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LIVER PROTEIN KINASE A ACTIVITY IS DECREASED DURING THE LATE HYPOGLYCEMIC PHASE OF SEPSIS

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ABSTRACT—Changes in protein kinase A (PKA, or cAMP-dependent protein kinase) activity in the rat liver during different metabolic phases of sepsis were investigated. Sepsis was induced by cecal ligation and puncture (CLP). Experiments were divided into 3 groups: control, early sepsis, and late sepsis. Early and late sepsis refer to those animals killed at 9 and 18 h, respectively, after CLP. Hepatic PKA was extracted and partially purified by acid precipitation, ammonium sulfate fractionation, and diethylaminoethyl (DEAE)-cellulose chromatography. PKA was eluted from DEAE-cellulose column with a linear NaCl gradient. Two peaks of PKA, type I (eluted at low ionic strength) and type II (eluted at high ionic strength), were collected and their activities were determined on the basis of the rate of incorporation of [γ-^{32}-P]ATP into histone. The results show that during early sepsis, both type I and type II PKA activities remained unchanged. During late sepsis, type I PKA activity was decreased by 40.7–53.6%, whereas type II PKA activity was unaffected. Kinetic analysis of the data on type I PKA during the late phase of sepsis reveals that the V_{max} (maximal velocity) values for ATP, cAMP, and histone were decreased by 40.7, 53.6, and 47.3%, respectively whereas the K_m (substrate concentration required for half-maximal enzymatic activity) values for ATP, cAMP, and histone were unaltered. These data indicate that type I PKA was inactivated during the late hypoglycemic phase of sepsis in the rat liver. Because PKA-mediated phosphorylation plays an important role in the regulation of hepatic glucose metabolism, an inactivation of PKA may contribute to the development of hypoglycemia during the late phase of sepsis.

INTRODUCTION

Protein kinase A (PKA, or adenosine 3′,5′-cyclic monophosphate [cAMP]-dependent protein kinase) plays an important role in the regulation of liver function and metabolism. PKA modulates liver function and metabolism by phosphorylating many intracellular enzymes and receptor proteins involved in the control of glycogenolysis, glycogenesis, gluconeogenesis, glycolysis, and Ca^{2+} homeostasis (1–6). These enzymes and receptor proteins may include phosphorylase b kinase (1), glycogen synthase (1, 2), pyruvate kinase (1), 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase bifunctional enzyme (1, 3), adenosine 5′-triphosphate (ATP)-dependent Ca^{2+} pump (4), and IP_3-receptor/channel (4–6). Phosphorylation of pyruvate kinase and glycogen synthase decreases glycolysis and glycogenesis, whereas phosphorylation of phosphorylase b kinase increases glycogenolysis (1, 2). Phosphorylation of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase bifunctional enzyme regulates glycolytic and glyconeogenetic fluxes (1, 3). Phosphorylation of ATP-dependent Ca^{2+} pump and IP_3-receptor alters intracellular Ca^{2+} concentration and distribution (4–6). Because the dynamics of various enzyme and receptor proteins mentioned above have been reported to be altered during endotoxin shock and sepsis (7–13) and because they all serve as common substrates for PKA phosphorylation, it is conceivable that PKA activity may be modified during the progression of sepsis. Accordingly, the present study dealing with the effect of sepsis on PKA activity in the rat liver was undertaken in an attempt to understand the pathogenesis of altered glucose and Ca^{2+} homeostasis during the progression of sepsis.

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MATERIALS AND METHODS

Materials

Diethylaminoethyl DEAE-cellulose, cAMP, and histone (type II-A) were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Other chemicals and reagents were of analytical grade.

