PARP-1 Inhibition Prevents Oxidative and Nitrosative Stress–Induced Endothelial Cell Death via Transactivation of the VEGF Receptor 2

Marlene T. Mathews, Bradford C. Berk

Objective—PARP-1, a DNA base repair enzyme, is activated by DNA breaks induced by oxidative (ROS) and nitrosative (RNS) stress. By consuming NAD⁺, PARP-1 activation can lead to ATP depletion and cell death. Studies suggest that inhibiting PARP-1 activity can attenuate pathologies associated with vascular smooth muscle and endothelial dysfunction. PARP-1 inhibition can also activate the prosurvival serine/threonine kinase, Akt. Vascular endothelial growth factor (VEGF) regulates endothelial cell survival via Akt activation downstream of VEGF receptor 2 (VEGFR2) activation. Here we investigated the hypothesis that PARP-1 inhibition protects human umbilical vein endothelial cells (HUVECs) from ROS- and RNS-induced cell death by limiting NAD⁺ depletion and by activating a prosurvival signaling pathway via VEGFR2 phosphorylation.

Methods and Results—We activated PARP-1 in HUVECs by treatment with hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻). Both depleted HUVECs of NAD⁺ and ATP, processes that were limited by the PARP-1 inhibitor, PJ34. ONOO⁻ and H₂O₂-induced cell death and apoptosis were attenuated in cells treated with PJ34 or PARP-1 siRNA. PARP-1 inhibition increased Akt, BAD, and VEGFR2 phosphorylation in HUVECs and in PJ34-treated rabbit aortas. The VEGFR2-specific tyrosine kinase inhibitor SU1498 decreased PARP-1 inhibition-mediated phosphorylation of VEGFR2 and Akt, and also reversed survival effects of PJ34. Finally, PARP-1 inhibition protected cells from death induced by serum starvation, evidence for a role in cell survival independent of energy protection.

Conclusions—PARP-1 inhibition prevents ROS- and RNS-induced HUVEC death by maintaining cellular energy in the form of NAD⁺ and ATP, and also by activating a survival pathway via VEGFR2, Akt, and BAD phosphorylation. (Arterioscler Thromb Vasc Biol. 2008;28:711-717)

Key Words: PARP inhibition ■ endothelial cell survival ■ VEGF receptor 2 ■ oxidative stress

Several studies have shown that inhibiting poly-ADP ribose polymerase-1 (PARP-1) attenuates organ dysfunction in settings such as postmyocardial infarction remodeling, ischemia-reperfusion injury, diabetic retinopathy, septic shock, diabetes, and atherosclerosis. A major feature of atherosclerosis includes increased levels of reactive nitrogen (RNS) and reactive oxygen species (ROS) associated with damage to cell membranes and DNA. It is not surprising therefore that PARP-1 inhibition was also shown to limit endothelial dysfunction and atherosclerosis in the ApoE⁻/⁻ mouse. High levels of ROS and RNS, such as occur in ischemia-reperfusion injury, inflammation, and diabetes mellitus, induce DNA single-strand breaks and activate poly-ADP ribose polymerase-1 (PARP-1).

PARP-1 is a zinc finger protein that belongs to a family of 18 identified genes that transcribe poly(ADP-ribose) polymerases, enzymes that catalyze the covalent transfer of poly-ADP units from NAD⁺ to acceptor proteins. PARP-1 has 3 functional domains: a DNA-binding domain (containing a nuclear localization signal), an automodification domain (which acts as an acceptor for poly ADP-ribose units), and a C terminus catalytic domain. Normally, PARP-1 contributes to DNA base excision repair and the maintenance of genomic stability. However, when overactivated by DNA damage induced by ROS/RNS, PARP-1 rapidly uses the substrate β-NAD⁺ to transfer poly ADP-ribose (PAR) to itself and to nuclear acceptor proteins. In an effort to resynthesize NAD⁺, the cell consumes its ATP pools and reaches an energy crisis, resulting in cell death.

