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STING-IRF3 Triggers Endothelial Inflammation in Response to Free Fatty Acid-Induced Mitochondrial Damage in Diet-Induced Obesity

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Abstract

Objective—Metabolic stress in obesity induces endothelial inflammation and activation, which initiates adipose tissue inflammation, insulin resistance, and cardiovascular diseases. However, the mechanisms underlying endothelial inflammation induction are not completely understood. Stimulator of interferon genes (STING) is an important molecule in immunity and inflammation. In the present study, we sought to determine the role of STING in palmitic acid (PA)-induced endothelial activation/inflammation.

Approach and Results—In cultured endothelial cells, PA treatment activated STING, as indicated by its perinuclear translocation and binding to interferon regulatory factor 3 (IRF3), leading to IRF3 phosphorylation and nuclear translocation. The activated IRF3 bound to the promoter of intercellular adhesion molecule 1 (ICAM-1) and induced ICAM-1 expression and monocyte–endothelial cell adhesion. When analyzing the upstream signaling, we found that PA activated STING by inducing mitochondrial damage. PA treatment caused mitochondrial damage and leakage of mitochondrial DNA (mtDNA) into the cytosol. Through the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS), the mitochondrial damage and leaked cytosolic mtDNA activated the STING-IRF3 pathway and increased ICAM-1 expression. In mice with diet-induced obesity, the STING-IRF3 pathway was activated in adipose tissue. However, STING deficiency

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(*Sting*$^{gt/gt}$) partially prevented diet-induced adipose tissue inflammation, obesity, insulin resistance, and glucose intolerance.

Conclusions—The mitochondrial damage-cGAS-STING-IRF3 pathway is critically involved in metabolic stress-induced endothelial inflammation. STING may be a potential therapeutic target for preventing cardiovascular diseases and insulin resistance in obese individuals.

Keywords

metabolic syndrome; vascular disease; endothelial inflammation; mitochondrial DNA; STING-IRF3 pathway; ICAM-1 expression

Introduction

Endothelial inflammation is a significant feature of obesity.[1] The proinflammatory state of the endothelial cells not only initiates cardiovascular diseases,[2, 3] but may also contribute to adipose tissue inflammation[1] and insulin resistance.[4, 5] In obesity, metabolic stress[6] from an increase in free fatty acids (FFAs) triggers endothelial inflammation and dysfunction.[7-9] However, the mechanisms underlying FFA-induced endothelial activation and inflammation are not completely understood.

Increasing evidence suggests that the stimulator of interferon genes protein (STING) is a critical signaling molecule in immunity[10, 11] and inflammation.[12, 13] STING can be activated by various stress signals, such as a viral infection,[10, 14] endoplasmic reticulum stress,[15, 16] or DNA leaked into the cytosol from damaged nuclei[17] or mitochondria.[18-24] When activated, STING recruits TANK-binding kinase 1 (TBK1) and the transcription factor interferon regulatory factor 3 (IRF3) and facilitates IRF3 phosphorylation and activation by TBK1.[25, 26] IRF3 then dimerizes and translocates into the nucleus, where it promotes the transcription of inflammatory factors, such as interferons.[27] The STING-IRF3 pathway plays a critical role in the innate immune response by promoting the expression of genes that suppress pathogen replication or facilitate adaptive immunity.[10, 11] Recent studies suggest that STING is also involved in tissue inflammation and destruction.[12, 13]

Given the importance of STING-IRF3 signaling in inflammation, we examined its role in metabolic stress-induced endothelial inflammation. We found that palmitic acid (PA), by inducing mitochondrial damage and release of mitochondrial DNA (mtDNA), activated the STING-IRF3 pathway, which in turn induced ICAM-1 expression and endothelial inflammation. In addition, STING was activated in adipose tissue from mice with diet-induced obesity and was involved in diet-induced adipose tissue inflammation and insulin resistance.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Palmitic Acid Activated the STING-IRF3 Pathway and Induced Endothelial Activation and Inflammation

To determine the role of STING-IRF3 signaling in metabolic stress-induced endothelial inflammation, we first examined whether the STING-IRF3 pathway could be activated by PA, the main type of FFA involved in obesity.[28] Human aortic endothelial cells (HAECS) were treated with PA at concentrations ranging from 0.1 to 0.4 mM (0.3 mM corresponds to the FFA levels found in obese and insulin-resistant individuals).[28] We found that PA had a minimal effect on the level of STING protein (Figure 1A). However, PA induced STING perinuclear translocation (Figure 1B), promoted STING binding to its target IRF3 (Figure 1C), and stimulated IRF3 phosphorylation (Figure 1A) and nuclear translocation (Figure 1D), indicating activation of the STING-IRF3 pathway. In addition, PA induced the expression of the inflammatory proteins VCAM-1, MCP1, IFN- γ, and IL-1 (Figure 1E), as well as the expression of ICAM-1, a critical molecule for inflammatory cell attachment to the endothelium, at both the protein (Figure 1F) and mRNA (Figure 1G) levels. Higher dose of PA significantly increased monocyte-endothelial cell adhesion (Figure 1H). Knocking down ICAM-1 (Figure 1I) reversed PA-induced monocyte-endothelial cell adhesion (Figure 1J), indicating that ICAM-1 has an essential role in endothelial inflammation. PA-induced activation of the STING-IRF3 pathway and stimulation of ICAM-1 expression were confirmed in HAECs from different donors (Figure S1). Together, these findings suggest that PA activates the STING-IRF3 pathway during the induction of endothelial inflammation.

