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Phosphorylation of Endothelial Nitric-Oxide Synthase Is Diminished in Mesenteric Arteries from Septic Rabbits Depending on the Altered Phosphatidylinositol 3-Kinase/Akt Pathway: Reversal Effect of Fluvastatin Therapy

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A running title: Statin on eNOS Regulation in Sepsis

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ABBREVIATIONS:

iNOS, inducible nitric-oxide synthase

NO, nitric oxide

eNOS, endothelial nitric-oxide synthase

LPS, lipopolysaccharide

PI3-K, phosphatidylinositol 3-kinase

HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A

PBS, phosphate-buffered saline

SECTION: Cardiovascular

Abstract

Endothelial dysfunction plays a crucial role in the pathophysiology of sepsis. Alterations in endothelial nitric-oxide synthase (eNOS) may contribute to the impaired endothelial function. We investigated whether the regulatory mechanism for eNOS phosphorylation and activation is altered in a rabbit lipopolysaccharide-induced septic model. Following induction of sepsis, a time-dependent marked reduction in eNOS phosphorylation was observed in mesenteric arteries, with a significant decrease in eNOS expression. Similarly, Akt phosphorylation was progressively and profoundly reduced, although total Akt remained unchanged. Furthermore, the amounts of the two subunits of phosphatidylinositol 3-kinase (PI3-K) in the membranous pool were diminished without changes in the total amount of the PI3-K heterodimer, indicating a decrease in translocation to the membranes. In vivo treatment with fluvastatin restored the decrease in eNOS phosphorylation in septic mesenteric vessels. This was possibly the result of the recovery of Akt phosphorylation. Treatment with the PI3-K inhibitor wortmannin partially inhibited the fluvastatin-induced increases in phosphorylation of Akt and eNOS, and the decrease in translocation of PI3-K heterodimer to the membranes during sepsis was slightly improved by fluvastatin. Sepsis-induced impairment of eNOS expression was also nearly normalized by fluvastatin. Importantly, rabbits treated with fluvastatin exhibited a dramatic improvement in sepsis survival. The present results showed vascular abnormalities of the PI3-K/Akt pathway involved in the impairment of eNOS phosphorylation and activation in sepsis. We also suggest that fluvastatin would ameliorate vascular endothelial dysfunction, in part, presumably via its recovery effect on Akt-dependent eNOS phosphorylation. It may be potentially useful for therapy of sepsis.

Introduction

Despite the systemic hypotension, organ failure, and morbidity that occur during sepsis being associated with overexpression of iNOS and excessive production of NO (Titheradge, 1999), a potential role of eNOS in the pathophysiology of sepsis is unsubstantiated. Previous work using eNOS knock-out mice has shown that they respond to endotoxin in an identical manner to wild-type littermates (Shesely et al., 1996), and very recently, less abundant expression of iNOS in LPS-treated eNOS knock-out mice have suggested that eNOS-derived NO is vital for facilitating iNOS expression in sepsis (Connelly et al., 2005). However, it has been revealed that mice overexpressing eNOS generate similar levels of plasma nitrite and nitrate to control animals in response to LPS but display resistance to LPS-induced hypotension, lung injury, and death (Yamashita et al., 2000). Moreover, we have recently demonstrated that eNOS expression is obviously diminished in blood vessels from rabbits (Matsuda et al., 2003) and in lung tissues from mice (Matsuda et al., 2004) following induction of sepsis with LPS. Since the relatively small amount of NO generated by eNOS provides important physiologic regulations, which are suggested to include those of vascular tone, bronchial tone, neurotransmission, or immune defense (Moncada et al., 1991; Gaston et al., 1994), impairment of expression and activity of eNOS, if any, may contribute to tissue injury in sepsis by hindrance of physiologic regulatory events with involvement of eNOS-derived NO.

Activation of PI3-K is an important proximal event in LPS signaling. Cell stimulation with LPS activates PI3-K and its downstream effector, Akt (Monick et al., 2001; Guha and Mackman, 2002; Connelly et al., 2004). Akt has been shown to phosphorylate eNOS leading to a persistent, Ca^{2+}-independent enzyme activation (Fulton et al., 1999; Dimmeler et al., 1999). In accordance, Akt may play a key role in

promoting endothelial NO synthesis. However, little is known about Akt-dependent eNOS phosphorylation in vascular endothelium following sepsis induction.

HMG-CoA reductase inhibitors are widely used for treatment of hyperlipidemia. This group of drugs is familiarly known as “statin” and their common mechanism of action is their ability to block hepatic conversion of HMG-CoA to L-mevalonate in the cholesterol biosynthetic pathway (Goldstein and Brown, 1990). However, a growing body of data suggests that HMG-CoA reductase inhibitors exhibit important immunomodulatory effects that are independent of their lipid-lowering ability (Kwak et al., 2000). These pleiotropic effects include anti-inflammatory actions, improvement of endothelial and microvascular functions, and modulation of eNOS (Lefer, 2002; Walter et al., 2004). Indeed, HMG-CoA reductase inhibitors have been shown to reverse the down-regulation of eNOS expression by hypoxia and oxidized low-density lipoprotein under cholesterol-clamped conditions (Laufs et al., 1997, 1998). Interestingly, treatment with an HMG-CoA reductase inhibitor, such as simvastatin, can profoundly improve survival in a murine model of sepsis (Merx et al., 2004). In the light of our previous report showing the diminished expression levels of eNOS in blood vessels from the animals after sepsis induction with LPS (Matsuda et al., 2003), it may be inferable that HMG-CoA reductase inhibitors could exert a protective effect against sepsis, at least in part, by virtue of their ability to ameliorate eNOS expression profile of endothelial cells thereby preventing vascular events.

The present study was aimed at verifying vascular changes in the signaling cascade mechanisms for Akt-dependent phosphorylation of eNOS in an *in vivo* rabbit model of endotoxin challenge. Additionally, we determined the therapeutic effect of the HMG-CoA reductase inhibitor, fluvastatin, on eNOS expression, eNOS phosphorylation, and activation of the PI3-K/Akt pathway in vascular tissues of this

septic model.

Materials and Methods

Sepsis Model.

Male New Zealand White rabbits weighing 2 to 2.5 kg were injected intravenously with LPS (100 μg/kg; *Escherichia coli* 055; List Biological Laboratories Inc., Campbell, CA) or an equivalent volume of sterile saline (sham; 2 ml/body) 3, 6 or 10 h before being killed by overdose of pentobarbital (60 mg/kg, i.v.). Our previous studies with this endotoxemic model have described the hemodynamic profile in detail. Thus, the animals injected with LPS show a marked but transient fall in blood pressure immediately after administration, and after a delay of about 2.5 h, hypotension and shock progressively develop over the following observation period (Matsuda et al., 2000, 2002). All animals received care in compliance with approved institutional animal care guidelines.

Drug Administration.

Rabbits received three intraperitoneal injections of 5 mg/kg of fluvastatin at 8 h intervals from 16 h before LPS treatment. This dose of fluvastatin was chosen because fluvastatin at this dose reportedly ameliorates endothelial dysfunction in obese Zucker rats (Nishimatsu et al., 2005). Fluvastatin was a gift from Novartis Pharma AG (Basel, Switzerland). It was dissolved in ethanol at a concentration of 20 mg/ml and diluted with saline at a rate of 1:3 to yield a final concentration of 5 mg fluvastatin/ml carrier. A control group of animals received the carrier only (carrier control). When wortmannin (1 mg/kg; BIOMOL International, Plymouth Meeting, PA) was used, it was intravenously given to the animal 1 h after LPS.

Preparation of Tissue Protein Extracts.

