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ORIGINAL ARTICLE

Rolipram impacts on redox homeostasis and cellular signaling in an experimental model of abdominal aortic aneurysm

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KEYWORDS
Abdominal aortic aneurysm;
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Abstract
Introduction: Cyclic nucleotide phosphodiesterases (PDEs) of the PDE4 subfamily are responsible for the hydrolysis and subcellular compartmentalization of cAMP, a second messenger that modulates vascular functionality. We had shown that PDE4B is induced in abdominal aortic aneurysms (AAA) and that PDE4 inhibition by rolipram limits experimental aneurysms. In this study we have delved into the mechanisms underlying the beneficial effect of rolipram on AAA.
Methods: AAA were induced in ApoE$^{-/-}$ mice by angiotensin II (Ang II) infusion. Aneurysm formation was evaluated by ultrasonography. The expression of enzymes involved in redox homeostasis was analyzed by real-time RT-PCR and the activation of signaling pathways by Western blot.
Results: Induction of PDE4B in human AAA has been confirmed in a second cohort of patients. In Ang II-infused ApoE$^{-/-}$ mice, rolipram increased the percentage of animals free of aneurysms without affecting the percentage of aortic ruptures. Quantitative analyses determined that this drug significantly attenuated aortic collagen deposition. Additionally, rolipram reduced the increased *Nox2* expression triggered by Ang II, exacerbated Sod1 induction, and normalized Sod3 expression. Likewise, PDE4 inhibition decreased the activation of both ERK1/2 and the canonical Wnt pathway, while AKT activity was not altered.
Conclusions: The inhibition of PDE4 activity modulates the expression of enzymes involved in redox homeostasis and affects cell signaling pathways involved in the development of AAA.

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PALABRAS CLAVE
Aneurisma de aorta abdominal;
Rolipram;
Cell signaling

Resumen
Introducción: Las fosfodiesterasas de nucleótidos cíclicos (PDEs) de la subfamilia PDE4 son responsables de la hidrólisis y compartimentalización subcelular del AMPc, segundo mensajero que modula la funcionalidad vascular. Habíamos demostrado que PDE4B se induce en el aneurisma de aorta abdominal (AAA) y que la inhibición de la actividad PDE4 mediante rolipram limita la formación de aneurismas experimentales. En este estudio hemos profundizado en los mecanismos que subyacen al efecto beneficioso del rolipram sobre el AAA.
Métodos: Se ha utilizado el modelo de inducción de AAA por infusión de angiotensina II (Ang II) en ratones ApoE$^{-/-}$. La formación de aneurismas se ha evaluado mediante ultrasonografía. La expresión de enzimas implicados en el control de la homeostasis rédox se ha analizado por RT-PCR en tiempo real y la activación de vías de señalización mediante Western blot.
Resultados: Se ha confirmado la inducción de la PDE4B en el AAA humano en otra cohorte de pacientes. En ratones ApoE$^{-/-}$ infundidos con Ang II el rolipram incrementó el porcentaje de animales libres de aneurisma sin afectar al de rotura aórtica. Análisis cuantitativos determinaron que este fármaco atenuó significativamente la deposición de colágeno en la aorta. Además, el rolipram redujo el incremento en la expresión de *Nox2* en respuesta a la Ang II, promovió una mayor inducción del nivel de mRNA de *Sod1* y normalizó la expresión de *Sod3*. Asimismo, la inhibición de PDE4 disminuyó la activación de la ERK1/2 y de la vía canónica de Wnt, mientras la actividad AKT no se vio alterada.
Conclusiones: La inhibición de la actividad PDE4 modula la expresión de enzimas implicados en la homeostasis rédox y afecta a vías de señalización celular implicadas en el desarrollo de la patología aneurismática.

