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Transcriptional Regulation of the Group IIA Secretory Phospholipase A2 Gene by C/EBP δ in Rat liver and its Relationship to Hepatic Gluconeogenesis during Sepsis

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Abstract

Background: The present study was undertaken to test hypothesis that altered transcription of secretory Phospholipase A2 (sPLA2) gene in rat liver is regulated by CCAAT/enhancer binding protein δ (C/EBPδ), and to assess its relationship to hepatic gluconeogenesis during the progression of sepsis.

Methods: Sepsis was induced by Cecal Ligation and Puncture (CLP). Experiments were divided into three groups, control, early sepsis (9 h after CLP), and late sepsis (18 h after CLP).

Results: DNA mobility and super shift assays reveal that C/EBP complexes in the liver consisted of at least three isoforms: C/EBPα, C/EBPβ, and C/EBPδ; and various C/EBP isoforms were capable of interacting with each other. Hepatocyte transfection experiments demonstrate that under normal conditions, binding of C/EBPδ to sPLA2 gene enhanced sPLA2 promoter activity and the binding resulted in an increase in hepatic gluconeogenesis. Under pathological conditions such as sepsis, binding of C/EBPδ to sPLA2 promoter increased during early and late phases of sepsis, and the increases in C/EBPδ binding correlated with increases in sPLA2 mRNA abundance and sPLA2 protein levels. Under otherwise the identical experimental conditions, hepatic gluconeogenesis was reduced during early and late phases of sepsis and the sepsis-induced reductions in liver gluconeogenesis were aggravated by binding of C/EBPδ to sPLA2 gene.

Conclusions: These results link C/EBPδ binding to altered sPLA2 promoter, and to hepatic gluconeogenesis under normal and pathological conditions. It is suggested that C/EBPδ-sPLA2- hepatic gluconeogenesis may function as a signalling axis affecting glucose homeostasis during the progression of sepsis.

Keywords: Secretory Phospholipase A2 (sPLA2); CCAAT/enhancer binding protein δ (C/EBPδ); Transcriptional factors; Transcriptional regulation; Liver gluconeogenesis; Sepsis

Introduction

Phospholipase A_2 (PLA_2), an enzyme protein that catalyzes the hydrolysis of phospholipids at the sn-2 position to generate lysophospholipids and free fatty acids, has been implicated to play a critical role in the pathogenesis of inflammatory disorders including shock and sepsis [1-10]. In sepsis patients, plasma PLA_2 activity was increased and the increased circulating PLA_2 correlated positively with severity of organ dysfunction and the eventual mortality [1-4]. In human volunteers, endotoxin challenge activated serum PLA_2 and elicited many features of sepsis syndrome [5,6]. In laboratory animals, secretory PLA_2 ($sPLA_2$) and cytosolic PLA_2 ($cPLA_2$) activities were increased in plasma and various organs including liver, heart, lung, spleen, thymus, and aorta following endotoxin administration [6-9] and the increased plasma $sPLA_2$ was proportional to the decrease in the mean arterial blood pressure [6]. The notion that PLA_2 plays an important role in the pathogenesis of sepsis and septic shock is further supported by recent finding that treatment of sepsis animals with antisense oligonucleotides targeting $sPLA_2$ and $cPLA_2$, in conjunction with antibiotics, decreased $sPLA_2$ and $cPLA_2$ protein expression in major organs, and the decreased tissue PLA_2 protein expression in multiple organs was accompanied by an absolute reduction of 30.8 % in 35-day mortality, in rats with sepsis [10].

Further studies on the underlying mechanism have revealed that the sepsis-induced over expression of $sPLA_2$ was regulated transcriptionally. In the rat model in which the animals exhibited a biphasic feature that closely resembling the clinical sepsis syndrome [11-13], the $sPLA_2$ activities were activated in the liver during early and late phases of sepsis [14,15] and in the heart during late phase of sepsis [16]. The activated $sPLA_2$ activities were found to correlate with concomitant increases in the steady-state level of $sPLA_2$ mRNA, the rate of transcription of $sPLA_2$ gene transcript, and $sPLA_2$ protein expression [15,16]. These findings indicate that $sPLA_2$ activity/expression was over expressed during sepsis and the sepsis-induced over expression was regulated at the transcriptional level [15,16].

Advances in the studies of molecular biology of $sPLA_2$ have indicated that $sPLA_2$ gene in the liver contains four distinct regulatory elements in the promoter region: A (-35 to -6), B (-125 to - 86), C (-209 to -176), and D (-247 to -211) [17-19]. Element C binds positive regulatory factors and element D binds a negative regulatory factor. Element C contains CCAAT/enhancer binding protein δ) (C/EBPδ)

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binding sites with high affinity [19]. Since C/EBPδ has been reported to function as a positive regulator for $sPLA_2$ gene transcription [19,20] and the C/EBPδ isoform expression has been reported to increase in multiple tissues following endotoxin administration [21-23], the present study was undertaken to test our hypothesis that the altered transcription of $sPLA_2$ gene is regulated by C/EBPδ in the liver, and to assess its relationship to hepatic glucose homeostasis, during the progression of sepsis. A network figure of regulation signalling pathway of factors mentioned above was illustrated in Figure 1.