Animal model

In conducting the research described in this report, the investigators adhered to the National Institutes of Health guidelines for the use of experimental animals. All procedures and protocols were approved by the Institutional Animal Care Committee of Saint Louis University School of Medicine. Experiments were performed on male Sprague-Dawley rats weighing from 270 to 350 g. All animals were fasted overnight with free access to water before the experiment. Sepsis was induced by cecal ligation and puncture (CLP) according to the method of Wichterman et al. (14) with slight modification. Under halothane anesthesia, a laparotomy was performed, and the cecum was ligated with a 3-0 silk ligature and punctured twice with an 18-gauge needle. The cecum was then returned to the peritoneal cavity, and the abdomen was closed in 2 layers. Control rats were sham operated (a laparotomy was performed, and

the cecum was manipulated but neither ligated nor punctured). All animals were resuscitated with 4 mL/100 g body weight of normal saline at the completion of surgery and also at 7 h postsurgery. Animals were fasted but had free access to water after operative procedures. Livers were removed from septic and control animals 9 or 18 h postoperation under chloralose and urethane anesthesia and were chilled immediately in ice-cold buffer A (2 mM ethylenediaminetetraacetic acid [EDTA], 50 mM dithiothreitol, 0.25 M sucrose, and 25 mM Tris-HC1, pH 7.4) for the extraction and purification of PKA. Early and late sepsis refer to those animals killed at 9 and 18 h, respectively, after CLP. Previous experiments showed that septic rats exhibit 2 metabolically distinct phases: a hypermetabolic/hyperglycemic phase during early sepsis followed by a hypometabolic/hypoglycemic phase during late sepsis (15, 16).

Extraction and purification of PKA

Liver PKA was extracted and partially purified by the method of Miyamoto et al. (17) with modification (18). Approximately 30–35 g of minced livers (pooled from 6–7 rats) trimmed free of visible fatty and vascular tissues were homogenized in 1.5 vol of buffer A with a Tekmar tissumizer (model SDT; Cincinnati, OH) operating at half-maximum speed. The homogenate was centrifuged at 27,000 g for 30 min, and the resultant supernatant was filtered through glass wool. The pH of the supernatant solution was adjusted to 4.8 by the stepwise addition of 1 N acetic acid. After 10 min of stirring, the precipitate was removed by centrifugation at 27,000 g for 30 min. The pH of the clear supernatant solution was adjusted to 7.4 with 0.5 M Tris-HC1. Solid ammonium sulfate (0.32 g/mL) was slowly added to the resultant solution.

After 60 min of stirring, the precipitate was collected by centrifugation at 16,000 g for 20 min and dissolved in 15 mL of buffer B (2 mM EDTA. 50 mM dithiothreitol, and 25 mM Tris-HC1, pH 7.4). The resultant solution was dialyzed against 100 vol of buffer B with 3 changes of buffer during 40 h. After dialysis, the solution was centrifuged at 27,000 g for 30 min, and the resultant supernatant was applied to a DEAE-cellulose column (1 × 18 cm) previously equilibrated with buffer B. The column was then washed with 250 mL of buffer B, and the enzyme was eluted with a linear gradient of 0–0.3 M NaC1. Fractions of 3 mL each were collected, and an aliquot (0.05 mL) was taken from each fraction for the determination of PKA activity. Two peaks of PKA, designated as type I (eluted at low ionic strength) and type II (eluted at high ionic strength), were collected separately, stored at −80°C in 10% (vol/vol) glycerol, and their activities were assayed within a week. The entire procedure was performed at 4°C unless otherwise stated.