At indicated times after injection of LPS or vehicle, the main and first branches of the mesenteric artery were carefully removed under induction of terminal anesthesia with pentobarbital, and the vessels were immediately frozen with liquid nitrogen. The blood vessels were then placed in ice-cold sterile water containing phosphatase and protease inhibitor cocktail (0.2 μl/100 mg tissue, Sigma, St. Louis, MO) with the exception of the experiments using anti-mouse protein phosphatase 2A, and cleaned of adhering fat and connective tissue under a microscope. Subsequently, the tissues were powdered under liquid nitrogen and solubilized in 3 ml of ice-cold sterile water which contained 0.1% Triton X-100. The lysates were centrifuged at 1,000 $\times$ g_{max} for 10 min at 4^{o}C to pellet any insoluble material. The membrane fractions were prepared as described previously (Matsuda et al., 1999). Thus, the supernatant was then spun at 100,000 $\times$ g_{max} for 30 min at 4^{o}C. The membrane pellet was resuspended in 100 μl of lysis buffer and saved. Protein concentrations were measured using a Bio-Rad protein assay kit.

Primary Antibodies Used.

Immunoblotting was performed with the following commercially available antibodies: anti-goat eNOS (R&D Systems, Minneapolis, MN), anti-mouse phospho-eNOS (Ser 1177) (BIOMOL International), anti-mouse Akt (BIOMOL International), anti-rabbit phospho-Akt 1/2/3 (Thr 308) (Acris Antibodies GmbH, Hiddenhausen, Germany), anti-mouse phosphor-Akt 1 (Ser 473) (Acris Antibodies GmbH), anti-mouse protein phosphatase 2A (Upstate Cell Signaling, Lake Placid, NY), anti-mouse PI3-K p85α (GeneTex, San Antonio, TX), anti-mouse PI3-K p110α (BD Biosciences, San Jose, CA), anti-mouse PI3-K p85β (Acris Antibodies GmbH), and anti-mouse actin (GeneTex).

Western Blot Analysis.

Samples (2-20 μg) were run on SDS-polyacrylamide gel electrophoresis, using 7.5-12.5% polyacrylamide gel, and electrotransferred to polyvinylidine difluoride filter membrane. To reduce nonspecific binding, the membrane was blocked for 60 min at room temperature in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) containing 1% bovine serum albumin. The membrane was then incubated for 60 min at 4°C with primary antibodies diluted at 1:100-500 in PBS containing 0.05% Tween 20. After an extensive washing with PBS-Tween buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse (eBioscience, San Diego, CA), anti-goat (eBioscience), anti-rabbit (Bio-Rad Laboratories, Hercules, CA) or anti-mouse (Bio-Rad Laboratories) antibody diluted at 1:1000-6000 in PBS-Tween buffer at room temperature for 60 min. The blots were washed twice in PBS-Tween buffer, developed using the enhanced chemiluminescence detection system (Amersham, Little Chalfont, Buckinghamshire, UK), exposed to X-ray film, and analyzed by NIH Image software produced by Wayne Rasband (National Institutes of Health, Bethesda, MD). To check for protein loading/transfer variations, all blots were stained with Ponceau red (washable, before incubation with antibodies) and with Coomassie Brilliant Blue (permanent, after the enhanced chemiluminescence detection system). Intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was observed between samples.

Survival Studies in Sepsis.

Additional groups of rabbits underwent LPS and were included in survival studies. Rabbits were randomly divided into two groups. One group of the animals performed

LPS injection was treated with fluvastatin in the same way as described above. The animals were allowed free access to food and water, and survival time of each rabbit was recorded for 60 h.

Statistical Analysis.

All data were presented in terms of means ± S.E. Statistical assessment of the data was made by Student's *t* test or one-way analysis of variance followed by Bonferroni's multiple comparison test when appropriate. A P value <0.05 was considered statistically significant for all analyses.

Results

In accordance with our previous study (Matsuda et al., 2003), a time-dependent decrease in the eNOS protein level was observed in mesenteric artery from the rabbit when sepsis was induced by intravenous injection of 100 μg/kg LPS (Fig. 1). Furthermore, sepsis induction dramatically reduced phosphorylation of native eNOS at serine 1177, as determined by Western blotting with the phospho-specific antibody. The ratio of phospho-eNOS to total eNOS decreased by 36-68% after 6 h and by 70-86% after 10 h.

Since activation of the serine/threonine kinase Akt phosphorylates and activates eNOS, which in turn induces NO production and vasorelaxation (Fulton et al., 1999; Dimmeler et al., 1999), the downstream phosphorylation of Akt was also studied under the same condition. The results of Western blot analysis revealed that vascular expression of Akt dually phosphorylated at threonine 308 and serine 473 was reduced in a time-dependent manner after induction of sepsis by LPS (Fig. 2A). Immunoblot analysis with antibodies against total Akt showed equal protein levels in all samples, thus demonstrating that the reduced amount of phosphorylated Akt following sepsis induction was not due to decreased expression of Akt. Indeed, a marked time-dependent reduction in the ratio of phospho-Akt to total Akt was found after sepsis induction (Fig. 2B and C).

A role for protein phosphatase 2A in Akt dephosphorylation has been demonstrated in vascular smooth muscle cells (Yellaturu et al., 2002). The expression level of protein phosphatase 2A was examined in vascular tissues from septic rabbits. Immunoblot analysis showed a slight but significant decrease rather than an increase in vascular expression of protein phosphatase 2A with the progression of sepsis (83 ± 4% of control at 10 h after LPS, $n = 5$, $P<0.05$).

Akt is located downstream of PI3-K (Datta et al., 1996; Klippel et al., 1997). Although the PI3-K family comprises at least 12 members (Vanhaesebroeck and Waterfield, 1999), we assessed PI3-Kα protein levels by Western blotting using its specific antibodies because of the importance of the α form in the regulation of downstream signaling molecules (Bi et al., 1999). As documented that PI3-K is a heterodimer phospholipids kinase composed of an 85-kDa regulatory subunit and 110-kDa catalytic subunit (Carpenter et al., 1990), the 85-kDa and 110-kDa bands corresponded to the PI3-Kα heterodimer were detected in rabbit mesenteric artery (Fig. 3). The total amount of the PI3-Kα heterodimer was substantially unchanged by sepsis induction. The protein kinase activity of PI3-Kα was determined by assessment of translocation of PI3-Kα to the membrane pool. Sepsis induction resulted in a time-dependent reduction in the amount of PI3-Kα heterodimer in the membrane fraction in vascular tissues (Fig. 3), suggesting that PI3-Kα was much less activated with the development of sepsis.

Additionally, we tested vascular changes in PI3-Kβ protein levels during sepsis. Western blot analysis using the specific antibody against the 85-kDa subunit of PI3-Kβ was performed in rabbit mesenteric artery. In similar to PI3-Kα, PI3-Kβ 85-kDa subunit in the membrane/total fractions was reduced by 34% after 6 h and by 20% after 10 h of sepsis (n = 3).

Statins may increase eNOS phosphorylation and expression through the PI3-K/Akt pathway (Kureishi et al., 2000). Effects of in vivo treatment of rabbits with fluvastatin on vascular expression and phosphorylation of Akt and eNOS were tested under control and septic conditions. The total expression levels of Akt and eNOS in vascular tissues were unchanged by in vivo fluvastatin treatment of control rabbits. However, fluvastatin induced a 2.6- and 2-fold increase in phospho-Akt and

phosphor-eNOS levels in vascular tissues from control rabbits, respectively (Fig. 4). Moreover, fluvastatin strongly prevented the prominent reduction in Akt and eNOS phosphorylation caused by 10 h of LPS-induced sepsis (8.9- and 9.6-fold increase, respectively) (Fig. 4). In addition, fluvastatin significantly improved a sepsis-induced reduction in total eNOS expression from 54 ± 3% to 85 ± 3% of control.

The reduced amount of PI3-Kα heterodimer in the membrane fraction in vascular tissues 10 h after induction of sepsis was significantly but incompletely attenuated by in vivo treatment with fluvastatin (Fig. 5). When the PI3-K inhibitor wortmannin (1 mg/kg) was given to the rabbit 1 h after LPS, this treatment resulted in a partial inhibition of the fluvastatin-induced increase in phosphorylation of Akt (Fig. 6). Furthermore, wortmannin treatment partially inhibited the fluvastatin-induced increase in phosphorylation of eNOS in septic vascular tissues (Fig. 7). The increased protein expression level of total eNOS in septic vascular tissues under fluvastatin therapy was significantly lowered by wortmannin, but this lowering effect was small (Fig. 7).

