ORIGINAL RESEARCH

Novel Mechanism for Nicotinamide Phosphoribosyltransferase Inhibition of TNF-α–mediated Apoptosis in Human Lung Endothelial Cells

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Abstract

Nicotinamide phosphoribosyltransferase (NAMPT) exists as both intracellular NAMPT and extracellular NAMPT (eNAMPT) proteins. eNAMPT is secreted into the blood and functions as a cytokine/enzyme (cytozyme) that activates NF-κB signaling via ligation of Toll-like receptor 4 (TLR4), further serving as a biomarker for inflammatory lung disorders such as acute respiratory distress syndrome. In contrast, intracellular NAMPT is involved in nicotinamide mononucleotide synthesis and has been implicated in the regulation of cellular apoptosis, although the exact mechanisms for this regulation are poorly understood. We examined the role of NAMPT in TNF-α–induced human lung endothelial cell (EC) apoptosis and demonstrated that reduced NAMPT expression (siRNA) increases EC susceptibility to TNF-α–induced apoptosis as reflected by PARP-1 cleavage and caspase-3 activation. In contrast, overexpression of NAMPT served to reduce degrees of TNF-α–induced EC apoptosis. Inhibition of nicotinamide mononucleotide synthesis by FK866 (a selective NAMPT enzymatic inhibitor) failed to alter TNF-α–induced human lung EC apoptosis, suggesting that NAMPT-dependent NAD^+ generation is unlikely to be involved in regulation of TNF-α–induced EC apoptosis. We next confirmed that TNF-α–induced EC apoptosis is attributable to NAMPT secretion into the EC culture media and subsequent eNAMPT ligation of TLR4 on the EC membrane surface. Silencing of *NAMPT* expression, direct neutralization of secreted eNAMPT by an NAMPT-specific polyclonal antibody (preventing TLR4 ligation), or direct TLR4 antagonism all served to significantly increase EC susceptibility to TNF-α–induced EC apoptosis. Together, these studies provide novel insights into NAMPT contributions to lung inflammatory events and to novel mechanisms of EC apoptosis regulation.

Keywords: apoptosis; nicotinamide phosphoribosyltransferase; NAD^+; acute lung injury

Previously known as a B-lymphocyte maturation factor (1), the nicotinamide phosphoribosyltransferase (NAMPT) enzyme, known as a pre–B-cell colony-enhancing factor, catalyzes the synthesis of nicotinamide mononucleotide (NMN) from nicotinamide and diphosphate (2). This intracellular enzymatic function reduces oxidant stress (3) and has been implicated in inhibiting cellular apoptosis (4). As a result, FK866, a noncompetitive intracellular NAMPT (iNAMPT) enzymatic inhibitor, is currently in phase II clinical trials as an anticancer agent for solid and advanced tumors via reductions in intracellular NAD^+ concentrations (5). The extracellular secreted form of NAMPT (eNAMPT) is included in the class of

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Clinical Relevance

We have identified nicotinamide phosphoribosyltransferase (NAMPT) as a secreted cytozyme involved in inflammatory responses to danger signals such as mechanical stress via ligation of Toll-like receptor 4. Intracellular enzymatic activity of NAMPT has been implicated in regulating cellular fate (retarding apoptosis), although the mechanistic basis for this speculation has been limited and somewhat controversial. In this report, we directly address *1*) the mechanism by which NAMPT influences endothelial apoptosis induced by TNF-α via gene silencing and *2*) comparisons with the pharmacological inhibition of its NAMPT activity (FK866, GNE-617, or GMX1778). We propose a novel model of NAMPT-mediated regulation of cellular fate that involves NAMPT secretion from endothelial cells and the downstream regulation of antiapoptotic and prosurvival mechanisms upon binding to its receptor.

cytokines/adipokines that initial studies suggested may exhibit insulin mimetic properties (6). Although this hypothesis remains unresolved (7–10), eNAMPT is secreted from multiple cells, including endothelium (11), adipocytes (12), macrophages (13), hepatocytes (14), placental cells (15), chondrocytes (16), and synovial fibroblasts (17). eNAMPT is also detected in synovial fluid (18), saliva (19), BAL fluid (11), and cerebrospinal fluid (20).

The relationship between NAMPT and cellular stress and stress responses in normal or pathological conditions is highly complex. Although an increase in iNAMPT and NMN synthesis is protective against genotoxic stress in lymphocytes (3), such increases were associated with insulin resistance in adipose tissue (13) and with inflammation in synovial tissue (18). Similarly, an increase in extracellular/circulating concentrations of NAMPT was linked to higher amounts of oxidative stress in cultured skeletal myocytes (21) and inflammation in cultured chondrocytes (22), whereas in cultured pancreatic β-cells, it was protective against palmitate-induced toxicity (23).