Although PARP-1 inhibition limits cellular energy depletion, recent evidence suggests that it activates a prosurvival signaling cascade through Akt phosphorylation as well. Little is known about the upstream mediators of Akt activation. In endothelial cells, VEGF is one of the primary regulators of cell survival, via activation of the VEGF receptor (VEGFR2), and subsequent activation of the PI3K/Akt axis. Here we investigated, using oxidative (H₂O₂) and nitrosative (ONOO⁻) stress, the mechanisms by which inhib...
iting PARP-1 inhibition protect against EC death. We demonstrate that PARP-1 inhibition attenuates ATP and NAD⁺ depletion and decreases EC death via a VEGFR2-mediated prosurvival pathway.

Materials and Methods

Materials
PARP-1 inhibitor PJ34 was purchased from Sigma. PARP-1 siRNA and anti–PARP-1 polyclonal antibody were from Santa Cruz Biotechnology. SU1498 and ATP luciferase assay kit were purchased from Calbiochem/EMD Biosciences. Antiphosphotyrosine 4G10 (pY-4G10) was from Upstate Biotechnologies. Antibodies to phospho-Akt (Ser-473), Flik-1 (VEGFR2), Akt, phospho-Bad (Ser-136), and Bad were from Cell Signaling Technologies. The NAD⁺/NADH assay kit was purchased from BioAssay Systems.

Cell Culture
HUVECs were isolated from human umbilical veins and seeded onto gelatin-coated 60-mm dishes maintained in Medium 200 (Cascade Biologics) with low serum growth supplement and 5% FBS as previously described. Cells were used at passages 2 to 5.

Peroxynitrite Synthesis
To make Na⁺ONOO⁻, 0.6 mol/L NaNO₂ and 0.7 mol/L acidified H₂O₂ were mixed via 2 15-mL syringes connected by rubber tubing and a stopcock. The mixture was quickly quenched in a beaker with 1.5 mol/L NaOH. The resulting yellow solution was stirred with MnO₂ until the disappearance of all bubbles, decanted, and placed in an Eppendorf tube and the concentration was determined by diluting it 1:1000 in 10 mmol/L NaOH and reading absorbance in a spectrophotometer (extinction coefficient=16700 mol/L⁻¹).

PARP-1 Activity Assay
PARP-1 activity was assayed (R&D Systems) based on the incorporation of biotinylated ADP-ribose onto histone proteins. Cell lysates from HUVECs containing 50 μg of protein were loaded into a 96-well plate coated with histones and biotinylated poly ADP-ribose, allowed to incubate for 1 hour, treated with strep-HRP, and read at 450 nm in a spectrophotometer.

Cell Death Assays
HUVECs were grown to confluence in 60-mm dishes, serum-starved overnight, treated with H₂O₂ and ONOO⁻, fixed with 3.7% formaldehyde 8 h after treatment, and stained with Hoescht dye. Cells were examined under a fluorescent microscope and counted for DNA content in 3 fields.

Measurement of NAD⁺
HUVECs were cultured in 60-mm plates and treated with varying concentrations of H₂O₂ and ONOO⁻ for 3 h. After exposure, cells were washed with cold PBS and pelleted. They were homogenized with NAD extraction buffer (BioAssay Systems), heated at 60°C for 5 min, and diluted in assay buffer (BioAssay Systems). Samples were vortexed and spun at 14 000 rpm for 5 min. 40 μL of sample was added to each well of 96-well microtiter plate. 80 μL of the working reagent (50 μL assay buffer, 1 μL alcohol dehydrogenase, 10 μL 1% ethanol, 14 μL PMS, and 14 μL MTT) was added quickly to each well. The reaction was measured at 565 nm at 37°C for 15 min. The difference in optical density between 0 and 15 min was used to calculate NAD⁺ with a prepared standard curve (0 to 10 μmol/L of NAD⁺). The calculated NAD⁺ levels for each experiment were average values of at least 4 measurements.