Rabbits were treated with fluvastatin or placebo. The animals injected intravenously with 100 μg/kg LPS without fluvastatin treatment died within 30 h. No deaths occurred in the animals that were injected intravenously with sterile saline instead of LPS. The survival curves for rabbits in which LPS was challenged clearly showed that fluvastatin significantly protected the animals against LPS-induced death (Fig. 8).

Discussion

The present study showed a time-dependent decrease in eNOS protein expression in mesenteric artery from the rabbit following induction of sepsis by LPS challenge. We have previously reported that LPS injection markedly up-regulated iNOS expression and down-regulated eNOS expression in this vascular tissue (Matsuda et al., 2003). In addition, the data presented in this study demonstrated that sepsis caused a progressive and profound reduction in phosphorylation of eNOS in rabbit mesenteric artery. Since phosphorylation of eNOS results in increased NO production, our results would suggest less production of NO by eNOS in sepsis. NO produced by eNOS is considered to serve as a ubiquitous mediator molecule involved in many physiologic regulations, including vascular tone, neurotransmission, and immune defense (Moncada et al., 1991; Gaston et al., 1994). Therefore, loss of eNOS-based NO synthesis may well contribute to vascular abnormalities, inflammatory sequalae, and physiologic dysfunction of organs in sepsis. Indeed, particular attention has been focused on endothelial cells which are a major target of sepsis-induced events and whose dysfunction plays a crucial role in the pathophysiology of septic shock (Peters et al., 2003).

The PI3-K/Akt pathway has been described to signal eNOS activation in response to shear stress (Fulton et al., 1999; Dimmeler et al., 1999). Thus, eNOS phosphorylation is downstream to Akt activation. Our study suggests that the sepsis-induced decrease in eNOS phosphorylation may be the result of reduced phosphorylation of Akt, because induction of sepsis was found to lead to a time-dependently marked reduction in Akt phosphorylation in rabbit mesenteric artery without any change in the total amount of Akt. A role of protein phosphatase 2A in dephosphorylation of Akt has been documented in vascular smooth muscle cells in response to the cellular stress condition (Yellaturu et al., 2002). This does not appear

to be the case, however, as vascular expression of protein phosphatase 2A was decreased rather than increased following sepsis induction, although we cannot completely exclude altered activity of protein phosphatase 2A that could be regulated not only by its expression but through other mechanisms. Activation of Akt occurs downstream of PI3-K (Datta et al., 1996; Klippel et al., 1997). Thus, activated PI3-K catalyzes phosphorylation of membrane inositol lipids and causes accumulation of phosphatidylinositol 3,4,5-trisphosphate and its phospholipid phosphatase product phosphatidylinositol 3,4-bisphosphate in the membrane. Such membrane changes allow docking of the lipid kinases phosphatidylinositol-dependent kinase 1 and Akt. Although PI3-K is a heterodimer lipid kinase consisting of an 85-kDa subunit bound to a 110-kDa catalytic subunit (Carpenter et al., 1990), the amounts of both subunits in the membranous pool were progressively and profoundly decreased in rabbit mesenteric artery following induction of sepsis. The mechanism of the decrease is probably due to a decrease in translocation. This possibility could be supported by the lack of changes in the total amount of the PI3-Kα heterodimer during sepsis, although it should be kept in mind that other subunits of Class IA PI3-K family members (Vanhaesebroeck and Waterfield, 1999) may be involved in sepsis-induced changes in this pathway. It is essential for PI3-K activation to allow translocation of the cytosolic PI3-K to the membranes where its lipid substrates and its binding monomeric G protein Ras reside (Vanhaesebroeck and Waterfield, 1999). Akt is activated following PI3-K recruitment to the inner surface of the plasma membrane. Therefore, it would be reasonable from the present data to conclude that PI3-K was much less activated with the development of sepsis. We thus indicate that impaired phosphorylation of Akt in vascular tissues during sepsis appears to be attributed to reduced activity of its upstream enzyme, PI3-K.

Contrary to the present findings in mesenteric arteries from septic rabbits, in vitro

studies have shown that LPS stimulation caused phosphorylation of Akt in human alveolar macrophages (Monick et al., 2001), human monocytic cells (Guha and Mackman, 2002), and human umbilical vein endothelial cells (Connelly et al., 2004), although its time-dependency is quite different from each other. At the present time, we do not have a clear understanding to reconcile these findings. However, the PI3-K/Akt pathway has been shown to regulate negatively nuclear factor-κB, a transcription factor activated by LPS, and overexpression of proinflammatory genes (Romashkova and Makarov, 1999; Vanhaesebroeck and Waterfield, 1999). Inhibition of PI3-K by wortmannin enhances LPS-induced NOS in murine peritoneal macrophages (Park et al., 1997), and activation of PI3-K/Akt can suppress LPS-induced lipoprotein lipase expression in J774.2 macrophages (Tengku-Muhammad et al., 1999). Induction of NOS by LPS in C6 glial cells and rat primary astrocytes has also been found to be regulated negatively by activation of PI3-K (Pahan et al., 1999). Finally, overexpression of Akt has been documented to improve survival in cecal ligation and puncture septic mouse model in association with prevention of sepsis-induced lymphocyte apoptosis (Bommhardt et al., 2004).

Interestingly, the HMG-CoA reductase inhibitor fluvastatin restored eNOS phosphorylation which was markedly impaired in mesenteric artery from septic rabbits, and this improvement was associated with the recovery of Akt phosphorylation. It is well established that Akt-dependent phosphorylation of eNOS is necessary for a full activation of eNOS and endothelium-dependent vasorelaxation (Fulton et al., 1999; Dimmeler et al., 1999). Statins can induce angiogenesis via activation of Akt and eNOS in vascular endothelial cells (Kureishi et al., 2000). Furthermore, statins appear to stimulate membrane translocation of Akt and its activation in endothelial cells (Skaletz-Rorowski et al., 2003). Thus, our results suggest that sepsis-induced

impairment of eNOS activation showed a substantial improvement by in vivo treatment with fluvastatin, at least in part, via its direct effect on Akt. However, we found that fluvastatin treatment was capable of attenuating the reduction in membrane translocations of the PI3-Kα heterodimer in septic vascular tissues, although this effect was not so pronounced. Moreover, in vivo treatment with the PI3-K inhibitor wortmannin led to a partial inhibition of increased phosphorylation of Akt and eNOS by fluvastatin treatment of the septic rabbit. These results suggest that the stimulatory effect of fluvastatin on Akt phosphorylation may partially involve PI3-K activation. Indeed, it has previously been demonstrated that inhibition of the PI3-K pathway by wortmannin and the additional PI3-K inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one) inhibited endothelial progenitor cell differentiation in mice treated with statins (Dimmeler et al., 2001). Furthermore, LY294002 could reportedly attenuate the increase in the level of heme oxygenase-1 by simvastatin in human and rat aortic smooth muscle cells (Lee et al., 2004).

We also found that fluvastatin restored vascular eNOS expression which was diminished following sepsis induction. Wortmannin treatment resulted in a significant inhibition of this restoration, suggesting that the effect of fluvastatin on total eNOS protein expression in septic vascular tissues may involve improvement of the PI3-K/Akt pathway. However, the inhibitory effect of wortmannin on the fluvastatin-induced increase in total eNOS in septic vascular tissues was evidently small. Statins inhibit the *Rho*-dependent pathway via suppression of *Rho* geranylgeranylation, which is an important posttranslational modification and is required for the membrane translocation and activation of *Rho,* and consequently stimulate eNOS expression (Laufs et al., 1998). It is conceivable that the recovery effect of fluvastatin on eNOS expression may be due to the inhibition of the *Rho*-dependent pathway, however, further investigations are

needed to prove this point. It would thus be expected that fluvastatin can improve sepsis-induced deteriorations of eNOS expression and activation, thereby ameliorating vascular endothelial dysfunction in sepsis. The ability of statins to restore the pathological endothelial dysfunction has been documented in animal models of stroke (Laufs et al., 2000) and diabetes (Nishimatsu et al., 2005).