Our prior genomics-intensive approaches originally identified *NAMPT* as a biomarker and candidate gene in preclinical and human inflammatory lung injury, particularly under conditions of excessive mechanical stress produced by mechanical ventilation (24). Further studies defined the involvement of STAT5-mediated *NAMPT* transcription and 3′ untranslated region epigenetic regulation (25, 26). Heterozygous *Nampt*$^{+/-}$ mice showed reductions in both inflammatory markers and lung injury when subjected to preclinical models of ventilator-induced lung injury (27). Furthermore, plasma concentrations of eNAMPT are a biomarker for acute respiratory distress syndrome and correlate with acute respiratory distress syndrome mortality (27–29). Recently, we clarified the mechanism by which eNAMPT exerts proinflammatory properties, and we identified Toll-like receptor 4 (TLR4) as the novel receptor for eNAMPT leading to the downstream activation of NF-κB–dependent signaling (30). Compared with the regulation of NMN synthesis by iNAMPT and recently elucidated eNAMPT signaling pathways, there is a paucity of mechanistic information regarding NAMPT regulation of cellular apoptosis (23, 31, 32), a critical event in inflammatory lung injury (33–35). We used human lung endothelial cells (ECs) to define NAMPT involvement in susceptibility to cytokine (TNF-α)–induced EC apoptosis as reflected by PARP-1 cleavage and caspase-3 activation. These studies demonstrated that TNF-α–induced EC apoptosis was enhanced by reductions in NAMPT expression (siRNA) and attenuated by NAMPT overexpression. NAMPT influence on EC apoptosis did not appear to involve iNAMPT nicotinamide phosphoribosyltransferase activity, because inhibition of NMN synthesis with FK866, a selective NAMPT inhibitor, failed to alter degrees of TNF-α–mediated EC apoptosis. Rather, NAMPT regulation of TNF-α–induced EC apoptosis appeared to be attributable completely to NAMPT secretion and subsequent eNAMPT ligation of TLR4 on the EC surface. Either EC silencing of *NAMPT* expression, direct neutralization of secreted eNAMPT by an NAMPT-specific polyclonal antibody (to prevent TLR4 ligation), or direct TLR4 antagonism all served to significantly increase EC susceptibility to TNF-α–induced EC apoptosis. Together, these studies provide novel insights into NAMPT contributions to lung inflammatory events and to novel mechanisms of EC apoptosis regulation.

Methods

A detailed Experimental Procedures section is available in the data supplement.

Results

Altered NAMPT Expression Significantly Influences TNF-α–mediated Human Lung EC Apoptosis

To explore precise mechanisms by which NAMPT modulates cell survival and apoptosis, human lung ECs were transfected with an NAMPT-specific siRNA, resulting in reduced NAMPT expression after 72 hours (Figure 1A). PARP-1 is a multifunctional regulator of transcription, chromatin structure, and genomic integrity activated by DNA breaks using NAD^+ as a substrate (36). During apoptosis, PARP-1 is cleaved by caspase-3 in two distinct fragments of 24 kD and 89 kD (37). *NAMPT* gene silencing in EC for 72 hours resulted in significant increases in PARP-1 expression, reflecting increased EC apoptosis (compared with a control silenced/scrambled siRNA group) (Figure 1A). Identical increases in EC apoptosis were observed in NAMPT-silenced EC that were subsequently treated for 5 hours with 30 ng/ml TNF-α, with increased PARP-1 cleavage compared with control silenced EC. To confirm that PARP-1 cleavage was indeed dependent on caspase-3 activation, unsilenced ECs were preincubated (6 h) with the known caspase-3 inhibitor z-DEVD-fmk before exposure to TNF-α, Fas ligand, or *tert*-butyl hydroperoxide (24 h), inducers of apoptosis. EC pretreated with z-DEVD-fmk and challenged with TNF-α demonstrated significantly reduced PARP-1 cleavage compared with EC pretreated with vehicle alone (DMSO) (Figure 1B). In contrast to results obtained by NAMPT silencing in EC that resulted in increased EC apoptosis, the transient plasmid-based overexpression of human *NAMPT*