Assay of PKA activity

PKA activity was assayed by the method of Miyamoto et al. (17) with modification (18). The standard reaction mixture in a final volume of 0.25 mL contained 40 mM Tris-HC1 (pH 7.4), 4 mM $MgC1_2$, 0.2 mg histone, 0.2 mM [γ-^{32}P]ATP with a radioactivity of ~7 × 10^5 cpm, and 0 or 2 μM cAMP. The reaction was initiated by the addition of partially purified PKA preparation containing 30 μg protein and was allowed to proceed for 3 min at 37°C. At the end of each incubation, the reaction was terminated by the addition of 1 mL of 15% trichloroacetic acid solution containing 2 mg of bovine serum albumin as a carrier protein. The mixture was centrifuged at 3000 g for 10 min. The resultant pellet was dissolved in 0.2 mL of 1 N NaOH, and the protein was reprecipitated with 2 mL of 15% trichloroacetic acid solution. The reprecipitation procedure was repeated one more time. The final pellet was dissolved in 0.5 mL of 1 N NaOH, and the radioactivity was determined by a liquid scintillation counter. The enzymatic activity was then calculated based on the incorporation of ^{32}P from [γ-^{32}P]ATP into histone. For kinetic analysis, the V_{max} (maximal velocity) and K_m (substrate concentration required for half-maximal enzyme activity) were obtained from Eadie-Hofstee plot (a plot of V against V/[S] in which V and [S] signify enzyme velocity and substrate concentration, respectively). The V_{max} was derived from the intercept of V axis and the slope, whereas K_m was the absolute value of the slope.

Protein determination

The protein content of enzyme preparations was determined by the method of Lowry et al. (19).

Statistics

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

RESULTS

Effects of time and protein dependency and of Mg^{2+} requirement on PKA activity were investigated in the control rat liver. The results indicate that both type I and type II PKA activities were increased linearly with incubation time for up to 4 min and with increasing concentrations of protein for up to 40μg/0.25 mL (data not shown). Mg^{2+} stimulated both type I and type II PKA activities. There was a sigmoidal relationship between PKA activities and the increasing concentrations of Mg^{2+} for up to 5 mM tested (data not shown). These results demonstrate that under our assay conditions, both type I and type II PKA activities measured were time- and protein-dependent and that Mg^{2+} was required for the full expression of enzymatic activities. On the basis of these observations, a standard assay protocol consisted of 3 min of incubation time, 30 μg of enzyme protein per reaction mixture, and 4 mM of Mg^{2+} was adopted for the comparison of enzyme kinetics between the control and septic experiments.

Fig. 1 shows changes in the V_{max} and K_m values for ATP for PKA in the rat liver during different phases of sepsis. The V_{max} for ATP for type I PKA remained relatively unaffected during the early phase of sepsis (9 h after CLP), whereas it was decreased by 40.7% ($P < 0.05$) during the late phase of sepsis (18 h after CLP) (Fig. 1A). The K_m values for type I PKA were unaltered during the early and the late phases of sepsis (Fig. 1B). For type II PKA, the V_{max} and K_m values for ATP remained constant during the early and the late phases of sepsis (Fig. 1A and B). These data indicate that type I PKA activity was inhibited in the rat liver during the late hypoglycemic phase of sepsis. It is noteworthy that the K_m values of type II PKA were similar to type I PKA; the V_{max} values of type II were <50 % of that of type I in the control group. The difference in the V_{max} values of type I vs. type II PKA is due to different subcellular location of regulatory subunits and different binding affinity of cAMP (20).

Fig. 2 depicts changes in the V_{max} and K_m for cAMP for PKA in the rat liver during the early and the late phases of sepsis. The V_{max} for cAMP for type I PKA was not affected during early sepsis, whereas it was decreased by 53.6% ($P < 0.01$) during late sepsis (Fig. 2A). The K_m values for type I PKA were unaltered during early and late phases of sepsis (Fig. 2B). For type II PKA, the V_{max} and K_m values for cAMP remained stable during the early and the late phases of sepsis (Fig. 2A and B). These results support the findings presented in Fig. 1 that type I PKA activity was inhibited in the rat liver during the late phase of sepsis. It is of interest to note that the K_m values for cAMP were 2 orders lower than that for ATP (comparison of K_m between Figs. 1B and 2B). The reason for the low K_m values for cAMP in comparison with that for ATP is most likely due to difference in the physiological concentrations between the two metabolites.

