Hypertension

ORIGINAL ARTICLE

Pharmacological Inhibition of Epidermal Growth Factor Receptor Prevents Intracranial Aneurysm Rupture by Reducing Endoplasmic Reticulum Stress

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BACKGROUND: Multiple pathways and factors are involved in the rupture of intracranial aneurysms. The EGFR (epidermal growth factor receptor) has been shown to mediate inflammatory vascular diseases, including atherosclerosis and aortic aneurysm. However, the role of EGFR in mediating intracranial aneurysm rupture and its underlying mechanisms have yet to be determined. Emerging evidence indicates that endoplasmic reticulum (ER) stress might be the link between EGFR activation and the resultant inflammation. ER stress is strongly implicated in inflammation and apoptosis of vascular smooth muscle cells, both of which are key components of the pathophysiology of aneurysm rupture. Therefore, we hypothesized that EGFR activation promotes aneurysmal rupture by inducing ER stress.

METHODS: Using a preclinical mouse model of intracranial aneurysm, we examined the potential roles of EGFR and ER stress in developing aneurysmal rupture.

RESULTS: Pharmacological inhibition of EGFR markedly decreased the rupture rate of intracranial aneurysms without altering the formation rate. EGFR inhibition also significantly reduced the mRNA (messenger RNA) expression levels of ER-stress markers and inflammatory cytokines in cerebral arteries. Similarly, ER-stress inhibition also significantly decreased the rupture rate. In contrast, ER-stress induction nullified the protective effect of EGFR inhibition on aneurysm rupture.

CONCLUSIONS: Our data suggest that EGFR activation is an upstream event that contributes to aneurysm rupture via the induction of ER stress. Pharmacological inhibition of EGFR or downstream ER stress may be a promising therapeutic strategy for preventing aneurysm rupture and subarachnoid hemorrhage. ***(Hypertension.* 2024;81:572–581. DOI: 10.1161/HYPERTENSIONAHA.123.21235.)** • **Supplement Material**.

Key Words: endoplasmic reticulum stress ■ epidermal growth factor ■ hypertension ■ intracranial aneurysm ■ mice ■ stroke ■ subarachnoid hemorrhage

Intracranial aneurysm rupture causes subarachnoid hemorrhage, resulting in severe mortality and morbidity.[1] Currently, available therapies for the prevention of aneurysm rupture are limited to invasive treatments such as surgical clipping and endovascular coiling.[1] Although these invasive therapies are well established, the adverse outcome rates from these procedures still present significant procedural risks.[1,2] Therefore, the pharmacological prevention of aneurysmal rupture is emerging as a potential alternative approach for patients with unruptured aneurysms.

Inflammation is emerging as a key component of the pathophysiology of intracranial aneurysms.[3–6] Given its potential as a therapeutic target, a better understanding

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NOVELTY AND RELEVANCE

What Is New?

We discovered that the activation of the EGFR (epidermal growth factor receptor) promotes intracranial aneurysm rupture in a mouse model of intracranial aneurysm.

We further confirmed that the effect of EGFR is through inducing endoplasmic reticulum stress and subsequent inflammatory responses in vascular walls.

What Is Relevant?

High blood pressure has been shown to play an important role in intracranial aneurysm rupture.

Currently available therapies for this devastating disease are limited to invasive treatments.

Clinical/Pathophysiological implications?

Our study firmly established that pharmacological inhibition of both EGFR and downstream endoplasmic reticulum stress was protective against intracranial aneurysm rupture. These pharmacological treatments may be a promising strategy for preventing aneurysm rupture and subarachnoid hemorrhage in a clinical setting.

Nonstandard Abbreviations and Acronyms

4-PBA	4-phenylbutyric acid
ATF4	activating transcription factor 4
CHOP	C/EBP homologous protein
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
GRP78	glucose-regulated protein 78
HIF-1α	hypoxia-inducible factor-1 alpha
IL-1β	interleukin-1 beta
IL-6	interleukin 6
TNF-α	tumor necrosis factor-alpha
UPR	unfolded protein response
VSMC	vascular smooth muscle cell
sXBP1	spliced X-Box binding protein 1

of molecular pathways involved in this inflammatory process may contribute to the development of medical therapies for the prevention of aneurysm rupture and subsequent subarachnoid hemorrhage.

Previous studies have consistently shown that the activation of the renin-angiotensin II system in the vascular walls of intracranial aneurysms induces aneurysm rupture, which is independent of hypertension.[7–9] Upon activation by angiotensin II, the epidermal growth factor receptor (EGFR) in arterial walls has been found to play a critical role in mediating inflammatory responses and promoting vascular damage.[10–12] EGFR, referring to a family of receptor tyrosine kinases, is expressed in vascular smooth muscle cells (VSMCs). Activation of EGFR has been found to stimulate phenotypic modulation of contractile VSMCs and promote the proliferation and migration of VSMCs.[13–15] EGFR was shown to mediate vascular diseases that involve an inflammatory component, including atherosclerosis and aortic aneurysm.[16–18] However, the role of EGFR in mediating intracranial aneurysm rupture and its underlying mechanisms remained to be determined.

