HHS Public Access

Author manuscript

Hypertension. Author manuscript; available in PMC 2021 July 01.

Published in final edited form as:

Hypertension. 2020 July ; 76(1): 267–276. doi:10.1161/HYPERTENSIONAHA.120.14686.

Mitochondrial Fission Mediates Endothelial Inflammation

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Conflict of Interest
None.

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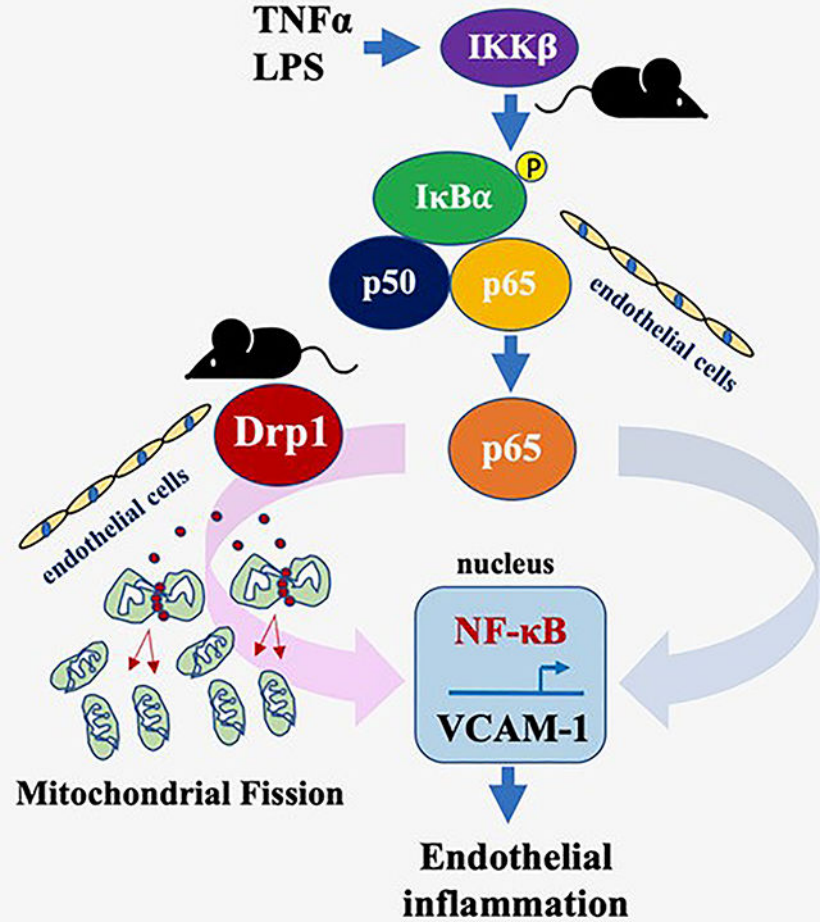
Abstract

Endothelial inflammation and mitochondrial dysfunction have been implicated in cardiovascular diseases, yet a unifying mechanism tying them together remains limited. Mitochondrial dysfunction is frequently associated with mitochondrial fission/fragmentation mediated by the GTPase dynamin-related protein 1 (Drp1). Nuclear factor (NF)-κB, a master regulator of inflammation, is implicated in endothelial dysfunction and resultant complications. Here, we explore a causal relationship between mitochondrial fission and NF-κB activation in endothelial inflammatory responses. In cultured endothelial cells, tumor necrosis factor-α or lipopolysaccharide induces mitochondrial fragmentation. Inhibition of Drp1 activity or expression suppresses mitochondrial fission, NF-κB activation, vascular cell adhesion molecule-1 induction, and leukocyte adhesion induced by these pro-inflammatory factors. Moreover, attenuations of inflammatory leukocyte adhesion were observed in Drp1 hetero-deficient mice as well as endothelial Drp1 silenced mice. Intriguingly, inhibition of the canonical NF-κB signaling suppresses endothelial mitochondrial fission. Mechanistically, NF-κB p65/RelA appears to mediate inflammatory mitochondrial fission in endothelial cells. In addition, the classical anti-inflammatory drug, salicylate, appears to maintain mitochondrial fission/fusion balance against tumor necrosis factor-α via inhibition of NF-κB. In conclusion, our results suggest a previously unknown mechanism whereby the canonical NF-κB cascade and a mitochondrial fission pathway interdependently regulate endothelial inflammation.

summary

We found that genetic reduction of Drp1 or pharmacological inhibition attenuated endothelial mitochondrial fission and inflammation in vitro as well as in mouse models. Inhibition of NF-kB also reduced endothelial mitochondrial fission in vitro and in mice with salicylate. Further investigation is needed to evaluate the translational potential of intervening in this pathway as a means to prevent inflammatory cardiovascular diseases.

Graphical Abstract



Keywords

Cell Signaling; Endothelium; Inflammation; Mitochondria; Nuclear Factor-κB; Basic Science Research

Introduction

Mitochondria are subcellular organelles that play a critical role in cellular metabolism in mammalian physiology. As part of their dynamic nature, mitochondria undergo continuous fission and fusion mainly mediated by dynamin-related protein 1 (Drp1) and mitofusins (Mfn1/2), respectively, in order to maintain mitochondrial quality and cellular homeostasis [1]. Disruptions in the fission/fusion balance (primarily a shift toward fission) perturb cellular physiology and have been implicated in a variety of diseases including those seen in the cardiovascular system [2]. Accordingly, various stressors such as high glucose, hypoxia and oxidative stress have been shown to induce Drp1-dependent mitochondrial fission in endothelial cells and mediate endothelial pathologies including impairment of endothelium-dependent relaxation, reduction in micro-vessels and defects in wound healing and

angiogenesis [3–5]. However, the mechanistic contribution of Drp1 as well as mitochondrial fission in endothelial inflammation has not been fully explored.