Fig. 3 shows changes in the V_{max} and K_m for histone for PKA in the rat liver during different phases of sepsis. For type I PKA, the V_{max} for histone remained unchanged during early sepsis, whereas it was decreased by 47.3% ($P < 0.05$) during the late phase of sepsis (Fig. 3A). The K_m values for histone for type I PKA were not affected during the early and the late

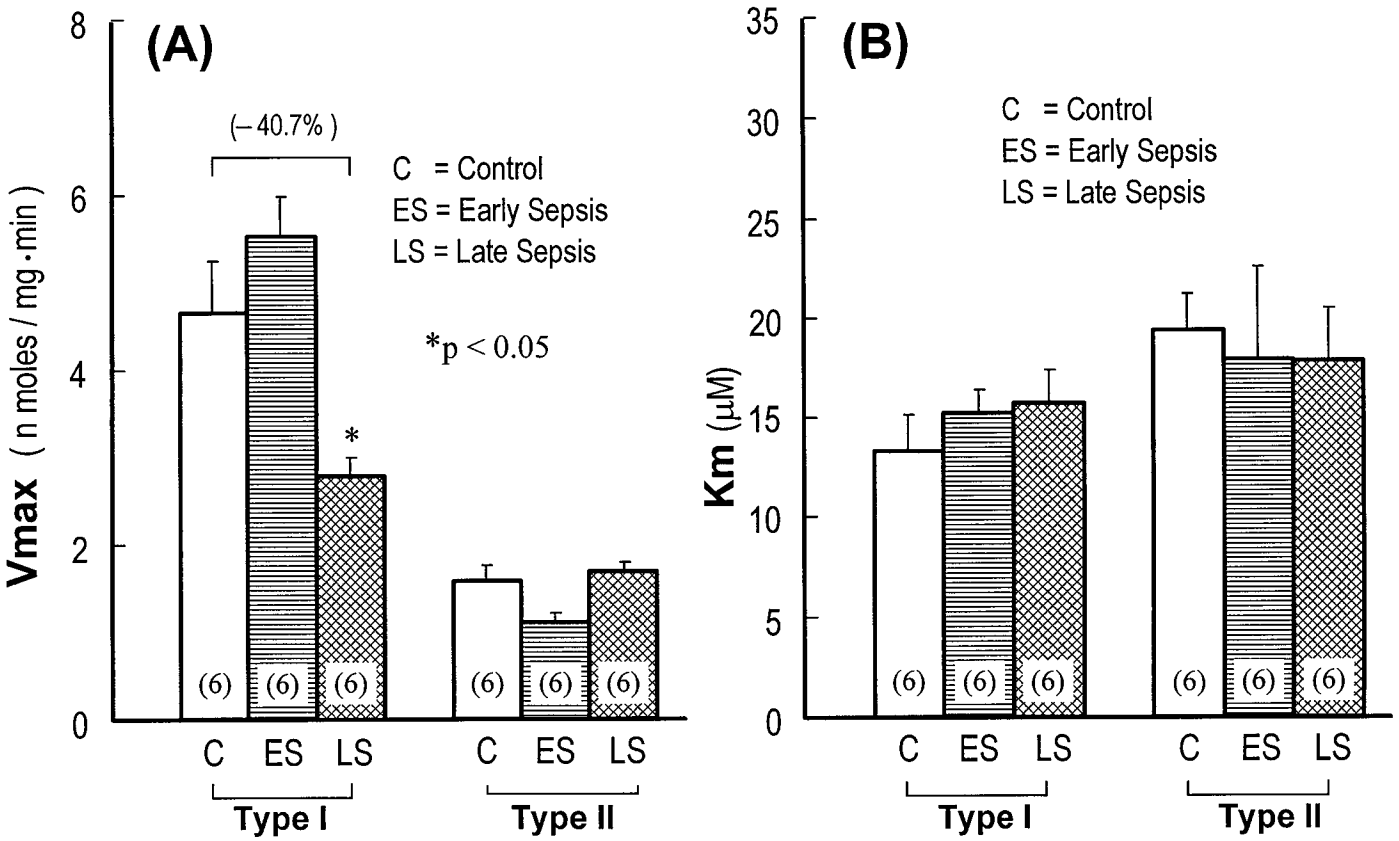

FIG. 1. **Changes in the V_{max} and K_m for ATP for protein kinase A in the rat liver during different phases of sepsis.** Protein kinase A activities were assayed as described under Materials and Methods except that ATP concentrations were varied from 10^{-7} to 10^{-3} M. V_{max} and K_m values were calculated from Eadie-Hofstee plots on the basis of different concentrations of ATP. Vertical bars indicate SEM. Number of experiments was shown in the parenthesis of each column.

phases of sepsis (Fig. 3B). For type II PKA, the V_{max} and K_m values for histone remained constant during the early as well as the late phases of sepsis (Fig. 3A and B). These results reinforce the findings presented in the previous 2 figures that type I PKA activity was inactivated in the rat liver during the hypoglycemic phase of sepsis.

DISCUSSION

PKA exists as type I and type II isozymes. The two isozymes are distinguished by their different regulatory (R) subunits, RI and RII, that interact with an identical catalytic (C) subunit. The RI and RII differ in molecular weight, protein sequence, isoelectric point, phosphorylation state, immunological characteristics, subcellular localization, tissue distribution, Kd for cAMP, and expression during development and transformation. Two isoforms of PKA serve distinct roles in different physiological processes, i.e., RI (type I PKA) being growth stimulatory, whereas RII (type II PKA) is a growth inhibitory and differentiation-inducing protein (see Ref. 20 for a review). The present