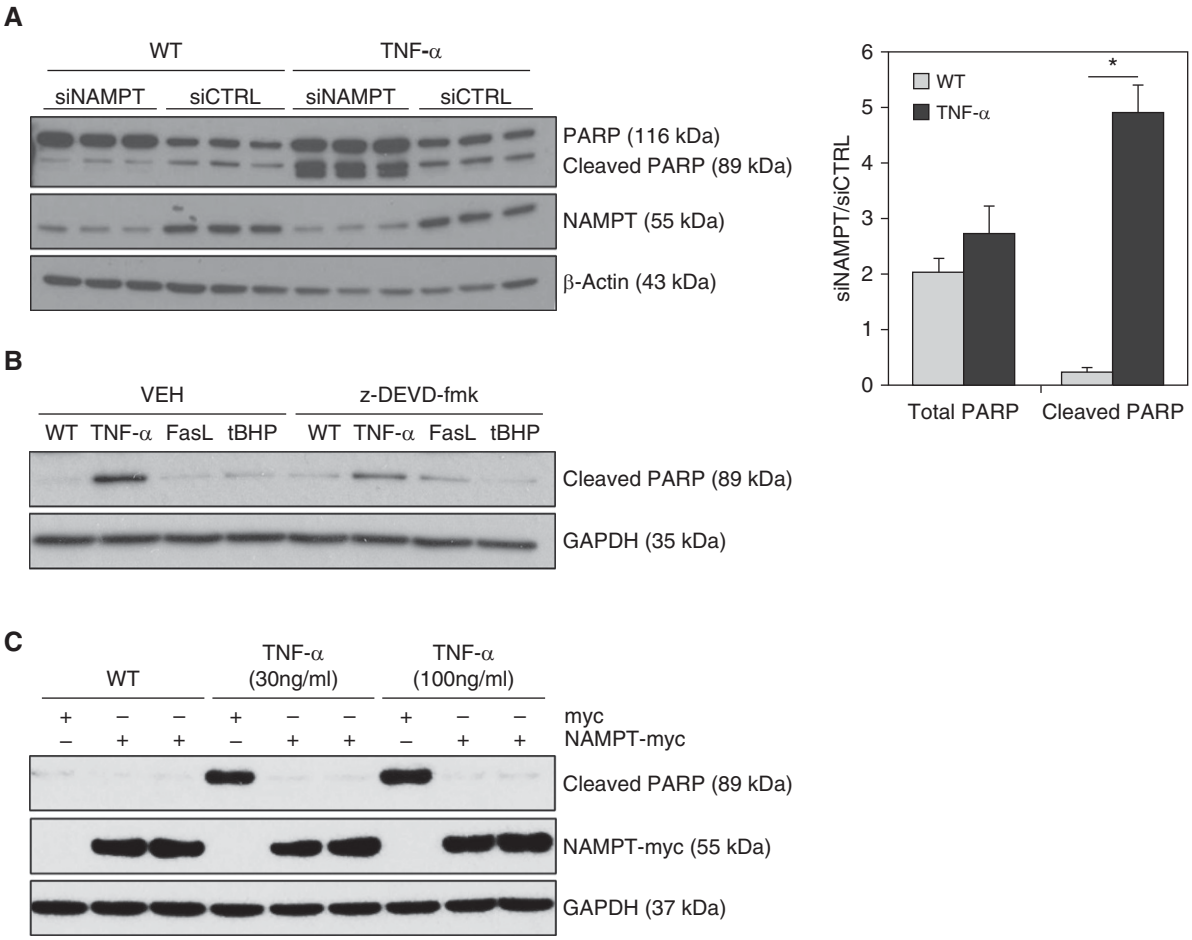

Figure 1. Nicotinamide phosphoribosyltransferase (NAMPT) expression modulates endothelial cell (EC) susceptibility to TNF-α–induced apoptosis. (*A*) EC transfection with *NAMPT* gene siRNA (72 h) reduced NAMPT expression and resulted in marked increases in full-length PARP-1 expression (compared with transfection with scrambled siRNAs) in both vehicle- and TNF-α–challenged ECs (30 ng/ml; 5 h). TNF-α challenge of NAMPT-silenced ECs markedly increased PARP-1 cleavage proteolysis, whereas scrambled siRNA-treated ECs exhibited minimal increases in PARP-1 cleavage. $^*P < 0.05$. (*B*) ECs were pretreated (6 h) with either vehicle (DMSO) or a caspase-3 inhibitor (z-DEVD-fmk; 20 μM) before 24-hour challenge with TNF-α (30 ng/ml), Fas ligand (FasL; 100 ng/ml), or *tert*-butyl hydroperoxide (tBHP; 5 mM). Inhibition of caspase-3 activity significantly reduced subsequent PARP-1 cleavage induced by TNF-α. (*C*) ECs were transfected with myc-tagged NAMPT plasmid, resulting in marked NAMPT overexpression. Compared with ECs transfected with myc-tag only, ECs overexpressing NAMPT exhibited virtual abolishment of PARP cleavage after TNF-α challenge (30 ng/ml or 100 ng/ml; 24 h). Error bars indicate SEM of three independent experiments. CTRL = control; siCTRL = scrambled silencing; siNAMPT = NAMPT silencing; VEH = vehicle; WT = wild type.

served to decrease concentrations of cleaved PARP-1 induced by TNF-α (Figure 1C), indicating that increases in NAMPT expression and protein concentrations reduce EC apoptosis.

NAD^+ Concentrations Fail to Influence TNF-α-mediated Human Lung EC Apoptosis

To determine whether pharmacological inhibition of NMN synthesis contributes to TNF-α–induced EC apoptosis, ECs were pretreated with DMSO or FK866 (NAMPT enzymatic inhibitor, 0.1–10 μM, 24 h), followed by TNF-α challenge (100 ng/ml, 24 h). Western blots showed that FK866 alone did not affect PARP-1 expression or cleavage and failed to alter PARP-1 cleavage in response to TNF-α (Figure 2A). Colorimetric measurements of caspase-3 activity confirmed that NAMPT-silenced ECs exhibit significantly higher degrees of caspase-3 activation in response to TNF-α. In addition, FK866 failed to mimic the effect of NAMPT silencing on TNF-α–induced EC apoptosis (Figure 2B), despite approximately 80% reductions in NAD^+ concentrations compared with only about a 50% reduction in NAD^+ concentrations by NAMPT silencing (*see* Figure E1 in the data supplement). Thus, increased TNF-α–induced EC apoptosis and PARP-1 expression and cleavage observed in NAMPT-silenced ECs are unlikely to be linked to NAD^+ depletion (Figure E2).