Emerging evidence indicates that endoplasmic reticulum (ER) stress might be the link between EGFR activation and the resultant inflammation in vascular diseases.[19–22] The ER is responsible for cellular protein synthesis and folding. Cellular stimuli that perturb ER homeostasis create a stress condition termed ER stress.[23,24] Under ER stress, unfolded protein aggregation and proteotoxicity induce proinflammatory responses via 3 unfolded protein response (UPR) pathways, IRE1 (inositol-requiring enzyme 1) , PERK (protein kinase RNA-Like ER kinase), and ATF6 (activating transcription factor 6).[23,24] Maladaptive UPR accompanied by excessive ER stress causes oxidative stress and inflammatory cell infiltration.[25,26] Literature suggests that ER stress and ensuing UPR are strongly implicated in the pathophysiology of vascular diseases involving VSMC inflammation and apoptosis.[23,27,28]

Taking this information together, we hypothesized that EGFR activation promotes the development of aneurysmal rupture by inducing ER stress. In this study, we examined the potential roles of EGFR and ER stress in developing aneurysmal rupture using a mouse model.

METHODS

Experiments were conducted following guidelines approved by the Institutional Animal Care and Use Committee. Details of experimental methods are available in Supplemental Materials.[5,7,8,19,29–45] Fisher exact test was used to analyze the incidences of aneurysm formation and subarachnoid hemorrhage. Log-rank (Mantel-Cox) test was used for the analysis of the survival rate. Real-time polymerase chain reaction data were analyzed by the Mann-Whitney *U* test.

RESULTS

Inhibition of EGFR Prevented the Development of Intracranial Aneurysm Rupture

As a first step to test the potential role of EGFR activation in the development of aneurysm rupture, we used an EGFR-specific inhibitor, erlotinib, in our well-established mouse model of intracranial aneurysm (Figure 1).[29,30] We used 2 treatment schemes. One was to give erlotinib 1 day before aneurysm induction (pretreatment), and the other was to administer erlotinib 6 days after aneurysm induction (posttreatment). This was because we have previously observed that aneurysms were formed in the first week in our model.[29,30] Therefore, the pretreatment scheme would confirm whether inhibition of EGFR would have any effect on aneurysm formation. The posttreatment scheme was designed to test the effect of EGFR inhibition on aneurysm rupture after aneurysms formed.

Pretreatment with erlotinib did not change the incidence of aneurysms (Figure 2A, vehicle versus erlotinib, 67% versus 54%, n=27 versus 28; P=0.41). In contrast, pretreatment with erlotinib significantly decreased the rupture rate compared with the vehicle treatment (Figure 2B, vehicle versus erlotinib, 72% versus 33%, n=18 versus 15; P<0.05). Mice treated with erlotinib also had a significantly better symptom-free survival rate than vehicle-treated mice (Figure 2C; P<0.05). There was no significant difference in blood pressure between the 2 groups (Table S2).

Similar to the results in the pretreatment scheme, posttreatment with erlotinib did not change the incidence of aneurysms (Figure 3A, vehicle versus erlotinib, 61% versus 43%, n=18 versus 16; P=0.49). However, posttreatment with erlotinib significantly decreased the rupture rate compared with the vehicle treatment (Figure 3B, vehicle versus erlotinib, 72% versus 14%, n=11 versus 7; P<0.05). Mice treated with erlotinib also had a significantly better symptom-free survival rate than vehicle-treated mice (Figure 3C; P<0.05). There was no significant difference in blood pressure between the 2 groups (Table S3).

These results indicate that inhibition of EGFR activation prevents aneurysm rupture but has minimal effect on aneurysm formation.

Inhibition of EGFR Reduced Gene and Protein Expression of ER-Stress Markers and Proinflammatory Cytokines

To further test our hypothesis that EGFR activation contributes to the development of intracranial aneurysm rupture through induction of ER stress, we tested the effect of the EGFR inhibitor, erlotinib, on the gene and protein expression of ER-stress markers in mice with induced aneurysms. We measured the levels of mRNA expression of 5 ER-stress markers (GRP78 [glucose-regulated protein 78], CHOP [C/EBP homologous protein], ATF4 [activating transcription factor 4], sXBP1 [spliced X-Box binding protein 1], and HERPUD [homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1]) in the cerebral arteries of mice treated with erlotinib or vehicle (Figure 4, top). Erlotinib treatment significantly decreased the mRNA expression levels of ER-stress markers, namely GRP78, CHOP, sXBP1, and HERPUD, as

