phases of sepsis (data not shown). Moreover, the RT-PCR product of PKCα mRNA in the liver of septic rat was significantly less than that of sham-operated rats. These observations provided strong evidence that suppressed expression and inactivation of PKCα might be involved in mediating liver functional failure during sepsis.

One possible cause of progressive organ failure in sepsis could be cell apoptosis (27, 28). PKCα plays an important role in cell proliferation and tumorogenecity (15, 19, 29). Although PKCα inactivation is concerned in apoptosis and subsequent functional failure of different cell types, it remains unclear how PKCα regulates nuclear events or modulates hepatic apoptosis during sepsis. Previous report indicated that a decrease of PKC activity is associated with apoptosis in freshly isolated rat hepatocytes (4). Furthermore, we identified the level of hepatic apoptosis during sepsis by many methods, such as annexin-V & PI stainings followed by flow-cytometry, DNA ladder electrophoresis and PARP-cleavage (30). Herein, the TUNEL positive cells were significantly increased at early and late stages of sepis (Fig. 8). Additionally, our recent results indicated that inactivation of PKCα may play an important role in modulating hepatic apoptosis during sepsis and this apoptosis is closely associated with the alterations of Bcl-2 family proteins (30). It has been reported that PKCα phosphorylates the lamin B, which participates in the activation of DNA fragmentation and nuclear apoptosis in HL60 cells (31). More interestingly, PKCα phosphorylates the CREB and up-regulate the bcl-2 expression (32) or cause a conformational change in the CREB

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protein, resulting in the activation of transcription (33). Furthermore, the down-regulation of PKCα gene expression resulted in the inhibition of Bcl-xL protein level by 83% and consequent induction of apoptosis (34, 35). These observations would support the notion that the activity and/or the expression of Bcl-2 family proteins may be under the control of PKCα signal pathway. The underlined mechanism of PKCα-inactivation in affecting its downstream effectors involved in triggering hepatic apoptosis during sepsis needs further investigation.

In conclusion, we present fundamental evidence of specific PKCα inactivation in rat liver during the progression of experimental sepsis. We suggest that PKCα might be the major candidate causing the hypometabolic phase through the failure in expression and/or translocation of the PKCα during sepsis. This finding provides certain insights into the molecular pathogenesis of liver dysfunction and denotes the necessity for searching for the possible therapeutic intervention of PKCα activator in treating such a disease entity.

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