Assay of Cellular ATP
ATP was measured using the ATP luciferase assay kit (Calbiochem). Cells were cultured in a 96-well microtiter plate and treated with varying concentrations of H₂O₂ and ONOO⁻ for 3 h. Culture medium was removed and cells were washed twice with PBS and treated with 100 μL nucleotide-releasing reagent. After 5 min at room temperature, 1 μL of luciferin-luciferase nucleotide-monitoring reagent was added and the plate loaded into a luminometer and read 1 min later. ATP levels were calculated against uninduced controls and expressed in relative light units.

LDH Assay
HUVECs were seeded into a 96-well plate at a density of 10⁴ cells in 120 mL of media and cultured for 24 h. They were treated with 200 μL of H₂O₂ or 150 μL ONOO⁻ for varying times. LDH activity was assayed using a kit (Cayman Chemical). Briefly, lactate and INT (a tetrazolium salt) were added to each well. The conversion of lactate to pyruvate and subsequent NADH production was monitored by the colorimetric reduction of INT at 490 nm.

PARP-1 siRNA
HUVECs were transiently transfected with siRNA designed for PARP-1 knockdown by the manufacturer (Santa Cruz Biotechnology) in Opti-MEM I Reduced Serum Medium (Invitrogen) using Lipofectamine 2000. Experiments were performed 48 h after the transfection. 10 ng of PARP-1 siRNA and scrambled siRNA were used.

Immunoprecipitation and Western Blotting
HUVECs were washed twice in cold PBS and harvested in lysis buffer containing 20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 μmol/L EGTA, 1% Triton X-100, 2.5 μmol/L sodium pyrophosphate, 1 μmol/L β-glycerophosphate, 100 mmol/L NaVO₄, 1 μmol/L NaF, and protease inhibitor. The protein concentration of the lysates was determined using the Bradford method (Bio-Rad). Equal amounts of protein were incubated with a specific antibody overnight at 4°C with gentle rotation. Then protein A/G PLUS-agarose (Santa Cruz) was added and incubated for additional 2 h. Then the beads were washed extensively with lysis buffer, and immune complexes were eluted in SDS-PAGE sample buffer. Total immune complex samples or protein samples from total cell lysate were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with appropriate primary antibodies. After washing and incubating with secondary antibodies (LiCor), immunoreactive proteins were visualized with the Odyssey LiCor Infrared Imaging System.

Perfusion Organ Culture
Animal experiments were performed according to the guidelines of the NIH and the American Heart Association for the care and use of laboratory animals and were approved by the University of Rochester Animal Care Committee. Male New Zealand White rabbits (2 to 3 kg; Covance Research Products Princeton, NJ) were anesthetized with ketamine (50 mg/kg, i.v.) and xylazine (2 mg/kg, i.v.). Arterial segments from the descending thoracic aorta were isolated and cannulated at a constant pressure (80 mm Hg) as described previously. To obtain a physiological fluid viscosity (0.04 poise), 5% dextran (Sigma-Aldrich) was added. Vessels were perfused with PBS containing 10 ng/mL VEGF or 10 mmol/L PJ34 for 30 min. To harvest endothelial cells, vessels were opened longitudinally and 0.2 mL of lysis buffer (20 mmol/L Tris-HCl pH 8.0, 0.05% Triton X-100, 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, and protease inhibitor cocktail (Sigma) was applied to the endothelial surface at room temperature for 6 min and collected.