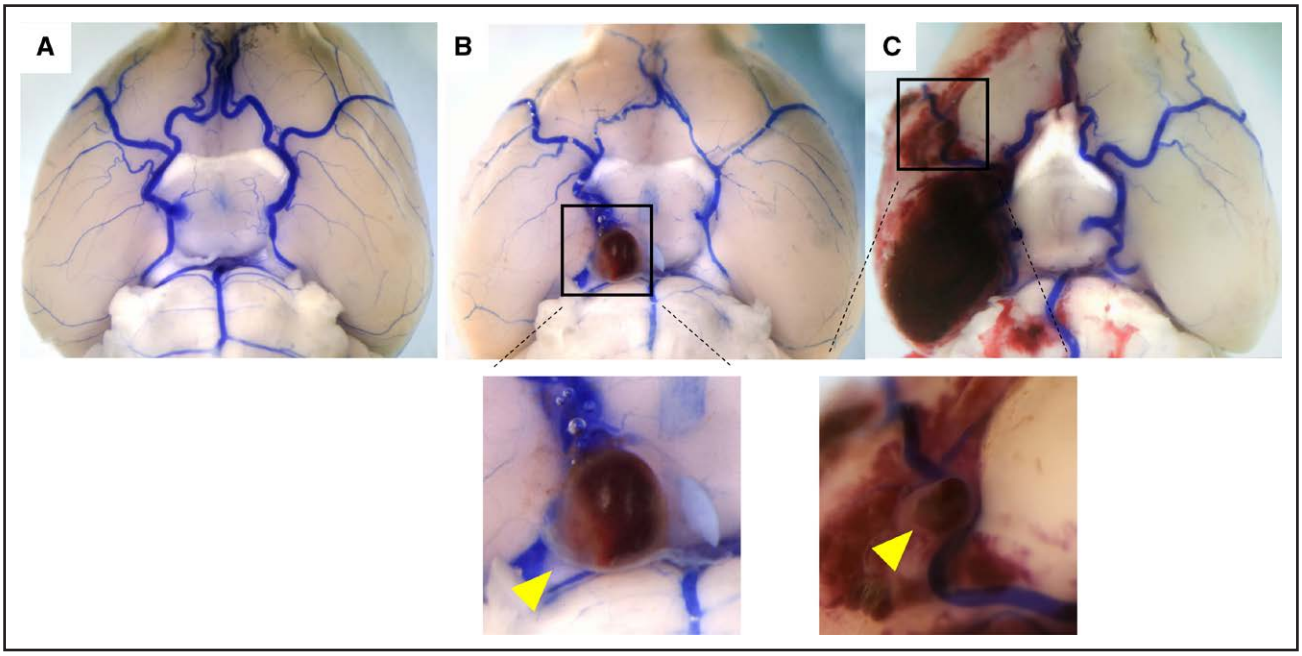

Figure 1. Representative images of unruptured and ruptured aneurysms.
Circle of Willis in mouse brains were perfused with bromophenol blue dye. **A**, No aneurysm. **B**, Unruptured aneurysm. **C**, ruptured aneurysm with subarachnoid hemorrhage. Arrowheads indicate intracranial aneurysms.

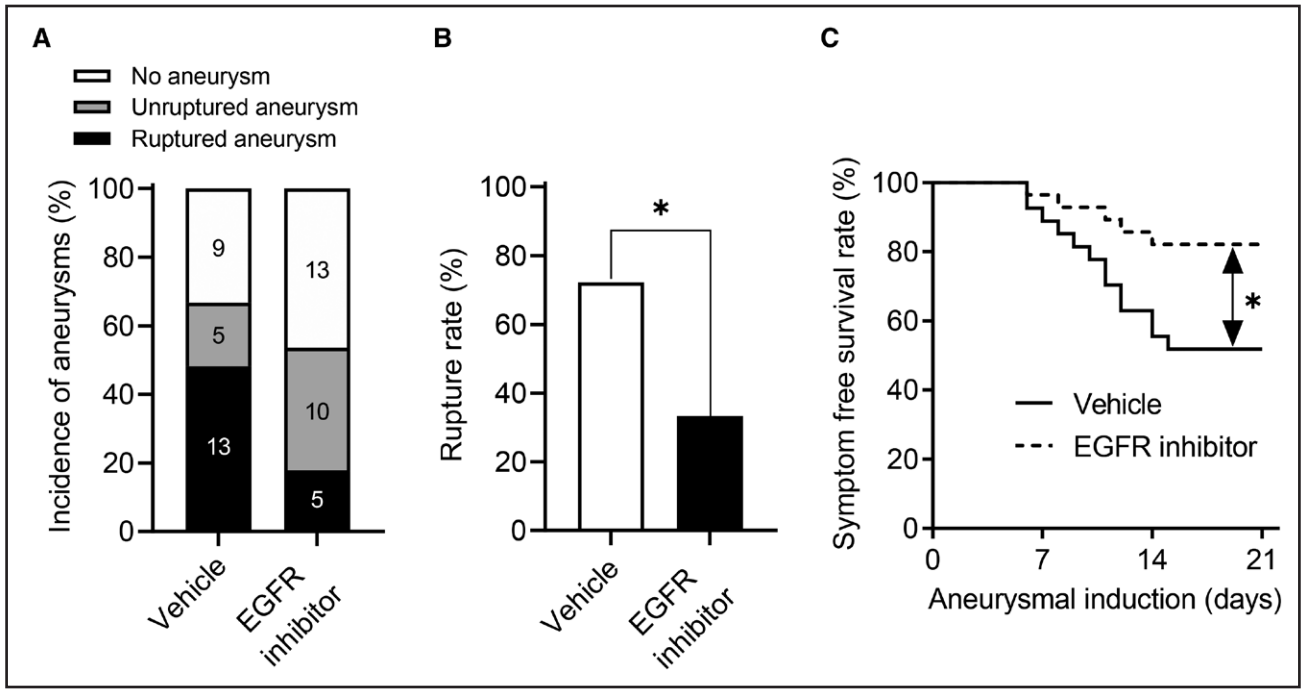

Figure 2. Inhibition of EGFR (epidermal growth factor receptor) activation (pretreatment) reduced the rate of aneurysm rupture. Pretreatment of the EGFR inhibitor, erlotinib, significantly decreased the rupture rate of intracranial aneurysms without altering the aneurysm formation rate (**A** and **B**). A significantly increased symptom-free survival rate (**C**) was seen in erlotinib-treated mice compared with vehicle-treated mice. Fisher exact test was used to analyze the rupture rate of aneurysms (**B**). Log-rank (Mantel-Cox) test was used for the analysis of the survival rate (**C**). *P<0.05.