STING Was Involved in PA-Induced ICAM-1 Expression and Endothelial Inflammation

We next determined whether the STING-IRF3 pathway played a role in PA-induced endothelial activation and inflammation. We found that knocking down STING with siRNA prevented PA-induced IRF3 phosphorylation (Figure 2A) and nuclear translocation (Figure 2B) and PA-induced increases in ICAM-1 protein (Figure 2C) and mRNA (Figure 2D) levels. Furthermore, the STING siRNA treatment attenuated PA-induced monocyte-endothelial cell adhesion (Figure 2E). These data indicate that STING is involved in PA-induced endothelial inflammation.

IRF3 Directly Bound to the ICAM-1 Promoter and Participated in PA-Induced ICAM-1 Expression and Endothelial Inflammation

We then investigated the role of IRF3 in endothelial inflammation. Silencing IRF3 with siRNA inhibited the PA-induced increases in ICAM-1 protein (Figure 3A) and mRNA (Figure 3B) levels, suggesting that IRF3 is involved in PA-induced ICAM-1 expression. An analysis with TFSEARCH (Searching Transcription Factor Binding Sites, version 1.3) has indicated that the 5′ untranslated region of the ICAM-1 gene contains several putative IRF3 binding sites (Figure 3C). Results from a chromatin immunoprecipitation (Chip) analysis showed that IRF3 bound to the ICAM-1 promoter (Figure 3D); this binding was significantly increased in PA-treated cells (Figure 3D), indicating that this binding may be involved in the PA-induced increase in ICAM-1 expression. Silencing STING prevented the binding between IRF3 and the ICAM-1 promoter, confirming the role of STING in promoting this binding and ICAM1 expression. Finally, IRF3 siRNA inhibited the PA-

induced increase in monocyte-endothelial cell adhesion (Figure 3E). Together, these findings suggest that IRF3 is an essential factor that mediates PA-induced ICAM-1 expression and endothelial inflammation.

Palmitic Acid Caused mtDNA to Leak into the Cytosol and Induced cGAS Activation

After establishing the role of the STING-IRF3 pathway in endothelial inflammation, we asked how PA activated this pathway. Recent studies suggest that STING is critically involved in inflammation in response to signals of cytosolic DNA that is released from viruses or damaged nuclei or mitochondria.[25, 29, 30] Through cyclic GMP-AMP synthase (cGAS), cytosolic DNA can be converted to the second messenger cGAMP, which then binds to and activates adaptor STING.[20, 23, 31]Because PA is a potent trigger for mitochondrial damage,[32, 33] we asked whether PA could activate STING by damaging mitochondria and causing mtDNA to leak into the cytosol.

Using double stranded DNA staining (dsDNA) and mitochondrial staining (MitoTracker), we detected mtDNA (dsDNA that co-localized with MitoTracker), nuclear DNA (dsDNA in the nucleus), and cytosolic DNA (dsDNA that did not co-localize with either mitochondria or the nucleus). Using this approach, we detected small dsDNA particles outside the mitochondria and the nucleus in PA-treated HAECs (Figure 4A), suggesting the presence of cytosolic DNA. However, cytosolic DNA was barely detectable in untreated HAECs. Because the cytosolic DNA could be either mtDNA released from damaged mitochondria or nuclear DNA that leaked from a damaged nucleus, we performed further tests to detect mtDNA in the cytosol fraction. Using PCR analysis with mtDNA sequences as primers, we detected mtDNA in the cytosolic fraction – ie, cytosolic mtDNA (Figure 4B). The amount of cytosolic mtDNA was significantly increased in cells treated with PA (Figure 4B). PA also induced an increase in the expression of cGAS (Figure 4C). Together, these data suggest that PA triggers mitochondrial damage and mtDNA leakage into the cytosol and activates the cytosolic DNA sensor cGAS.

mtDNA and cGAS Were Involved in PA-Induced Activation of the STING-IRF3 Pathway

We further determined the role of mtDNA and cGAS in PA-induced STING-IRF3 activation and endothelial inflammation. We showed that treating HAECs with mtDNA isolated from PA-treated HAECs induced STING perinuclear translocation (Figure 5A) and IRF3 phosphorylation (Figure 5B) and nuclear translocation (Figure 5C). mtDNA also increased ICAM-1 expression (Figure 5B). Inducing mitochondrial damage with carbonyl cyanide m-chlorophenylhydrazone (CCCP) also stimulated STING perinuclear translocation (Figure 5D) and IRF3 nuclear translocation (Figure 5E), indicating that mitochondrial damage directly activates STING-IRF3. Importantly, knocking down cGAS with siRNA significantly inhibited the PA-induced increases in IRF3 phosphorylation and ICAM-1 expression (Figure 5F), indicating that cGAS is involved in PA-induced STING activation. Together, these observations suggest that PA activates the STING-IRF3 pathway by causing mitochondrial damage and the release of mtDNA into the cytosol.