Endothelial dysfunction is an early independent risk factor for cardiovascular diseases such as hypertension and coronary artery disease. Endothelial inflammation and oxidative stress are the major characteristics associated with endothelial dysfunction [6]. While the evolutionarily conserved nuclear factor-κB (NF-κB) pathway is indispensable to maintaining cellular homeostasis in response to transient stress [7], chronic NF-κB activation appears to mediate vascular inflammation and exaggerate endothelial dysfunction [8]. Canonical signaling leading to NF-κB-dependent gene induction involves an upstream kinase, IκB kinase beta (IKKβ), and its substrate, IκBα. Phosphorylation and subsequent degradation of IκBα releases NF-κB to translocate to the nucleus where it induces genes involved in inflammation, immune responses, and tissue remodeling [7]. Of importance to NF-κB-dependent cell adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) appears critical in vascular inflammation [9].

Among several cytokines implicated in endothelial inflammation, tumor necrosis factor-α (TNF-α) appears to be critical to induce endothelial dysfunction and expression of cell adhesion molecules and chemokines via NF-κB activation, thus contributing to leukocyte recruitment [10]. While activation of the toll like receptors (TLRs) involved in the innate immune system are the first line of host defense mechanism in mammals, chronic activation of the endothelial TLRs, which are activated by microbial endotoxins including lipopolysaccharide (LPS) as well as damage-associated molecular pattern molecules, has also been implicated in NF-κB-driven vascular inflammation [11].

Given the association between vascular inflammation and perturbations in mitochondrial homeostasis in human and animal models of cardiovascular diseases [12, 13], we aimed to study the potential mechanisms that tie the mitochondrial fission signaling to endothelial inflammation such as those induced by TNF-α and LPS.

Methods

This article adheres to the American Heart Association Journals implementation of the Transparency and Openness Promotion Guidelines. The data that support the findings of this study are available from the corresponding author on reasonable request. Detailed methods and materials are available in the online-only Data Supplement and Table S1.

Results

TNF-α induces mitochondrial fission in endothelial cells via Drp1

In primary rat aortic endothelial cells (ECs), we observed a time-dependent (1 hour~6 hours) increase in mitochondrial fragmentation in response to TNF-α with maximum responses occurring at 3 hours (Figure 1A). Drp1 is a critical positive regulator of mitochondrial fission in mammalian cells [14]. Genetic inhibition of Drp1 using adenoviruses encoding the GTPase deficient dominant-negative $Drp1^{K38A}$ mutant [15] (Figure 1B) or Drp1 small interfering RNA (siRNA), siDrp1, (Figure 1C) suppressed TNF-α-induced mitochondrial

fission in cultured ECs. Pharmacologic inhibition of mitochondrial fission using mdivi1 [16] also suppressed TNF-α-induced mitochondrial fragmentation (Figure 1D). Drp1 Ser616 phosphorylation is known to enhance Drp1 activity and mitochondrial fission. In contrast, Drp1 Ser637 phosphorylation is known to be inhibitory [17]. A prior study reported that TNF-α increased both total and Ser616 phosphorylated Drp1 in H9C2 cells [18]. TNF-α also down-regulated Mfn2 in 3T3 cells [19]. In cultured ECs, TNF-α transiently down-regulated expression of IκBα. However, TNF-α did not alter Drp1 expression, Drp1 phosphorylation or Mfn2 expression in ECs (Figures S1), suggesting that the mechanism of Drp1 activation and fission induced by TNF-α may be cell type specific.

Drp1 and its mitochondrial receptor Mff mediate inflammatory NF-κB activation and VCAM-1 induction in endothelial cells

NF-κB is a master regulator of inflammation [7]. ECs transduced with $Drp1^{K38A}$ showed significant inhibition in TNF-α-induced NF-κB-driven promoter activity (Figure 2A). Accordingly, we aimed to test the potential contribution of mitochondrial fission in the inflammatory VCAM-1 induction. TNF-α induced VCAM-1 induction in ECs. This VCAM-1 induction was attenuated by overexpression of $Drp1^{K38A}$ (Figure 2B) or silencing of Drp1 (Figure 2C). In contrast, silencing of Drp1 was unable to block TNF-α-induced p65 nuclear translocation in ECs (Figure 2D).

Drp1 mediates mitochondrial fission via its mitochondrial outer membrane receptors including mitochondria fission factor (Mff) [14]. Over-expression of dominant negative Mff serine 155/172 alanine substitution mutant ($Mff^{S155/172A}$) [20] or silencing Mff expression suppressed TNF-α-induced mitochondrial fragmentation (Figures S2A and S2B). Dominant negative Mff attenuated TNF-α-induced NF-κB promoter activation (Figures S2C) and VCAM-1 induction (Figures S2D) in ECs. Silencing of Mff also attenuated TNF-α-induced VCAM-1 induction (Figures S2E).

TLR activation by LPS induces endothelial VCAM-1 via NF-κB activation [21]. Accordingly, we have further tested if Drp1 and Mff are required for LPS responses in cultured ECs. Transduction of $Drp1^{K38A}$ or $Mff^{S155/172A}$ attenuated LPS-induced mitochondrial fragmentation and VCAM-1 induction in ECs (Figures S3).

Drp1 inhibition attenuates monocyte adhesion and mitochondrial ROS production induced by TNF-α in endothelial cells

Endothelial NF-κB activation and VCAM-1 expression are causative factors in leukocyte adhesion and vascular inflammation [21, 22]. In response to TNF-α, cultured ECs showed an increase in monocyte adhesion. ECs transduced with $Drp1^{K38A}$ (Figure 3A) or $Mff^{S155/172A}$ (Figure 3B) were protected from TNF-α-induced monocyte adhesion. mdivi1 also attenuated TNF-α-induced monocyte adhesion in ECs (Figure 3C).

Since mitochondrial ROS production has been shown to enhance endothelial leukocyte adhesion [23] and ischemia/reperfusion-induced mitochondrial fission in human umbilical vein ECs [24], we have further studied the relationship between mitochondrial fission and oxidative stress. Genetic inhibition or silencing of Drp1 blocked TNF-α-induced mitochondrial oxidative stress as measured with a mitochondrial protein oxidization indicator, MitoTimer

[25] (Figures S4A and S4B). However, scavenging of mitochondrial superoxide generation by superoxide dismutase 2 mimic mitoTempo did not alter TNF-α-induced VCAM-1 expression in ECs (Figure S4C), although it reduced mitochondrial oxidative stress (Figure S4D) and monocyte adhesion (Figure S4E). Taking these data together, the mitochondrial fission pathway via Drp1 appears to be critical for monocyte adhesion to ECs, which likely involves NF-κB-dependent VCAM-1 induction as well as mitochondrial oxidative stress.

