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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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. Likewise, 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. It is noteworthy that 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.

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 knockout mice has shown that they respond to endotoxin in an identical manner to wild-type littermates (Shehery et al., 1996), and it has been recently shown that less abundant expression of iNOS in LPS-treated eNOS knockout mice suggests 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. Because 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 the involvement of eNOS-derived NO.

Activation of PI3-K is an important proximal event in LPS

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 LY294002, 2-(4-morpholino)-8-phenyl-1(4H)-benzopyran-4-one; Flv, fluvastatin.
signaling. Cell stimulation with LPS activates PI3-K and its downstream effector, Akt (Monick et al., 2001; Guha and Mackman, 2002; Connelly et al., 2005). Akt has been shown to phosphorylate eNOS, leading to a persistent, Ca<sup>2+</sup>-independent enzyme activation (Dimmel et al., 1999; Fulton et al., 1999). In accordance with this, 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 the drugs’ common mechanism of action is their ability to block hepatic conversion of HMG-CoA to 3-7-mevalonate in the cholesterol biosynthetic pathway (Goldstein and Brown, 1990). However, a growing body of data suggest 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. In addition, 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 approximately 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 the approved institutional animal care guidelines.

**Drug Administration.** Rabbits received three intraperitoneal injections of 5 mg/kg fluvastatin at 9-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 of fluvastatin/ml of 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 of 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. Thereafter, the tissues were powdered under liquid nitrogen and solubilized in 3 ml of ice-cold sterile water that contained 0.1% Triton X-100. The lysates were centrifuged at 100,000 g for 10 min at 4°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 g for 30 min at 4°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 (Bio-Rad Laboratories, Hercules, CA).

**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 (Thr308) (BD Biosciences, San Jose, CA), anti-rabbit phospho-Akt 1 (Ser 473) (BD Biosciences), 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 to 12.5% polyacrylamide gel, and electrotransferred to polyvinylidene 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 Na2HPO4, and 1.5 mM KH2PO4) containing 1% bovine serum albumin. The membrane was then incubated for 60 min at 4°C with primary antibodies diluted at 1:100 to 1:500 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), or anti-rabbit (Bio-Rad Laboratories), antibody diluted at 1:1000 to 1:6000 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 injection and were included in survival studies. Rabbits were randomly divided into two groups. One group of the animals that underwent LPS injection was treated with fluvastatin in the same way as described above. The animals were allowed free access to food and water, and the survival time of each rabbit was recorded for 60 h.

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Statistical Analysis. All data were presented in terms of means ± S.E. Statistical assessment of the data were 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 Ser1177, as determined by Western blotting with the phosphospecific antibody. The ratio of phospho-eNOS to total eNOS decreased by 36 to 68% after 6 h and by 70 to 86% after 10 h.

Because activation of the serine/threonine kinase Akt phosphorylates and activates eNOS, which in turn induces NO production and vasorelaxation (Dimmeler et al., 1999; Fulton 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 Thr308 and Ser473, 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. 2A, B 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; Klippe, 1997). Although the PI3-K family comprises at least 12 members (Vanhasebroeck 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, PI3-Kα is a heterodimer phospholipid kinase composed of an 85-kDa regulatory subunit and 110-kDa catalytic subunit (Carpenter et al., 1990); the 85- and 110-kDa bands that 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.

In addition, 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. Similar to PI3-Kα, the 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 phospho-eNOS levels in vascular tis-
Fig. 3. Sepsis-induced reductions in membrane translocations of the p110 and p85 subunits of PI3-Kα in rabbit mesenteric artery. In the top 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, 6, and 10 h are shown in comparison with those obtained in control. In the bottom trace, a bar graph summarizing the immunoblot data is shown. 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 top 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 bottom trace, a bar graph summarizing the immunoblot data is shown. 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 Flv on sepsis-induced reductions in membrane translocations of the p110 and p85 subunits of PI3-Kα in rabbit mesenteric artery. In the top 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 bottom trace, a bar graph summarizing the immunoblot data is shown. 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.

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. Because 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 sequelae, 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 (Dimmel et al., 1999; Fulton 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 seem to be the case, however, as vascular expression of protein phosphatase 2A was decreased rather than increased following sepsis induction; 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., 1996), 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 the 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 (Vanhasebroeck 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 seems 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 mononuclear cells (Guaia and Mackman, 2002), and human umbilical vein endothelial cells (Connelly et al., 2005), although the time dependence of LPS-induced Akt phosphorylation for each is quite different. 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; Vanhasebroeck 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 (Dimmel et al., 1999; Fulton et al., 1999). Statins can induce angiogenesis via activation of Akt and eNOS in vascular endothelial cells (Connelly et al., 2005), although the time dependence of LPS-induced Akt phosphorylation for each is quite different. 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; Vanhasebroeck 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).

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