Secreted NAMPT Regulates TNF-α–mediated Human Lung EC Apoptosis

The divergence between iNAMPT pharmacological inhibition and reductions in *NAMPT* expression (silencing) suggested either the involvement of secreted NAMPT in TNF-α–induced EC apoptosis or the potential for additional iNAMPT functional effects beyond NMN synthesis. To determine the role of EC-secreted eNAMPT, IP of eNAMPT from EC culture medium was followed by Western blotting (Figure E3), studies that validated the presence of secreted

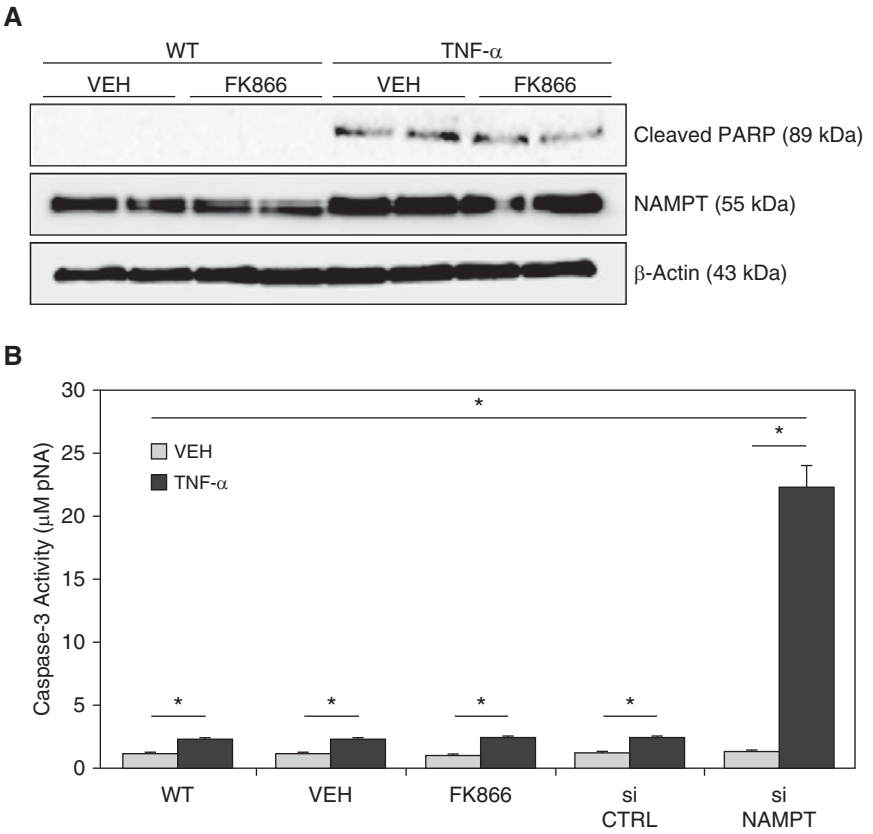

Figure 2. Pharmacological inhibition of EC NAMPT and reduced nicotinamide mononucleotide synthesis fails to alter TNF-α–induced PARP cleavage. (*A*) ECs were pretreated (24 h) with FK866 (10 μM) or vehicle (DMSO), followed by TNF-α challenge (100 ng/ml; 24 h). FK866 failed to produce significant differences in the amounts of TNF-α–induced PARP-1 cleavage. (*B*) Comparisons of TNF-α–induced caspase-3 activity (colorimetric assay) in ECs silenced for NAMPT (72 h) or treated with FK866 (10 μM; 24 h). Only NAMPT silencing (siRNA) significantly increased TNF-α–induced caspase-3 activity compared with controls, with FK866 failing to alter caspase-3 activation upon subsequent TNF-α challenge compared with DMSO. *$P < 0.05$. Error bars indicate SEM of three independent experiments. pNA = para-nitroaniline.

eNAMPT at the same molecular size as iNAMPT detected in EC lysates. EC *NAMPT* gene silencing reduced eNAMPT secretion (Figure 3A), as verified via an NAMPT ELISA (Figure 3B).

We next determined whether secreted eNAMPT increases prosurvival mechanisms in EC. The effects of culture medium derived from NAMPT-silenced EC (donor group) were evaluated for effects on EC apoptosis and compared them with effects in culture medium obtained from control silenced EC (Figure 4A). The addition of culture medium from unsilenced EC to TNF-α–challenged, NAMPT-silenced EC significantly reduced caspase-3 activation (Figure 4B). In contrast, these reductions in TNF-α–induced caspase-3 activation by conditioned media were significantly less when culture medium from NAMPT-silenced EC (with reduced concentrations of NAMPT in the conditioned media) was used, suggesting that a secreted factor or factors reduce EC apoptosis, with NAMPT being the likely candidate evoking these responses. The incubation of control nonsilenced EC with various concentrations of FK866 (1–100 μM) for 24 hours failed to affect basal NAMPT secretion (Figure E4), indicating that changes in NAD^+ concentrations are not the cause of the reduced apoptosis.