Materials and Methods

Materials

Expression plasmids including C/EBPα (5649 bp), C/EBPβ (5254 bp), and C/EBPδ (4529 bp), cloned individually into BamHI-HindIII sites of plasmid pMEX, were obtained from Dr. Peter F. Johnson, National Cancer Institute, National Institutes of Health. The P2 (-1614 to +20) promoter for $sPLA_2$ gene constructed into XbaI-HindIII sites of native pUC-SH-CAT plasmid, was obtained from Dr. Jean Luc Olivier, Universite Pierre et Marie Curie, France. Rat group II PLA_2 cDNA cloned into SmaI and EcoRI sites of plasmid pGEM3Z was a gift of Dr. Jun Ishizaki, Shionogi, Japan. The pSV-β-galactosidase (β-gal) reporter vector, pCAT (CAT=chloramphenicol acetyltransferase) basic vector, pCAT enhancer vector, and pGL3 basic vector, were purchased from Promega. C/EBPα, C/EBPβ, C/EBPδ, and CREB (cAMP response element binding protein) antibodies were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody specific to $sPLA_2$ IIa and mouse monoclonal antibody against β-actin were products of Abcan (Cambridge, MA) and Sigma-Aldrich (St. Louis, MO), respectively.

Rat sepsis model

All animal experiments were performed with the approval of the Animal Care Committee of Saint Louis University School of Medicine, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague- Dawley rats weighing from 270 to 300 g were used. They were divided into three groups: control, early sepsis and late sepsis.

Number of animals included in each group was 6. All animals were fasted overnight with free access to water. Sepsis was induced by Cecal Ligation and Puncture (CLP) as described by Wichterman et al. [24] with minor modification [15,25]. Under isoflurane anesthesia, a laparotomy was performed and the cecum was ligated and punctured twice with an18-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) and time-matched. The values obtained at three time points (0, 9, and 18 h) for sham-operated controls were virtually identical. All animals were resuscitated subcutaneously with 4 mL/100 g body wt of normal saline at the completion of surgery and also at 7 h post-surgery. Early and late sepsis refers to those animals sacrificed at 9 and 18 h, respectively, after CLP. Previous experiments show that septic rats were in hyperdynamic/hyperglycemic state (characterized by increases in body temperature, heart rate and cardiac output, and with elevated blood glucose, lactate, epinephrine and norepinephrine concentrations) during early sepsis while they were in hypodynamic/hypoglycemic state (characterized by decreases in body temperature, heart rate, cardiac output, mean arterial blood pressure, blood glucose level, and with increased blood lactate, epinephrine and norepinephrine concentrations) during late sepsis [25]. The mortality rates were 0% for control, 9% for early sepsis, and 20% for late sepsis. Only those animals that survived at each designated time point were included in the experiments.

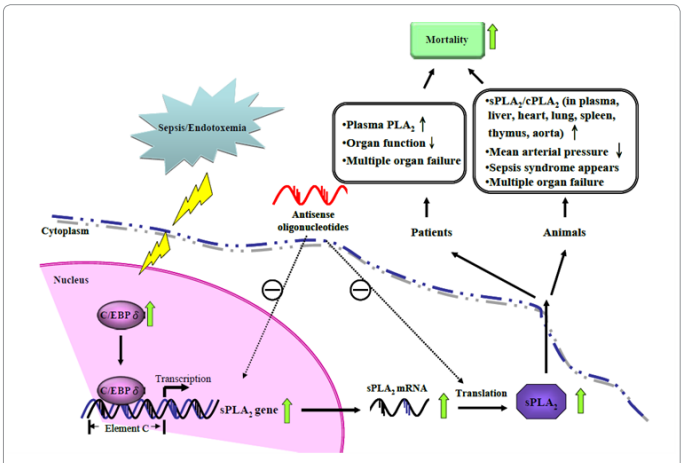

Figure 1: A network figure of regulation signaling pathway of factors mentioned in the introduction section. Minus (–) signs indicate inhibition.

Determination of sPLA2 protein level by Western blot analysis

Western blot analysis was performed according to the method of Ausubel et al. [26] with modification as previously described by us [15]. Samples of liver homogenate were denatured and subjected to Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10-20% polyacrylamide gradient gel). Proteins separated by SDS-PAGE were transferred to polyvinylidene fluoride membranes (Bio-Rad) and nonspecific binding sites were blocked with 10% nonfat dry milk in Tris- Buffered Saline (TBS). Blots were washed three times with TBST (TBS containing 0.5% Tween 20) followed by incubation with specific antibodies against $sPLA_2$ IIa and β-actin for 2.5 and 1 h, respectively, at room temperature. β-actin was used as an internal standard. Subsequently, the blots were washed and incubated with immunoglobulin, peroxidase-linked species-specific secondary antibodies (Amersham Life Science) for 1 h at room temperature. Blots were developed using an Enhanced Chemiluminescent (ECL) detection reagent (Amersham Life Science) and finally exposed to Hyperfilm-ECL (Amersham Life Science). Protein bands on the film were scanned and quantified, and the relative densities were normalized for β-actin expression.

