Maintenance of Bad Phosphorylation Prevents Apoptosis of Rat Hepatic Sinusoidal Endothelial Cells *in Vitro* and *in Vivo*

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To elucidate the mechanism of apoptosis of liver sinusoidal endothelial cells (SECs), we examined the phosphorylation status of Bad and its upstream signaling molecules during apoptosis in culture and after ischemia-reperfusion injury. Rat SECs were isolated by the immunomagnetic method, and 2 days after culture, most SECs underwent apoptosis, which was associated with decreased tyrosine phosphorylation of cellular proteins. Addition of orthovanadate (OV), a protein tyrosine phosphatase inhibitor, sustained cellular protein phosphorylation and strongly inhibited apoptosis. Bad was dephosphorylated at Ser-112 and Ser-136 during apoptosis, but the phosphorylation status of Bad was maintained in the presence of OV. OV activated the Akt, extracellular signal-regulated protein kinase, and p38 mitogen-activated protein kinase pathways, which are involved in Bad phosphorylation. In the absence of OV, depletion of Bad by RNA interference conferred resistance to apoptosis. Hepatic injury after ischemia-reperfusion was alleviated by OV treatment, with significant inhibition of SEC apoptosis. SEC apoptosis *in vivo* was associated with dephosphorylation of Bad, Akt, and extracellular signal-regulated protein kinase, which was blocked by OV treatment. Our data suggest that maintenance of Bad phosphorylation is important in the prevention of SEC apoptosis and that the anti-apoptotic property of OV might have therapeutic utility. (*Am J Pathol* 2006, 168:1097–1106; DOI: 10.2353/ajpath.2006.050462)

Sinusoidal endothelial cells (SECs) of the liver are specialized endothelial cells that play important roles in

maintaining normal liver functions through interaction with hepatocytes and other nonparenchymal cells.^{1,2} Various pathological conditions of the liver, such as septic shock and reperfusion injury after ischemia, have been shown to be associated with death of SECs.^{1–4} Many reports have demonstrated that SEC damage during cold or warm preservation of the liver hampers the outcome of liver transplantation.^{5–7} Several lines of evidence have shown that death of SECs in those adverse conditions is executed by apoptosis.^{8–10} Prevention of SEC apoptosis could be a therapeutic strategy for liver diseases and beneficial to postoperative graft function. However, at present, little is known about the mechanisms that drive the apoptotic process and represent targets for intervention.

We previously reported that cultured SECs spontaneously undergo apoptosis after several days even in the presence of vascular endothelial growth factor (VEGF), the most potent growth factor for SECs.¹¹ Other investigators have suggested possible involvement of protein kinase C δ overexpression and oxidative stress in the VEGF-resistant apoptosis of cultured SECs.^{12,13} However, because our preliminary experiments showed that protein tyrosine phosphorylation levels of cellular proteins were high in freshly isolated SECs but dramatically decreased during culture, we speculated that the decrease of protein tyrosine phosphorylation might be important in apoptosis of SECs. It has been demonstrated that several signaling pathways regulated by protein tyrosine phosphorylation are involved in inhibition of the pro-apoptotic protein Bad through phosphorylation at specific serine residues.^{14–18} Thus, it is possible that decreased protein tyrosine phosphorylation might lead to activation of Bad, which has been implicated in the mechanism for apoptosis of human umbilical vein endothelial cells.^{19,20}

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In the present study, to investigate the significance of tyrosine phosphorylation of cellular proteins in SEC survival, we examined the effect of sodium orthovanadate (OV) on apoptosis of primary-cultured SECs. OV is a phosphate analog that binds to the active center of protein tyrosine phosphatases and inhibits their activity, thereby inducing sustained tyrosine phosphorylation of cellular proteins.^{21,22} We have found that early apoptosis of SECs, which is associated with marked dephosphorylation of Bad, is almost completely prevented by OV. The anti-apoptotic effect is mediated through the activation of various protein tyrosine phosphorylation pathways, which in turn inactivates Bad by phosphorylation of distinct serine residues. Furthermore, in the ischemia-reperfusion liver injury model of the rat, we have demonstrated that Bad is also dephosphorylated and activated in apoptotic SECs and that systemic treatment with OV maintains Bad phosphorylation and promotes SEC survival. Our study highlights the importance of Bad phosphorylation for the survival of SECs and suggests that OV might be therapeutically effective against SEC apoptosis *in vivo*.