To confirm that secreted eNAMPT is the effector regulating EC apoptotic responses, we next used a goat polyclonal antibody raised against NAMPT that abolishes TLR4 binding and NF-κB activation (27). NAMPT-silenced EC incubated with control conditioned medium supplemented with the NAMPT neutralizing antibody exhibited increased degrees of TNF-α–induced caspase-3 activation compared with EC treated with conditioned medium supplemented with control IgG or conditioned medium alone (Figure 4C). To further examine the mechanisms by which secreted eNAMPT alters TNF-α–induced apoptosis, ECs were transfected with an NAMPT-hemagglutinin (NAMPT-HA) plasmid and conditioned media containing ectopically secreted fusion NAMPT protein sampled after 48 hours with HA-tagged NAMPT detected in supernatant by IP and by ELISA (data not shown). In a separate set of experiments, ECs were pretreated with LPS from the photosynthetic bacterium *Rhodobacter sphaeroides* (1 μg/ml) or with vehicle PBS for 1 hour, followed by supplementation with conditioned medium obtained from transfected EC or with empty endothelial growth medium-2 for 48 hours. After 24 hours of exposure to conditioned media, ECs were challenged with TNF-α (100 ng/ml) for 24 hours and assayed for caspase-3 activity. EC exposed to conditioned media containing high concentrations of HA-NAMPT demonstrated reduced TNF-α–induced caspase-3 activation compared with EC exposed to endothelial growth medium-2 alone. This protective effect of HA-NAMPT–containing conditioned media was attenuated by EC pretreatment with a TLR4 inhibitor, LPS from the photosynthetic bacterium *Rhodobacter sphaeroides*. These studies indicated that secreted eNAMPT is a critical determinant in the regulation of TNF-α–induced EC apoptosis via ligation of TLR4 receptor, as we reported previously (27). The TLR4 receptor ligand LPS increased EC caspase-3 activation; however, preincubation of NAMPT-silenced cells with LPS (10 ng/ml) before TNF-α challenge not only failed to confer antiapoptotic protection but also was additive in augmenting caspase-3 activation (Figure E6). These studies indicate that NAMPT exerts antiapoptotic protection via unknown mechanisms that are distinct from those of other TLR4 agonists.

Recombinant Human NAMPT Protects Human Lung EC against TNF-α–induced EC Apoptosis

To further validate the role of eNAMPT in the regulation of TNF-α–induced EC

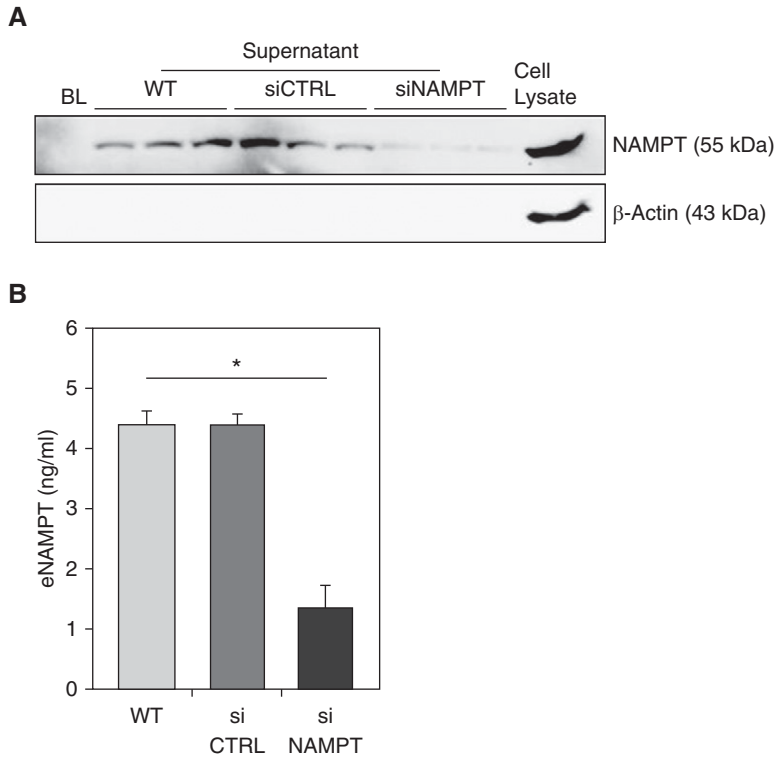

Figure 3. ECs secrete extracellular NAMPT (eNAMPT), with *NAMPT* gene silencing reducing subsequent amounts of eNAMPT secretion. (*A*) ECs were silenced for NAMPT (72 h), followed by sampling of supernatant (culture media). Secreted eNAMPT was enriched by IP and detected by Western blotting. eNAMPT concentrations in NAMPT-silenced media were reduced compared with silenced CTRL and untransfected cells. (*B*) ELISA quantitative measurement of NAMPT concentrations in EC supernatants. Shown is the reduction in NAMPT in conditioned media (EC supernatant) derived from NAMPT-silenced ECs compared with the unchallenged ECs and CTRL-silenced ECs. $^*P < 0.05$. Error bars indicate SEM of three independent experiments. BL = baseline.

apoptosis, ECs were pretreated with 100 ng/ml recombinant human NAMPT (rhNAMPT) for 24 hours followed by TNF-α challenge (24 h) with assessment of concentrations of the immunoreactive PARP-1–cleaved fragment ($DEVD^{214}$). rhNAMPT alone failed to alter basal amounts of PARP-1 cleavage; however, rhNAMPT pretreatment reduced TNF-α–mediated PARP-1 cleavage compared with TNF-α–challenged EC without rhNAMPT pretreatment (Figure 5A). In addition, EC pretreated with rhNAMPT followed by TNF-α challenge exhibited reduced DNA strand breaks (TUNEL assay, fluorescein) compared with TNF-α–challenged EC without rhNAMPT pretreatment (Figure 5B). rhNAMPT pretreatment effects on TNF-α–mediated PARP-1 cleavage were dose dependent (5–80 ng/ml) (Figure E7). These results demonstrate that rhNAMPT confers resistance to proapoptotic stimuli such as TNF-α.