Determination of the steady-state level of sPLA2 mRNA by Northern blot analysis

Northern blot analysis was performed according to the method of Sambrook et al. [27] with modification as previously described by us [15]. Total cellular RNAs were extracted from liver tissues with acid quanidinum thiocyanate-phenol-chloroform mixture using a RNA isolation kit (bulletin 1, TEL-TEST "B"). Poly(A)$^+$ mRNAs were isolated from total RNA by column chromatography on oligo(dT)-cellulose type 7. RNA and poly(A)$^+$ mRNA concentrations were determined by absorbance at 260 nm, and the purities were assayed by the 260/280 nm ratio. Samples containing poly(A)$^+$ mRNA were denatured, size fractionated, and then transferred to nylon membranes (Micron Separations). The membranes were ultraviolet cross-linked, baked, and then hybridized by cDNA probes labeled with [α-^{32}P]dCTP. The specific probe used was a 750 bp EcoRI-PstI fragment purified from the rat group IIA cDNA. After hybridization, the membranes were washed thoroughly and the $sPLA_2$ hybridization signals were scanned and quantified.

DNA mobility shift assay (DMSA) and supershift assay

Nuclear extracts used for DMSA and supershift assay were prepared by the method of Roy et al. [28]. Liver tissues were minced, homogenized, and centrifuged (1,000 g × 8 min). The resultant pellets were lysed, homogenized, and centrifuged (12,000 g × 8 min) again. The final supernatant was dialyzed for 1 h against a buffer (50 mM KCl, 4 mM $MgCl_2$, 20 mM K_3PO_4, 1 mM β-mercaptoethanol), stored at -70°C, and then used for DMSA and supershift assay. DMSA and supershift assay were performed according to the method of Gao et al. [29]. For DMSA, the reaction mixture in a final volume of 20 µl contained 1 ng of ^{32}P-labeled probe, 10 µg of nuclear extract, 20 mM Tris-HCl, pH 7.9, 1.5 % glycerol, 1 mM DTT, 0.5 mM PMSF, and 2 µg of poly(dI-dC). In competition experiments, radioactive probe and competitor were mixed prior to the addition of nuclear extract. Reactions were allowed to proceed for 20 min at 25°C, and DNA bindings were subsequently analyzed by electrophoresis (10 % polyacrylamide gel). The ^{32}P-labeled oligonucleotide used was 5'-TGC AGA TTG CGC AAT CTG CA-3' and the ^{32}P labeling was achieved by using T4 kinase and 50 µCi of [r-^{32}P] ATP. For supershift assay, the experiments were carried out in the same manner as for DMSA except that appropriate antibodies against C/EBPα, C/EBPβ, or C/EBPδ, were added to the binding reactions. For supershift analysis of the interaction between various C/EBP isoforms and CREB, CREB antibody and CREB oligonucleotide probe were used.

Measurement of sPLA2 promoter activity (hepatocyte culture, transfection experiment, CAT assay, and β-gal assay)

$sPLA_2$ promoter activity was determined based on the activity ratio of chloramphenicol acetyltransferase (CAT) to β-galactosidase (β-gal) in hepatocytes upon transfection with various promoter-reporter plasmids [29]. Hepatocytes used for transfection experiments were isolated according to a collagenase perfusion protocol [30]. Freshly isolated hepatocytes were washed and plated onto polylysine-coated culture dishes in attachment medium (GIBCO). After 3 h, the medium was changed to DMEM containing 5 % fetal calf serum, 1×10^{-8} M dexamethasone, 10 ng/ml EGF, 5 µg/ml insulin, 2.5 µg/ml fungizone, 50 µg/ml gentamycin, 67 µg/ml penicillin, and 100 µg/ml streptomycin. The cells were then transfected with various expression plasmids harboring $sPLA_2$ P2 promoter, CAT, β-gal, C/EBPα, C/EMPβ, or C/EBPδ coding regions. Transfections were performed using Lipofection (GIBCO/BRL) as a transfecting reagent and were allowed to proceed for 6 h. After transfection, the cells were incubated in a reduced serum medium for 4 h and then changed to normal growth medium for 60 h. At the end of transfection experiments, the cell were harvested, lysed, and then assayed for CAT and β-gal activities [19,29]. For $sPLA_2$ promoter activity assay, parallel plates were transfected with TK-CAT basic and TK-CAT control plasmids to serve as negative and positive controls, respectively. For β-gal assay, parallel plates were transfected with pSV basic and pSV control plasmids to serve as negative and positive controls, respectively. All values of CAT activities in hepatocyte extract were normalized to β-gal activities in the same extract.