Our survival studies clearly showed that fluvastatin protected the rabbits against LPS-induced septic death. This finding is in good agreement with the result by Merx et al. (2004), who have reported that simvastatin significantly improves survival in a cecal ligation and puncture septic mouse model. They have stated the importance of increased mononuclear cell adhesiveness in septic mice to be reversed by simvastatin treatment (Merx et al., 2004). In the light of the recent report that overexpression of Akt can improve survival in sepsis (Bommhardt et al., 2004), we interpret the present results to suggest that the profound improvement in survival may be associated with up-regulation of the Akt pathway by statins. However, other mechanisms, which may also involve important cellular effects independent of lipid lowering, cannot be excluded.

In summary, phosphorylation of eNOS was markedly diminished in rabbit mesenteric artery following LPS-induced sepsis. This seemed to be associated with the impaired PI3-K/Akt pathway, although direct experimental evidence to support this mechanism awaits further study. Fluvastatin restored sepsis-induced impairment of eNOS phosphorylation in association with the recovery of Akt phosphorylation, and dramatically improved survival in sepsis. Although being well established in the treatment of lipid disorders, statins may have a new potential of being therapeutic for subjects with sepsis.

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Footnotes

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Figure Legends

Fig. 1. Sepsis-induced reduction in eNOS phosphorylation at serine 1177 in rabbit mesenteric artery. In the upper trace, representative Western blots of phospho-eNOS (top) and total eNOS (bottom) after induction of sepsis by LPS for 3 h, 6 h, and 10 h are shown in comparison with those obtained in control. In the lower trace, bar graph summarizing the immunoblot data is represented. Bands were quantified in arbitrary units by densitometry. Values are means ± S.E. (n = 5) of phospho-eNOS/eNOS expressed as percentage of the respective sham control result. *P<0.05 versus control by t test.

Fig. 2. Sepsis-induced reductions in Akt phosphorylation at threonine 308 and at serine 473 in rabbit mesenteric artery. A, representative Western blots of phospho-Akt at threonine 308 (top) and at serine 473 (middle) and of total Akt (bottom) after induction of sepsis by LPS for 3 h, 6 h, and 10 h in comparison with those obtained in control. B and C, bar graphs summarizing the immunoblot data. Bands were quantified in arbitrary units by densitometry. Values represent means ± S.E. (n = 5) of phospho-Akt at threonine 308 (B) and at serine 473 (C)/Akt expressed as percentage of the respective sham control result. *P<0.05 versus control by t test.

Fig. 3. Sepsis-induced reductions in membrane translocations of the p110 and p85 subunits of PI3-Kα in rabbit mesenteric artery. In the upper trace of each panel, representative Western blots of p110 subunit (A) or p85 subunit (B) of PI3-Kα in the membrane fraction (top) and the total fraction (bottom) after induction of sepsis by LPS for 3 h, 6 h, and 10 h are shown in comparison with those obtained in control. In the lower trace, bar graph summarizing the immunoblot data is represented. Bands were

quantified in arbitrary units by densitometry. Values are means ± S.E. (n = 5) of p110α (A) and p85α (B) in membrane/total fractions expressed as percentage of the respective sham control result. *P<0.05 versus control by t test.

Fig. 4. Effects of in vivo treatment with fluvastatin (Flv) on Akt and eNOS phosphorylation in mesenteric arteries from control and 10-h septic rabbits. Fluvastatin (5 mg/kg) was injected intraperitoneally at 8 h intervals from 16 h before induction of sepsis by LPS. In the upper trace of each panel, representative Western blots of phospho-Akt and total Akt (A) or phospho-eNOS and total eNOS (B) 10 h after induction of sepsis by LPS with and without fluvastatin treatment are shown in comparison with those obtained in control. In the lower trace, bar graph summarizing the immunoblot data is represented. Bands were quantified in arbitrary units by densitometry. Values are means ± S.E. (n = 5) of phospho-Akt/Akt (A) and phospho-eNOS/eNOS (B) expressed relative as percentage of the respective sham control result. *P<0.05 versus control; #P<0.05 versus 10 h of sepsis.

Fig. 5. Effect of in vivo treatment with fluvastatin (Flv) on sepsis-induced reductions in membrane translocations of the p110 and p85 subunits of PI3-Kα in rabbit mesenteric artery. In the upper trace of each panel, representative Western blots of p110 subunit (A) or p85 subunit (B) of PI3-Kα in the membrane fraction (top) and the total fraction (bottom) 10 h after induction of sepsis by LPS with and without fluvastatin treatment are shown in comparison with those obtained in control. In the lower trace, bar graph summarizing the immunoblot data is represented. Bands were quantified in arbitrary units by densitometry. Values are means ± S.E. (n = 5) of p110α (A) and p85α (B) in membrane/total fractions expressed as percentage of the respective sham control result.

*P<0.05 versus control; #P<0.05 versus 10 h of sepsis.

Fig. 6. Inhibitory effect of wortmannin (Wt) on increased phosphorylation of Akt in mesenteric arteries by in vivo treatment with fluvastatin (Flv) of 10-h septic rabbits. Wortmannin (1 mg/kg) was intravenously injected 1 h after LPS. A, representative Western blots of phospho-Akt at threonine 308 (top) and at serine 473 (middle) and of total Akt (bottom) 10 h after induction of sepsis when treated with fluvastatin and fluvastatin/wortmannin in comparison with those obtained in sepsis alone. B and C, bar graphs summarizing the immunoblot data. To standardize between experiments, an arbitrary density of 1 was assigened to be the band obtained from the vascular sample of the 10-h septic rabbit. Values are means ± S.E. (n = 5) of phospho-Akt at threonine 308 (B) and at serine 473 (C)/Akt expressed relative to the respective result from sepsis alone. *P<0.05 versus 10 h of sepsis alone; #P<0.05 versus sepsis with fluvastatin treatment.

Fig. 7. Inhibitory effect of wortmannin (Wt) on increased phosphorylation and protein expression of eNOS in mesenteric arteries by in vivo treatment with fluvastatin (Flv) of 10-h septic rabbits. Wortmannin (1 mg/kg) was intravenously injected 1 h after LPS. A, representative Western blots of phospho-eNOS (top), total eNOS (middle) and actin (bottom) 10 h after induction of sepsis when treated with fluvastatin and fluvastatin/wortmannin in comparison with those obtained in sepsis alone. B and C, bar graphs summarizing the immunoblot data. To standardize between experiments, an arbitrary density of 1 was assigened to be the band obtained from the vascular sample of the 10-h septic rabbit. Values are means ± S.E. (n = 5) of phospho-eNOS/eNOS (B) and eNOS/actin (C) expressed relative to the respective result

from sepsis alone. *P<0.05 versus 10 h of sepsis alone; #P<0.05 versus sepsis with fluvastatin treatment.

Fig. 8. Kaplan-Meier survival curves. Fluvastatin (5 mg/kg) was injected intraperitoneally at 8 h intervals from 16 h prior LPS treatment. Control rabbits were injected intravenously with sterile saline instead of LPS. Survival was recorded for 60 h. Six rabbits were used for each group.