Materials and Methods

Isolation and Culture of SECs

Livers of adult male Fischer 344 rats (150 to 250 g) were digested with collagenase (Wako, Osaka, Japan) by the two-step perfusion technique, and a nonparenchymal cell fraction was obtained after repeated low-speed centrifugation to remove hepatocytes. SECs were isolated from the fraction by the immunomagnetic method using 4.5- μ m-diameter magnetic beads (Dynabeads, CELLlection Pan Mouse IgG kit; Dynal, Oslo, Norway) conjugated with SE-1 monoclonal antibody,²³ which specifically recognizes an antigen expressed in rat SECs. As described previously, this method enabled us to isolate SECs with high purity (98%), which was better than that obtained by the conventional Percoll method.¹¹ The protocols for animal experimentation were previously approved by the Animal Research Committee, Akita University. All animal experiments adhered to the Guidelines for Animal Experimentation of the University.

The isolated SECs were suspended in EBM-2 medium (BioWhittaker, Walkersville, MD) containing 2% fetal bovine serum and seeded on type I collagen-coated multiwell plastic plates. Because higher serum concentrations (>5%) have been reported to be harmful for rat SECs,²⁴ the low concentration was used throughout the *in vitro* studies. After 3 hours (designated as "time 0"), medium was replaced with new medium with or without OV or VEGF. The number of cells per well after 96 hours of culture was estimated by the Hoechst 33258-based DNA fluorometric assay.²⁵ In some experiments, a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002; Cell Signaling Technology, Beverly, MA), a mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059; Calbiochem, San Diego, CA), p38 mitogen-activated protein kinase (MAPK) inhibitors (SB202190, SB203580, and

SB202474 [inactive control analog]; Calbiochem) were also added to the medium.

Detection of Apoptosis and Caspase Activation in Cultured SECs

Apoptosis of cultured SECs was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method (Apoptag; Intergen, Purchase, NY) and agarose electrophoresis of DNA. Caspase activity was measured using fluorogenic peptide substrates (Ac-DEVD-MCA and Ac-LEHD-MCA for caspase-3 and -9, respectively; Peptide Institute, Osaka, Japan).

Western Blot Analysis

Cells were lysed in a radioimmunoprecipitation assay buffer, and protein samples (30 μ g/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-phosphotyrosine (PY20; TaKaRa Biochemicals, Tokyo, Japan), anti-caspase-3 (H-277; Santa Cruz Biotechnology, Santa Cruz, CA), anti-activated (cleaved) caspase-9 (Cell Signaling Technology), anti-Bad (BD Transduction Laboratories, Franklin Lakes, NJ), anti-phospho-Bad (Ser-112) (Cell Signaling Technology), anti-phospho-Bad (Ser-136) (Oncogene Research Products, San Diego, CA), anti-14-3-3 proteins (K-19; Santa Cruz Biotechnology), anti-phospho-Akt (Cell Signaling Technology), anti-phospho-extracellular signal-regulated protein kinase (ERK) (E-4; Santa Cruz Biotechnology), anti-phospho-p90RSK (Cell Signaling Technology), anti-phospho-p38 MAPK (Cell Signaling Technology), anti-phospho-mitogen- and stress-activated protein kinase 1 (MSK1) (Cell Signaling Technology), anti-Bcl-2 (N-19; Santa Cruz Biotechnology), anti-Bcl-X_L (Cell Signaling Technology), and anti- β -actin (Sigma) antibodies.

Co-Immunoprecipitation Analysis

Cells were lysed in a buffer containing 0.2% Nonidet P-40, and protein samples (500 μ g each) were immunoprecipitated with 6 μ g of anti-14-3-3 antibody (K-19; Santa Cruz Biotechnology) followed by precipitation with protein A-Sepharose. The resulting immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-Bad (BD Transduction Laboratories), anti-phospho-Bad (Ser-112) (Cell Signaling Technology), anti-phospho-Bad (Ser-136) (Oncogene Research Products), and anti-14-3-3 proteins (K-19; Santa Cruz Biotechnology) antibodies.

Depletion of Bad Expression in Cultured SECs with RNA Interference

A 21-nucleotide short interfering RNA (siRNA) duplex with 3'dTdT overhangs corresponding to Bad mRNA (5'-

GAAUGAGCGAUGAAUUUGA-3') was synthesized (Japan Bio Service, Saitama, Japan). We also used two commercially available siRNA duplexes (Silencer Pre-designed siRNA [52431 and 189465]; Ambion, Austin, TX) for Bad silencing. At 4 hours after plating, cells were transfected with each siRNA by the siPORT amine protocol (Ambion). For negative control experiments, a control siRNA (Ambion), which is nonhomologous to any known gene sequence, was applied to cells.