Determination of gluconeogenesis

Gluconeogenesis was determined based on the ability of hepatocyte to produce glucose from alanine [31,32]. Following the completion of transfection experiment, hepatocytes (4×10^6 cells) were incubated in 1 ml of Krebs-Ringer bicarbonate buffer saturated with 95% O_2-5% CO_2. The incubation was proceeded in the presence or absence of 20 mM alanine for 50 min at 37°C under 95% O_2-5% CO_2. At the end of incubation, hepatocytes were deproteinized with barium hydroxide and zinc sulfate solution. The mixture was centrifuged at 14,000 g for 2 min. The resultant supernatant was neutralized and its glucose content was subsequently assayed based on colorimetric glucose oxidase procedure [32]. Values obtained in the presence were subtracted for those in the absence, of alanine, and used as gluconeogenesis activities.

Statistical analysis

Results were presented as mean ± SEM. Number of experiments was 6 for each group. Statistical analysis of the data was performed using one-way analysis of variance with a post hoc analysis using Student-Newman-Keuls tests. All calculations were performed using the standard statistical software SPSS 14.0 (Armonk, NY). A p value of less than 0.05 was accepted as statistically significant.

Results

Figure 2 shows DMSA and supershift analysis of C/EBP isoforms and their interaction with each other and with CREB in the control rat liver. Hepatic nuclear extracts prepared from control rat liver bound ^{32}P-labeled C/EBP probe, forming C/EBP: DNA complex bands (lanes 3, 5, 7, and 9). These C/EBP complex bands were almost completely displaceable by nonlabeled C/EBP probe (comparison between lanes 3 and 1), and furthermore, they were supershifted by antibodies specific to C/EBPβ (lane 4), C/EBPα (lane 7), and C/EBPδ (lane 9). These results demonstrate that C/EBP complex was composed of various isoforms including C/EBPα, C/EBPβ, and C/EBPδ. In addition to reacting with antibodies specific to C/EBPα, C/EBPβ, and C/EBPδ isoforms, the C/EBP complex band was supershifted by antibody specific to CREB (comparison between lanes 3 and 5) and displaceable by nonlabeled CREB oligonucleotide (comparison between lanes 3 and 2). These findings indicate that C/EBP complex in the liver was composed of at least three isoforms: C/EBPα, C/EBPβ, and C/EBPδ; and furthermore, various C/EBP isoforms were capable of interacting with each other and with CREB.

Figure 3 depicts binding of C/EBPδ to $sPLA_2$ promoter (Figures 3A and 3B) and its relationship to changes in steady-state level of $sPLA_2$

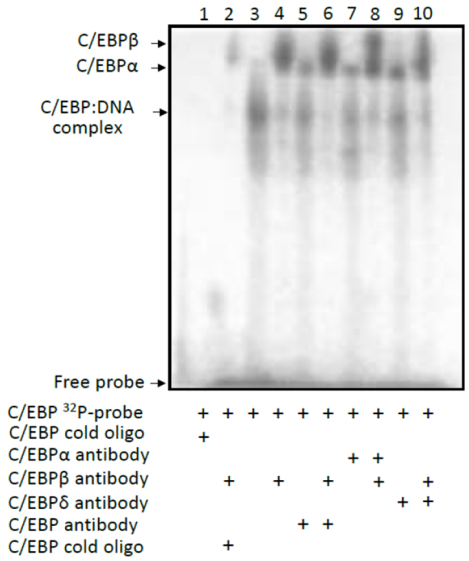

Figure 2: DNA mobility shift assay (DMSA) and supershift assay of C/EBP isoforms and their interaction with each other and with CREB in the control rat liver. DMSA and supershift assay were performed as described under Materials and Methods. + indicates presence of 32P-probe, antibody, and/or oligonucleotide in the reaction mixture.

mRNA (Figure 3C) and $sPLA_2$ protein level (Figure 3D) in rat liver during the progression of sepsis. DMSA and supershift assay reveal that binding of C/EBPδ to $sPLA_2$ promoter was increased by 36 % ($p<0.01$) and 86 % ($p<0.01$) during early and late phases, respectively, of sepsis (Figures 3A and 3B). Northern blot analysis shows concomitant increases in the steady-state level of $sPLA_2$ mRNA, i.e., 38 % ($p<0.01$) and 105 % ($p<0.01$) increases during early and late phases, respectively, of sepsis (Figure 3C). Similarly, Western blot analysis depicts parallel increases [+45 % ($p<0.01$) during early phase and +98 % ($p<0.01$) during late phase] in $sPLA_2$ protein level during the progression of sepsis (Figure 3D). These results demonstrate that binding of C/EBPδ to $sPLA_2$ promoter in the liver was increased during the progression of sepsis, and the sepsis-induced increases in C/EBPδ binding to $sPLA_2$ promoter were correlated with concomitant increases in $sPLA_2$ gene transcript and protein level.