Critical Role of STING in Diet-Induced Adipose Vascular Inflammation and Insulin Resistance

Finally, we examined the role of STING in diet-induced endothelial inflammation in mice. We evaluated the endothelial inflammation and macrophage infiltration in adipose tissue,[1] which is known to trigger insulin resistance in obesity.[1, 3, 34] We used STING-deficient (C57BL/6J-Tmem173gt/J, *Sting*$^{gt/gt}$) mice, in which a point mutation in the *Sting* gene (T596A) causes STING dysfunction and degradation.[35]

Wild-type (WT) mice and *Sting*$^{gt/gt}$ mice were fed a chow diet (CD) or a HFD for 12 weeks. Low levels of STING in the adipose tissue of the *Sting*$^{gt/gt}$ mice were confirmed (Figure 6A). Phosphorylated IRF3 and ICAM-1 protein were significantly increased in the epididymal adipose tissue (Figure 6A) and aortic wall (Figure S2) of the HFD-fed WT mice, compared with those of the CD-fed WT mice. However, the levels of phosphorylated IRF3 and ICAM-1 in adipose tissue were significantly lower in the HFD-fed *Sting*$^{gt/gt}$ mice than in the HFD-fed WT mice (Figure 6A). Consistently, immunostaining showed lower ICAM-1 expression (Figure 6B) and macrophage infiltration (Figure 6C) in the adipose vessels of the HFD-fed *Sting*$^{gt/gt}$ mice, as compared with that of the HFD-fed WT mice. Furthermore, in the adipose tissue of the HFD-fed WT mice, we observed significant collagen deposition (Figure 6D) and crown-like structures (CLSs) (Figure 6E), which are formed by aggregations of macrophages around a single adipocyte and indicative of adipose tissue inflammation.[4] HFD-induced collagen deposition and CLS formation were significantly reduced in the HFD-fed *Sting*$^{gt/gt}$ mice, suggesting a critical role for STING in diet-induced adipose inflammation.

Because adipose tissue inflammation contributes to insulin resistance,[4, 5] we examined whether insulin resistance could be improved in STING-deficient mice. We found that, compared with HFD-fed WT mice, HFD-fed *Sting*$^{gt/gt}$ mice had a lower body weight (Figure 6F), lower plasma FFA levels (Figure 6G), improved insulin sensitivity (Figure 6H), and better glucose tolerance/homeostasis (Figure 6I). Together, these findings support the notion that STING plays a critical role in HFD-induced adipose inflammation and insulin resistance.

Discussion

In this study, we have provided evidence that STING is an important signaling molecule in metabolic stress-induced endothelial inflammation and insulin resistance. We show that PA, by causing mitochondrial damage and mtDNA leakage into the cytosol, activates cGAS, STING, and IRF3, which translocates to the nucleus, binds directly to the ICAM-1 promoter, and mediates PA-induced ICAM-1 expression and endothelial inflammation. In STING-deficient mice, the effects of diet-induced obesity, including endothelial inflammation (in adipose tissue), insulin resistance, and glucose intolerance, were alleviated.

Obesity is associated with a chronic, low-grade proinflammatory state that is characterized by endothelial inflammation[1] and macrophage infiltration.[34] Inflammation plays a critical role in the development of insulin resistance/diabetes[36] and the associated cardiovascular complications.[6] Understanding the pathogenesis and identifying the pathways leading to

endothelial inflammation is important for developing therapeutic strategies to prevent and treat vascular diseases and insulin resistance in obese individuals. Recently, increasing evidence has suggested that STING plays a critical role in inflammatory diseases, including vascular inflammation.[12, 13] Gain-of-function mutations in STING have been identified in patients with an autoinflammatory disease characterized by early-onset systemic inflammation and vasculopathy[13] and in patients with a familial inflammatory syndrome.[37] In the current study, we have shown that STING is an essential molecule for obesity-associated endothelial inflammation and insulin resistance. In our *in vivo* experiments, the HFD challenge significantly increased STING levels in the vascular walls and adipose tissues in WT mice. STING deficiency reduced the HFD-induced ICAM-1 expression in the endothelium and macrophage infiltration in the adipose tissue. Together, our findings indicate that STING plays an important role in vascular inflammation and suggest that this pathway could serve as a potential therapeutic target for preventing the development of vascular diseases and insulin resistance in obese individuals.