Statistical Analysis
Group differences were analyzed using the standard Student t test. All values are expressed as mean±SE. P<0.05 was considered statistically significant.
PARP-1 Activity, Which Is Inhibited by PJ34

Hydrogen Peroxide and Peroxynitrite Increase PARP-1 Activity

Results

Hydrogen Peroxide and Peroxynitrite Increase PARP-1 Activity, Which Is Inhibited by PJ34

Because both H₂O₂ and ONOO⁻ have DNA-damaging capacities, we studied their ability to activate PARP-1. HUVECs were exposed to H₂O₂ and ONOO⁻ for 3 h, and PARP-1 activity was assayed. H₂O₂ activated PARP-1 in a dose-dependent manner with an EC₅₀ of 200 μmol/L, and a maximum 8-fold increase; ONOO⁻ at equimolar concentrations was 2.5 times more effective (Figure 1A). To inhibit PARP-1 we used PJ34, a water-soluble phenanthridinone-derived PARP-1 inhibitor.¹⁶ PJ34 inhibited both H₂O₂ and ONOO⁻-induced PARP-1 activity in a dose-dependent manner with an IC₅₀ of ~6.5 μmol/L (supplemental Figure IA).

Hydrogen Peroxide and Peroxynitrite Induce NAD⁺ Depletion That Is Reversed by PARP-1 Inhibition

After confirming that H₂O₂ and ONOO⁻ activate PARP-1 whereas PJ34 inhibits this enzyme in HUVECs, we examined their effects on energy consumption. PARP-1 uses NAD⁺ as a substrate to generate branching polymers of ADP-ribose, leading to ATP and NAD⁺ loss. To monitor intracellular NAD⁺, we used an enzymatic assay based on alcohol dehydrogenase activity. We found that NAD⁺ levels dropped 68% after a 3-hour exposure to H₂O₂ (Figure 1B) and 77% after a 3-hour exposure to ONOO⁻ (Figure 1B). This NAD⁺ loss was limited to 20% for H₂O₂ and 40% for ONOO⁻ with PJ34 (10 μmol/L) pretreatment (Figure 1B).

In addition to NAD⁺ content, we also studied ATP as a measure of energy consumption. We used an assay based on luciferase-catalyzed oxidation of D-luciferin in the presence of ATP and oxygen, whereby the amount of ATP is quantified by the amount of light produced. To inhibit PARP-1, we used both PARP-1 siRNA and PJ34. PARP-1 siRNA successfully decreased PARP-1 expression in HUVECs to 15% (Figure 1B). HUVECs with H₂O₂ at varying concentrations for 3 h and found that at all concentrations of H₂O₂, PARP-1 siRNA, and PJ34 significantly increased ATP content (Figure 1C).

ROS- and RNS-Induced HUVEC Death Can Be Attenuated by PARP-1 Inhibition

To rule out the possibility of cell leakage contributing to ATP and NAD⁺ depletion, we assayed HUVECs for LDH release after H₂O₂ and ONOO⁻ treatment (supplemental Figure IIA). There was no significant LDH leakage 3 h after ROS/RNS treatment. Because NAD⁺ depletion and ATP consumption are associated with cell death,¹⁰ we assayed the effect of PARP-1 inhibition on H₂O₂ and ONOO⁻-mediated cell death. PARP-1 siRNA-transfected HUVECs experienced a ~60% reduction in cell death compared to scrambled siRNA-transfected controls (Figure 2A). In addition, when apoptosis was measured using Hoescht stain, PARP-1 siRNA reduced the number of apoptotic nuclei after 8 h by ~25% (Figure 2B). PJ34 also reduced HUVEC death induced by H₂O₂ (67% decrease, Figure 2A) and by ONOO⁻ (60% decrease, supplemental Figure II B). Cells transfected with PARP-1 siRNA, and then treated with PJ34 exhibited a decrease in apoptosis (Figure 2B) that did not differ significantly from the effects of PJ34 or PARP-1 siRNA alone. This result suggests that PARP-1 inhibition attenuates apoptosis primarily through the inhibition of PARP-1 ADP-ribosylation activity.