Discussion

We have determined a novel new mechanism by which NAMPT regulates signaling pathways that influence major cellular fate decisions such as survival and programmed cell death. Our data clearly indicate that NAMPT-mediated effects on EC apoptosis are not triggered by iNAMPT-dependent NMN synthesis, but rather involve eNAMPT secretion and subsequent engagement of TLR4 to produce reductions in EC apoptosis. This conclusion is supported by the effects of rhNAMPT on TNF-α–induced EC apoptosis (caspase-3 cleavage) and by the inability of pharmacological inhibition of iNAMPT phosphoribosyltransferase activity (FK866) to alter degrees of TNF-α–induced EC apoptosis. Furthermore, supplementation with NAMPT-containing conditioned media from control EC, but not NAMPT-depleted media from NAMPT-silenced EC, resulted in attenuation of EC susceptibility to TNF-α–induced EC apoptosis. This protection observed with NAMPT-containing conditioned media was abolished by the addition of an eNAMPT neutralizing antibody as well as by TLR4 inhibition.

Reductions in NMN concentrations via FK866-mediated inhibition of NAMPT enzymatic activity induce a diverse range of biological effects, including effects on autophagy (38), oncosis (39), and endoplasmic reticulum stress (40). In addition to iNAMPT regulation of intracellular NMN synthesis, iNAMPT may exert homeostatic effects that are dependent on protein–protein interactions involved in the regulation of oxidative stress and inflammation, such as NADH dehydrogenase subunit 1, IFN-induced transmembrane protein 3, γ-glutamyltransferase, ubiquitin-conjugating enzyme E2L6, and ferritin light chain, that were detected by our prior yeast two-hybrid studies (41). Although the precise impact of these potential interactions on cellular processes, including regulation of prosurvival mechanisms independently of NMN synthesis, remains poorly understood, reductions in iNAMPT protein concentrations would be predicted to alter the magnitude of these interactions and thus alter EC susceptibility to cell death.

Our studies indicate very strong linkages between reductions in NAMPT expression and reduced eNAMPT secretion and, consequently, reduced autocrine/paracrine effects on cytokine-mediated apoptosis. NAMPT overexpression leads to increased eNAMPT secretion and enhanced autocrine/paracrine effects shown to be relevant to IL-1β–induced articular chondrocyte secretion of proinflammatory cytokines (IL-6, TNF-α) (16) by hepatocytes with increased *NAMPT* gene expression (14, 42, 43) and by transgenic mice overexpressing NAMPT with observed reductions in cardiac ischemia/reperfusion injury (44). Our proposed model adds clarity to observed increases in IL-8 secretion in TNF-α–challenged alveolar epithelial cells overexpressing NAMPT (45), reduced permeability in IL-1β–stimulated alveolar epithelium with reduced *NAMPT* expression (46), and reduced inflammation in human pulmonary