Systemic as well as endothelial Drp1 silencing prevents leukocyte adhesion in mice

To determine in vivo significance of mitochondrial fission in regulation of vascular inflammation, heterozygous Drp1 mice (Drp1+/−) or control Drp1+/+ mice were infused with TNF-α. Subsequently, intravital microscopy was utilized to measure leukocyte adhesion in mouse mesenteric postcapillary venules. Compared with the control Drp1+/+ mice, TNF-α-induced leukocyte adhesion in mouse mesenteric postcapillary venules was attenuated in Drp1+/− mice (Figure 4A). Moreover, inducible silencing of endothelial Drp1 in the $Drp1^{flox/flox}$ $VeCad^{TRE}$ Cre mice attenuated TNF-α-induced leukocyte adhesion in mesenteric micro-circulation (Figure 4B and 4C). mdivi1 pretreatment also suppressed TNF-α-induced increases in leukocyte adhesion in C57BL/6 mice (Figure 4D). Taking these data together, Drp1 activation and subsequent mitochondrial fission appear to be prerequisites for NF-κB activation and subsequent endothelial inflammatory responses in vitro and in vivo.

Drp1 activity is necessary for proinflammatory proteome induction in endothelial cells

To further explore contribution of this pathway in overall pro-inflammatory re-programming in ECs, we took advantage of shotgun proteomics combined with gene ontology analysis. Rat aortic ECs transduced with $Drp1^{K38A}$ or control GFP adenovirus were stimulated with TNF-α and cell lysates were analyzed with a mass spectroscopy (Figure S5A and Table S2). In ECs expressing control GFP, TNF-α significantly up-regulated specific groups of functional proteins including those involved with aging, wound healing, and apoptotic mitochondria. Enrichment of mitochondrial proteins was also observed. Kyoto Encyclopedia of Genes and Genomes/KEGG pathway analysis further suggest a premature aging condition with proteotoxicity induced by TNF-α in ECs. Significant interaction is predicted among the enriched proteins in mitochondria and those implicated in aging and age associated diseases (Figure S5B). In sharp contrast, these specific alterations by TNF-α were not observed in ECs transduced with $Drp1^{K38A}$, whereas proteins involved in collagen fibril organization and poly(A) RNA binding were reduced. These data suggest that the endothelial pro-inflammatory responses including those associated with inflamm-aging [26] and metabolic reprogramming are dictated by the status of mitochondrial fission and Drp1 activity.

Inter-dependent relationship exists between NF-κB activation and mitochondrial fission in endothelial cells

Our findings suggest a potential cross-communication between the canonical NF-κB cascade and mitochondrial fission machinery involving Drp1. Therefore, we further explored the regulatory role of the NF-κB cascade for endothelial mitochondrial fragmentation. We found that rat aortic ECs pretreated with Bay 11–7085, the inhibitor of IκBα phosphorylation and degradation, showed a decrease in TNF-α-induced mitochondrial fragmentation. Similar inhibitions in IκBα degradation and mitochondrial fragmentation

were noted in ECs pre-treated with the IKKβ inhibitor TPCA-1 (Figure 5A, S6A and S6B). Adenoviruses encoding IκBα serine 32 and 36 alanine ($IκBα^{S32/36A}$) [27] and IKKβ serine 177 and 181 alanine ($IKKβ^{S177/181A}$) [28] dominant-negative substitution mutants were used to verify the contribution of IκBα and IKKβ in mitochondrial fission regulation. $IκBα^{S32/36A}$ and $IKKβ^{S177/181A}$ transduction in rat aortic ECs attenuated TNF-α-induced mitochondrial fragmentation (Figure 5B). In addition, transduction of the Rel homology domain of p65 [29], a dominant negative inhibitor of NF-κB attenuated mitochondrial fragmentation induced by TNF-α in ECs (Figure S6C). These data suggest that the canonical NF-κB cascade is required for TNF-α-induced mitochondrial fission. It has been reported that the components of NF-κB are present at mitochondria [30, 31]. Subcellular fractionation of rat aortic ECs demonstrated expression of IκBα and p65 in the mitochondria fraction. TNF-α induced mitochondrial IκBα degradation that was attenuated with TPCA-1 (Figure S6D).

Salicylate prevents endothelial mitochondrial fission *in vitro* and *in vivo*

To explore translational relevance of the NF-κB inhibition in endothelial mitochondrial fission and subsequent inflammatory responses, we examined the effect of a non-steroidal anti-inflammatory drug, sodium salicylate, which inhibits IKKβ [32]. Sodium salicylate attenuated TNF-α-induced mitochondrial fragmentation, VCAM-1 induction and IκBα degradation in rat aortic ECs (Figure 6A – 6C). Sodium salicylate further prevented increases in leukocyte adhesion induced by TNF-α in mouse mesenteric micro-circulation (Figure 6D). Sodium salicylate or mdivi1 treatment also prevented reduction in mitochondrial aspect ratio in the TNF-α-treated mouse aorta (Figure 6E). Thus, these data uncover a new pharmaco-mechanism of a classical anti-inflammatory reagent, salicylate, to maintain mitochondrial fission fusion balance under an inflammatory stress.

Discussion

Diseases associated with chronic vascular inflammation are typically marked by mitochondrial dysfunction and persistent NF-κB activation [33, 34]. Mitochondrial regulation is critical for innate immune responses which involve mitochondrial antiviral signaling protein/MAVS to induce NF-κB activation [35]. However, information regarding the potential cross-communication between the NF-κB cascade and mitochondrial morphological adaptation in inflammatory conditions has been not fully explored in the vascular system [36]. Here we reconcile these two seemingly independent pathways by showing enhanced mitochondrial fission is not only a required element for the vascular inflammation (NF-κB promoter activation, VCAM-1 induction and leukocyte adhesion) but is also mediated by the the NF-κB component p65 (Figure 6F). However, several critical questions still remain unanswered in the potential cascade.