We observed that, compared with the WT controls, HFD-fed *Sting*$^{gt/gt}$ mice had a lower body weight, lower plasma FFA levels, improved insulin sensitivity, and better glucose tolerance, supporting an important role for STING in the development of insulin resistance. It is important to suggest that STING may contribute to the development of insulin resistance through multiple mechanisms, such as inducing chronic inflammation, interfering with lipid metabolism/lipolysis, and directly inhibiting insulin signaling (unpublished data). Further studies should delineate the role of STING in these different mechanisms. Nevertheless, inducing endothelial inflammation in adipose tissue may be one of the many mechanism by which STING contributes to insulin resistance.

STING regulates gene expression by promoting the phosphorylation and translocation of several transcription factors, including STAT1, STAT6,[38] NF-kB,[27] and IRF3.[16, 25-27, 38] Among these factors, IRF3 is a well-studied target. IRF3 promotes inflammation by inducing the transcription of proinflammatory factors, such as interferons.[13, 27, 29, 38, 39] IRF3 is also involved in apoptosis by inducing the expression of Bax, a proapoptotic protein.[16] In this study, we identified ICAM-1 as a novel target of IRF3 in inflammation. We found that IRF3 binds to the promoter of ICAM-1 and enhances ICAM-1 expression, leading to increases in monocyte adhesion and inflammation. Consistent with our findings, recent reports have shown an association between a STING gain-of-function mutation and an increase in ICAM-1 expression.[13, 40,41] Our study provides direct evidence linking the STING-IRF3 pathway with ICAM-1 expression and inflammation.

It is well known that mitochondrial damage plays an essential role in cardiovascular inflammation and diseases.[42] However, the underlying mechanisms are not completely understood. Recent studies have suggested that cGAS-STING serves as a sensor of cytosolic DNA,[20-23] which can originate from damaged nuclei or mitochondria. We show here that PA increased mtDNA in the cytosol and induced the expression of cGAS, which was involved in PA-induced activation of the STING-IRF3 pathway and stimulation of ICAM-1 expression. Although cytosolic DNA from nuclear origin may also activate STING, our findings raise the possibility that PA induces mitochondrial damage and leakage of mtDNA into the cytosol, which activates the cGAS-STING-IRF3 pathway, leading to increases in ICAM-1

expression and endothelial inflammation. Thus, the cytosolic mtDNA-cGAS-STING-IRF3 pathway is a novel link between metabolic stress-induced mitochondrial damage and endothelial inflammation.

Conclusions

In summary, we have demonstrated that the cytosolic mtDNA-cGAS-STING-IRF3 mechanism is critically involved in metabolic stress-induced endothelial inflammation. Palmitic acid induces mitochondrial damage and leakage of mtDNA into the cytosol, thereby activating the cGAS-STING-IRF3 pathway, which in turn triggers endothelial activation/inflammation. STING plays a critical role in diet-induced adipose inflammation and insulin resistance. Thus, STING may be an important therapeutic target for preventing cardiovascular diseases and insulin resistance in obese individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

cGAS	cyclic GMP-AMP synthase
FFA	free fatty acid
HAECs	human aortic endothelial cells
HFD	high-fat diet
ICAM-1	intercellular adhesion molecule 1
IRF3	interferon regulatory factor 3

mtDNA	mitochondrial DNA
PA	palmitic acid
STING	stimulator of interferon genes
TBK1	TANK-binding kinase 1
WT	wild type

Highlights

- Mitochondria damage-cytosolic mtDNA-cGAS-STING-IRF3 pathway is an important mediator of metabolic stress-induced endothelial inflammation.
- STING plays a critical role in diet-induced adipose vascular inflammation and insulin resistance *in vivo*.
- STING may be an important therapeutic target for preventing cardiovascular diseases and insulin resistance in obese individuals.