Partial Hepatic Ischemia-Reperfusion Injury

Adult male Wistar rats (180 to 220 g) were anesthetized with sodium pentobarbital and subjected to occlusion of the left lateral and median lobes of the liver by clamping the vascular pedicle with a clamp for 120 minutes. OV (5 mg/kg body weight) was administered intraperitoneally at 30 minutes before ischemia and immediately after reperfusion. Liver injury was estimated by plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Plasma levels of hyaluronic acid (HA) were measured using an enzyme-linked immunosorbent assay (Chugai Diagnostics Science, Tokyo, Japan). SECs were isolated from the control livers (sham-operated) and livers after 2-hour ischemia followed by 1-hour reperfusion and examined for the activation of caspase-3 and -9 and the phosphorylation status of Bad (Ser-112 and Ser-136), Akt, ERK, p90RSK, MSK1, and p38 MAPK by Western blotting as described above.

Immunofluorescent Microscopy

Acetone-fixed frozen sections of the livers were treated with 5% skim milk in phosphate-buffered saline and then incubated with anti-activated caspase-9 (Cell Signaling Technology) and SE-1 antibodies. The sections were then incubated with tetramethylrhodamine isothiocyanate-conjugated swine anti-rabbit antibody (for activated caspase-9) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (for SE-1).

Results

Spontaneous Death of Cultured Rat SECs Is Associated with Decreased Tyrosine Phosphorylation of Cellular Proteins and Is Prevented by OV

SECs spontaneously underwent cell death after 2 days, but most cells survived in the presence of OV (Figure 1A). Although the cell death was accompanied by a marked decrease in protein tyrosine phosphorylation, OV induced a marked increase in phosphorylation (Figure 1B). Although VEGF (50 ng/ml) promoted cell growth and induced an elongated cell morphology, it exerted only a modest effect on cell death (Figure 1A) and did not exert any discernible effects on protein tyrosine phosphorylation (Figure 1B). Because of prevention of cell death, OV

Figure 1. Death of cultured rat SECs is associated with a decrease in tyrosine phosphorylation of cellular proteins. SECs were cultured for 3 hours, and then various concentrations of OV or VEGF were added (the time of addition was designated as time 0). **A:** Phase-contrast micrographs (original magnification, $\times 200$). SECs were cultured for 48 hours with or without OV (40 $\mu\text{mol/L}$) or VEGF (50 ng/ml). **B:** Western blot analysis of freshly isolated and cultured SECs for tyrosine-phosphorylated proteins using an anti-phosphotyrosine antibody (PY20). **C:** The estimation of cell number per well by DNA fluorometric assay. SECs were treated with various concentrations of OV (up to 60 $\mu\text{mol/L}$) and cultured for 96 hours. Each point represents the mean $\pm$ SEM of five independent experiments.

increased the cell number per well in a dose-dependent manner with the maximal effect at 40 $\mu\text{mol/L}$ (Figure 1C).

The Death of Cultured SECs Is Accompanied by Nucleosomal DNA Fragmentation and the Activation of Caspase-9 and -3

The death of cultured SECs was associated with DNA fragmentation, which was revealed by the TUNEL method (Figure 2A) and agarose electrophoresis of DNA (Figure 2B). Addition of OV in SEC cultures decreased the number of TUNEL-positive cells (Figure 2A) and strongly inhibited nucleosomal DNA fragmentation in a dose-dependent manner (Figure 2B). The effect of VEGF (50 ng/ml) was weaker compared with that of OV (40 $\mu\text{mol/L}$) (Figure 2B).

Cleaved and activated forms and increased activities of caspase-9 and -3 were detected in SECs cultured for 48 hours (Figure 2, C [Western blotting] and D [caspase activity assay]). The caspase activation was reduced by OV in a dose-dependent manner, whereas it was only slightly reduced by VEGF (Figure 2, C and D). We could detect neither activated caspase-8 nor tBid, which is generated on proteolytic cleavage of Bid by caspase-8,^{26,27} by Western blotting (data not shown).

Figure 2. Cultured SECs undergo apoptotic cell death associated with nucleosomal DNA fragmentation and activation of caspase-9 and -3. **A:** Detection of apoptotic cells by TUNEL method. SECs were cultured for 48 hours in the absence (control) or presence of OV (40 μ M/L). **Arrowheads** indicate TUNEL-positive cells. **B:** Agarose electrophoresis for detection of nucleosomal DNA fragmentation. Genomic DNA were isolated from time 0 SECs and SECs cultured further for 48 hours without (control) or with various concentrations of OV (10–40 μ M/L) or VEGF (50 ng/mL). **C:** Western blot analyses of caspase-9 and -3 using specific antibodies for the activated (cleaved) forms. **D:** Measurement of caspase activities using fluorogenic peptide substrates (Ac-DEVD-MCA and Ac-LEHD-MCA for caspase-3 and -9, respectively). SECs were treated with various concentrations of OV (5–40 μ M/L) or VEGF (20, 50 ng/mL) and cultured for 48 hours. Each point represents the mean $\pm$ SEM of three independent experiments. ** P < 0.01; *** P < 0.001 (compared with control, one-way analysis of variance).