study demonstrates that type I PKA activity was inactivated during the late phase of sepsis in the rat liver. We previously found that the CLP-induced septic rat, an identical animal model used in the current study, exhibits two distinct metabolic phases: an initial hyperglycemia followed by a progressive hypoglycemia (15, 16). The present finding that type I PKA activity was inactivated during late sepsis may have a pathophysiological significance in contributing to the development of hypoglycemia during the late stage of sepsis. Activation of PKA is known to stimulate glycogen phosphorylase, the rate-limiting enzyme for glycogen breakdown, resulting in an increased production of glucose from liver (1). In contrast, an inactivation of PKA would result in a decrease in glycogen breakdown and a subsequent reduction in the release of glucose from the liver. Our finding that type I PKA activity was inactivated in the liver during the late phase of sepsis thus may provide a biochemical explanation as to why animals in the late stage of sepsis are hypoglycemic.

PKA phosphorylates other intracellular enzymes such as pyruvate kinase, which were involved in the regulation of glucose homeostasis. It has been reported that pyruvate kinase activity was stimulated in dog liver after endotoxin administration (21). The stimulation in pyruvate kinase activity can be explained by the inactivation of type I PKA as reported in this study during late-stage sepsis, because phosphorylation of pyruvate kinase by PKA is known to decrease pyruvate kinase activity (1). This finding is further supported by previous findings that phosphorylation of pyruvate kinase in response to catecholamine was severely impaired in hepatocytes of septic and endotoxemic rats (7).

Ca^{2+} transport in liver plasma membranes involves formation of a phosphorylated intermediate of Ca^{2+}-ATPase (22–24). The level of phosphorylated intermediate of Ca^{2+}-ATPase in