Introduction

Cyclic nucleotide phosphodiesterases (PDEs) are a superfamily of highly conserved enzymes that regulate cellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), second messengers that play a critical role in intracellular signalling.[1–3] These enzymes are phosphohydrolases that selectively catalyse the hydrolysis and inactivation of cAMP and cGMP, thereby regulating the magnitude and duration of their signalling, along with multiple physiological and metabolic processes, from inflammation, ion channel-dependent signalling, cell differentiation and apoptosis, muscle contraction, lipogenesis, glycogen synthesis, and gluconeogenesis.[4] Altered activity or expression of these enzymes has been linked to various pathologies including cardiovascular disease.

There are 11 families of PDEs (PDE1 to PDE11) that exhibit different selectivity towards their substrates.[5,6] The members of the PDE4 subfamily constitute the majority of cAMP-selective PDEs in different organs and cell types and are characterised by their sensitivity to inhibition by rolipram.[7–10] This enzyme subfamily has four members (PDE4A-D) with multiple variants that are expressed in a large number of tissues and have been implicated in numerous pathophysiological processes. Through control of the rate of cAMP degradation these enzymes are key in the regulation of vascular function by modulating endothelial permeability, angiogenesis, monocyte/macrophage activation, and the regulation of vascular smooth muscle cell (VSMC) proliferation, migration, differentiation, and contraction.[7,8]

Abdominal aortic aneurysm (AAA) is a chronic degenerative disease consisting of localised dilation of the aorta resulting from weakening of the vascular wall.[11,12] It is a vascular disorder that is often asymptomatic, but rupture leads to severe internal bleeding associated with high morbidity and mortality. The clinical management of patients with AAA is limited to surgical intervention of high-risk aneurysms, which is not complication-free and carries a high health care cost.[13] However, there are no pharmacological strategies available to limit progression and reduce the risk of aneurysm rupture,[14] and therefore it is essential to further investigate the pathophysiology of these diseases to identify new therapeutic targets to improve the treatment of these patients.[14,15]

Our previous studies in samples of aneurysmal aorta from both human and experimental models show increased vascular expression of PDE4B and how the inhibition of PDE4 activity by rolipram attenuates the development of AAA in a preclinical model.[16] In the present study, we confirm the induction of PDE4B in human aneurysm in a second cohort of patients and further investigate the mechanisms involved in the beneficial effect of rolipram on AAA in an experimental model, demonstrating how this drug modulates the expres-

sion of enzymes involved in redox homeostasis and affects cell signalling pathways.

Material and methods

Studies in human samples

Human samples corresponding to aneurysmal lesions were obtained from patients undergoing open surgical repair of AAA at the Hospital de la Santa Creu i Sant Pau (HSCSP), Barcelona; n = 27). Healthy abdominal aorta samples from multi-organ donors that showed no signs of aneurysmal or atherosclerotic pathology were also included in the study, as previously described (n = 15).[16–18] Patients with AAA were male, of whom 85.1% were smokers.[17] The HSCSP Ethics Committee (12/031/1316) approved the use of these samples, which was undertaken following the guidelines set by the Declaration of Helsinki. Written informed consent was obtained from patients and control individuals or their legal representatives. Aortic samples were rapidly stored at −80 °C for ribonucleic acid (RNA) extraction or processed appropriately for immunohistochemical analyses.

Animal model

Studies were performed in apolipoprotein E-deficient mice (ApoE$^{-/-}$; B6.129P2-Apoetm1Unc/J) kept in a pathogen-free environment under standard lighting (12 -h light/dark cycles) and temperature (21 °C ± 1 °C) conditions in the animal experimentation unit of the *Institut de Recerca del Hospital de la Santa Creu i Sant Pau* (IRHSCSP), Barcelona, Spain, with ad libitum access to food and drink throughout the procedure. The study was performed in males due to the sexual dimorphism that characterises this disease with a higher prevalence in males in both humans and animal models. AAA was induced by infusion of angiotensin II (Ang II; 1000 ng/kg/min) with micro-osmotic pumps (model 1004, Alzet; Durect Corporation) implanted subcutaneously.[16,17,19] The animals were randomised into three groups: saline-infused mice, Ang II-infused animals, and Ang II-infused mice treated with rolipram daily (3 mg/kg; i.p.; SelleckChem) from the day before implantation of the micro-pump. Saline +1% dimethyl sulfoxide (DMSO) was used as vehicle and both control animals and the Ang II-infused group of mice received an equivalent volume of the vehicle. After four weeks, the animals were anaesthetised with ketamine (150 mg/kg) and medetomidine (1 mg/kg), sacrificed by thoracotomy, and the aortas were removed and processed appropriately for further studies.