Figure 4 shows interaction between various subtypes of C/EBP expression plasmids and $sPLA_2$ promoter activities in hepatocytes isolated from control rats. Co-transfection of control hepatocytes with $sPLA_2$ promoter-reporter vector and C/EBPδ expression plasmid enhanced $sPLA_2$ promoter activity by 39 % ($p<0.01$), while co-transfection with $sPLA_2$ promoter-reporter vector and C/EBPα expression plasmid, or with $sPLA_2$ promoter-reporter vector and C/EBPβ expression plasmid, had no effect. The C/EBPδ-induced enhancement in $sPLA_2$ promoter activity was further increased from 39 to 84 % when C/EBPδ and C/EBPα expression plasmids were co-transfected. Similarly, the C/EBPδ-induced enhancement in $sPLA_2$ promoter activity was further potentiated from 39 to 107 % when C/EBPδ and C/EBPβ expression plasmids were co-transfected. These results demonstrate that in the control liver, C/EBPδ in its monomeric form functions as an activator for $sPLA_2$ promoter while C/EBPα or C/EBPβ in its monomeric form had no effect. In addition, the activation of PLA_2 promoter by monomeric C/EBPδ was further potentiated by its heterodimeric complex with C/EBPα or C/EBPβ isoform.

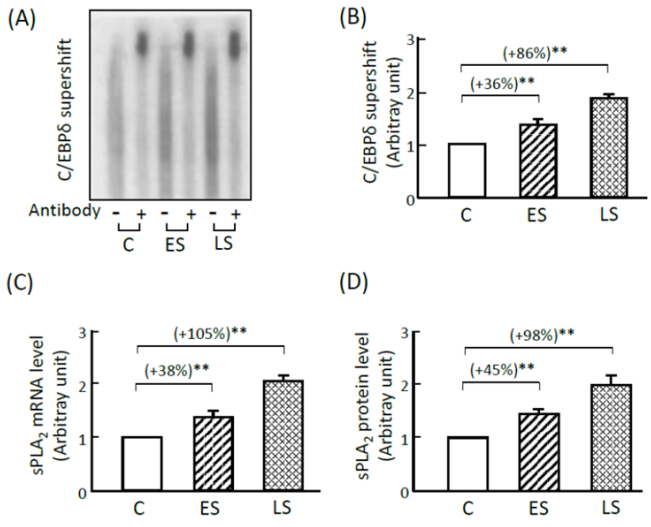

Figure 3: Binding of C/EBPδ to sPLA2 promoter (Figures 3A and 3B) and its relationship to changes in steady-state level of sPLA2 mRNA (Figure 3C) and sPLA2 protein level (Figure 3D) in rat liver during the progression of sepsis. Super shift analysis of C/EBPδ binding to sPLA2 promoter and measurements of sPLA2 mRNA abundance and sPLA2 protein expression were carried out as described under Materials and Methods. Values were mean ± standard errors of the mean. Number of experiments was 6 for each group. + indicates presence of antibody. Early Sepsis (ES) and Late Sepsis (LS) refer to those animals sacrificed at 9 and 18 hr, respectively, after CLP. C indicates control experiments. Vertical bars represent standard errors of the mean. $^{**}P < 0.01$.

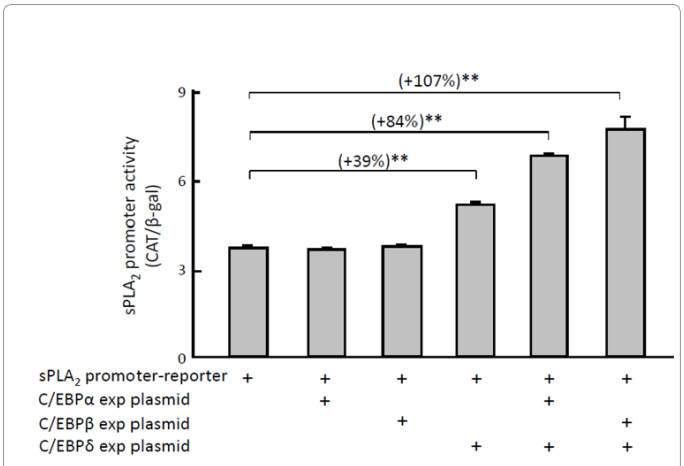

Figure 4: Interaction between various subtypes of C/EBP expression plasmids and sPLA2 promoter activity in hepatocytes isolated from control rats. Hepatocyte isolation and culture, co-transfection of sPLA2 promoter-reporter vector and C/EBP expression plasmids, and assay of sPLA2 promoter activity were performed as described under Materials and Methods. Values were mean ± standard errors of the mean. Number of experiments was 6 for each group. + indicates transfection or co-transfection. One unit of CAT (chloramphenicol acetyltransferase) transfers 1 nmole of acetate to chloramphenicol in 1 min at 37 °C. One unit of β-gal (β-galactosidase) hydrolyzes 1 μmole of o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and galactose per min at 37°C. $^{**}P < 0.01$.