The Pro-Apoptotic Protein Bad Is Dephosphorylated and Dissociated from 14-3-3 Proteins in Apoptosis of Cultured SECs

We then examined the effects of OV on the phosphorylation status of Bad, which is crucial in the activation of caspase-9 and -3. Bad was strongly phosphorylated at Ser-112 in the cells soon after isolation or after culturing for 3 hours (time 0), but it was dephosphorylated after 48 hours (Figure 3A). OV (40 μ M/L) maintained the phosphorylation status, whereas the effect of VEGF was insignificant (Figure 3A). OV slightly increased Bcl-xL expression, although it did not affect the protein expression levels of Bad, 14-3-3 proteins, and Bcl-2 (Figure 3A). Co-immunoprecipitation of Bad with 14-3-3 proteins showed that apoptosis was associated with dissociation of Bad from 14-3-3 proteins (Figure 3B). OV treatment maintained the association of these proteins, and the co-immunoprecipitated Bad was strongly phosphorylated at Ser-112 and at Ser-136 (Figure 3B).

Figure 3. Bad is dephosphorylated and dissociates from 14-3-3 proteins in SECs during apoptosis. **A:** Western blot analyses for phosphorylated Bad (P-Bad) (Ser-112), Bad, 14-3-3 proteins, Bcl-xL, and Bcl-2. Time 0 SECs and SECs cultured without (control) or with OV (20 and 40 μ M/L) or VEGF (20 and 50 ng/mL) for 48 hours were analyzed. **B:** Co-immunoprecipitation of Bad with 14-3-3 proteins. Lysates from time 0 SECs and SECs cultured without (control) or with OV (40 μ M/L) or VEGF (50 ng/mL) for 48 hours were incubated with 14-3-3 antibodies and immunoprecipitated, followed by Western blotting for Bad, P-Bad (Ser-112), P-Bad (Ser-136), and 14-3-3 proteins.

Akt, ERK, p90RSK, MSK1, and p38 MAPK Are Markedly Dephosphorylated in Apoptosis of Cultured SECs

Next, we examined the signaling pathways involved in the phosphorylation of Bad by Western blotting. It has been reported that Bad phosphorylation at Ser-136 is promoted by the PI3K/Akt pathway,^{14,15} which can be activated by various receptor tyrosine kinases (RTKs). On the other hand, Bad phosphorylation at Ser-112 has been shown to be controlled by the Ras/MEK/ERK/p90RSK and p38 MAPK pathways.^{16–18} ERK and p38 MAPK have been demonstrated to activate MSK1,²⁸ which then phosphorylates Ser-112 of Bad.¹⁸ Although Akt, p90RSK, MSK1, and p38 MAPK were strongly phosphorylated in SECs at time 0, the phosphorylation levels of all of these signal transduction molecules were markedly decreased after 48 hours (Figure 4). ERK was highly phosphorylated in freshly isolated SECs (see Figure 9C), but it was rapidly

Figure 4. Dephosphorylation of Akt, ERK, p90RSK, MSK1, and p38 MAPK occurs in SECs during apoptosis. Western blot analysis was performed to detect phosphorylated Akt, ERK, p90RSK, MSK1, and p38 MAPK in cultured SECs. Proteins were extracted from time 0 SECs and SECs cultured without (control) or with OV (20, 40 μ M/L) or VEGF (20, 50 ng/mL) for 48 hours and analyzed.

Figure 5. The anti-apoptotic effect of orthovanadate is partially or completely abrogated by specific inhibitors of signal transduction molecules. Effects of various inhibitors of signal transduction molecules on OV-induced suppression of the caspase-3 activity in cultured SECs. SECs were cultured in the absence or presence of various concentrations of the PI3K inhibitor (LY294002) (A), MEK inhibitor (PD98059) (B), LY294002 and/or PD98059 (C), or p38 MAPK inhibitor (SB203580) (D), with or without OV (40 μ M/L), for 48 hours. Each point represents the mean $\pm$ SEM of three independent experiments. * P < 0.05; *** P < 0.001 (compared with SECs treated only with OV, one-way analysis of variance).

dephosphorylated in SECs after being cultured (Figure 4). OV treatment maintained phosphorylation of these molecules at high levels (Figure 4). VEGF only slightly increased ERK and p90RSK phosphorylation, but it did not affect the phosphorylation status of Akt, MSK1, and p38 MAPK (Figure 4).