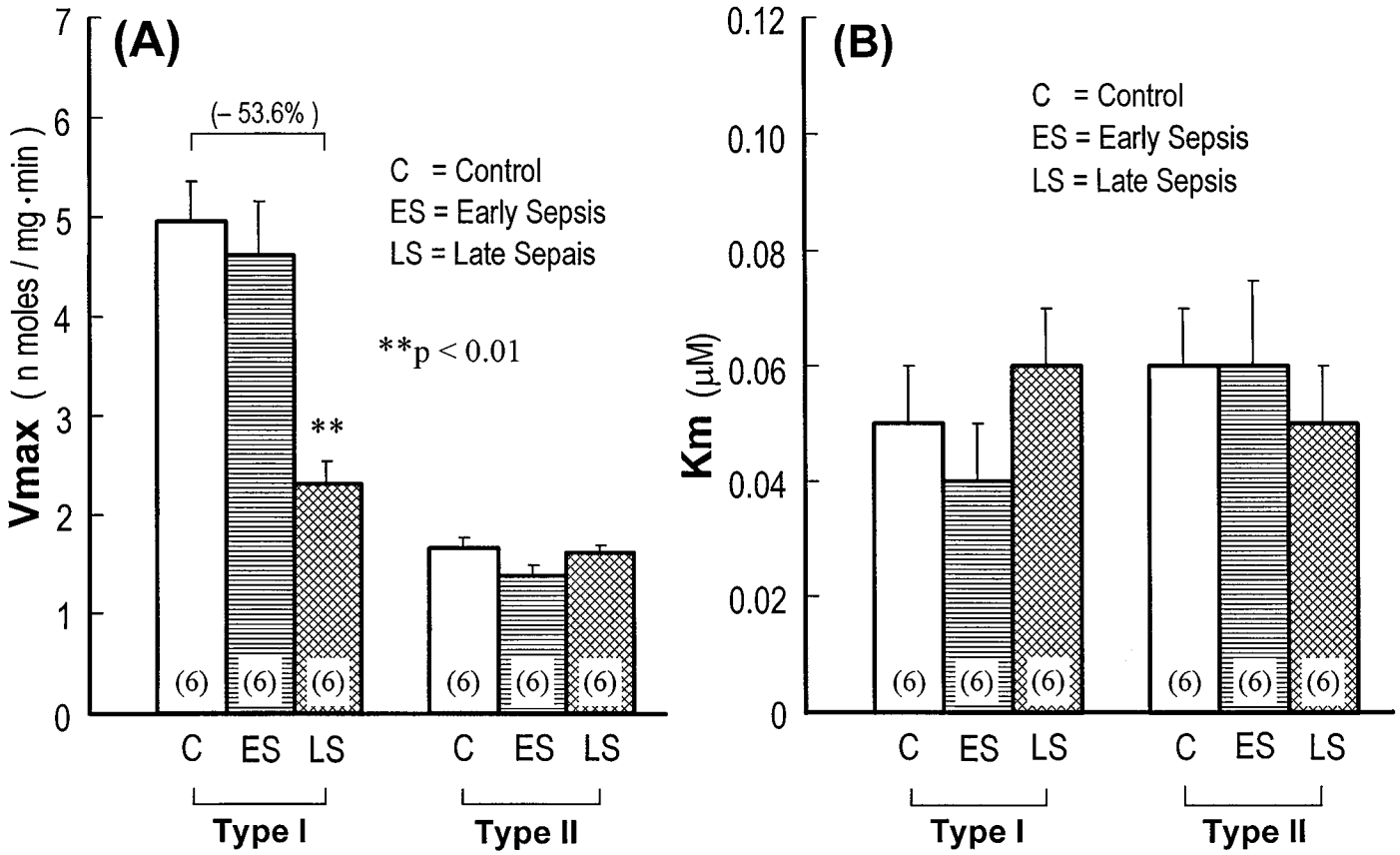

FIG. 2. **Changes in the V_{max} and K_m for cAMP for protein kinase A in the rat liver during the early and the late phases of sepsis.** Protein kinase A activities were measured as described under Materials and Methods except that cAMP concentrations were varied from 10^{-9} to 10^{-6} M. V_{max} and K_m values were obtained from Eadie-Hofstee plots based on different concentrations of cAMP. Vertical bars indicate SEM. Number of experiments was shown in the parenthesis of each column.

liver microsomal preparation was reduced significantly during septic shock (25). The ATP-dependent Ca^{2+} transport by liver plasma membrane was unaffected during early sepsis (9 h after CLP) but was decreased by 34–63% during late sepsis (18 h after CLP) (12). Furthermore, the ATP-dependent Ca^{2+} uptake in the rough endoplasmic reticulum of rat liver was impaired during sepsis (13). Thus, the current finding that type I PKA activity was decreased 18 h after CLP may have a pathophysiological significance in contributing to the development of the impairment of ATP-dependent Ca^{2+} transport in rat liver during the late phase of sepsis.