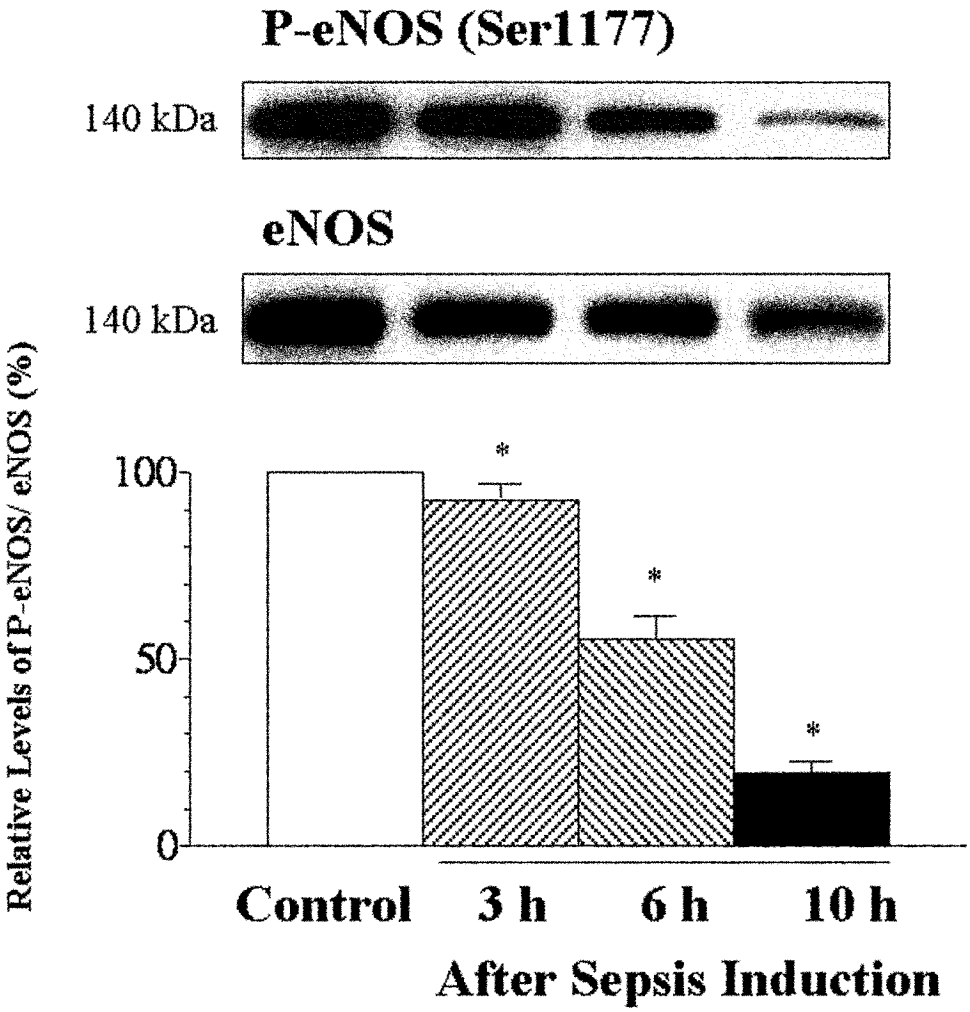
P-eNOS (Ser1177)
140 kDa
eNOS
140 kDa
Relative Levels of P-eNOS/ eNOS (%)
100
50
0
*
*
*
Control
3 h
6 h
10 h
After Sepsis Induction

Figure 1

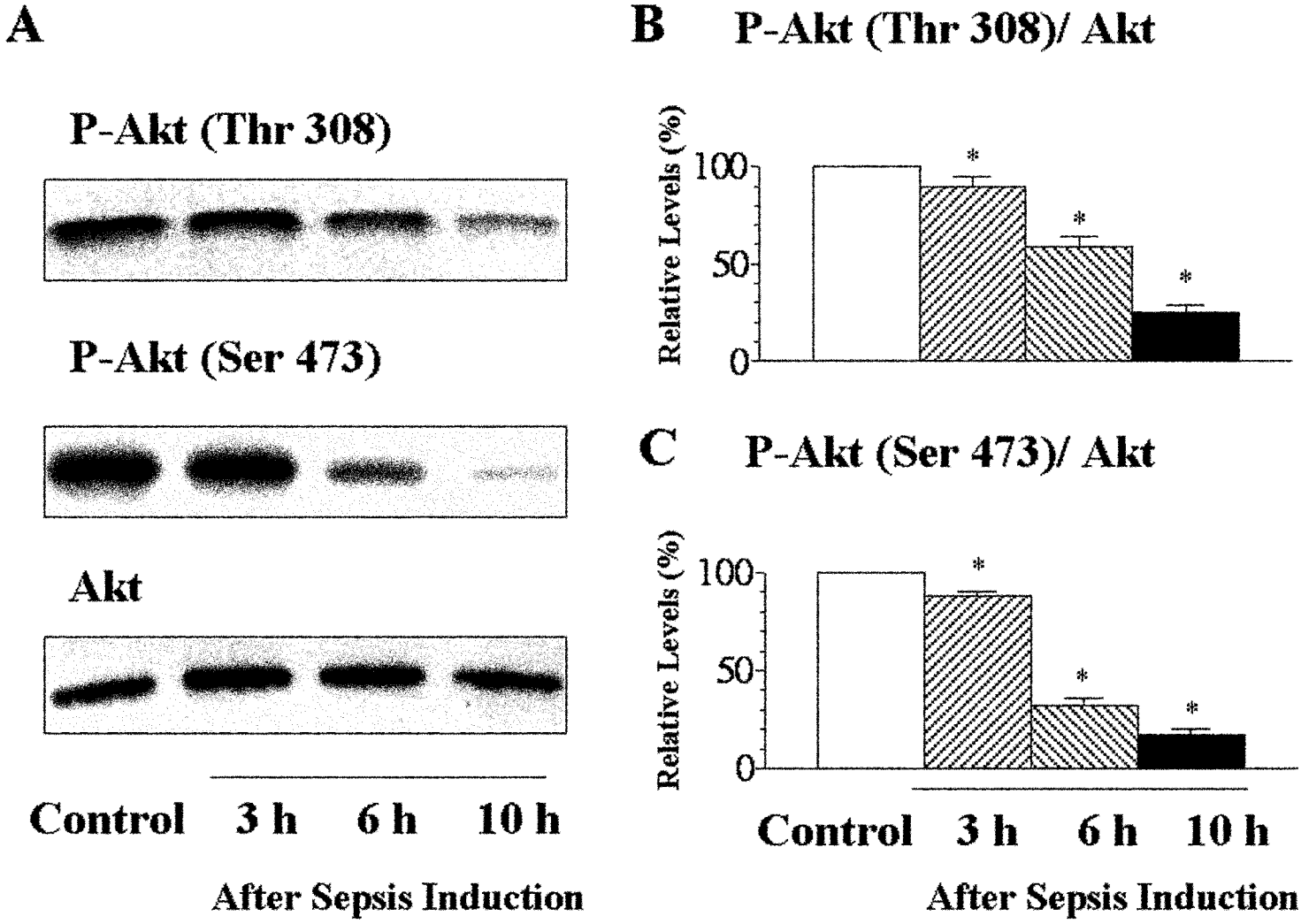
A
P-Akt (Thr 308)
P-Akt (Ser 473)
Akt
Control
3 h
6 h
10 h
After Sepsis Induction
B
P-Akt (Thr 308)/ Akt
Relative Levels (%)
100
50
0
*
C
P-Akt (Ser 473)/ Akt
Relative Levels (%)
100
50
0
*
Control
3 h
6 h
10 h
After Sepsis Induction