The research was conducted in accordance with the Spanish Animal Protection Policy RD53/2013, which complies with Animal Research: Reporting of in Vivo Experiments (ARRIVE), and the European Union Directive 2010/63/EU on the protection of experimental animals. All procedures were approved by the IRHSCSP Ethics Committee and the *Departament d'Acció Climàtica, Alimentació i Agenda Rural de la Generalitat de Catalunya* (project 11483).

Measurement of aortic diameter

The diameter of the abdominal aorta was measured by ultrasonography the day before micropump implantation and weekly after Ang II infusion. For this purpose, the animals were anaesthetised with 2% isofluorane and placed on a heated platform in the supine position. The precordium was then shaved, and abdominal ultrasound was performed using the Vevo 2100 ultrasound with a 30 MHz transducer to record the abdominal aorta as previously described.[16,17,19,20] Diameters greater than 1.5 mm were considered aneurysms. The percentage of aneurysm-free animals and the percentage that were free of aortic rupture were established throughout the procedure.

Real-time PCR expression analysis

Total RNA was isolated using TriPure Isolation Reagent (Roche Diagnostics) and reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and random hexamers. The mRNA level was quantified using the ABI PRISM 7900 H T sequence detection system and specific oligonucleotides and probes provided by Integrated DNA Technologies or the TaqMan™ gene expression assays-on-demand (Applied Biosystems) to detect mRNA levels in human samples of *PDE4B* (Hs00277080_m1) and *PDE4D* (Hs01579625_m1). The expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (*Nox1;* Mm00549170_m1), *Nox2* (Mm01287743_m1), *Nox4* (Mm00479246_m1) and the superoxide dismutases *Sod1;* Mm.PT.58.12368303), *Sod2* (Mm.PT.58.14276358), and *Sod3* (Mm.PT.58.12048855) was analysed in mouse samples. Human β-actin *ACTB* (β-actin; HS99999903_m1) and *TATA-binding protein* (*Tbp*; Mm00446973_m1) were used as normalisation genes in human and murine samples, respectively. Amplifications were made in duplicate, and the relative mRNA level was determined using the $2^{-\Delta\Delta CT}$ method.

Western blot

Protein lysates were subjected to electrophoresis on polyacrylamide gels (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride membranes (IPVH00010; Immobilon, Merck-Millipore). These membranes were blocked in a solution containing 5% skimmed milk in Tris-HCl buffer with .05% Tween 20 (TBST) at room temperature for one hour. The membranes were then incubated overnight at 4 °C and shaken with antibodies against active β-catenin (#88145; Cell Signaling), extracellular Signal-Regulated Kinase 1/2 (ERK1/2) total (#9102; Cell Signaling) and phosphorylated (#9106; Cell Signaling), and the Ser473-phosphorylated form of protein kinase B or AKT (#9271; Cell Signaling). After several washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Dako Products, Agilent) and then the bound antibodies were detected by the addition of the reagent Luminata™ Western Horseradish Peroxidase (HRP) Substrate (Immobilon, Merck-Millipore). The chemiluminescent signal was detected by autoradiographic films (CURIX RP2 plus, Agfa). The size of