compared with the vehicle treatment (vehicle versus erlotinib, GRP78: 1.0±0.48 versus 0.70±0.39; P<0.05; CHOP: 1.0±0.38 versus 0.71±0.19; P<0.05; sXBP1: 1.0±0.38 versus 0.71±0.34, P<0.05; HERPUD: 1.0±0.43 versus 0.72±0.43; P<0.05). Additionally, there was a significantly decreased expression level of the oxidative stress marker HIF-1α (hypoxia-inducible factor-1 alpha) in mice treated with erlotinib compared with the vehicle control (1.0±0.22 versus 0.69±0.22; P<0.05). We did not find a significant difference between erlotinib and vehicle treatment on the expression levels of ATF4, catalase, inducible nitric oxide synthase, SOD-1 (*superoxide dismutase 1*), and nuclear factor-kappa B (NF-κB) (Figure S1).

In a separate set of experiments, using immunofluorescence staining, we confirmed that EGFR is highly expressed in smooth muscle cells in mice with induced aneurysms (Figure S2). Aneurysm induction caused notable apoptosis of smooth muscle cells in mice treated with vehicle. In contrast, erlotinib significantly reduced the apoptosis of smooth muscle cells (Figure S3). Erlotinib also reduced the infiltration of macrophages (Figure S4). Using immunohistochemical staining, we evaluated the protein expression levels of ER-stress markers in the vessels of the circle of Willis from aneurysm-induced mice. Compared with vehicle, erlotinib treatment significantly reduced the protein expression of CHOP (Figure S5) and sXBP1 (Figure S6). In addition, erlotinib also significantly reduced the protein expression of the proinflammatory proteinase MMP9 (matrix metalloproteinase-9) (Figure S7).

To determine whether the resultant ER stress from EGFR activation confers inflammation that is known to lead to the rupture of aneurysms, we tested the effect of EGFR inhibition on gene and protein expression of proinflammatory cytokines in our mouse model (Figure 4, lower). Erlotinib treatment significantly decreased the expression levels of MMP9, TNF-α (tumor necrosis factor-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) as compared with the vehicle treatment (vehicle versus erlotinib, MMP9: 1.0±0.34 versus 0.62±0.57; P<0.05; TNF-α: 1.0±0.47 versus 0.49±0.51; P<0.05; IL-1β: 1.0±0.81 versus 0.61±0.66, P<0.05; IL-6: 1.0±0.82 versus 0.44±0.59; P<0.05).

In a separate set of experiments, we assessed the protein levels of TNFα, IL-1β, and IL-6 in tissue homogenate of the Circle of Willis from mice treated with vehicle or erlotinib using enzyme-linked immunosorbent assay. Erlotinib treatment significantly decreased the protein levels of TNF-α, IL-1β, and IL-6 as compared with the vehicle treatment (Figure S8, vehicle versus erlotinib, TNF-α: 1.0±0.24 [n=11] versus 0.65±0.32 [n=12]; P<0.05; IL-1β: 1.0±0.16 [n=5] versus 0.37±0.12 [n=6]; P<0.05; IL-6: 1.0±0.24 [n=5] versus 0.47±0.13 [n=6]; P<0.05).

Inhibition of ER Stress Prevented the Development of Intracranial Aneurysm Rupture

To establish the direct link between ER stress and aneurysmal rupture, we used a well-established ER-stress