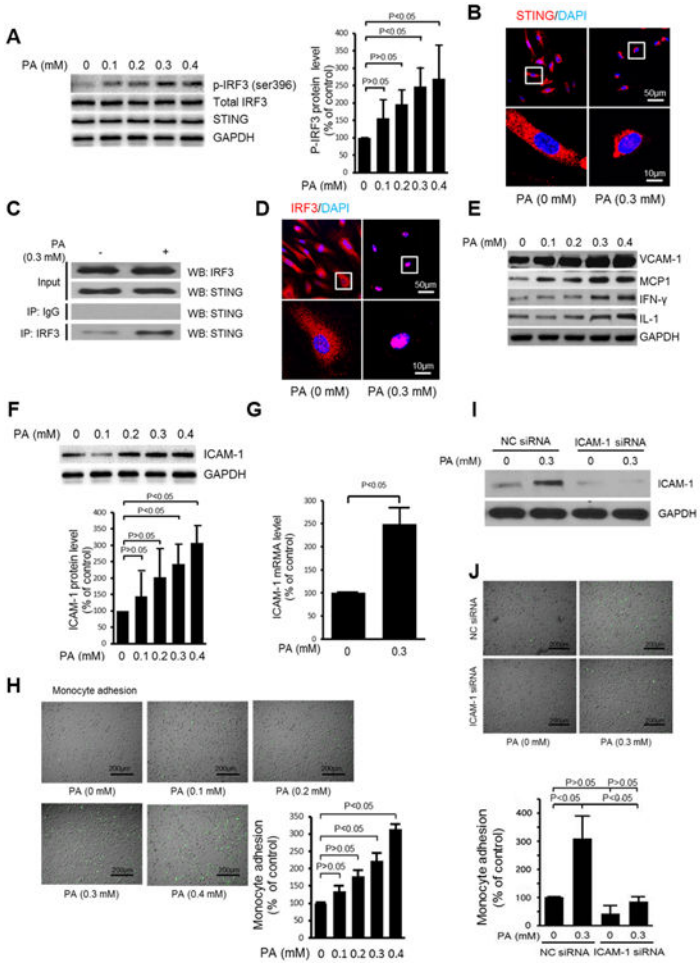

Figure 1. Palmitic acid (PA) activated the STING-IRF3 pathway and induced endothelial inflammation

Human aortic endothelial cells were treated with PA for 24 hours. **A,** Western blot analysis showed that PA increased the levels of phospho-IRF3 (n=5 biological repeats). **B,** Representative images of immunostaining demonstrated that STING was mainly in perinuclear area in PA treated cells (n=5 biological repeats). **C,** Representative co-immunoprecipitation analysis indicated that PA induced STING-IRF3 binding (n=5 biological repeats). **D,** Representative images of immunostaining demonstrated that PA induced IRF3 translocation from the cytoplasm to the nucleus (n=5 biological repeats). **E,** Western blot analysis showed that PA increased the levels of inflammatory proteins (VACM-1, MCP1, IFNγ, and IL-1). **F,** Western blot analysis showed that PA increased ICAM-1 protein level (n=5 biological repeats). **G**, RT-PCR demonstrated that PA induced ICAM-1 mRNA (n=6 biological repeats). **H**, Monocyte adhesion assay showed an increase in monocyte adhesion to PA-treated endothelial cells (n=3 biological repeats). Knocking down ICAM-1 in endothelial cells **(I)** significantly reduced monocyte adhesion to PA-treated cells (n=3 biological repeats) **(J)**.

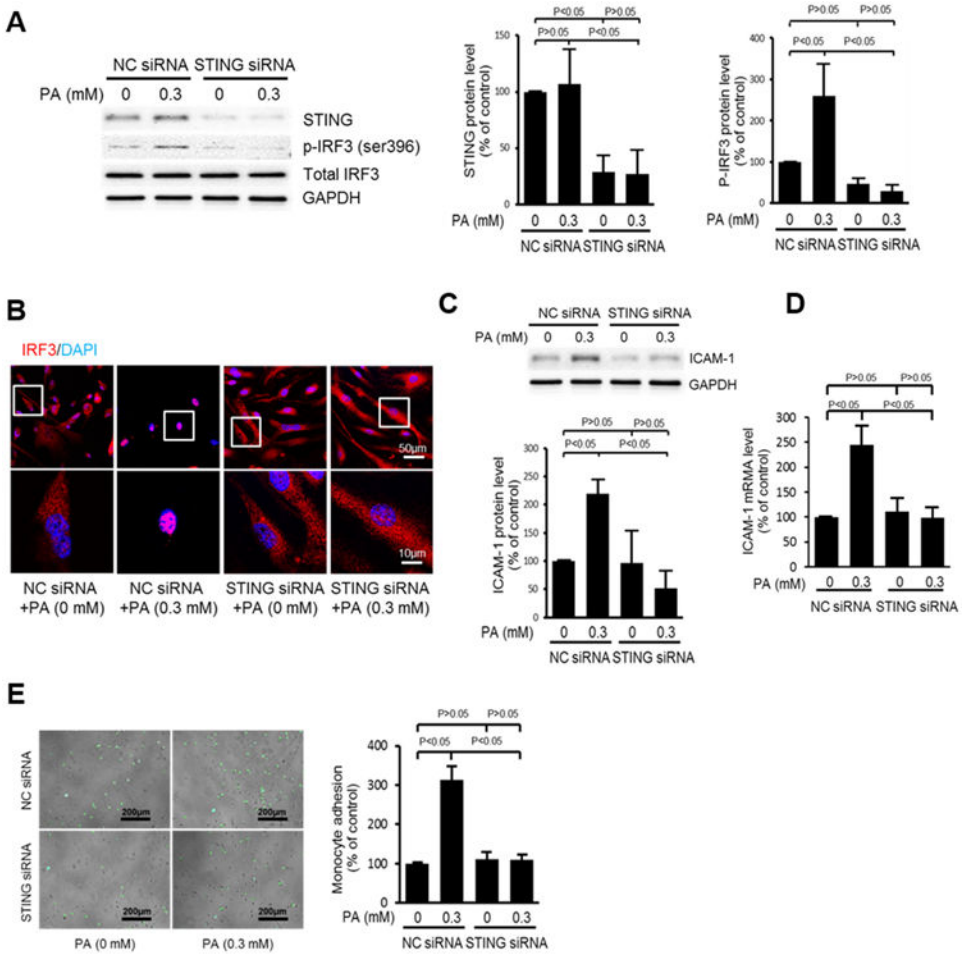

Figure 2. STING was involved in palmitic acid (PA)-induced IRF3 activation, ICAM-1 expression and endothelial activation/inflammation