How does p65 feed into Drp1 and promote mitochondrial fission? In addition to Drp1 Ser616 phosphorylation or Ser637 de-phosphorylation, several post translational modifications such as sumoylation and nitrosylation are known to be involved in Drp1 activation [17]. It is therefore likely that there is a signal cross-talk between the mitochondrial p65 and Drp1, which leads to the post translational modification(s) of Drp1 in mediating

mitochondrial fission. In parallel to our findings, receptor-interacting serine/threonine-protein kinase 3/RIPK3 mediates Drp1-dependent fission downstream of the TNF-α receptor [37]. In addition, NF-κB-inducing kinase activates Drp1 and induces mitochondrial fission in the absence of IKKs in mouse embryonic fibroblasts [38]. In contrast, opposing regulatory relationships between NF-κB activity and mitochondrial fission were reported in cardiomyocytes [39]. It is likely that the signaling mechanism to determine a regulatory relationship between Drp1 and NF-κB activation is dependent on the given cell type. Alternatively, it is possible that TNF-α or LPS alters expression of other fission/fusion machineries directly, or indirectly via other mitochondrial proteins in ECs. For examples, IKKα, a member of noncanonical NF-κB pathway, regulates mitochondrial fission via induction of optic atrophy 1 protein in fibroblasts [40]. IκBα has been shown to regulate voltage-dependent anion channel 1 (VDAC1) at the outer mitochondrial membrane independently of NF-κB retention [31]. Mitochondrial translocation of p65 via mortalin negatively regulates mitochondrial gene transcription and function [41], whereas positive regulation of mitochondrial respiration via p65 has also been reported [42]. Further investigation is clearly needed to test some of these possibilities regulating Drp1 activation and mitochondrial fission in endothelial cells such as with assessments on protein-protein interactions and post translational modifications.

How do Drp1 (and/or mitochondrial fission) and p65 intersect and coordinate NF-κB activation in ECs? As p65 still translocates to the nucleus with Drp1 inhibition, it is likely that Drp1 may positively regulate NF-κB activation such as via an enhancer system [43] independent of p65 as illustrated in Fig 6F. Increase in mitochondrial energy production in response to mitochondrial fission may also fulfill the need for enhanced transcription independently of p65. Drp1 is abundant in cytosol and also functions independently from mitochondria [14]. However, our confirmation that inhibition of a mitochondrial Drp1 receptor Mff attenuates NF-κB activation and VCAM-1 induction support the role of mitochondrial Drp1 and fission in regulation of inflammation. Thus, our data extend a new mitochondrial branch of the NF-κB regulation via Drp1/Mff-dependent mitochondrial fission which dictates the canonical NF-κB signaling to transmit vascular inflammation. Our concept of the vital role of Drp1 in promotion of stress-induced inflammatory responses is supported by the literature demonstrating the requirement of Drp1 in T cell proliferation and immune responses [44, 45].

Is mitochondrial fission necessarily detrimental to EC according to our data? In general, mitochondrial fission fusion machineries are both important to maintain mitochondrial health and quality control [1]. In ECs, Drp1 appears indispensable for angiogenesis [5]. NF-κB mediates critical defense mechanisms in the immune systems including those interacting with endothelium [7]. Thus, mitochondrial fission is not necessarily detrimental to EC but may provide us with an alternative way to potentially control undesired inflammatory response in certain chronic conditions. It is well accepted that mitochondria orchestrate cell reprogramming from a metabolic perspective. Recent studies demonstrated that Drp1-dependent mitochondrial fission may be critical in such cell reprograming [46]. Available literature [47] as well as our proteomic data indicate that the metabolic re-programming of endothelial cells is also vital to the development of vascular pathology. In addition, our data suggest that activation of Drp1 and/or mitochondrial fission contributes to mitochondrial

superoxide production, which is most likely due to enhanced mitochondrial oxygen consumption and ATP production. The enhanced mitochondrial superoxide production via Drp-1 activation has also been reported in other cell systems [48]. However, these data cannot exclude the possibility that Drp1 and mitochondrial fission also affect oxidative stress in mitochondria via alteration on mitochondrial antioxidant defense mechanisms such as Sirt3 expression/activity [49], glutathione levels [50] as well as changes in mitochondrial NAD+ levels [51].

What is the clinical significance of the salicylate data? Salicylate is one of the most frequently utilized anti-inflammatory reagents known to suppress inflammatory responses such as those with NF-κB activation [32]. Salicylate is also known to inhibit mitochondrial ATP production and activate AMP-activated protein kinase/AMPK [52]. Our findings indicate that salicylate may be effective to maintain mitochondrial fission fusion balance under inflammatory condition. Thus, salicylate may also be beneficial in other pathological conditions, where disruptions of the fission fusion balance have been observed.

Perspectives

The present study has demonstrated a previously unknown mechanism whereby the NF-κB cascade and a mitochondrial fission pathway operated by Drp1 interdependently regulate endothelial inflammation. Therefore, endothelial Drp1 appears to be a unique therapeutic target against inflammatory cardiovascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by National Institute of Health grants, HL128324 (S.E. and V.R.), HL133248 (S.E.), DK111042 (R.S. and S.E.), NS109382 (S.E.), GM123266 (H.S.), F31HL127971 (S.J.F.), F31HL146081 (M.J.B.) and F30HL146006 (H.A.C.), American Heart Association grants, 16GRNT30130013 (V.R.), 16GRNT30410007 (S.E.), 19PRE34430038 (M.J.B.) and 19PRE34430037 (H.A.C), and Japan Grant-in-Aid for Scientific Research, 18KK0437 (M.M.).

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Novelty and Significance

What is new?

A GTPase, dynamin-related protein-1 (Drp1) and mitochondrial fission are important players in endothelial NF-kB activation and inflammation.

What is relevant?

Inhibiting Drp1 and mitochondrial fission prevented endothelial inflammatory responses in endothelial culture and mouse models which introduces a potential therapeutic target, Drp1 or mitochondrial dynamics in general, as a possible approach.