In addition to facilitating the ATP-dependent Ca^{2+} transport, PKA may also play a role in the IP_3-induced intracellular Ca^{2+} release (4). IP_3 has been proposed to be a second messenger for Ca^{2+} mobilization. Addition of IP_3 at low concentration causes Ca^{2+} release from microsomal stores in rat hepatocytes. Under pathological conditions such as endotoxin shock and sepsis, Ca^{2+} release in response to IP_3 challenge was found to be impaired (26, 27), and furthermore, the binding activity of IP_3 receptor in the endoplasmic reticulum was unaffected during early phase of sepsis but was significantly depressed by 40–50% during the late phase of sepsis (9). Because PKA is known to activate the permeability properties of the IP_3 for receptor/channel opening (4), it is conceivable that the observed impairment of the Ca^{2+} release from intracellular stores due to the depressed IP_3 binding to endoplasmic reticulum (9, 26, 27), may be a result of the inactivation of type I PKA, as reported in this study.

The exact mechanism responsible for the decrease in the type I PKA activity in the rat liver during the hypoglycemic phase of sepsis is not completely understood. Binding of catecholamine agonists to the β-adrenergic receptor rapidly activates adenylate cyclase via the stimulating quanine nucleotide regulatory protein Gs, resulting in a rise in cellular level of cAMP and consequently activating PKA activity. Although we have not determined cAMP concentration in the current study, changes in hepatic cAMP content reported by other investigators during endotoxic and septic shock have been contradictory. Hepatic cAMP level has been reported to decrease in endotoxic dog and mouse (28, 29), increase in rat infected with Diplococcus pneumoniae (30), and not change in rat after endotoxin administration (31). Previous studies from this laboratory indicated that the binding affinity and the number of β-adrenergic receptor were decreased (32), and the decrease in the dynamics of β-adrenergic receptor was accompanied by a reduction in the adenylate cyclase activity (33) in dog liver after endotoxin administration. More recently, we found that β-adrenergic receptor was underexpressed in the rat liver during the early and late phases of sepsis (34). Thus, the reduction in the density of β-adrenergic receptor (32, 34) and the inactivation of its coupling system (33) may contribute to the observed decrease in the type I PKA activity during the late phase of