Human aortic endothelial cells were transfected with STING siRNA or scrambled siRNA (NC) and then treated with PA for 24 hours. **A,** Western blot analysis showed that silencing STING decreased the PA-induced IRF3 phosphorylation (n=6 biological repeats). **B,** Representative images of immunostaining showing that knocking down STING prevented PA-induced IRF3 nuclear translocation. **C,** Western blot analysis showed that silencing STING reversed the PA-induced increase in ICAM-1 protein level (n=6 biological repeats). **D,** RT-PCR analysis demonstrated that STING siRNA attenuated the PA-induced increase in ICAM-1 mRNA level (n=5 biological repeats). **E,** Representative images of the monocyte adhesion assay showing that STING siRNA reduced the monocyte-endothelial cell adhesion induced by PA (n=3 biological repeats).

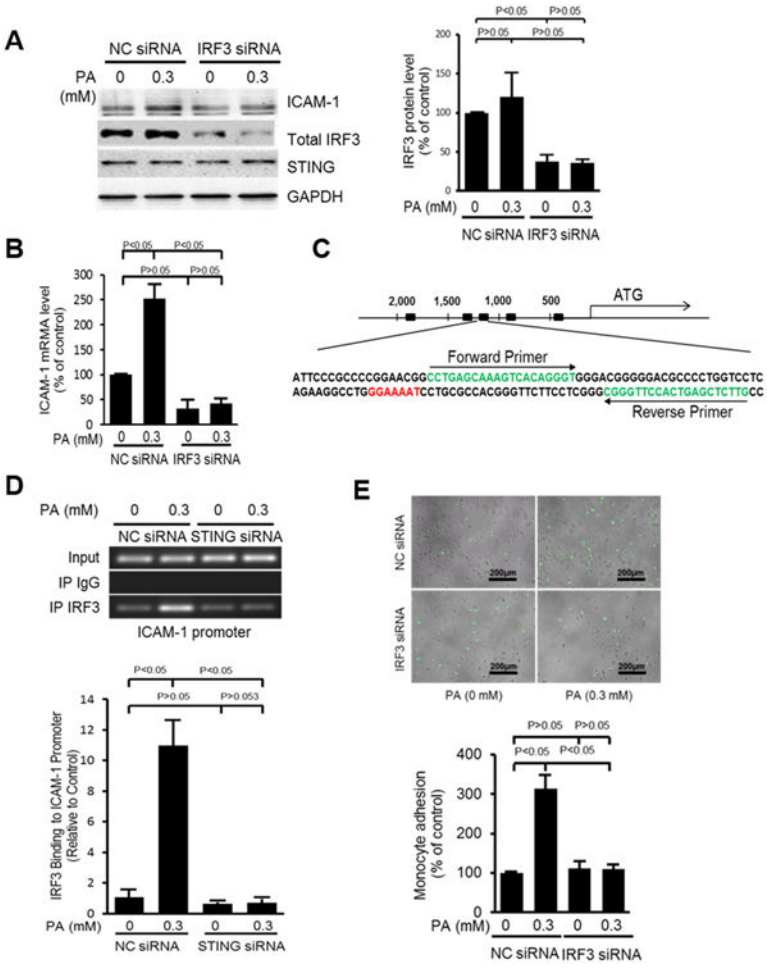

Figure 3. IRF3 directly bound to the ICAM-1 promoter and participated in PA-induced ICAM-1 expression and endothelial activation/inflammation

Human aortic endothelial cells were transfected with IRF3 siRNA or scrambled siRNA (NC) and then treated with PA for 24 hours. **A-B,** Western blot analysis (n=6 biological repeats) and RT-PCR (n=5 biological repeats) showed that knocking down IRF3 inhibited the PA-induced increases in ICAM-1 protein **(A)** and mRNA **(B)** levels. **C,** A putative IRF3 binding site upstream of the 5′UTR of ICAM-1 is shown. **D,** Chromatin immunoprecipitation assay showed that IRF3 bound to the ICAM-1 promoter in unstressed cells. The binding was increased in PA-treated cells and abolished by inhibiting STING expression (n=5 biological repeats). **E,** The monocyte adhesion assay showed that knocking down IRF3 attenuated the PA-induced monotype-endothelial cell adhesion (n=3 biological repeats).