Figure 2

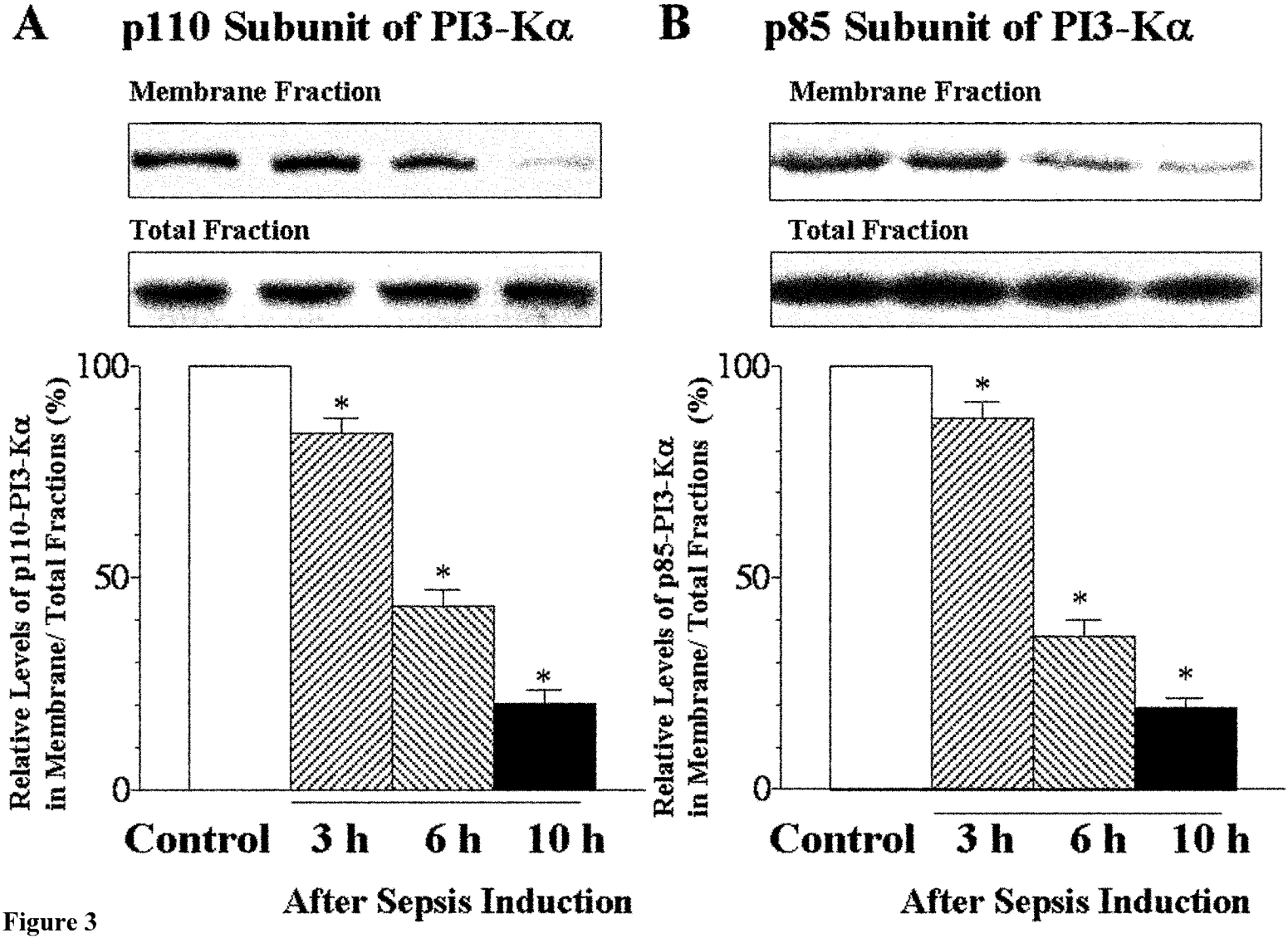
A
p110 Subunit of PI3-Kα
Membrane Fraction
Total Fraction
Relative Levels of p110-PI3-Kα in Membrane/ Total Fractions (%)
100
50
0
*
*
*
Control
3 h
6 h
10 h
After Sepsis Induction
B
p85 Subunit of PI3-Kα
Membrane Fraction
Total Fraction
Relative Levels of p85-PI3-Kα in Membrane/ Total Fractions (%)
100
50
0
*
*
*
Control
3 h
6 h
10 h
After Sepsis Induction

Figure 3

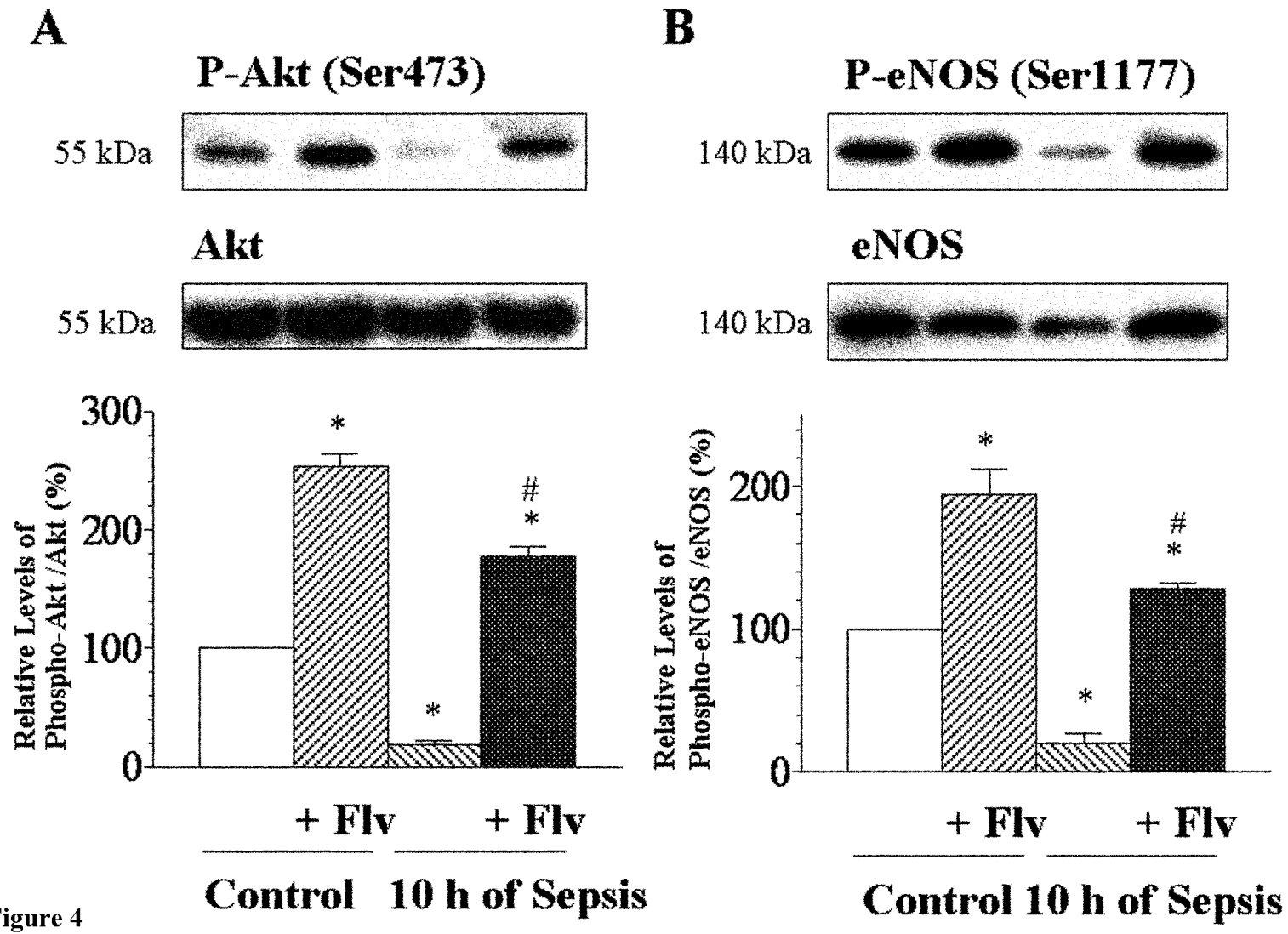
A
P-Akt (Ser473)
55 kDa
Akt
55 kDa
Relative Levels of Phospho-Akt /Akt (%)
300
200
100
0
*
*
#
*
+ Flv
+ Flv
Control
10 h of Sepsis
B
P-eNOS (Ser1177)
140 kDa
eNOS
140 kDa
Relative Levels of Phospho-eNOS /eNOS (%)
200
100
0
*
*
#
*
+ Flv
+ Flv
Control
10 h of Sepsis