The Anti-Apoptotic Effect of OV Is Partially or Completely Abrogated by Specific Inhibitors of Signal Transduction Molecules

To identify relevant signal transduction pathways that maintain SEC survival, we examined the effect of specific pharmaceutical inhibitors of signal transduction molecules on caspase-3 activity in cultured SECs either treated or untreated with OV. Although the PI3K inhibitor (LY294002) and the MEK inhibitor (PD98059) only showed marginal effects when used alone (Figure 5, A and B), they almost completely abrogated the effect of OV when applied in combination (Figure 5C). In contrast, the p38 MAPK inhibitor (SB203580) alone efficiently abrogated the effect of OV (Figure 5D). A similar effect was observed with another p38 MAP kinase inhibitor (SB202190) but not with the inert control compound with a similar structure (SB202474) (data not shown). These inhibitors did not significantly affect caspase-3 activity in untreated cells (Figure 5, A, B, and D).

We then examined the effects of inhibitors on the phosphorylation status of the signal transduction molecules and Bad in the OV-treated cells. LY294002 induced dephosphorylation of Akt, resulting in specific dephosphorylation of Ser-136 of Bad (Figure 6A). PD98059 inhibited

Figure 6. Abrogation of the anti-apoptotic effect of OV requires Bad dephosphorylation at Ser-112 and at Ser-136. **A:** Western blot analyses for phosphorylated forms of Akt, ERK, p90RSK, MSK1, p38 MAPK, Bad (Ser-112), and Bad (Ser-136) in cultured SECs treated with various inhibitors of signal transduction molecules. SECs were cultured in the absence or presence of LY294002 (40 μ M/L), PD98059 (40 μ M/L), SB202190 (20 μ M/L), or SB203580 (20 μ M/L), with or without OV (40 μ M/L), for 48 hours. For comparison, a sample of time 0 SECs was included in the analysis (culture period, 0 hours). **B:** Co-immunoprecipitation of Bad with 14-3-3 proteins. Lysates were prepared from SECs cultured without (control) or with OV (40 μ M/L) in the absence or presence of the indicated inhibitors for 48 hours, after which they were incubated with 14-3-3 antibodies and immunoprecipitated, followed by Western blotting for Bad, P-Bad (Ser-112), P-Bad (Ser-136), and 14-3-3 proteins. **C:** Western blot analyses for phosphorylated forms of Akt, ERK, Bad (Ser-112), and Bad (Ser-136). SECs were cultured in the absence or presence of LY294002 (40 μ M/L), PD98059 (40 μ M/L), or LY294002 (40 μ M/L) + PD98059 (40 μ M/L), with or without OV (40 μ M/L), for 48 hours.

phosphorylation of ERK and its downstream proteins (p90RSK and MSK1), resulting in specific dephosphorylation of Ser-112 but not of Ser-136 (Figure 6A). Although Bad was co-immunoprecipitated with 14-3-3 proteins in the presence of OV and LY294002, Ser-136 was dephosphorylated, whereas phosphorylation of Ser-112 was well maintained (Figure 6B). In Bad co-immunoprecipitated with 14-3-3 proteins in the presence of OV and PD98059, dephosphorylation of Ser-112 but not Ser-136 was observed (Figure 6B). As expected, when cells were simultaneously treated with LY294002 and PD98059, ERK and Akt were inhibited, resulting in the suppression of Bad phosphorylation at Ser-112 and at Ser-136 (Figure 6C). The p38 MAPK inhibitors (SB203580 and SB202190) suppressed phosphorylation of p38 MAPK and MSK1 by OV and resulted in dephosphorylation of Ser-112 of Bad (Figure 6A). However, the p38 MAPK inhibitors also suppressed Akt phosphorylation and thus induced dephosphorylation of Ser-136 (Figure 6A).

Figure 7. Depletion of Bad expression with siRNA enhances survival of SECs. **A:** Phase-contrast micrographs (original magnification, $\times 200$). SECs were transfected with control siRNA or Bad siRNA and cultured for 48 hours. **B:** Measurement of the caspase-3 activity and Western blot analysis for Bad and β -actin (loading control) expression. Protein lysates were prepared from SECs either transfected or nontransfected with siRNAs, in the absence or presence of OV ($40 \mu\text{mol/L}$). In the **top panel**, each point represents the mean $\pm$ SEM of three independent experiments. *** $P < 0.001$ (compared with SECs transfected with control siRNA, one-way analysis of variance).