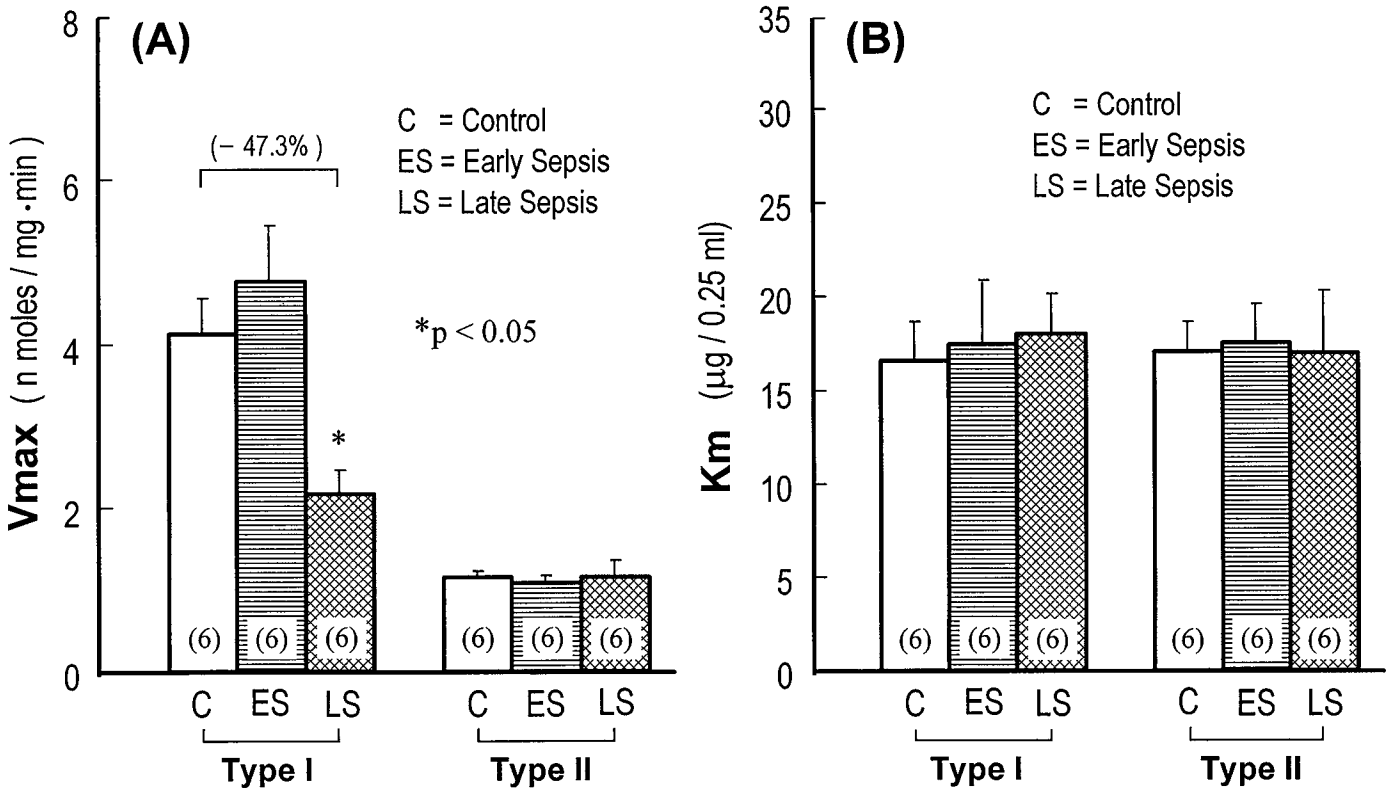

FIG. 3. **Changes in the V_{max} and K_m for histone for protein kinase A in the rat liver during the early and the late phases of sepsis.** Protein kinase A activities were measured as described under Materials and Methods except that histone concentrations were varied from 10^{-6} to 10^{-4} g/0.25 mL. V_{max} and K_m values were obtained from Eadie-Hofstee plots based on different concentrations of histone. Vertical bars indicate SEM. Number of experiments was shown in the parenthesis of each column.

sepsis. Alternatively, the reported decrease in hepatic ATP content (35, 36) during late sepsis (not determined in this study) may inactivate type I PKA activity. However, this possibility may be less likely because a 65% reduction in hepatic ATP content (36) does not appear to be sufficient to affect the K_m for ATP for PKA (the level of ATP after 65% reduction in late sepsis is about 1 mM ,which is 50 times higher than the K_m for ATP for type I PKA; calculated from Table 1 of Ref. 36 and Fig. 1B of this study); furthermore, the K_m for ATP for type I PKA was unaffected during late sepsis (Fig. 1B). In addition to changes in hepatic cAMP content in association with altered β-adrenergic receptor dynamics and changes in hepatic ATP content, changes in the phospholipase A activity may play a role in the sepsis-induced inactivation of PKA. Because activation of phospholipase A decreases the activity of PKA (37) and phospholipase A activities are activated in the liver in endotoxin shock (38) and sepsis (39, 40), it is conceivable that the observed decrease in type I PKA activity during the late phase of sepsis is a result of activation of phospholipase A. Further investigation is needed to clarify the role of phospholipase A in the regulation of the decrease in type I PKA activity in the rat liver during the late hypoglycemic phase of sepsis.

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