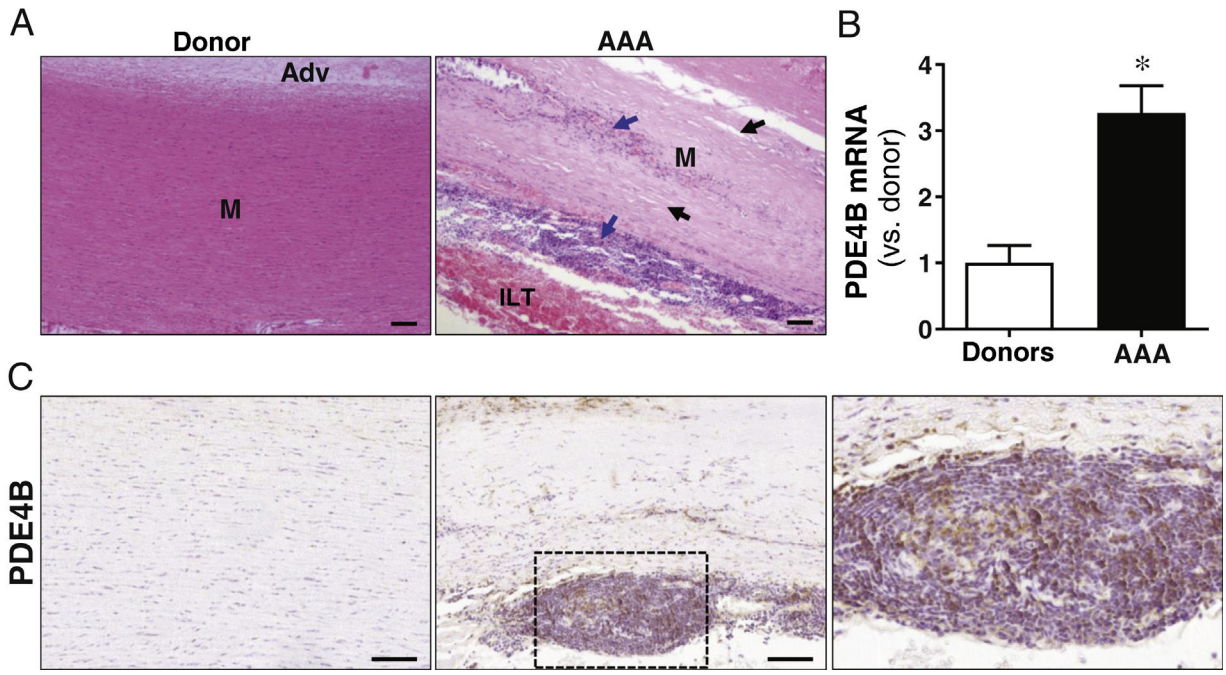

Figure 1 PDE4B expression is increased in human AAA. (A) Representative images of haematoxylin-eosin staining of abdominal aortic samples from healthy donors (Donor) and AAA patients (AAA) showing the disorganisation of the vascular wall in this disease characterised by significant inflammatory infiltrate (blue arrows) and loss of VSMC content (black arrows). The position of the media (M), adventitia (Adv), and intraluminal thrombus (ILT) detected in the aneurysmal lesion is indicated (Bars: 100 μm). (B) Quantification of PDE4B mRNA level in abdominal aorta from donors (n = 15) and AAA patients (n = 27). Results are shown as mean ± SEM. *p < .001 vs. donors. (C) Immunohistochemical analysis for PDE4B in these samples. The magnified region corresponding to the inflammatory infiltrate positive for PDE4B is indicated with an inset (Bars: 100 μm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

detected proteins was estimated using a molecular weight marker (HyperPAGE Prestained Protein Marker; Bioline) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels; MAB374 confirmed uniform loading per lane. The films were scanned in the GS-800 densitometer (BIO-RAD) and the relative quantification of the bands using Quantity One software (BIO-RAD).