Depletion of Bad Expression with siRNA Enhances Survival of SECs

Our results suggested that Bad might play a crucial role in the mechanism of SEC apoptosis *in vitro*. To further test this hypothesis, we examined whether specific depletion of Bad protein by the RNA interference approach affected SEC apoptosis. When SECs were transfected with Bad siRNA, the extent of cell death was markedly reduced compared with those transfected with control siRNA (Figure 7A). Bad expression was reduced to 9.5% of the control level by Bad siRNA, whereas control siRNA showed no effect (Figure 7B, bottom panel). Bad depletion significantly inhibited activation of caspase-3 ($45.4 \pm 3.2\%$ of the control level), but OV treatment did not demonstrate any additive anti-apoptotic effect in the presence of Bad siRNA (Figure 7B, top panel). To confirm the effect of specific Bad depletion, we also tested two commercially available Bad siRNA duplexes (52431 and 189465). Although both duplexes suppressed Bad ex-

pression, siRNA 52431 was more effective than 189465 (reduction to 11.5 and 55.1% of the control level by 52431 and 189465, respectively). The caspase-3 activation was more strongly inhibited by siRNA 52431 than 189465 (38.9 ± 1.5 and $62.3 \pm 2.6\%$ of the control level by 52431 and 189465, respectively), suggesting a correlation between the degree of depletion of Bad expression and the effect on caspase-3 inhibition.

Ischemia-Reperfusion Causes SEC Apoptosis with Marked Bad Dephosphorylation, Which Can Be Prevented by Systemic Treatment with OV

We then examined the mechanism of SEC apoptosis and the effect of OV *in vivo* using a model of ischemia-reperfusion injury of the rat liver. There was a marked increase in plasma levels of transaminases (ALT and AST) after reperfusion (Figure 8A; AST, data not shown), as well as of HA, indicating that the function of SECs was deteriorated (Figure 8B). Interestingly, systemic OV treatment significantly suppressed the increase of both transaminases and HA (Figure 8, A and B). Furthermore, the extent of coagulation necrosis, which was evident at 24 hours after reperfusion, was much lower in the OV-treated animals (Figure 8C). Morphometric analysis of the necrotic areas revealed that there was a statistically significant difference between control and OV-treated animals (Figure 8D).

After reperfusion for 1 hour, immunohistochemistry for activated caspase-9 demonstrated many apoptotic cells in the liver, most of which corresponded to SE-1-positive SECs (Figure 9A). OV treatment dramatically reduced the number of apoptotic cells with activated caspase-9 (Figure 9A), which was confirmed by quantitative analysis (Figure 9B). Suppression of activation of caspase-9, as well as of caspase-3, by OV treatment was demonstrated by Western blot analyses of SECs isolated from the livers after 2 hours of ischemia followed by 1 hour of reperfusion (Figure 9C). Ischemia and reperfusion resulted in marked dephosphorylation of Bad (Ser-112, Ser-136), Akt, ERK, p90RSK, and MSK1, although they did not affect the phosphorylation status of p38 MAPK in apoptotic SECs (Figure 9C). Consistent with the anti-apoptotic effect, OV treatment maintained phosphorylation of Bad (Ser-112 and Ser-136), Akt, ERK, p90RSK, and MSK1 in SECs (Figure 9C).

Discussion

Spontaneous cell death of cultured SECs showed several apoptotic properties, including nucleosomal DNA fragmentation, TUNEL positivity, and activation of caspase-9 and -3. Because there was no detectable activation of caspase-8 and cleavage of its substrate protein Bid during apoptosis, it is conceivable that the apoptosis of SECs is mainly executed by the mitochondrial pathway, not by the death receptor pathway. Although there have been several reports suggesting that the death receptor

Figure 8. OV protects the rat liver against ischemia-reperfusion injury. Male Wistar rats were subjected to 70% partial hepatic ischemia for 120 minutes, followed by reperfusion. OV (OV [+]) or saline (OV [-]) was administered (intraperitoneally) at 30 minutes before ischemia and immediately after reperfusion. **A:** Plasma ALT levels before ischemia (-2 hours), at the end of ischemia (0 hours), and after reperfusion (1, 3, 6, and 24 hours). The data represent the means $\pm$ SEM from four to eight animals per group for each time point. Values of sham-operated animals are also shown at 1 and 3 hours after reperfusion. $*P < 0.05$; $**P < 0.01$ (compared with OV [-] at each time point, one-way analysis of variance). **B:** Plasma levels of HA. Samples collected after ischemia (0 hours) and after reperfusion (3 and 6 hours) were analyzed for HA levels. The data represent the means $\pm$ SEM from five to eight animals per group for each time point. $*P < 0.05$; $***P < 0.001$ (compared with OV [-] at each time point, one-way analysis of variance). **C:** Representative photomicrographs of livers subjected to 2 hours of ischemia and reperfusion for 24 hours (hematoxylin and eosin). N, an area of coagulation necrosis. **D:** Quantitative assessment of necrotic areas. Areas of coagulation necrosis were measured in 10 randomly selected fields for each animal. The data represent the means $\pm$ SEM from five or six animals per group. $**P < 0.01$ (compared with OV [-], one-way analysis of variance).

pathway could be active in SECs when stimulated by tumor necrosis factor- α or Jo-2 antibodies,²⁹⁻³² it has also been shown that lipopolysaccharide treatment does not induce apoptosis of SECs in primary cultures, even though it enhances expression of Fas and its ligand.³³