PARP-1 Inhibition Increases Akt and BAD Activation in Response to ROS and RNS

Although our data support the hypothesis that PARP-1 inhibition limits energy consumption and mitigates ROS- and RNS-induced cell death, we were interested in the possibility of other PARP-1-mediated survival mechanisms. Specifically, PARP-1 inhibition increases Akt phosphorylation in...
Neuronal and liver cells exposed to oxidative stress. To investigate the relevance of this signaling pathway in endothelial cells, we treated HUVECs with H₂O₂ and PJ34 and measured Akt phosphorylation. Akt phosphorylation was enhanced nearly 9-fold by PJ34 (Figure 3A). To confirm that this result was specific to PARP-1 inhibition, we also transfected cells with PARP-1 siRNA. Similar to PJ34, PARP-1 knockdown increased Akt phosphorylation by ~9-fold after H₂O₂ exposure (supplemental Figure IIIA).

Because PARP-1 inhibition increased Akt phosphorylation, we hypothesized that PARP-1 inhibition would also increase the phosphorylation of downstream Akt substrates, such as BAD. We confirmed a 7-fold increase in BAD phosphorylation (pBAD) in PJ34-treated HUVECs stimulated with H₂O₂ (Figure 3A). A 7-fold increase in pBAD was also observed when we used PARP-1 siRNA to inhibit PARP-1 (supplemental Figure IIIB).

We next determined whether PARP-1 inhibition activated Akt in the setting of nitrosative stress. We treated HUVECs with ONOO⁻ and PJ34 and assayed for pAkt and pBAD. Similar to the results with H₂O₂, ONOO⁻ in conjunction with PARP-1 inhibition increases Akt and BAD phosphorylation. A and B, HUVECs were serum-starved for 12 h, treated with PJ34 (10 μmol/L), and exposed to 300 μmol/L H₂O₂ in (A) or 150 μmol/L ONOO⁻ in (B) for 15 min. Lysates were probed for pAkt (Ser 473) and total Akt or pBAD (Ser 136) and total BAD. Quantitative analysis of protein phosphorylation was normalized by arbitrarily setting the ratio of densitometry of control cells pAkt/Akt or pBAD/BAD to 1.0 (n=3 for all experiments).

**Figure 3.** PARP-1 inhibition increases Akt and BAD phosphorylation in HUVECs. A and B, HUVECs were serum-starved for 12 h, treated with PJ34 (10 μmol/L), and exposed to 300 μmol/L H₂O₂ in (A) or 150 μmol/L ONOO⁻ in (B) for 15 min. Lysates were probed for pAkt (Ser 473) and total Akt or pBAD (Ser 136) and total BAD. Quantitative analysis of protein phosphorylation was normalized by arbitrarily setting the ratio of densitometry of control cells pAkt/Akt or pBAD/BAD to 1.0 (n=3 for all experiments).

**PARP-1 Inhibition Increases the Association of BAD With 14-3-3**

When phosphorylated on Ser136, BAD associates with the multifunctional phosphoserine-binding protein 14-3-3. This association inhibits BAD-induced cell death. To confirm that the phosphorylation of BAD elicited by PJ34 was part of this signaling cascade, we assayed for the association of BAD with 14-3-3 after H₂O₂ and PJ34 treatment (supplemental Figure IIIC). Under control conditions, the basal association of BAD and 14-3-3 was enhanced 2-fold by PJ34. This interaction was increased slightly by H₂O₂, but rose significantly to nearly 7-fold when cells were pretreated with PJ34. These data indicate that PARP-1 inhibition can phosphorylate BAD and increase its association with 14-3-3, thus inhibiting its apoptotic effects.

**PARP-1 Inhibition Activates the VEGF Receptor 2**

To determine the mechanism by which PARP-1 inhibition stimulates Akt and BAD phosphorylation, we studied proteins upstream of Akt involved in survival pathways. VEGF is a well-established vascular survival factor, and its activity can be modulated by poly-ADP ribosylation (PARsylation). Recent data have also implicated PARP-1 inhibition in angiogenesis, a process mediated in part by VEGFR2. We therefore studied the effects of PARP-1 inhibition on VEGFR2 phosphorylation to define the mechanism of Akt phosphorylation.