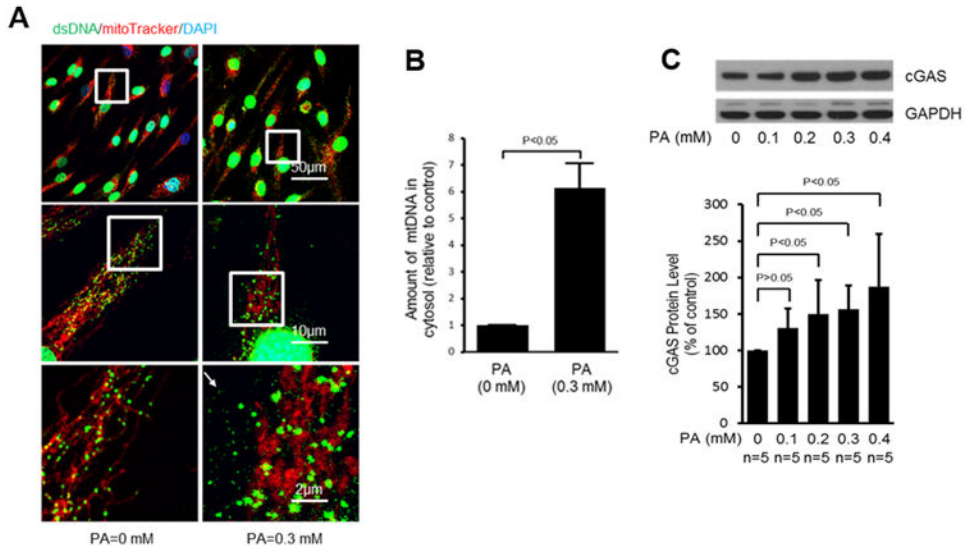

Figure 4. Palmitic acid (PA) significantly increased the levels of cytosolic mtDNA and cGAS protein

Human aortic endothelial cells were treated with PA for 24 hours. **A,** Representative images of double staining (n=5 biological repeats) with dsDNA antibody and MitoTracker showing mtDNA (dsDNA that co-localized with MitoTracker), nuclear DNA (dsDNA in the nucleus), and cytosolic DNA (dsDNA that did not co-localize with either mitochondria or the nucleus). PA treatment increased the amount of cytosolic White arrow points to cytosolic DNA. **B,** PCR analysis of cytosolic mtDNA. DNA in the cytosolic fraction was isolated, and the copy number of mtDNA (mtDNA sequences as primers) was measured and normalized with the copy number of nuclear DNA (nuclear DNA sequences as primers). The results showed that PA increased the amount of mtDNA in the cytosol (n=5 biological repeats). **C,** PA treatment increased cGAS protein (n=5 biological repeats).