Apoptosis of cultured SECs was associated with a marked decrease in tyrosine phosphorylation of cellular proteins. The potent protein tyrosine phosphatase inhibitor OV enhanced protein tyrosine phosphorylation and at the same time strongly inhibited apoptosis. Our data suggest that tyrosine phosphorylation of cellular proteins might be important in SEC survival. Interestingly, OV has been reported to be effective in preventing apoptosis of human umbilical vein endothelial cells induced by serum deprivation.^{34,35} Our study also demonstrated that VEGF, the most potent growth factor for SECs, showed a modest cell-protecting effect without affecting the entire level of

Figure 9. OV maintains Bad phosphorylation and inhibits SEC apoptosis in the ischemia-reperfusion injury model of the rat. **A:** Photomicrographs of immunofluorescent double staining with antibodies specific for SE-1 (green) and caspase-9 (red) of the liver tissues subjected to 2 hours of ischemia and reperfusion for 1 hour (original magnification, $\times 200$). OV was administered (intraperitoneally) at 30 minutes before ischemia and immediately after reperfusion. **B:** Quantitative assessment of activated caspase-9-positive SECs in the liver 1 hour after reperfusion. Positive cells were counted in 12 randomly selected fields for each animal. The data represent the means $\pm$ SEM from seven or eight animals. $**P < 0.01$ (compared with OV [-], one-way analysis of variance). **C:** Western blot analyses for activated caspase-9, caspase-3, P-Bad (Ser-112), P-Bad (Ser-136), Bad, P-Akt, P-ERK, P-p90RSK, P-MSK1, and P-p38 MAPK in SECs isolated from the control livers (sham-operated) and livers after 2-hour ischemia followed by 1-hour reperfusion (I/R).

tyrosine phosphorylation. It is not clear whether this is due to another distinct mechanism for SEC survival or to masking of cell death by concomitant cell proliferation. In contrast with the effect of VEGF, OV did not stimulate proliferation of SECs (N. Ohi and Y. Nishikawa, unpublished observations).

During apoptosis of cultured SECs, the pro-apoptotic protein Bad was dephosphorylated at Ser-112 and Ser-136, either of which is known to be required for interaction with 14-3-3 proteins.^{14,36-38} The anti-apoptotic effect of OV was associated with Bad phosphorylation at both serine residues. It has been shown that 14-3-3 proteins sequester phosphorylated Bad from mitochondria where it exerts the pro-apoptotic actions. In contrast, when Bad is dephosphorylated, unleashed Bad can interact with Bcl-xL or Bcl-2 in the mitochondria, thereby inactivating these anti-apoptotic proteins and inducing apoptosis.³⁹ Our data showing that specific depletion of Bad with RNA interference significantly inhibited the apoptosis of SECs highlighted the critical role of Bad. Because OV treatment did not demonstrate an additive effect in the presence of Bad siRNA, it is conceivable that the anti-apoptotic effect of OV was mainly mediated through Bad modification.

Figure 10. The proposed mechanism for SEC apoptosis and the anti-apoptotic effect of OV. SEC apoptosis *in vitro* and *in vivo* is associated with inactivation of the PI3K/Akt and Ras/MEK/ERK pathways, which results in dephosphorylation of the pro-apoptotic protein Bad at Ser-136 and Ser-112, respectively. Dephosphorylated Bad then triggers the mitochondrial pathway of apoptosis through interaction with pro-survival Bcl-2 family members, such as Bcl-xL. OV stimulates RTKs by its inhibitory action on protein tyrosine phosphatases, and then activates the PI3K/Akt and Ras/MEK/ERK pathways, thereby maintaining phosphorylation of Bad. OV also stimulates the p38 MAPK pathway, which is involved in Ser-112 phosphorylation of Bad. Bad phosphorylated either at Ser-112 or Ser-136 interacts with 14-3-3 proteins and is sequestered from the mitochondria.

Bad phosphorylation is known to be governed by several distinct signaling pathways, in which protein tyrosine phosphorylation plays crucial roles. It has been established that the PI3K/Akt pathway is involved in phosphorylation at Ser-136,^{14,15} whereas the Ras/MEK/ERK/p90RSK and p38 MAPK/MSK1 pathways are involved in phosphorylation at Ser-112.^{16–18} Our study revealed that apoptosis of cultured SECs was closely related to the inactivation of multiple signal transduction molecules, including Akt, ERK, p90RSK, MSK1, and p38 MAPK, which led to Bad dephosphorylation. As summarized in Figure 10, we hypothesized that the anti-apoptotic effect of OV might be exerted through maintaining the signal transduction molecules in the active state, thereby enabling phosphorylation of Bad and its sequestration from mitochondria. OV has been shown to stimulate autophosphorylation of many RTKs, including receptors for epidermal growth factor, hepatocyte growth factor, and insulin, by inhibiting protein tyrosine phosphorylation.²² Akt activation through serine phosphorylation might be executed by PI3K, which could be activated by various RTKs.