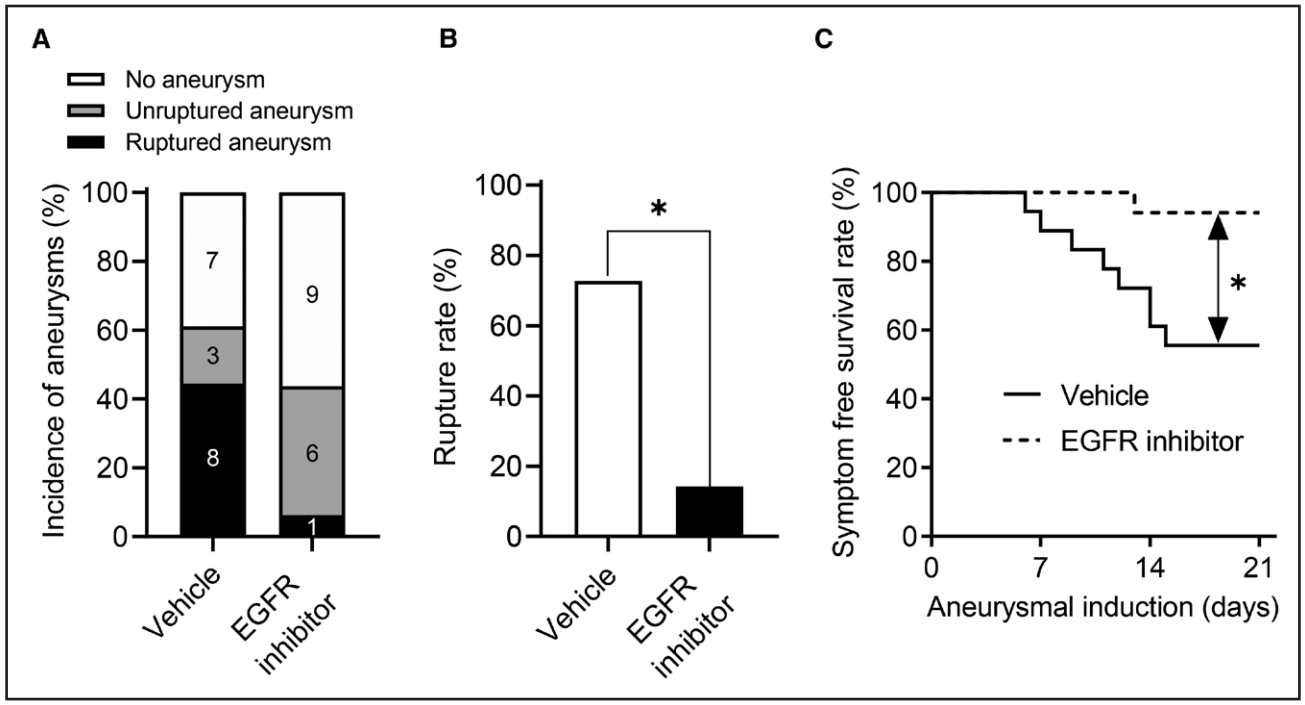

Figure 3. Inhibition of EGFR (epidermal growth factor receptor) activation (posttreatment) reduced the rate of aneurysm rupture.

Posttreatment of the EGFR inhibitor significantly decreased the aneurysm rupture rate without altering the formation rate (**A** and **B**). Compared to mice in the control group, a significantly increased symptom-free survival rate was observed in mice treated with erlotinib (**C**). Fisher exact test was used to analyze the rupture rate of aneurysms (**B**). Log-rank (Mantel-Cox) test was used for the analysis of the survival rate (C). *P<0.05.

reducer, 4-phenyl butyric acid (4-PBA).[45] Following the same protocol for erlotinib pretreatment, we started the treatment with vehicle or 4-PBA 1 day before aneurysm induction. The ER-stress reducer did not affect the formation of aneurysms, as indicated by the lack of difference in the overall incidence of aneurysms (Figure 5A, vehicle versus 4-PBA, 94% versus 87%, n=17 versus 23; P=0.62). However, the ER-stress reducer significantly decreased the rupture rate (Figure 5B, vehicle versus 4-PBA, 88% versus 50%, n=16 versus 20; P<0.05). Mice treated with 4-PBA also had a significantly better symptom-free survival rate than mice treated with the vehicle (Figure 5C; P<0.05). There was no significant difference in blood pressure between the 2 groups (Table S4).

Induction of ER Stress Nullified the Protective Effect of EGFR Inhibition on Aneurysm Rupture

Finally, to establish a link between EGFR inhibition and ER-stress reduction in the prevention of aneurysm rupture, we treated mice with the EGFR inhibitor erlotinib alone or in combination with tunicamycin, the ER-stress inducer. The rationale was that if EGFR activation directly induces ER stress, then treatment of tunicamycin would nullify the protective effects of erlotinib on aneurysm rupture. As expected, there was no significant difference in aneurysm formation rate between erlotinib treatment with or without the addition of tunicamycin (Figure 6A, erlotinib versus tunicamycin, 92% versus 93%, n=13 versus 14; P=0.96). Erlotinib reduced the aneurysm rupture rate (in comparison, the rupture rate was 69% for the vehicle control); however, this effect was completely abolished by the tunicamycin treatment (Figure 6B, erlotinib versus tunicamycin, 45% versus 92%, n=11 versus 13; P<0.05, Fisher exact test). The addition of the ER-stress inducer decreased the symptom-free survival rate to a near-significant level compared with the EGFR inhibitor treatment alone (Figure 6C; P=0.055). There was no significant difference in blood pressure among these groups (Table S5).

DISCUSSION

In this study, using a well-established mouse model, we showed that both inhibition of EGFR activation and ER stress significantly reduced the rupture rate of intracranial aneurysms. Furthermore, the inhibition of EGFR activation reduced the gene and protein expression levels of ER-stress markers and proinflammatory cytokines. These data suggest that EGFR activation is an upstream event that contributes to aneurysm rupture via the induction of ER stress. We confirmed this notion directly with experiments showing that pharmacological induction of ER stress abolished the protective effect of EGFR on aneurysmal rupture.