Figure 5 depicts changes in gluconeogenesis following co-transfection of $sPLA_2$ promoter-reporter vector and various subtypes of C/EBP expression plasmids in hepatocytes isolated from control rats. It is note-worthy that the experimental protocols for Figure 5 were identical to those for Figure 4 except that the end-point measurements were different: namely, gluconeogenesis for Figure 5 versus $sPLA_2$ promoter activity for Figure 4. As depicted in Figure 5, co-transfection of control hepatocytes with PLA_2 promoter-reporter vector and C/EBPδ expression plasmid enhanced hepatic glucose production by 29.2 % ($p<0.01$), while co-transfection with $sPLA_2$ promoter-reporter vector and C/EBPα expression plasmid, or with PLA_2 promoter-reporter vector and C/EBPβ expression plasmid, failed to affect hepatic gluconeogenesis. Although the C/EBPδ-induced enhancement in hepatic gluconeogenesis remained elevated, no further potentiation was observed when C/EBPδ was complexed with C/EBPα or C/EBPβ expression plasmid. These data together with those presented in Figure 4 demonstrate that binding of C/EBPδ, in its monomeric form, to $sPLA_2$ gene in the control liver has a functional impact in regulating hepatic glucose homeostasis, i.e., stimulating hepatic glucose production.

Figure 6 shows changes in gluconeogenesis following co-transfection of $sPLA_2$ promoter-reporter vector and C/EBPδ expression plasmid in hepatocytes isolated from control and septic rats. When control and septic hepatocytes were transfected with $sPLA_2$ promoter-reporter vector, glucose production was reduced by 16.7 % ($p<0.01$) and 34.8 % ($p<0.01$) during early and late phases, respectively, of sepsis (comparison of empty columns in Figure 6A). When control and septic hepatocytes were co-transfected with $sPLA_2$ promoter-reporter vector and C/EBPδ expression plasmid, the reduction in gluconeogenesis was expanded from 16.7 to 36.1% during early sepsis and from 34.8 to 55.4 % during late sepsis (comparison of shaded columns in Figure 6A). When data presented in Figure 6A were re-plotted as shown in Figure 6B, it is apparent that C/EBPδ binding to $sPLA_2$ promoter plays a significant role in aggravating the reduction in hepatic gluconeogenesis during the progression of sepsis. These results together with those presented

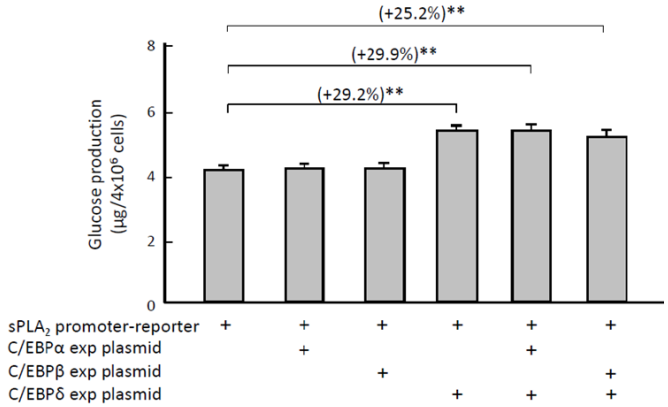

Figure 5: Changes in gluconeogenesis following co-transfection of sPLA2 promoter-reporter vector and various subtypes of C/EBP expression plasmids in hepatocytes isolated from control rats. Hepatocyte isolation and culture, co-transfection of sPLA2 promoter-reporter vector and C/EBP expression plasmids, and assay of gluconeogenesis were carried out as described under Materials and Methods. Values were mean ± standard errors of the mean. Number of experiments was 6 for each group. + indicates transfection or co-transfection. $^{**}P < 0.01$.

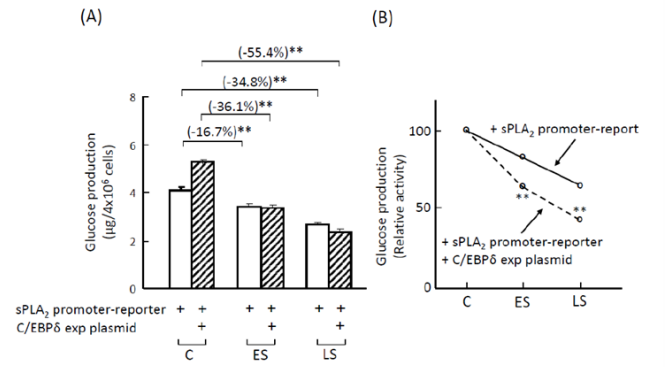

Figure 6: Changes in gluconeogenesis following co-transfection of sPLA2 promoter-reporter vector and C/EBPδ expression plasmid in hepatocytes isolated from control and septic rats. Experiments were carried out in the same manner as described in the legend to Figure 5 except that hepatocytes used were isolated from both the control and septic rats. Values were mean ± standard errors of the mean. Number of experiments was 6 for each group. + indicates transfection or co-transfection. C = Control; ES = Early Sepsis; LS = Late Sepsis. $^{**}P < 0.01$.

in previous figures provide an experimental evidence linking C/EBPδ binding to the altered sPLA2 promoter activity, and consequently impairing hepatic glucose homeostasis during the progression of sepsis. Based on these data, it is suggested that C/EBPδ-sPLA$_2$ gene transcription-hepatic gluconeogenesis may function as a signaling axis contributing to the formation of hypoglycemia during the progression of sepsis.