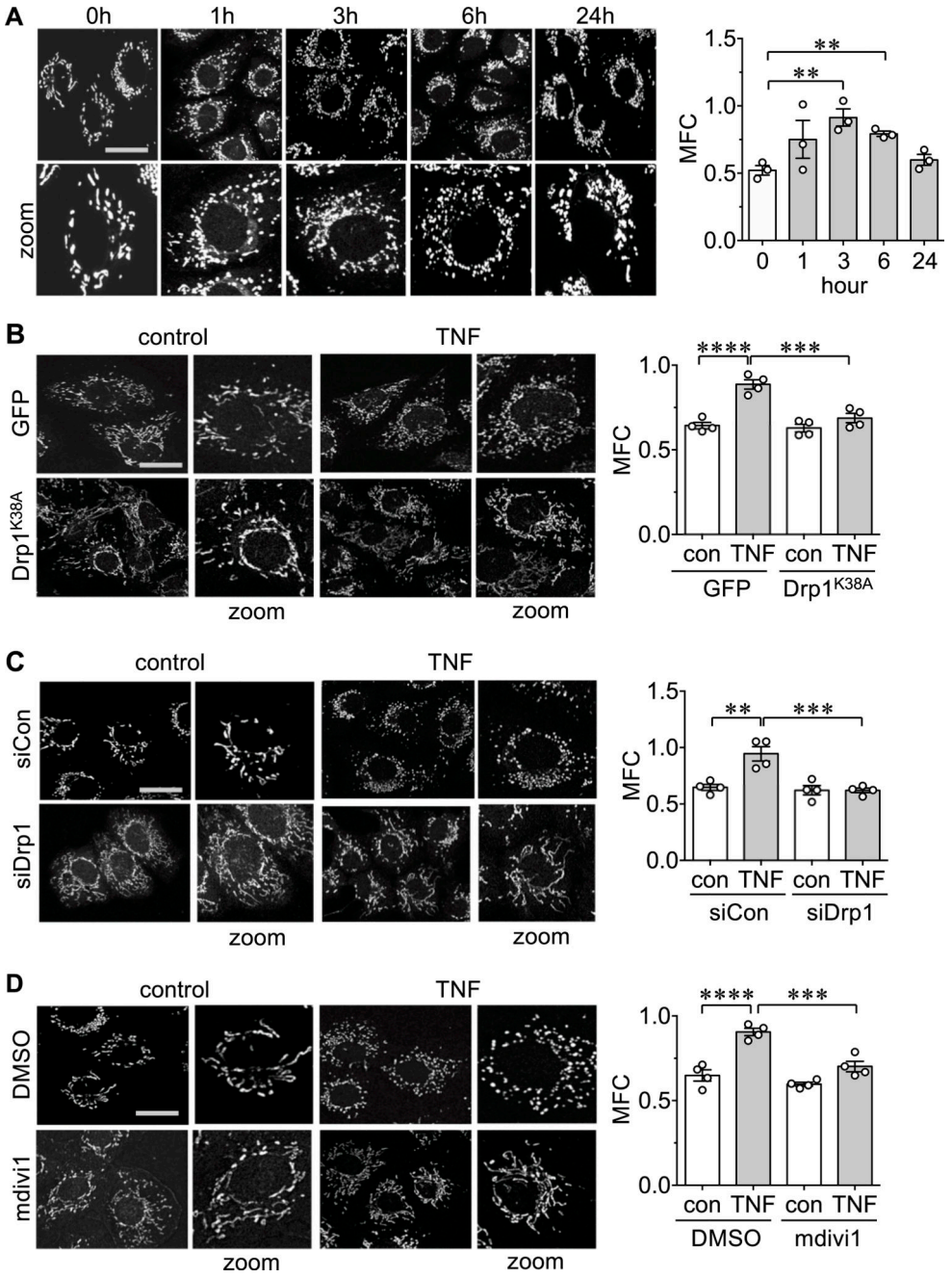

Figure 1. Mitochondrial fission inducer Drp1 mediates TNF-α)-induced mitochondrial fragmentation in endothelial cells.

A. Rat aortic ECs were stimulated with 10 ng/mL rat TNF-α for indicated times and mitochondrial fragmentation count (MFC) was measured. **B**. Rat aortic ECs transduced with adenovirus encoding dominant-negative Drp1^{K38A} mutant or control GFP at 100 multiplicity of infection (moi) for 48 hours were stimulated with 10 ng/mL TNF-α for 3 hours and MFC was measured. **C**. Rat aortic ECs transduced with adenovirus encoding siDrp1 or control non-silencing RNA (siCon) at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α for 3 hours and MFC was measured. **D.** Rat aortic ECs pretreated with 50 μmol/L

mitochondrial division inhibitor-1 (mdivi1) or vehicle (0.1% DMSO) for 1 hour were stimulated with 10 ng/mL TNF-α (TNF) for 3 hours and MFC was measured. con: basal control. The bars show the mean±SEM from 3~4 independent experiments as indicated. Scale bar is 15 μm and 4x zoomed pictures are included. **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (1-way ANOVA).