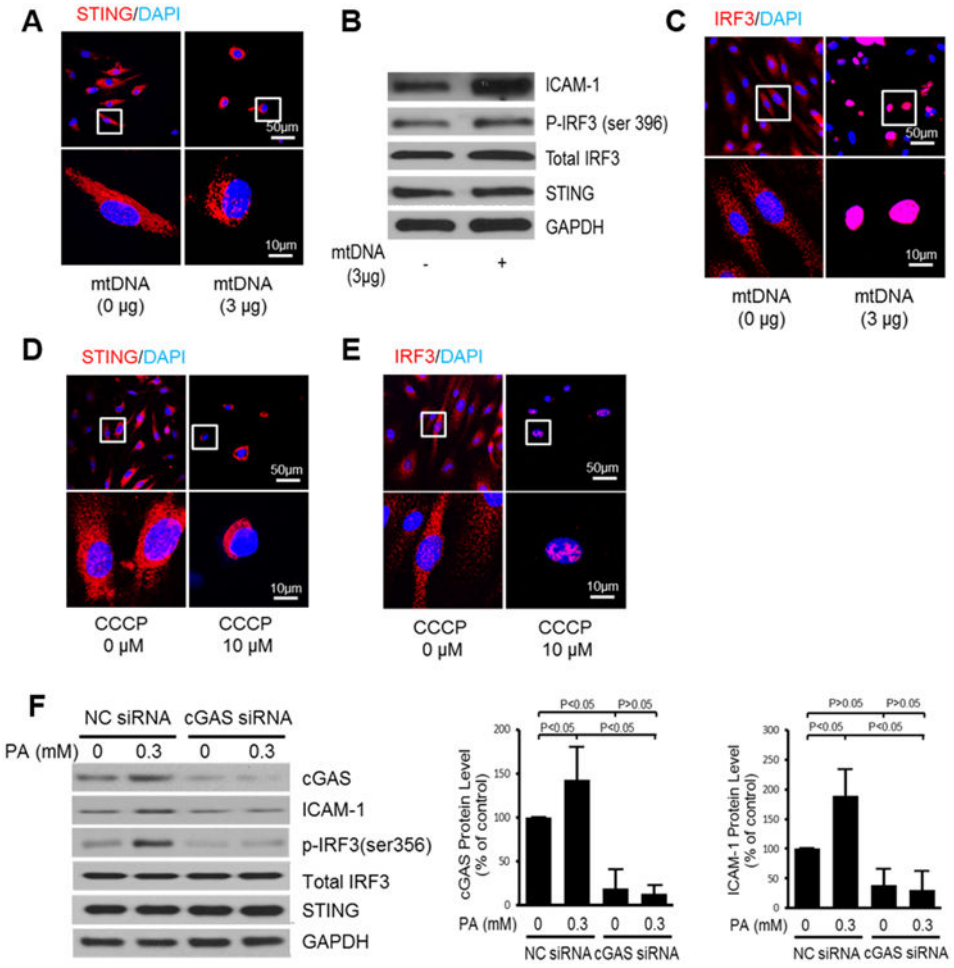

Figure 5. Mitochondrial damage and cytosolic mtDNA were potent triggers for STING-IRF3 activation

A, Human aortic endothelial cells transfected with mtDNA showed increases in STING perinuclear translocation (n=5 biological repeats). **B,** Western blot results (n=5 biological repeats) proved that mtDNA transfection increased IRF3 phosphorylation and the ICAM-1 protein level. **C,** Immunostaining (n=5 biological repeats) demonstrated that mtDNA transfection increased IRF3 nuclear translocation. **D-E,** Representative images of immunostaining showing that carbonyl cyanide m-chlorophenyl hydrazine (CCCP) stimulated STING perinuclear translocation (n=5 biological repeats) and IRF3 nuclear translocation (n=5 biological repeats). **F,** Knocking down cGAS with siRNA partially prevented the PA-induced increases in IRF3 phosphorylation and ICAM-1 expression (n=5 biological repeats).

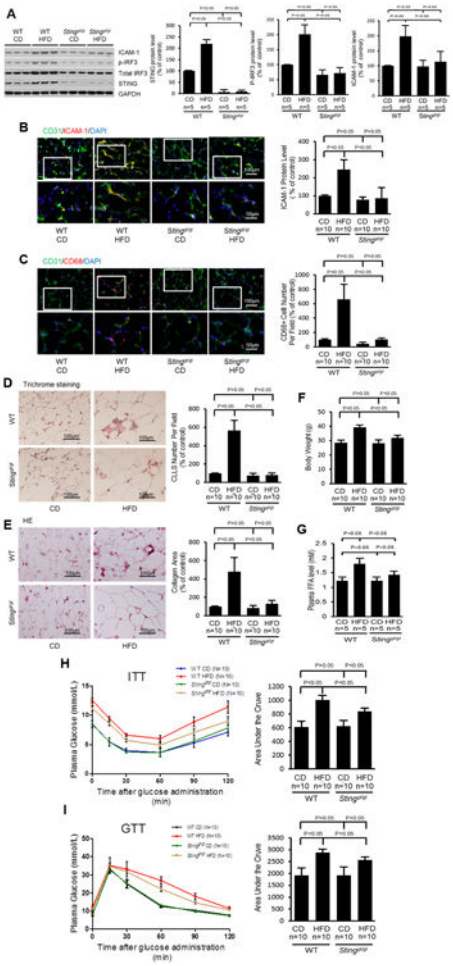

Figure 6. Sting deficiency alleviated high-fat diet (HFD)-induced endothelial inflammation in adipose tissue and insulin resistance

Four-week-old wild-type (WT) and STING deficient (*Sting*$^{gt/gt}$) mice were fed either a chow diet (CD) or a HFD for 12 weeks (n=10 in each group). **A,** Western blot analysis of epididymal adipose tissue showed that IRF3 phosphorylation and ICAM expression were less in HFD-fed S*ting*$^{gt/gt}$ mice than in HFD-fed WT mice. **B-C,** Immunofluorescent staining indicated that there was less ICAM-1 expression in endothelial cells **(B)** and macrophage infiltration **(C)** in the adipose vessels of the HFD-fed *Sting*$^{gt/gt}$ mice than in those of the HFD-fed WT mice. **D-E,** Hematoxylin and eosin (HE) staining and trichrome staining showed that the amount of collagen deposition **(D)** and the number of crown-like structures (CLS) **(E)** were less in the adipose tissue from HFD-fed *Sting*$^{gt/gt}$ mice than in that from the HFD-fed WT mice. **F-G,** HFD-fed *Sting*$^{gt/gt}$ mice had a lower body weight **(F)** and lower plasma free fatty acid levels **(G)** than the HFD-fed WT mice. **H-I,** The insulin tolerance test (ITT) **(H)** and the glucose tolerance test (GTT) **(I)** showed improved insulin sensitivity and glucose tolerance in HFD-fed *Sting*$^{gt/gt}$ mice, as compared with that in HFD-fed WT mice. Area under the curve was compared between groups.