Cells were treated with VEGF, H₂O₂, or PJ34 and lysates were probed for phosphotyrosine using the 4G10 antibody. Phosphorylation of a 230-kDa band increased 22-fold after 15 min of VEGF treatment (Figure 4, lane 2), but was attenuated by pretreatment with the VEGFR2-specific tyrosine kinase inhibitor SU1498 (Figure 4, lane 3). Phosphorylation of a band of identical molecular weight to the VEGFR2 similarly

**Table 1.** PARP-1 Inhibition Activates the VEGF Receptor 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>pAkt/Akt</th>
<th>pBAD/BAD</th>
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<tr>
<td>H₂O₂ alone</td>
<td>-</td>
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<tr>
<td>PJ34 alone</td>
<td>-</td>
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<tr>
<td>H₂O₂ + PJ34</td>
<td>+</td>
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<tr>
<td>PARP-1 siRNA alone</td>
<td>-</td>
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<tr>
<td>H₂O₂ + PARP-1 siRNA</td>
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increased after 15 min of PJ34 exposure alone (Figure 4, lane 4), which was also decreased by SU1498 pretreatment (Figure 4, lane 5). Akt phosphorylation was increased by PJ34 (Figure 4, lane 4), but attenuated by SU1498 (Figure 4, lane 5) indicating that PJ34-mediated Akt phosphorylation correlates with VEGFR2 phosphorylation (Figure 4, lane 2).

Next, we immunoprecipitated HUVEC lysates with antiphosphotyrosine (4G10) and probed for VEGFR2. Treatment with VEGF increased immunoprecipitation of a 230-kDa band consistent with the VEGFR2 phosphorylation by 10-fold (Figure 4, lane 2). Akt phosphorylation was increased by 1.9-fold (Figure 4, lane 2), but attenuated by SU1498 (Figure 4, lane 5) indicating that PJ34-mediated Akt phosphorylation correlates with VEGFR2 phosphorylation (Figure 4, lane 2).

To confirm the in vivo relevance of this signaling cascade, we isolated arterial segments from the descending thoracic aorta of rabbits and perfused them ex vivo with the PARP-1 inhibitor, PJ34, for 30 min. We found that PJ34 prevented stress-induced death. To confirm that PARP-1 inhibition could confer a survival effect independent of energy protection, we induced HUVEC death using serum starvation. After pretreating HUVECs with varying concentrations of PJ34 and serum-starving cells for 24 h, we found that PARP-1 inhibition conferred a statistically significant survival advantage at >10 μmol/L and above (supplemental Figure IVB). To prove further the energy independent effects of PARP-1, we studied knockdown of PARP-1 with siRNA. Similar to PJ34, PARP-1 siRNA protected HUVECs from serum starvatin-induced death (supplemental Figure IVC).

**Discussion**

The major findings of this study are that PARP-1 inhibition stimulates tyrosine phosphorylation of VEGFR2 and activation of Akt, thereby promoting endothelial cell survival.
PARP-1 inhibition promoted plaque stability in ApoE−/− mice by inhibiting foam cell apoptosis. The PJ34 protective effect on ischaemic cell death, its related inflammation and survival signals.

In conclusion, we believe that PARP-1 inhibition will improve endothelial function by preventing energy depletion, enhancing a survival pathway via VEGFR2/Akt/BAD phosphorylation, and by limiting inflammation via NFκB, thus making PARP-1 a valid therapeutic target for vascular disease.

Disclosures

None.