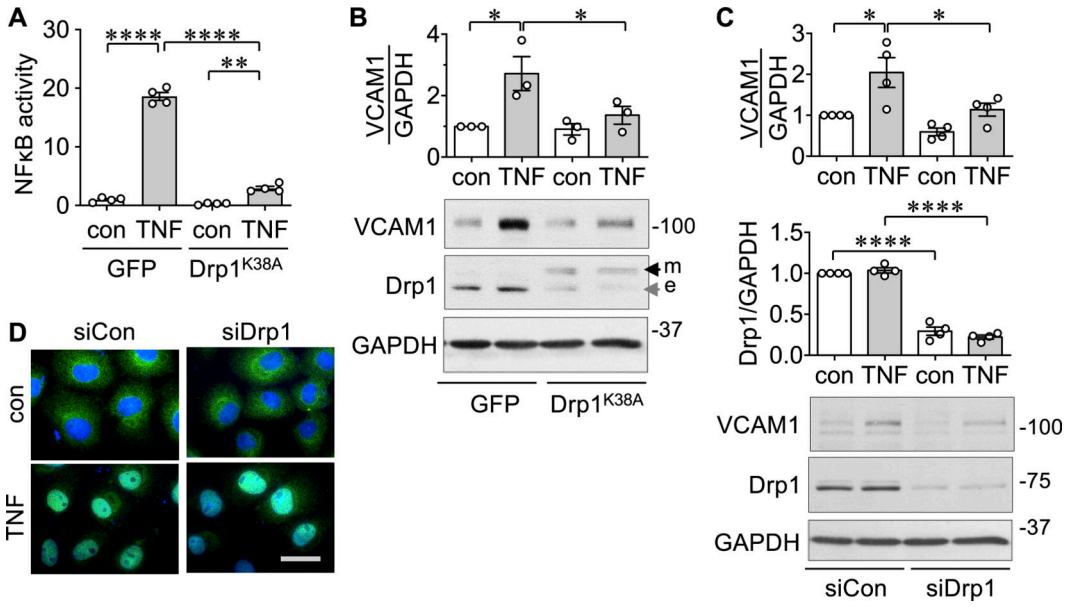

Figure 2. Drp1 mediates TNF-α-induced NF-κB activation and VCAM-1 induction in endothelial cells.

A. Rat aortic ECs transduced with adenovirus encoding dominant-negative Drp1^{K38A} mutant or control GFP at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α for 24 hours and NF-κB luciferase activity (fold) was measured. **B**. Rat aortic ECs transduced with adenovirus encoding dominant-negative Drp1^{K38A} mutant or control GFP at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α (TNF) for 6 hours and VCAM-1 expression was measured. con: basal control. e: endogenous Drp1, m: mutant Drp1. For quantification, densitometry analysis was performed and normalized with corresponding GAPDH density. **C**. Rat aortic ECs transduced with adenovirus encoding siDrp1 or control non-silencing RNA (siCon) at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α for 6 hours and VCAM-1 expression was measured. The bars show the mean±SEM from 3~4 independent experiments as indicated. *p<0.05, ****p<0.0001 (1-way ANOVA). **D.** Rat aortic ECs transduced with adenovirus encoding siDrp1 or control non-silencing RNA (siCon) at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α for 20 min and p65 nuclear translocation was measured.

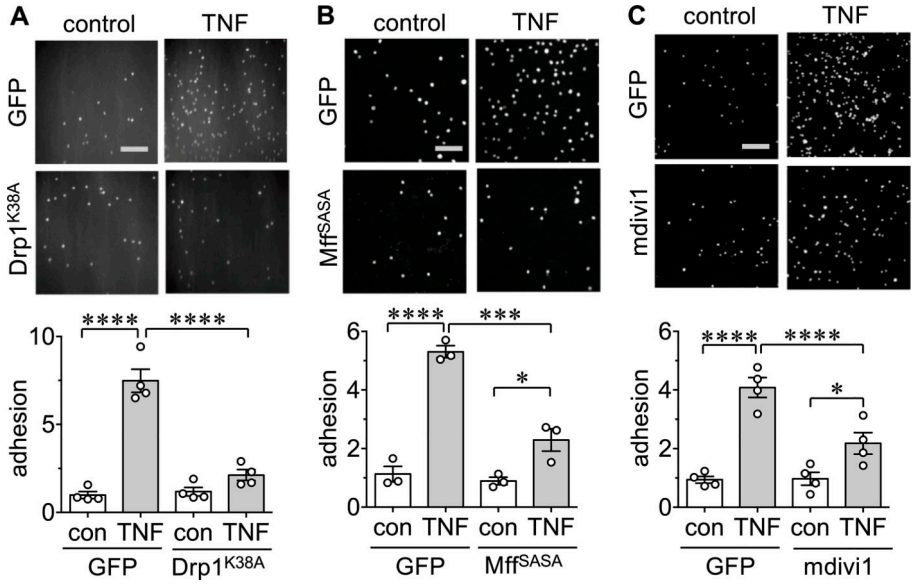

Figure 3. Drp1 mediates leucocyte endothelial adhesion *in vitro*.

A. aortic ECs transduced with adenovirus encoding dominant-negative Drp1^{K38A} mutant or control GFP at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α (TNF) for 6 hours and THP-1 monocyte adhesion (fold) was measured. con: basal control. **B**. Rat aortic ECs transduced with adenovirus encoding dominant-negative Mff$^{S155/172A}$ mutant or control GFP at 100 moi for 48 h were stimulated with 10 ng/mL TNF-α for 6 hours and THP-1 monocyte adhesion (fold) was measured. **C**. Rat aortic ECs pretreated with 50 μmol/L mdivi or vehicle (0.1% DMSO) for 1 hour were stimulated with 10 ng/mL TNF-α (TNF) for 6 hours and THP-1 monocyte adhesion (fold) was measured. Scale bar is 100 μm. The bars show the mean±SEM from 3~4 independent experiments as indicated. *p<0.05, ***p<0.005, ****p<0.001 (1-way ANOVA).