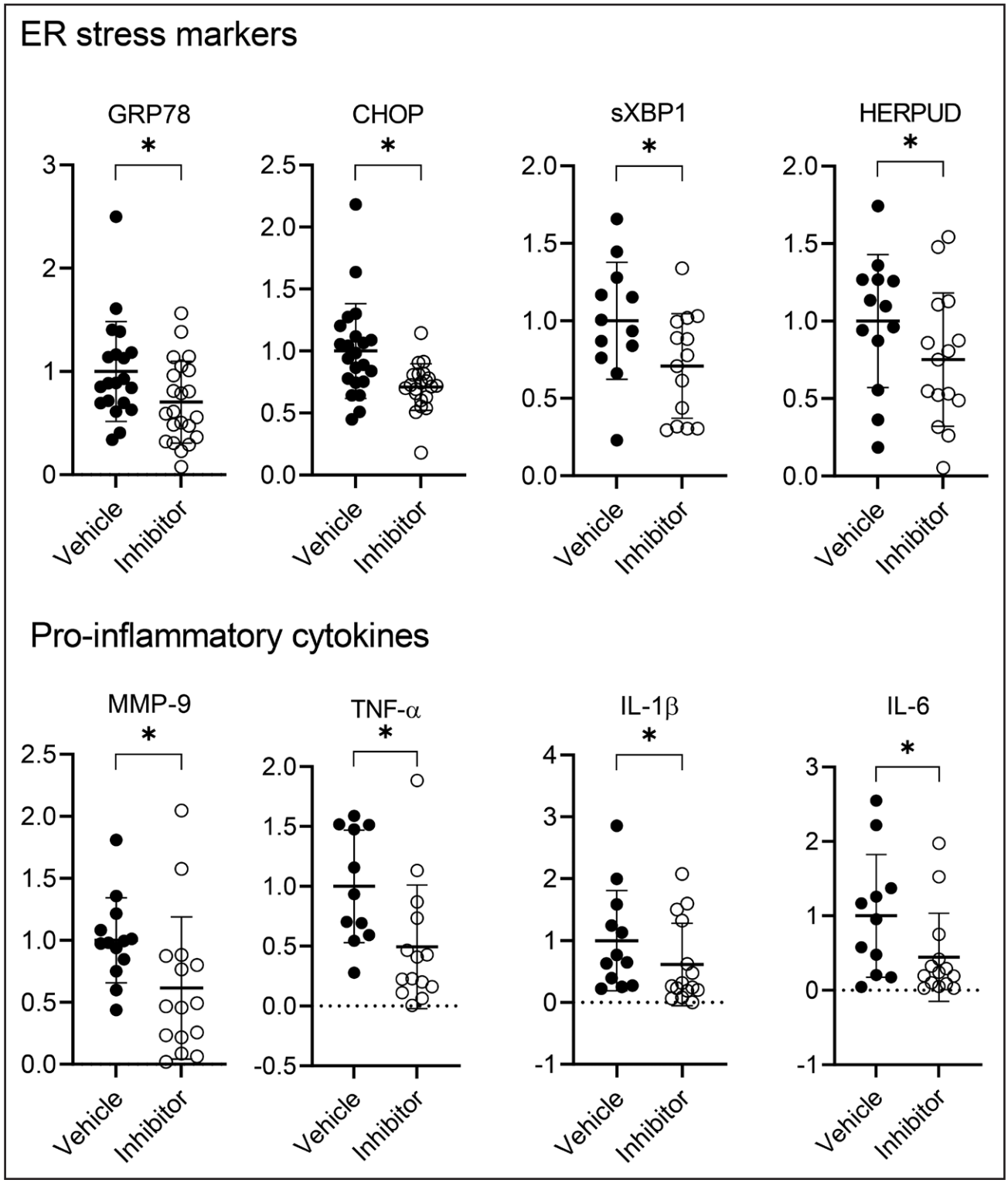

Figure 4. Inhibition of EGFR (epidermal growth factor receptor) activation decreased mRNA (messenger RNA) expression of endoplasmic reticulum (ER) stress markers and proinflammatory cytokines in cerebral arteries.
EGFR inhibitor-treated mice had significantly reduced mRNA expression of ER-stress markers GRP78 (glucose-regulated protein 78), CHOP (C/EBP homologous protein), sXBP1 (spliced X-Box binding protein 1), and HERPUD (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1) compared with vehicle-treated controls. Erlotinib-treated mice also had significantly decreased mRNA expression of proinflammatory cytokines MMP9 (matrix metalloproteinase-9), TNF-α (tumor necrosis factor-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), compared with vehicle-treated controls. Data are expressed as mean±SD, the Mann-Whitney *U* test, **P*<0.05.