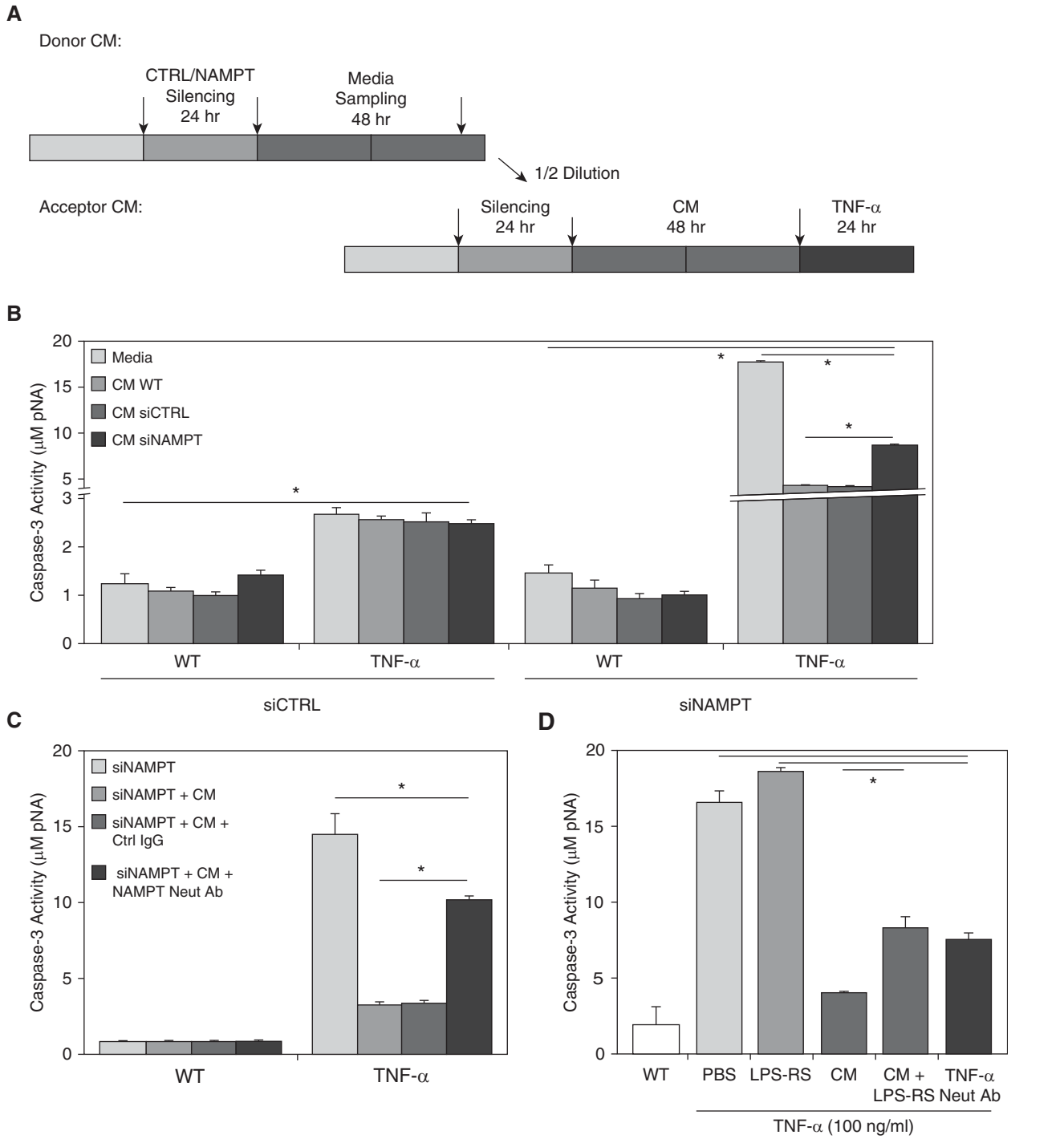

Figure 4. eNAMPT is responsible for TNF-α–induced cell death. (*A*) Schematic representation of the conditioned medium (CM) strategy. Donor CM was obtained from either CTRL-silenced ECs or ECs silenced for NAMPT (24 h) (donor group), collected after 48 hours. Figure 3B depicts the reduced NAMPT concentrations in CM from NAMPT-silenced ECs. CM was prepared by 50% CM (donor group) and 50% fresh endothelial growth medium-2 to treat another set of ECs (acceptor group), which had already been silenced for NAMPT expression (24 h). After 48 hours of continuous incubation with CM, the acceptor group ECs were further treated with TNF-α for another 24 hours. (*B*) Supplementation of the CM from NAMPT-silenced ECs significantly blunted the protection offered by CM against TNF-α in NAMPT-silenced EC (100 ng/ml; 24 h)-activated caspase-3 activity compared with the CTRL CM. $^*P < 0.05$. (*C*) Protection against TNF-α–activated caspase-3 activity in NAMPT-silenced ECs induced by the CM from CTRL cells was attenuated by goat polyclonal anti-NAMPT antibody (1 μg/ml) but not by coincubation with normal CTRL goat IgGs. (*D*) Incubation with CM from ECs transfected with NAMPT-hemagglutinin reduced the activation of caspase-3 by TNF-α (100 ng/ml; 24 h), and the effect was significantly impaired by preincubation with LPS from the photosynthetic bacterium *Rhodobacter sphaeroides* (1 μg/ml). Coincubation of TNF-α with a monoclonal anti–TNF-α antibody with neutralizing ability served as a positive CTRL. $^*P < 0.05$. Error bars indicate SEM of three independent experiments. LPS-RS = LPS from *Rhodobacter sphaeroides*; Neut Ab = anti-NAMPT neutralizing antibody.