Immunohistochemistry

Human and murine samples were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections (5 μm) were prepared with the Jung RM2055 microtome (Leica Microsistemas S.L.U). Samples were deparaffinised and rehydrated in a gradient of ethanol solutions. After antigen unmasking in citrate buffer (10 mM pH 2.5), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution in methanol. The samples were then incubated with 10% serum, endogenous avidin/biotin was blocked (Vector Laboratories), and incubated overnight at 4 °C with an antibody specific for PDE4B (NBP2-01171, Novus Biologicals). Conventional haematoxylin-eosin and Mason's trichrome staining was performed.

Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). Significant differences (for $p < .05$) were established by one-way ANOVA (Sidak's post hoc analysis). When the data distribution did not follow the Agostino-Pearson normality test, the Mann–Whitney U test or the Kruskal–Wallis test was used. The Mantel-Cox test was used to analyse occurrence of an event (aneurysm-free and rupture-free percentage) between two populations. Data were analysed with GraphPad Prism version 6.01 (GraphPad by Dotmatics).

Results

PDE4B expression in aortic aneurysm

Our group has previously shown that PDE4B expression is increased in human AAA.[16] These results were confirmed in a second cohort of patients. Compared to the healthy aorta, which shows a compact structure in which VSMCs are arranged in an orderly fashion, the vascular wall of AAA patients is partially disorganised and shows a significant inflammatory infiltrate as well as a loss of VSMCs (Fig. 1A). In these samples, real-time RT-PCR assays showed a significant increase in PDE4B mRNA level in aneurysmal lesions compared to healthy aortas from organ donors (Fig. 1B). Immunohistochemical studies confirmed the induction of PDE4B expression in AAA, where this enzyme is mainly localised in inflammatory cells (Fig. 1C). Expression in donor aorta was almost negligible (Fig. 1C).

Inhibition of PDE4 activity attenuates aneurysm formation and vascular remodelling in a preclinical model

Administration of rolipram in the ApoE$^{-/-}$ mouse model of AAA induction by Ang II infusion attenuated aneurysm formation (Fig. 2A).[16] Indeed, histological analyses showed that this drug markedly limited vascular remodelling (Fig. 2B) and significantly reduced Ang II-induced collagen deposition as assessed by Mason's trichrome staining (Fig. 2C). After Ang II administration, the percentage of aneurysm-free animals decreased progressively throughout the experimental procedure, and at the end of treatment was around 25% of the animals (Fig. 3A). Treatment with rolipram attenuated this response. In fact, the time pattern in the group of animals in which PDE4 activity was inhibited differed from that shown by the group infused with Ang II, and after 28 days this percentage was 75%, significantly higher than in the animals receiving Ang II alone (Fig. 3A). In contrast, no significant differences were observed in the percentage of animals free of aortic rupture (Fig. 3B).

Rolipram regulates the expression of enzymes involved in the control of redox homeostasis

Our previous studies had shown that rolipram decreases superoxide radical production in the abdominal aorta of the ApoE$^{-/-}$ mouse infused with Ang II.[16] We therefore characterised the expression of two of the enzyme systems that contribute to redox homeostasis in the vasculature. We specifically analysed the expression of different isoforms of NADPH oxidase and Sod family enzymes. In our experimental model, Ang II did not modify the mRNA level of *Nox1* and *Nox4* but significantly induced the expression of *Nox*2, an increase that was partially decreased by rolipram administration (Fig. 4A). In turn, Ang II induced vascular expression of *Sod1*, *Sod2*, and *Sod3* (Fig. 4B). Inhibition of PDE4 activity with rolipram exacerbated the increase in *Sod1* mRNA level and normalised *Sod3* expression without affecting the increase in *Sod2* expression (Fig. 4B).

Inhibition of PDE4 activity modulates cell signalling in the experimental AAA model

The ERK1/2 signalling pathway plays a fundamental role in vascular inflammation and activation of metalloproteases (MMPs), key processes in the development of AAA. Our analyses demonstrate that ERK1/2 activity detected in aneurysmal lesions of Ang II-infused ApoE$^{-/-}$ mice is significantly decreased in rolipram-treated animals (Fig. 5). In contrast, we observed no significant differences in AKT activation, which was not affected by treatment with this drug. In turn, the activation of the canonical Wnt or β-catenin-dependent pathway in AAA, previously described by our group,[21] was significantly reduced by rolipram treatment (Fig. 6).