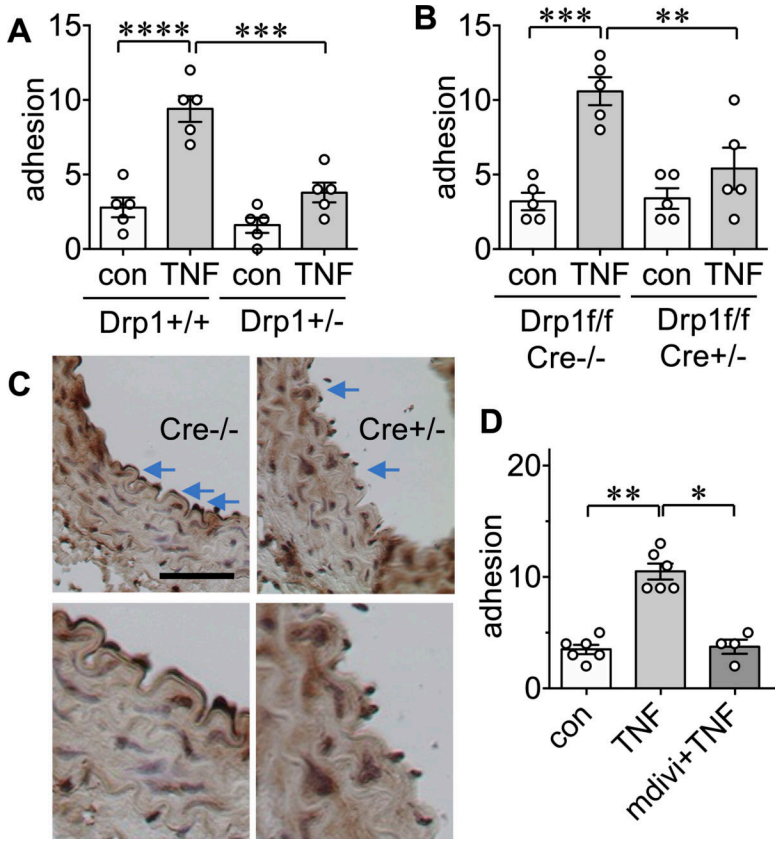

Figure 4. Drp1 mediates leucocyte endothelial adhesion *in vivo*.

A. Leukocyte adhesion was evaluated by intravital microscopy of mesenteric micro-circulation in 8~10 week old male Drp1+/− and control Drp1+/+ mice treated with 20 ng/g TNF-α or saline control for 6 hours. **B**. Leukocyte adhesion was evaluated by intravital microscopy of mesenteric micro-circulation in 8~10 week old male $Drp1^{flox/flox}$ $VeCad^{TRE}$ Cre+/− (Drp1f/f Cre+/−) and control $Drp1^{flox/flox}$ $VeCad^{TRE}$ Cre−/− (Drp1f/f Cre−/−) mice treated with 20 ng/g TNF-α or saline control for 6 hours. **C**. Immunohistochemical confirmation of endothelial Drp1 silencing in mouse aorta. Scale bar is 50 μm. 4x zoomed pictures are included. **D.** Leukocyte adhesion (fold) was evaluated by intravital microscopy of mesenteric micro-circulation in 8~10 week old male C57BL/6 mice treated with 20 ng/g mouse TNF-α (TNF) or saline control (con) for 6 hours with or without pretreatment of 25 μg/g mdivi1 for 18 hours. The bars show the mean±SEM from 5~6 independent experiments as indicated. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 (1-way ANOVA).

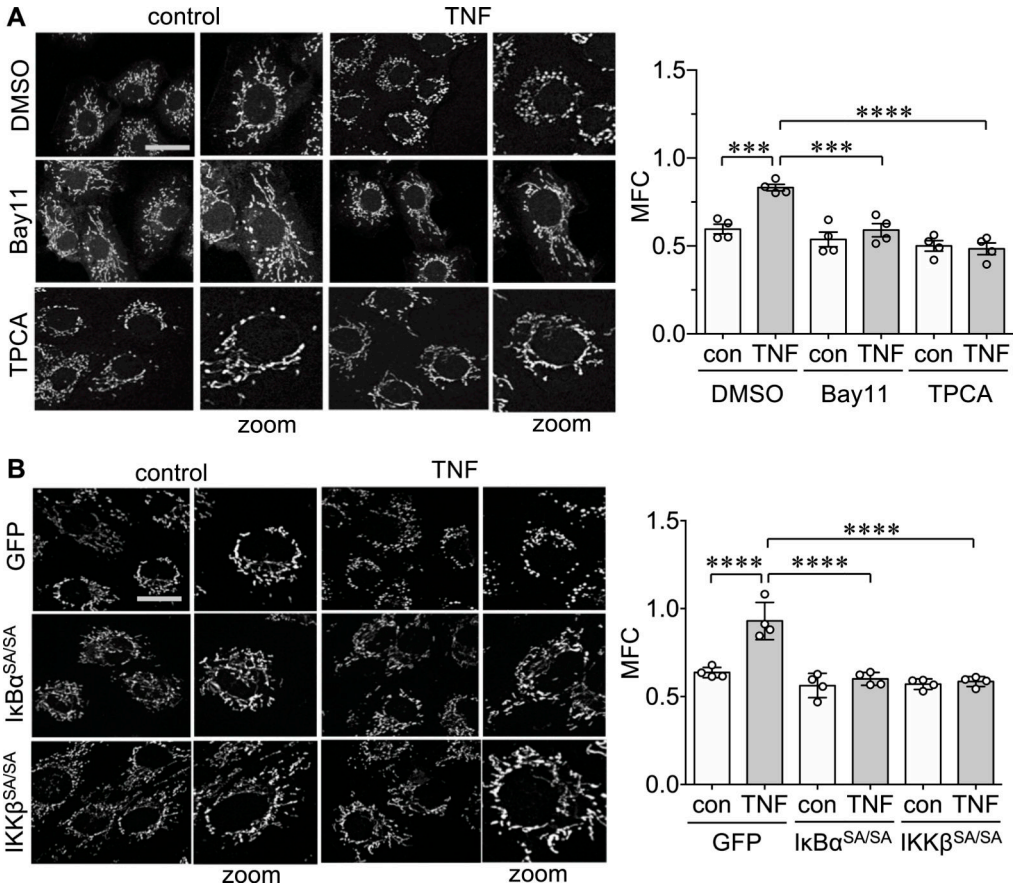

Figure 5. NF-κB activation mediates mitochondrial fission in response to TNF-α in endothelial cells.

A. Rat aortic ECs pretreated with NF-κB inhibitors (5 μmol/L TPCA-1 or 10 μmol/L Bay11–7085,) for 30 min were stimulated with 10 ng/mL TNF-α for 3 hours and MFC was measured. Scale bar is 15 μm and 4x zoomed pictures are included. **B.** Rat aortic ECs transduced with adenovirus encoding IκBα$^{S32/36A}$ (IκBα$^{SA/SA}$), IKKβ$^{S177/181A}$ (IKKβ$^{SA/SA}$), or control GFP at 100 moi for 48 hours were stimulated with 10 ng/mL TNF-α for 3 hours and MFC was measured. The bars show the mean±SEM from 4 independent experiments as indicated. ***p<0.005, ****p<0.001 (1-way ANOVA).