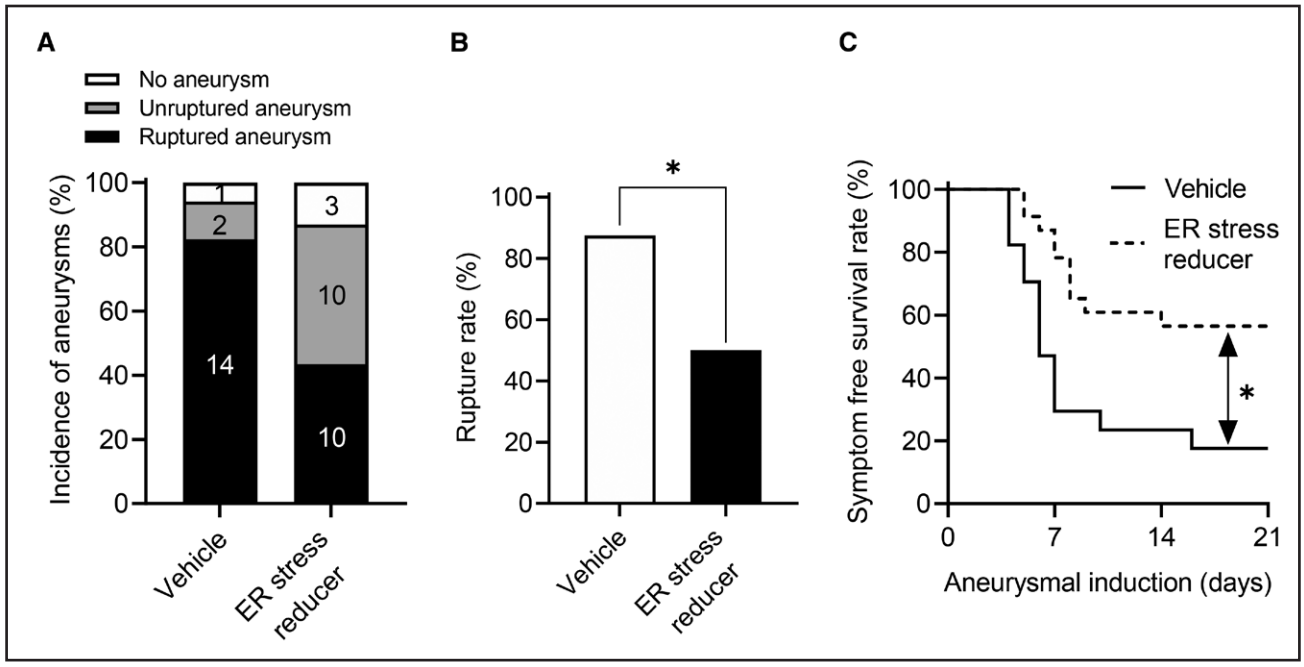

Figure 5. Inhibition of endoplasmic reticulum (ER) stress decreased the rate of aneurysm rupture.
ER-stress reduction with 4-phenyl butyric acid (4-PBA) significantly decreased the aneurysm rupture rate without altering the formation rate (**A** and **B**). A significantly increased symptom-free survival rate was found in mice treated with 4-PBA compared with control mice (**C**). Fisher exact test was used to analyze the rupture rate of aneurysms (**B**). Log-rank (Mantel-Cox) test was used for the analysis of the survival rate (**C**). **P*<0.05.

Although limited information has been available about the role of ER stress in intracranial aneurysm rupture, previous studies have suggested a potential mechanistic link between ER stress and subsequent arterial wall disruption. Prolonged ER stress can trigger apoptotic cell death, which is mediated by a caspase-12 dependent