Discussion

There are currently no pharmacological strategies to limit the development of AAA. The only therapeutic measure available is surgery (open or endovascular) for aneurysms at high risk of rupture (aortic diameter >5–5.5 cm).[13,14] Although it has been suggested that statins, doxycycline, cyclooxygenase-2 (COX-2), or angiotensin-converting enzyme inhibitors, among others, could reduce progression of AAA, none have shown a conclusive clinical benefit.[14] The lack of useful drugs in the treatment of AAA is a major obstacle to the clinical management of these patients, and therefore there is a clear need to identify and develop new pharmacological tools to treat and prevent this pathology.

Alteration of the cAMP level in the vascular wall could affect key processes involved in aneurysmal disease. Indeed, it has been shown that in human AAA there is a decrease in the expression of the alpha Gs subunit (GSα), which reduces cAMP production and aggravates the development of Ang II-induced aneurysms.[22] In this context, the PDE4 enzyme subfamily may also play an important role in the development of this disease due to its ability to regulate cAMP levels. PDE4 is considered one of the main therapeutic targets in inflammatory diseases, although its potential utility in aneurysm had not been explored in depth until our research that demonstrated the induction of PDE4B in both human and experimental AAA.[16]

This study has corroborated the increased expression of PDE4B in aneurysmal lesions in a second cohort of patients. In these lesions, PDE4B was mainly localised in the inflammatory infiltrate, consistent with this isoform being the main PDE,[4] enzyme expressed in inflammatory cells.[7] Our previous studies had discounted a relevant role of the PDE4D isoform in aneurysmal disease, although other authors later detected increased expression of PDE4D in VSMCs in patients with AAA, in murine aneurysmal lesions, without alteration of PDE4B.[23] The discrepancies between the two studies could be, related to the low number of human samples analysed by Gao et al.,[23] their heterogeneity, well as|as, differences in the animal models of AAA. In any case, both previous studies, the present study demonstrate that inhibition of PDE,[4] activity by rolipram attenuates the development of AAA.[16,23] In fact, rolipram increased the percentage of AAA-free animals. In contrast, we observed no significant difference in the incidence of aortic rupture, which is the main cause of mortality in this animal model. Sympathetic activation has been linked to aortic dissection,[19] since rolipram could increase the synthesis, release of noradrenaline[24] this phenomenon could be, involved in the lack of benefit of this drug in aortic rupture.

We reported that rolipram has a beneficial effect on vascular remodelling in AAA by preserving elastin integrity, decreasing MMP activity, and attenuating the increase in the level of collagen 1A1 mRNA.[16] The present study confirms the benefit of rolipram on extracellular matrix remodelling, as PDE4 inhibition decreased the increase in collagen in the aneurysmal lesion, an adaptive and compensatory response aimed at maintaining the mechanical stability of the aneurysmal wall that occurs in advanced stages of this disease.[25]