Table 1 depicts yields and viabilities of hepatocytes isolated from control, early sepsis, and late sepsis rats during various steps of co-transfection experiment. Yields of hepatocytes (9.1-9.3×10^6 cells/g wet wt.) were virtually identical among control, early sepsis, and late sepsis groups. Viabilities of hepatocytes were indifferent among three experimental groups (control, early sepsis, and late sepsis) at any given time points during the co-transfection procedure, although they were decreased from 95.2-96.1 % before the transfection (3 h post-isolation) to 48.7-50.2 % after the transfection (9 h post-transfection), and remained at 44.3-46.9 % prior to lysis (79 h post-isolation). These data indicate that changes observed in sPLA$_2$ promoter activity (Figure 3) and gluconeogenesis (Figure 6) during the progression of sepsis was not experimental artifacts due to hepatocyte yield and viability.

Figure 7 shows uptake efficiencies of transfecting multiple vectors in hepatocytes isolated from control, early sepsis, and late sepsis animals. Transfection efficiencies were negligible in all three experimental groups (control, early sepsis, and late sepsis) when hepatocytes were transfected with pSV-β-gal basic vector. The transfection efficiencies were increased by approximately 40-fold in all experimental groups when hepatocytes were transfected with β-gal reporter vector, and they remained at the same levels when hepatocytes were co-transfected with two (β-gal reporter + sPLA$_2$ promoter-reporter), three (β-gal reporter + sPLA$_2$ promoter-reporter + C/EBPα expression plasmid), and four (β-gal reporter + sPLA$_2$ promoter-reporter + C/EBPα expression plasmid + C/EBPδ expression plasmid) vectors. There were no differences in uptake efficiencies among control, early sepsis, and late sepsis groups when hepatocytes were transfected with one, two, three, or four transfecting molecules. These results reinforce the notion that the sepsis-induced alterations in sPLA$_2$ promoter activity (Figure 3) and hepatic glucose production (Figure 6) were not experimental artifacts due to transfection (uptake) efficiency.

Discussion

In this study, the DNA mobility shift and supershift assays have revealed that C/EBP complexes in rat liver consisted of at least three isoforms: C/EBPα, C/EBPβ, and C/EBPδ (Figure 2), and various C/EBP isoforms were capable of interacting with each other and with CREB (Figures 2 and 4). Subsequent hepatocyte culture and co-transfection experiments demonstrated that under physiological (control) conditions, binding of C/EBPδ to sPLA$_2$ gene in the liver enhanced sPLA$_2$ promoter activity (Figure 4) and the binding had a functional significance in regulating liver glucose metabolism, i.e., stimulating hepatic gluconeogenesis (Figure 5). Further experiments revealed that under pathological conditions such as sepsis, binding of C/EBPδ to sPLA$_2$ promoter increased consecutively during early and late phases of sepsis, and the increases in C/EBPδ binding to sPLA$_2$ gene correlated with concomitant increases in sPLA$_2$ mRNA abundance and sPLA$_2$

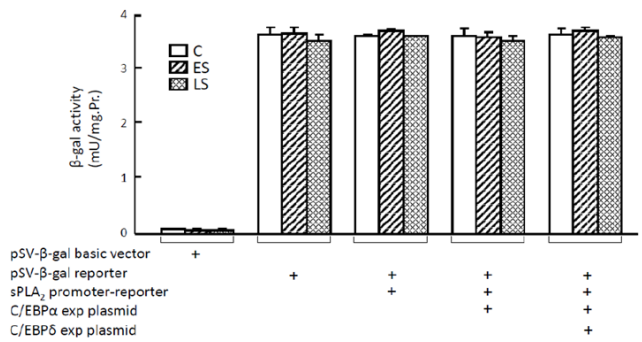

Figure 7: Uptake efficiencies of transfecting multiple vectors in hepatocytes isolated from control, early sepsis, and late sepsis rats. Transfection experiments were performed as described under Materials and Methods. Values were mean ± stand errors of the mean. Number of experiments was 6 for each group. + indicate transfection or co-transfection. C = Control; ES = Early Sepsis; LS = Late Sepsis. One unit of β-galactosidase activity is defined as hydrolysis of 1 μmole of o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and galactose per minute at PH 7.5 at 37°C.

	n	Yield (10^6 cells/ g wet wt)	Viability (%)		
			Before transfection (3 h post- isolation)	After transfection (9 h post- isolation)	Before lysis (79 h post- isolation)
Control	6	9.2 ± 0.8	96.1 ± 0.8	50.2 ± 2.4	46.9 ± 0.9
Early sepsis	6	9.1 ± 0.6	96.1 ± 0.7	48.9 ± 3.6	44.3 ± 1.3
Late sepsis	6	9.3 ± 0.8	95.2 ± 0.8	48.7 ± 1.2	45.0 ± 0.7

Values were mean ± standard errors of the mean. n = number of experiments. Hepatocytes were transfected with $sPLA_2$ P2 promoter-reporter vector using Lipofectin as a transfecting reagent

Table 1: Yields and viabilities of hepatocytes isolated from control, early sepsis, and late sepsis rats during various steps of co-transfection experiment.

protein level (Figure 3). Under otherwise the identical experimental conditions, hepatic gluconeogenesis was successively reduced during early and late phases of sepsis and the sepsis-induced reductions in hepatic glucose production were aggravated by the binding of C/EBPδ to $sPLA_2$ promoter (Figure 6). These results, to our knowledge, provide the first experimental evidence linking C/EBPδ binding to $sPLA_2$ promoter, and to the altered hepatic glucose homeostasis under normal as well as the pathological conditions. Furthermore, the results suggest that C/EBPδ-$sPLA_2$-hepatic gluconeogenesis may function as a signaling axis affecting glucose homeostasis during the progression of sepsis.