The treatment with a MEK inhibitor (PD98059) or a PI3K inhibitor (LY294002) only partially antagonized the anti-apoptotic effect of OV, but addition of both inhibitors, which lead to dephosphorylation of Ser-112 and Ser-136 of Bad, completely abrogated the anti-apoptotic effect. Furthermore, the effect of OV was efficiently abrogated by the p38 MAPK inhibitor (SB203580 or SB202190) that inhibited not only p38 MAPK and MSK1 but also Akt phosphorylation, thereby leading to Bad dephosphorylation at Ser-112 and at Ser-136. Similar interaction between the PI3K/Akt and p38 MAPK pathways has recently been described in aortic endothelial cells.⁴⁰ Taken together, our results indicated that abrogation of the anti-apoptotic effect of OV required Bad dephosphorylation at both serine residues and thus suggested that phosphorylation of Bad either at Ser-112 or Ser-136 might be sufficient for SEC survival. Our inhibitor experiments also showed that Ser-112 phosphorylation of Bad took place only when both ERK and p38 MAPK were phosphorylated, suggesting the requirement of both of these pathways for proper phosphorylation of Ser-112 in SECs.

Whether the endothelial death after ischemia-reperfusion is apoptotic or necrotic has been a subject of debate.^{8–10,41–43} However, the early cell death of SECs after reperfusion in our model was associated with the activation of caspase-9, supporting the notion that the death was apoptotic. OV dramatically reduced the number of active caspase-9-positive SECs in the liver after reperfusion. The protective effect of OV was further confirmed by suppression of the increase in plasma HA levels after reperfusion. Because approximately 90% of HA is normally removed and degraded by SECs, the increase in the plasma concentration reflects SEC damage *in vivo*.⁴⁴ We also demonstrated that OV was effective in alleviating the tissue damage in the ischemia-reperfusion model of the rat liver. The suppression of subsequent hepatocyte injury might be due to maintenance of proper microcirculation in OV-treated animals. However, because OV promotes growth and affects differentiation of cultured rat hepatocytes,⁴⁵ it is also possible that a direct influence on hepatocytes might play a part in the protective effects of OV.

In SECs undergoing apoptosis after ischemia-reperfusion, Bad was dramatically dephosphorylated at Ser-112 and at Ser-136. Bad dephosphorylation was accompanied by inactivation of the PI3K/Akt and Ras/MEK/ERK pathways, but not the p38 MAPK pathway. Although phosphorylation of p38 MAPK was unaffected after ischemia-reperfusion, the inactivation of Ras/MEK/ERK pathway might be sufficient to cause Bad dephosphorylation at Ser-112, because our *in vitro* results suggested that Ser-112 phosphorylation only took place when both Ras/MEK/ERK and p38 MAPK pathways were activated in SECs. Thus, our data indicated that Bad dephosphorylation caused by inactivation of the relevant upstream pathways might be a common mechanism for SEC apoptosis *in vitro* and *in vivo*. Furthermore, systemic treatment with OV maintained the active state of the PI3K/Akt and Ras/MEK/ERK pathways and prevented Bad dephosphorylation induced by ischemia-reperfusion. OV has been shown to affect cellular functions by enhancing various

signaling pathways when administered systemically. It has been reported that OV is able to augment insulin signaling by activation of the PI3K/Akt and p38 MAPK pathways,⁴⁶ thereby exerting an insulin-like action *in vitro* and *in vivo*.^{47–50} Interestingly, recent studies have shown that OV treatment rescues neuronal death of the gerbil and rat hippocampus after transient ischemia via PI3K/Akt and MAPK pathways.^{51,52} It has also been reported that OV protects cardiomyocytes from ischemia-reperfusion injury in the rat heart through Akt activation.⁵³ Our data are consistent with a recent report demonstrating that adenoviral gene transfer of the constitutively active form of Akt protects the rat liver from ischemia-reperfusion injury with increased phosphorylation of Bad at Ser-136.⁵⁴

In conclusion, our study has demonstrated that Bad phosphorylation regulated by multiple signaling pathways is critical in survival of SECs *in vitro* and *in vivo*. Bad might be a target for therapeutic intervention in various pathological conditions in which SEC apoptosis is involved. We have shown here that it is feasible to maintain Bad phosphorylation in SEC and prevent their apoptosis by application of OV, which activates various protein tyrosine phosphorylation pathways. Thus, OV treatment could be a strategy for protecting the liver from ischemia-reperfusion injury, and it might also be beneficial in preserving the liver before transplantation.

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