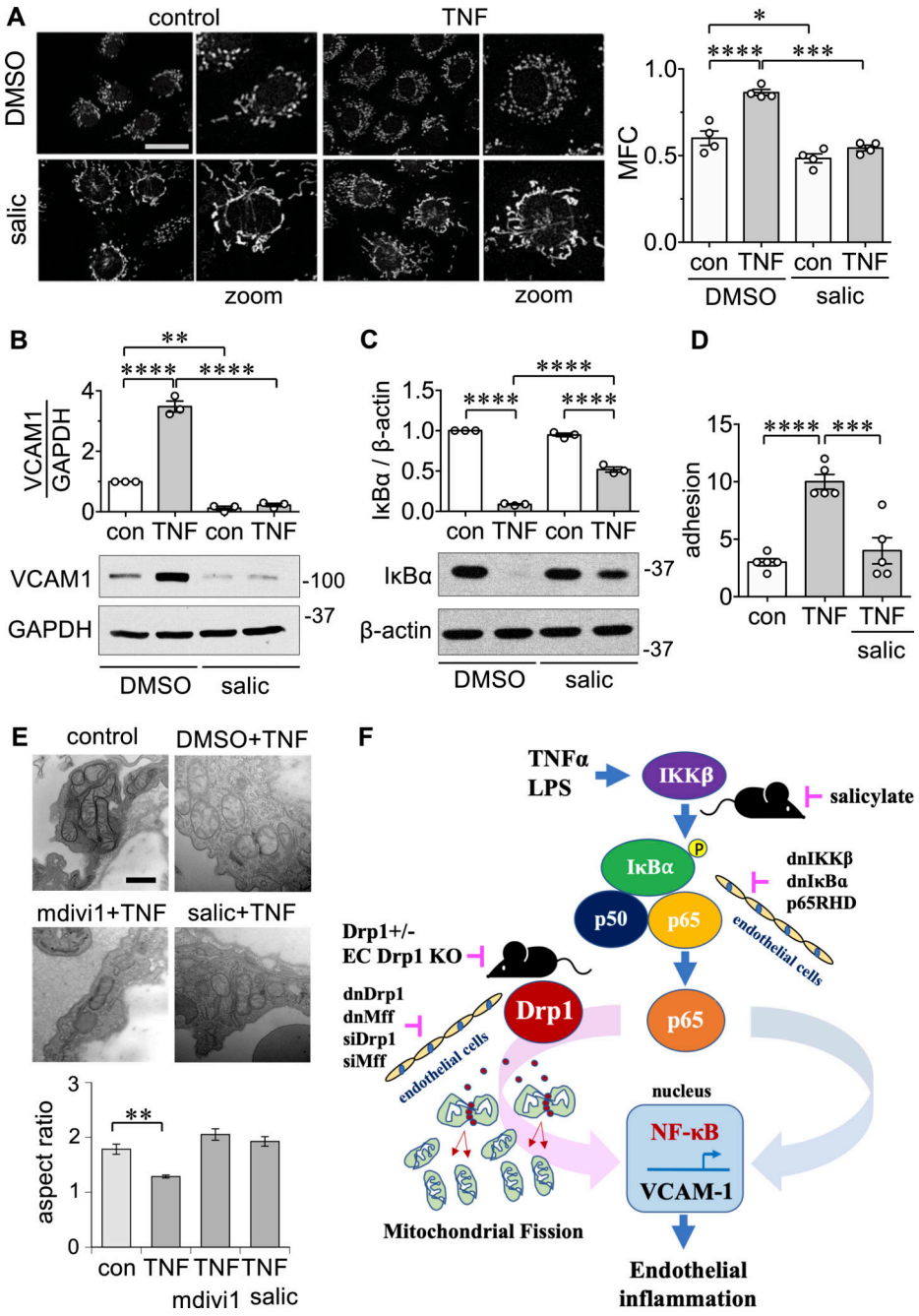

Figure 6. Salicylate targets mitochondrial fission in endothelial cells.

A. Rat aortic ECs pretreated with 50 mM salicylate (salic) or vehicle (0.1% DMSO) for 30 min were stimulated with 10 ng/mL TNF-α (TNF) for 3 hours and MFC was measured. con: basal control. Scale bar is 15 μm and 4x zoomed pictures are included. **B.** Rat aortic ECs pretreated with 50 mM salicylate or vehicle (0.1% DMSO) for 30 min were stimulated with 10 ng/mL TNF-α for 6 hours and VCAM-1 expression was measured. **C.** Rat aortic ECs pretreated with 50 mM salicylate or vehicle (0.1% DMSO) for 30 min were stimulated with 10 ng/mL TNF-α for 20 min and IκBα degradation was measured with immunoblotting. **D.** Leukocyte adhesion was evaluated by intravital microscopy of mesenteric micro-circulation

in mice treated with 20 ng/g TNF-α for 6hours with or without 200 μg/g sodium salicylate pretreatment for 7 hours. **E**. Endothelial mitochondrial aspect ratios were evaluated by electron microscopy in mouse aortas treated with 20 ng/g TNF-α for 6h with or without 200 μg/g sodium salicylate pretreatment for 7 hours or 25 μg/g mdivi1 for 18 hours. Scale bar is 50 nm. The bars show the mean±SEM from 3~5 independent experiments as indicated except E (41–57 mitochondria were analyzed from 15 pictures). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (1-way ANOVA). **F**. Graphical representation of the overall findings. Left side description: In cultured endothelial cells, pharmacological and genetic inhibition of Drp1 activity attenuated TNF-α or LPS-induced NF-κB activation and subsequent inflammatory responses (VCAM-1 expression and THP-1 adhesion) in addition to mitochondrial fission inhibition. Presence of this cascade is also supported in mesenteric microcirculation with Drp1 knockout animals. Right side description: Intriguingly, inhibition of the canonical NF-κB cascade at multiple points (IKKβ, IκBα and p65) also attenuated mitochondrial fission in cultured ECs. Presence of this cascade is also supported in mesenteric microcirculation in mice with treatment of an IKKβ inhibitor, salicylate. Accordingly, our findings demonstrate an interdependent relationship between the canonical NF-κB cascade and the mitochondrial fission mechanism via Drp1 in mediating inflammatory responses in endothelial cells.