Figure 4

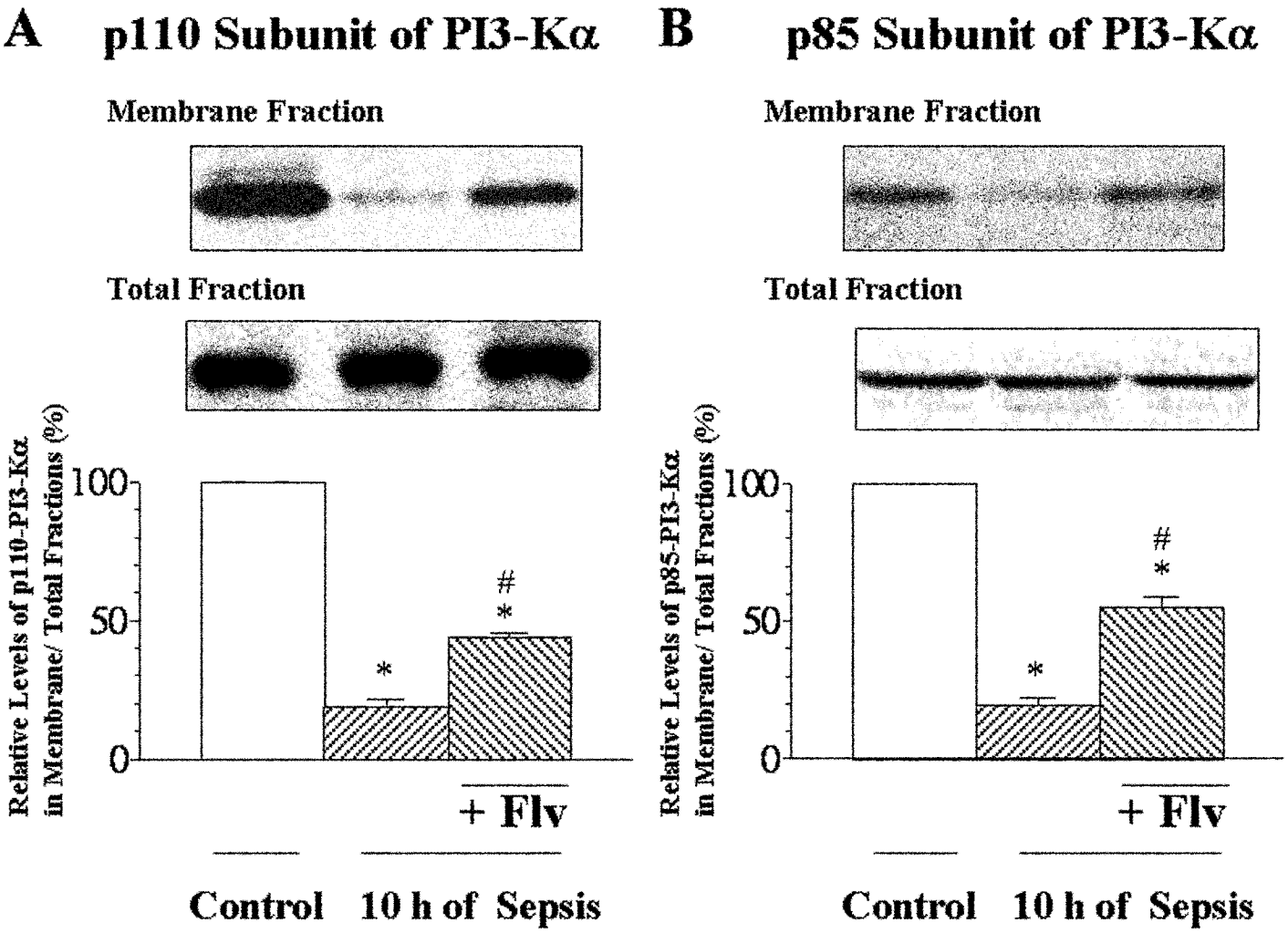
A
p110 Subunit of PI3-Kα
Membrane Fraction
Total Fraction
Relative Levels of p110-PI3-Kα in Membrane/ Total Fractions (%)
100
50
0
*
#
*
+ Flv
Control
10 h of Sepsis
B
p85 Subunit of PI3-Kα
Membrane Fraction
Total Fraction
Relative Levels of p85-PI3-Kα in Membrane/ Total Fractions (%)
100
50
0
*
#
*
+ Flv
Control
10 h of Sepsis

Figure 5

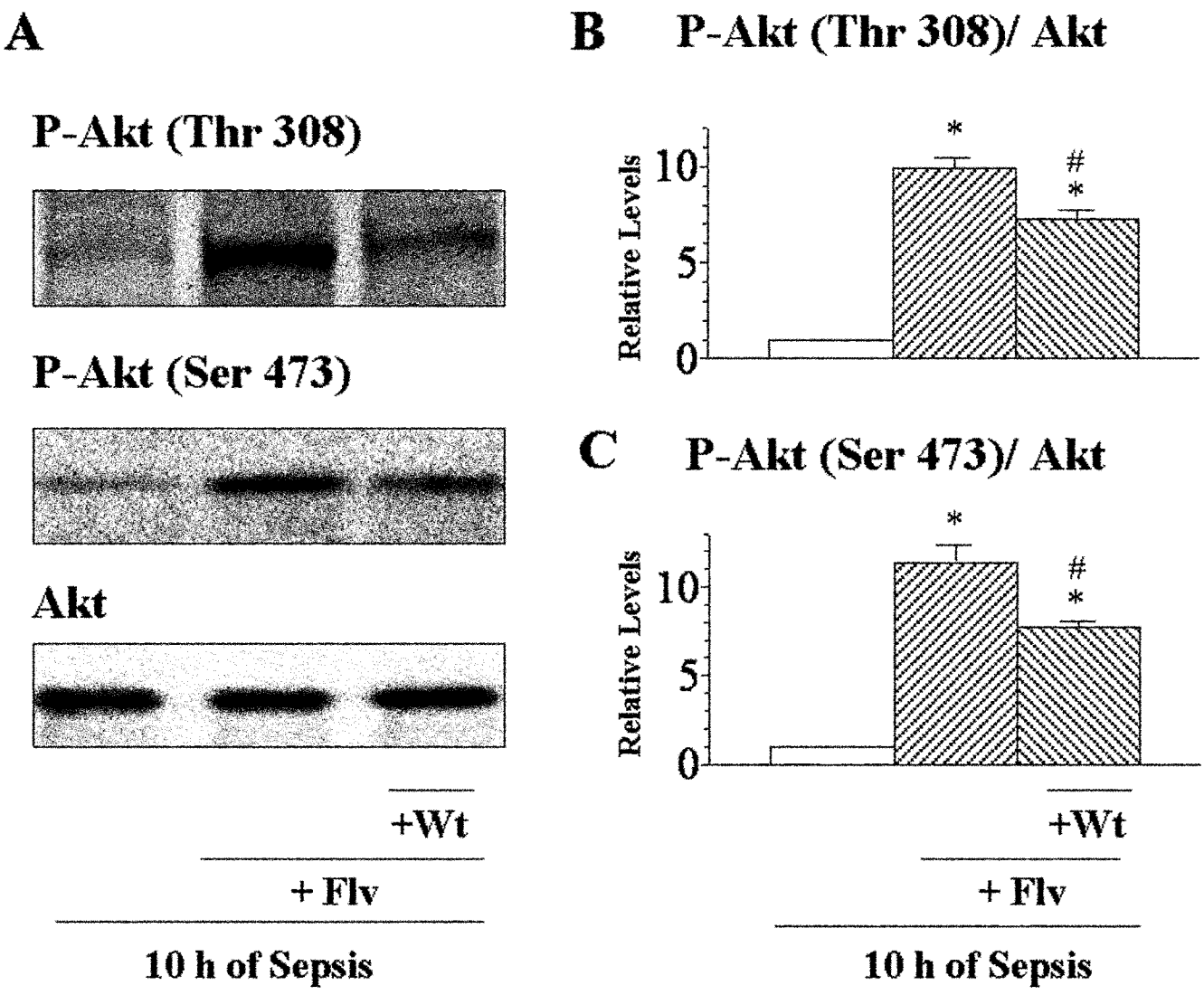
A
P-Akt (Thr 308)
P-Akt (Ser 473)
Akt
+Wt
+ Flv
10 h of Sepsis
B
P-Akt (Thr 308)/ Akt
Relative Levels
10
5
0
*
#
*
C
P-Akt (Ser 473)/ Akt
Relative Levels
10
5
0
*
#
*
+Wt
+ Flv
10 h of Sepsis