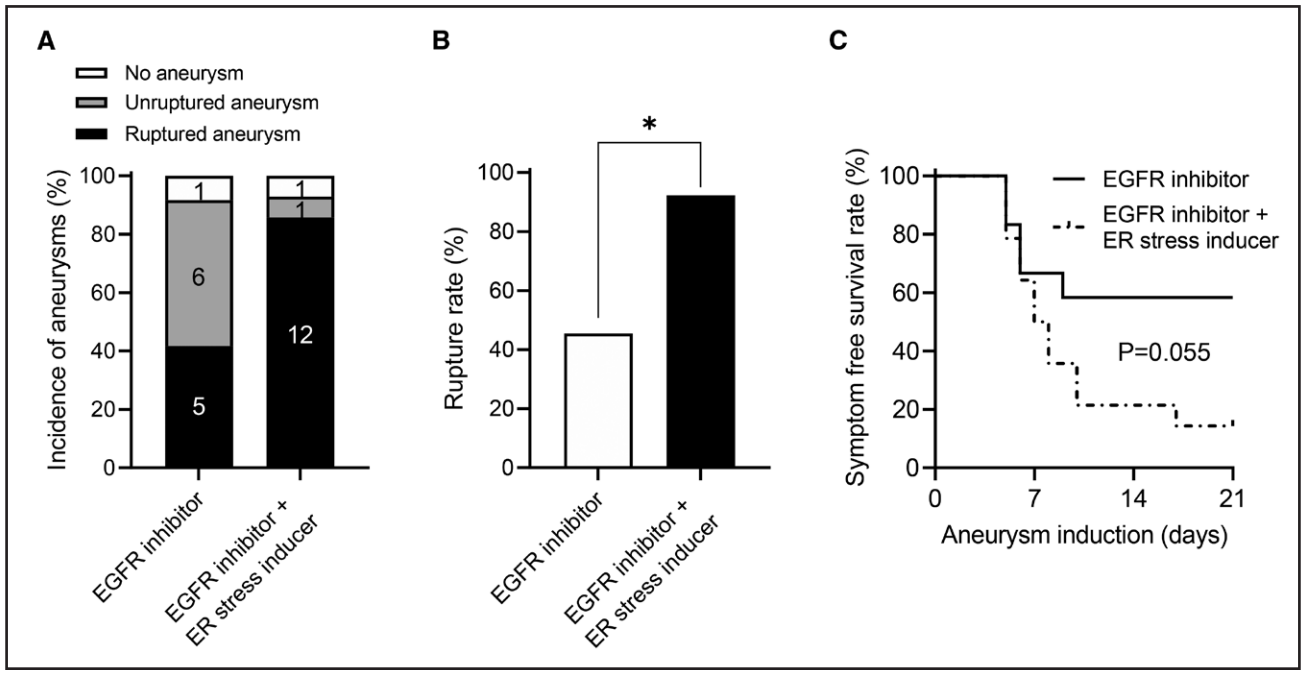

Figure 6. The induction of endoplasmic reticulum (ER) stress nullified the protective effect of EGFR (epidermal growth factor receptor) inhibition on aneurysm rupture.
The reduced aneurysmal rupture rate afforded by EGFR inhibitor treatment (in comparison, 69% for vehicle control) was completely abolished by ER-stress induction using tunicamycin (**A** and **B**). The addition of the ER-stress inducer decreased the symptom-free survival rate to a near-significant level compared with the erlotinib treatment alone (**C**). Fisher exact test was used to analyze the rupture rate of aneurysms (**B**). Log-rank (Mantel-Cox) test was used for the analysis of the survival rate (**C**). **P*<0.05.

pathway and by transcriptional induction of CHOP, and by activation of c-Jun N-terminal kinase.[46–48] VSMC apoptosis has been shown to induce medial expansion associated with increased elastic lamina breaks and abnormal matrix deposition in humans.[28,49] VSMC apoptosis, a potential sign and consequence of the maladaptive UPR, has been observed both in human and animal models of intracranial aneurysm.[50–55] Furthermore, ER stress and all 3 pathways of UPR are responsible for the phenotypic modulation of VSMCs.[13,14] The remodeling of SMCs at the aneurysm wall has been shown to be associated with aneurysm rupture in humans.[56,57] These studies support ER stress and subsequent UPR being directly involved in intracranial aneurysm rupture.

Our data showed that EGFR inhibition via erlotinib not only significantly decreased the rupture rate of the intracranial aneurysm but also decreased gene expression levels of ER-stress markers of GRP78, sXBP1, HERPUD, and CHOP. GRP78 is a master regulator of ER stress that modulates downstream UPR pathways. sXBP1 is upregulated by IRE1 arm activation, and HERPUD is upregulated by ATF6 arm activation, respectively.[25,26] CHOP is the molecule at the converging point of PERK/ATF4 and ATF6 UPR pathways and is mainly related to ER-stress–induced apoptotic cell death.[26,58] These results indicate that EGFR activation induces ER stress through activating all 3 UPR pathways. The data on ER-stress induction increasing the aneurysm rupture rate further reaffirms that ER stress plays a critical role in the rupture of intracranial aneurysm. The nullification of EGFR's protective effect on aneurysm rupture by ER-stress induction suggests that ER stress is a downstream event of EGFR activation in our mouse model.

In addition to proinflammatory cytokines, our data also showed a significant decrease in HIF-1α due to EGFR inhibition. HIF-1α is a marker of oxidative stress. Activation of vascular EGFR produces reactive oxygen species through Rac activation, causing oxidative stress.[10] EGFR activation was also shown to produce HIF-1α in VSMCs, and HIF-1α can trigger ER stress and CHOP-mediated apoptosis.[19] These are in agreement with our current findings. Therefore, as a known risk factor,[59–62] oxidative stress might compound with overall EGFR activation and ER-stress induction on the aneurysm site, eventually contributing to the rupture of aneurysms.

This study has several limitations. First, the animal model may not completely replicate all biological events that lead to aneurysm rupture, as aneurysms were induced rather than spontaneously formed. Vascular inflammation is known to play a key role in the pathophysiology of intracranial aneurysms in both humans and animals. There may be significant differences in the triggering factors of vascular inflammation between human aneurysms and this model. However, the phenotypes of intracranial aneurysms in the model closely mimic that of intracranial aneurysms in humans[5,30] More importantly, this model shares the end phenotypes, aneurysmal rupture, and associated neurological symptoms with human aneurysms, indicating its similarity of the underlying biological processes to human intracranial aneurysms.[7,30]

Second, we used only male mice in this study, though we have previously examined the sex differences in intracranial aneurysms in our model.[32,63] To fully model human aneurysms, the experimental protocol that utilizes aged female mice, especially reproductively senescent female mice or female mice with long-term estrogen depletion, may be desirable in the future.[64–66] However, at this point, the inclusion of these aged female mice will make the study scope too expansive and too ambitious.

Another limitation of this study is that, although our data support the notion of EGFR activation inducing ER stress in our model, we can only postulate the pathways of ER stress involved based on mRNA expression data. Future studies using ER-stress pathway-specific blockers/promoters or transgenic mice may further map out the specific pathways of ER stress responsible for promoting aneurysm rupture.

PERSPECTIVES

Our findings suggest that EGFR activation promotes intracranial aneurysm rupture by inducing ER stress in vascular walls. Future clinical studies will need to validate these findings to confirm the relationship between aneurysm rupture and EGFR-ER-stress pathways.

CONCLUSIONS

This study showed the potential role of EGFR and ER stress in the development of intracranial aneurysm rupture. Pharmacological inhibition of EGFR or downstream ER stress may be a promising strategy for preventing aneurysm rupture and subarachnoid hemorrhage.

ARTICLE INFORMATION

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Disclosures

None.

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