Alteration in hepatic glucose homeostasis in one of the key metabolic features during the progression of sepsis. The altered hepatic glucose metabolism is characterized by a rapid depletion of hepatic glycogen content, an impaired glycogenesis, an accelerated glycogenolysis, and a depressed gluconeogenesis [11,33]. The ultimate result of these metabolic alterations is the development of hyperglycemia during the initial phase of sepsis followed by a transition from hyper- to hypo-glycemia during late phase of sepsis [11,33]. Regulation of liver glucose metabolism is a complicated process that includes numerous hormonal regulatory factors such as catecholamines [α_1 adrenergic receptor (α_1AR) and β_2 adrenergic receptor (β_2AR) agonists], glucagon, vasopressin, angiotensin, and insulin. α_1AR agonist, vasopressin, and angiotensin stimulate gluconeogenesis and glycogenolysis while they inhibit glycolysis via changes in intracellular Ca^+/calmodulin-linked protein kinases and phosphorylation of a number of protein substrates [34,35]. β_2AR agonist and glucagon enhance gluconeogenic and glucolytic fluxes through activation of cAMP-dependent protein kinases and interaction with membrane receptors. Insulin, in contrast, opposes the actions of the above-mentioned hormones via phosphorylation of various protein substrates [34,35]. Our findings that binding of C/EBPδ to $sPLA_2$ enhances $sPLA_2$ promoter activity, activates $sPLA_2$ gene transcription, increases $sPLA_2$ protein expression, and finally depressing hepatic glucose production, may have a physiological significance in contributing to the understanding of the altered hepatic glucose metabolism during sepsis, because it provides an additional facet that hepatic glucose dyshomeostasis can be regulated via a non-hormonal route, i.e., C/EBPδ-$sPLA_2$ -hepatic gluconeogenesis signaling axis.

C/EBPδ is a member of the C/EBP family of transcription factors and it has been implicated to play an important role in the inflammatory responses such as sepsis and endotoxemia [22,23,36]. C/EBPδ mRNA and protein are expressed in normal tissues at a low level but are rapidly and drastically induced in many tissues by bacterial lipopolysaccharide or numerous proinflammatory mediators [21-23,36-40]. C/EBPδ expression was increased in human umbilical vein endothelial cells after in vitro incubation of cells with lipopolysaccharide [39].

C/EBPδ:DNA binding activity and C/EBPδ protein expression were upregulated in skeletal muscles following induction of sepsis in rats [40]. C/EBPδ mRNA levels were increased in multiple organs including kidney, spleen, brain, heart, intestine, lung, testes, and fat in mice after treatment with lipopolysaccharide [21]. C/EBPδ mRNA abundance and protein level were augmented in liver, lung, and kidney tissues in mice upon priming and challenge with endotoxin, and furthermore, the C/EBPδ-deficiency mice decreased endotoxin-induced systemic inflammation and partly protected against mortality [22,23]. These observations together with those presented in current study demonstrate that C/EBPδ is an important transcription factor contributing to the pathogenesis of sepsis.

C/EBPδ-binding motifs have been identified in the regulatory regions of various proinflammatory genes including those encoding $sPLA_2$, IL-6, IL-8, IL-1β, TNF-α, inducible nitric oxide synthase, etc. [19,20,36-38]. Of particular relevance is the induction of $sPLA_2$ during the development of sepsis [6,14-16] and the therapeutic implication on the improvement of clinical outcome upon neutralization of the over-transcribed $sPLA_2$ gene [10]. Treatment of septic rats with antisense oligonucleotides targeting $sPLA_2$ and $cPLA_2$, in conjunction with antibiotics, reduced target protein expression in multiple organs including liver, heart, and kidney, resulting in an absolute reduction of 30.8 % in 35-day mortality [10]. Since the sepsis-induced activation of $sPLA_2$ is considered to be solely secondary to the transcriptional activation of its gene [15-17] and that C/EBPδ has been identified to function as a positive regulator for $sPLA_2$ gene transcription [19,20], it is possible that activation of C/EBPδ would translate into increases in $sPLA_2$ mRNA abundance and protein expression. This possibility is confirmed by present findings that sepsis-induced increases in C/EBPδ binding to $sPLA_2$ promoter correlated positively with concomitant increases in $sPLA_2$ gene transcription and protein expression during the progression of sepsis. Of particular significance is that the activated C/EBPδ-$sPLA_2$ signaling resulted in a deranged hepatic function by reducing its ability to produce glucose. These findings thus open a therapeutic option by targeting C/EBPδ, in addition to $sPLA_2$, for the treatment of sepsis.

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