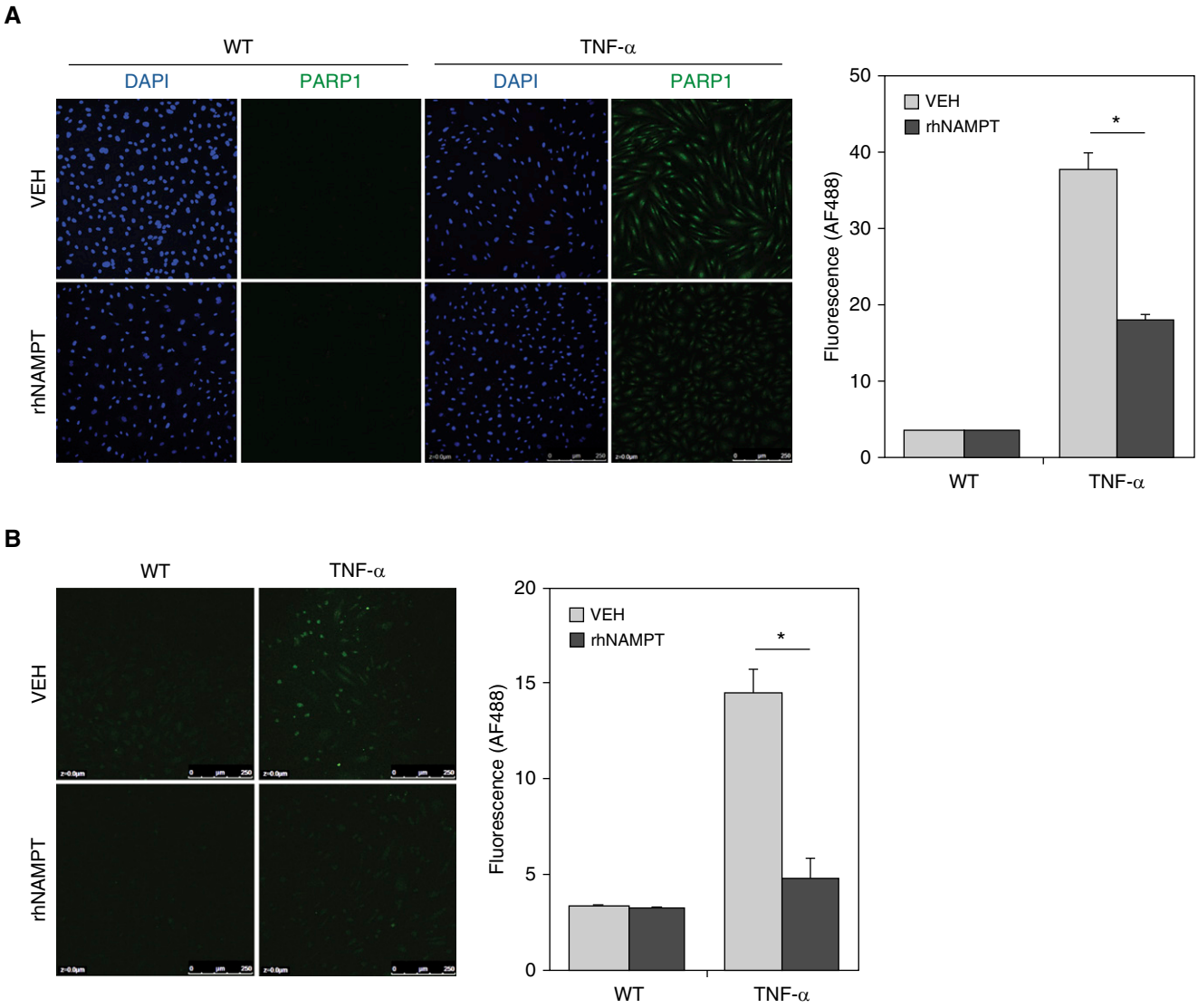

Figure 5. Recombinant human NAMPT (rhNAMPT) attenuates TNF-α–induced apoptosis markers. (*A*) ECs were pretreated with rhNAMPT (100 ng/ml; 24 h), then challenged with TNF-α (100 ng/ml; 24 h). ECs were next fixed and stained for the cleaved fragment of PARP-1 (89 kD). ECs pretreated with rhNAMPT and challenged with TNF-α showed reduced fluorescent signal (Alexa Fluor 488 [AF488]; Thermo Fisher Scientific) compared with ECs without rhNAMPT pretreatment. (*B*) ECs were pretreated with rhNAMPT (100 ng/ml; 24 h), followed by exposure to TNF-α (100 ng/ml; 24 h). Cells were assayed by the TUNEL method and showed a reduction in the fluorescein signal in the samples pretreated with rhNAMPT followed by TNF challenge. There were no differences between control and rhNAMPT-treated ECs. *$P < 0.05$. Error bars indicate SEM of three independent experiments.

microvascular EC infected with the H1N1 influenza strain (31).

We also observed that inhibition of NMN synthesis failed to alter EC caspase-3 activation, whereas *NAMPT* silencing in TNF-α–challenged EC increased caspase-3 activation and PARP-1 cleavage compared with controls. FK866 is widely used as an antitumor agent (5) because NAD^+ is a cofactor/substrate in PARP1-dependent DNA repair, and NAD^+ requirements are increased in cancer cells repairing unstable genomic DNA. The multipotent mouse fibroblast cell line C3H10T1/2 and the omnipotent preosteoblast cell line MC3T3-E1 express higher concentrations of iNAMPT during osteogenic differentiation (47), and reduced NAMPT expression in C3H10T1/2 cells enhances adipogenesis and reduces osteogenesis (48). In cultured EC progenitor cells, exogenous NAMPT significantly increased the translocation of NF-κB in nuclei, with subsequent impairment of migration and adhesion capacity (49).

Our ELISA-derived data validated that eNAMPT secretion into EC cultured medium is significantly reduced in NAMPT-silenced EC and increased in NAMPT-overexpressing EC. We have shown previously that the eNAMPT receptor is TLR4, with robust downstream NF-κB signaling (30) resulting in altered expression of multiple targets involved in inflammation, oxidative stress, and regulation of cell death and survival pathways (50). Secreted eNAMPT therefore

may exert an overall protective survival effect in cells exposed to proapoptotic stimuli such as serum withdrawal, cytokines (TNF-α), LPS, hypoxia, or excessive mechanical stress (25). Reductions in secreted concentrations of eNAMPT (after *NAMPT* silencing) interfere with such autocrine or paracrine effects, decreasing cellular resistance to subsequent cellular stress. The capacity of conditioned medium from control EC (but not from NAMPT-silenced EC), supplemented by silenced EC, to restore the cellular defenses against cytokine challenge supports this underlying hypothesis. Although it is possible that reduced NAMPT expression (due to silencing) indirectly reduces expression and/or secretion of cytokines that impact cell survival as a result of decreased NMN synthesis, our studies employing an eNAMPT neutralizing antibody to reduce TLR4 ligation verified that secreted eNAMPT is responsible for reduced EC apoptotic responses to TNF-α.

In summary, we have identified a distinct and novel role for eNAMPT in regulating EC survival and apoptosis, with iNAMPT-mediated NMN synthesis being uninvolved in regulation of TNF-α–induced EC apoptosis. Secretion of eNAMPT and subsequent ligation of the NAMPT receptor TLR4 provides a novel mechanism for NAMPT in activating prosurvival mechanisms in endothelium as a stress response mechanism during inflammatory lung injury. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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