Figure 6

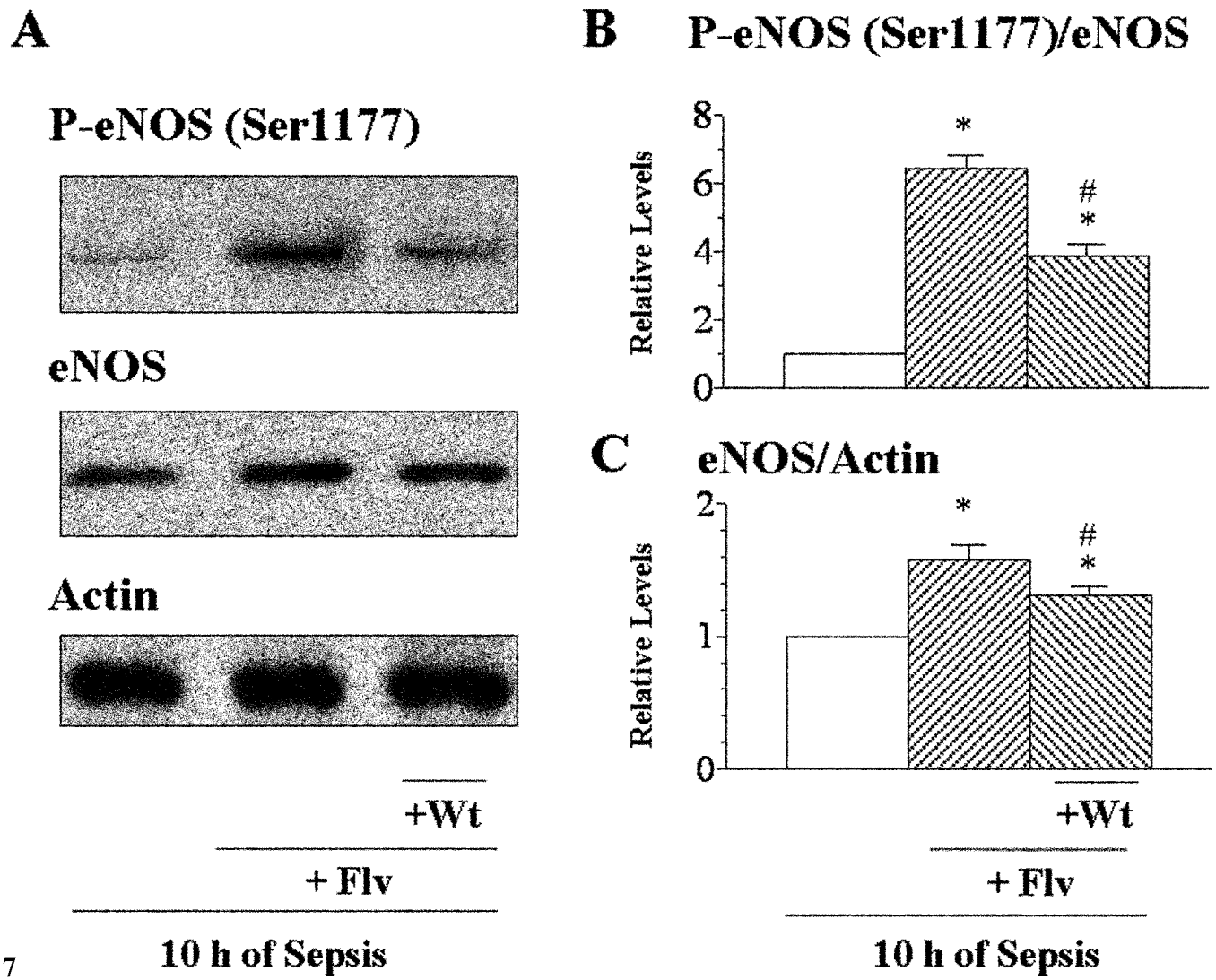
A
P-eNOS (Ser1177)
eNOS
Actin
+Wt
+ Flv
10 h of Sepsis
B
P-eNOS (Ser1177)/eNOS
Relative Levels
0
2
4
6
8
*
#
*
C
eNOS/Actin
Relative Levels
0
1
2
*
#
*
+Wt
+ Flv
10 h of Sepsis

Figure 7

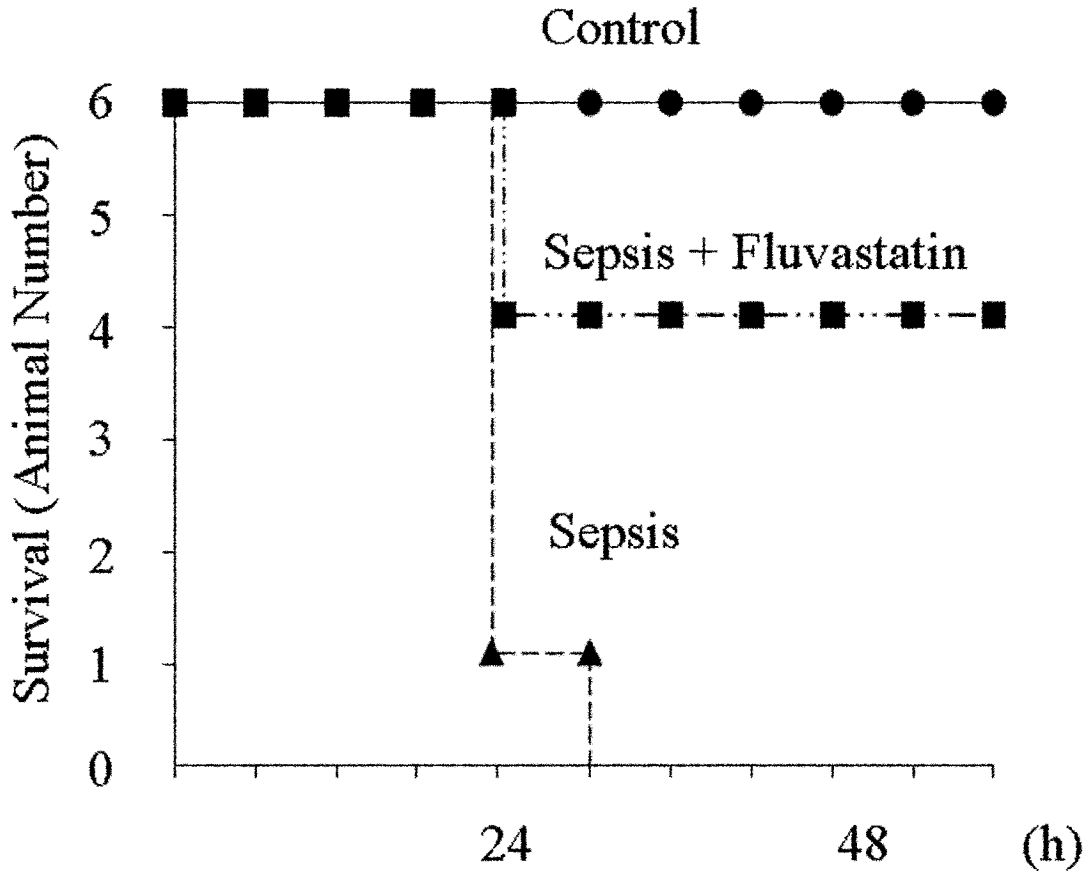
Control
Sepsis + Fluvastatin
Sepsis
Survival (Animal Number)
0
1
2
3
4
5
6
24
48
(h)

Figure 8