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PARP-1 inhibition may prove beneficial in limiting the initiation and progression of vascular disease because of its abilities to protect against ROS/RNS-induced energy depletion, to stimulate prosurvival signaling pathways, and to prevent NFκB activation and the transcription of inflammatory genes. A therapeutic concern may be the effect of chronic PARP-1 inhibition on genomic stability because of the role of PARP-1 in DNA repair and genomic stability. However, PARP-1 knockout mice are viable and only susceptible to genetic damage when exposed to high levels of radiation. DNA base excision repair involves a number of enzymes, many of which likely compensate for PARP-1, including PARP-2. Therefore, we believe that inhibiting PARP-1 will have minimal effects on genomic stability under physiological conditions. The long-term effects of PARP-1 inhibition on endothelial function will undoubtedly involve changes in gene expression and inflammation, consequences that deserves careful consideration in the future.

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Supplemental figure I. PARP-1 inhibition using PJ34 and PARP-1 siRNA.
A, Cells were pre-treated with the PARP-1 inhibitor PJ34, exposed to 300µM H₂O₂ or 150µM ONOO⁻, and assayed for PARP-1 activity after 3 hours (n=4). B, HUVEC were transfected with either scrambled (scr) siRNA or PARP-1 siRNA. PARP expression in the cell homogenates was detected by Western blotting. Equal protein loading was confirmed by blotting for actin.
Supplemental figure II. PARP-1 inhibition limits ROS and RNS-induced cell death independent of membrane integrity. A, HUVEC were pre-treated with H₂O₂ or ONOO⁻. Culture media was assayed for LDH at the indicated times (n=3; *p<0.001 with one-way ANOVA and Fisher’s PLSD). B, HUVEC were either pre-treated with PJ34 (10µM) or transfected with PARP-1 siRNA and exposed to ONOO⁻. After 24 hours, cells were trypsinized and counted in three fields with trypan blue in a hemocytometer (n=3; *p<0.003 with one-way ANOVA and Fisher’s PLSD).
Supplemental figure III. PARP-1 inhibition enhances ROS-mediated Akt and BAD phosphorylation and BAD-14-3-3 interaction. A and B, HUVEC were serum-starved for 12 hours and treated with PJ34 (10μM) or transfected with either scrambled or PARP-1 siRNA before being exposed to 300μM H₂O₂ for 15 minutes. Cell lysates were run on an SDS-PAGE gel and western-blotted for pAkt (Ser 473) and total Akt or pBAD (Ser 136) and total BAD. Quantitative analysis of protein phosphorylation was normalized by arbitrarily setting the ratio of densitometry of control cells pAkt/Akt or pBAD/BAD to 1.0 (n=3 for all experiments). C, HUVEC were serum-starved for 12 hours, treated with 10μM PJ34, then exposed to 300 μM H₂O₂ for 30 minutes. Lysates were immunoprecipitated (IP) with anti-14-3-3 and immunoblotted with anti-BAD. Quantitative analysis of protein precipitation was normalized by arbitrarily setting the ratio of control-condition immunoprecipitate to 1.0 (n=3). TCL: total cell lysates, IgG: control rabbit IgG, * p<0.05.
Supplemental figure IV. PARP-1 inhibition increases HUVEC survival independent of energy conservation. A, PARP-1 inhibition increases VEGFR2 phosphorylation. HUVEC were serum-starved and treated for 15 minutes with VEGF (10ng/ml), H₂O₂ (300µM), SU1498 (10µM), PJ34 (10µM) or a combination of these agonists. A, Lane 1: control; lane 2: VEGF; lane 3: VEGF+SU1498; lane 4: PJ34; lane 5: PJ34+SU1498; lane 6: PARP-1 siRNA; lane 7: PARP-1 siRNA+SU1498. Cell lysates were immunoprecipitated (IP) with 4G10 antibody, and immunoblotted (IB) with anti-VEGFR2. Lysates were saved and run on a separate SDS-PAGE gel and probed with actin to confirm equal protein input (n=3). TCL, total cell lysates; IgG, control mouse IgG. B, Cells were pre-treated with varying concentrations of PJ34 and serum-starved for 24 hours, then counted. C, Cells were transfected with either scrambled or PARP-1 siRNA (20nM) and serum-starved for 24 hours in varying concentrations of serum and counted using trypan blue.