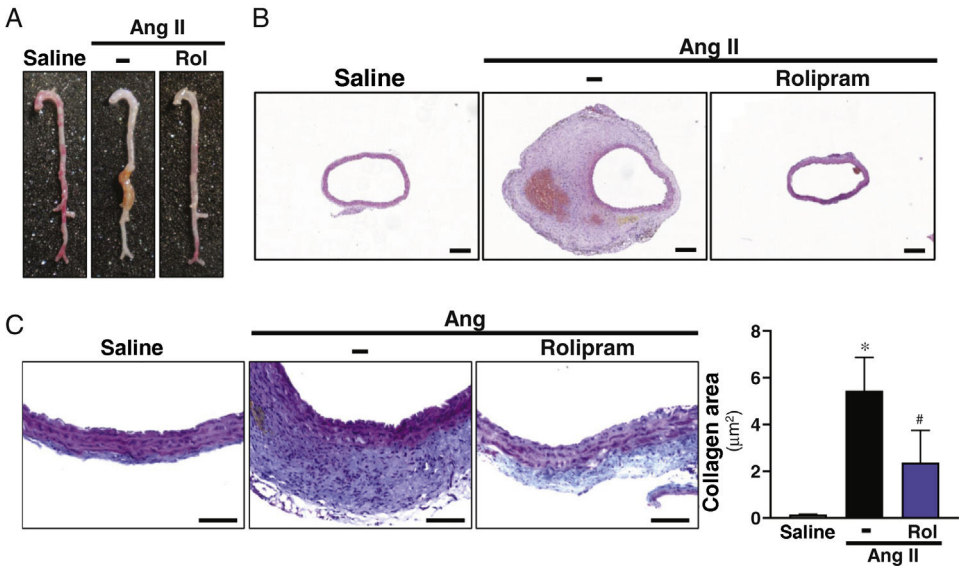

Figure 2 Rolipram treatment attenuates AAA formation in Ang II-infused ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were infused with saline or Ang II (1000 ng/kg/min) for 28 days and treated or not with rolipram (Rol; 3 mg/kg/day; i.p.). (A) Macroscopic images of aortas from each of the experimental groups. (B) Representative images of haematoxylin-eosin staining of sample sections from the groups indicated in A (Bars: 200 μm). (C) Representative images of Mason's trichrome staining of sample sections from the groups indicated in A (Bars: 100 μm). The histogram shows the quantification of the collagen-positive area. Results are shown as mean ± SEM $p < .05$: * vs. saline; # vs. Ang II. (saline, n = 5; Ang II, n = 10; rolipram, n = 6).

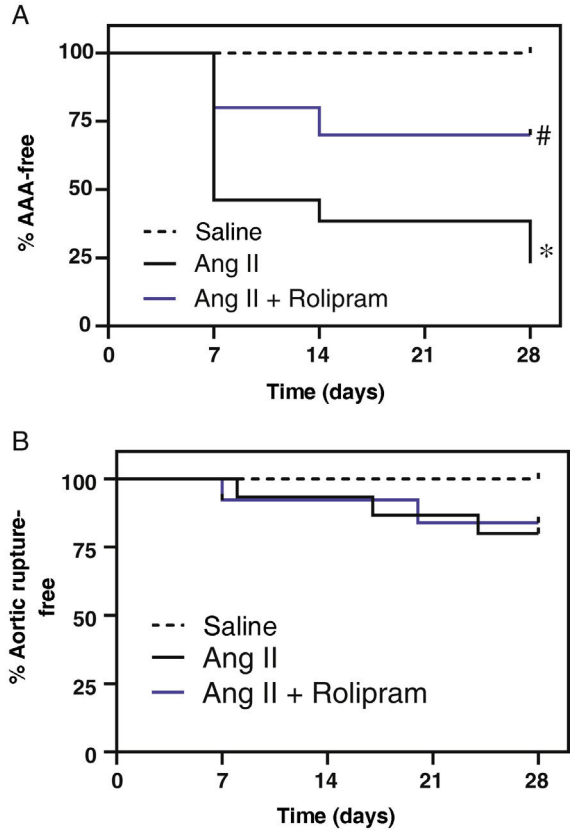

Figure 3 Rolipram attenuates AAA development in Ang II-infused ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were infused with saline or Ang II (1000 ng/kg/min) for 28 days and treated or not with rolipram (3 mg/kg/day; i.p.). (A) Percentage of AAA-free animals throughout the experimental period in each group is shown $p < 0.05$: * vs. saline; # vs. Ang II. (B) Percentage of animals free of aortic rupture (saline, n = 9; Ang II, n = 15; rolipram, n = 13).

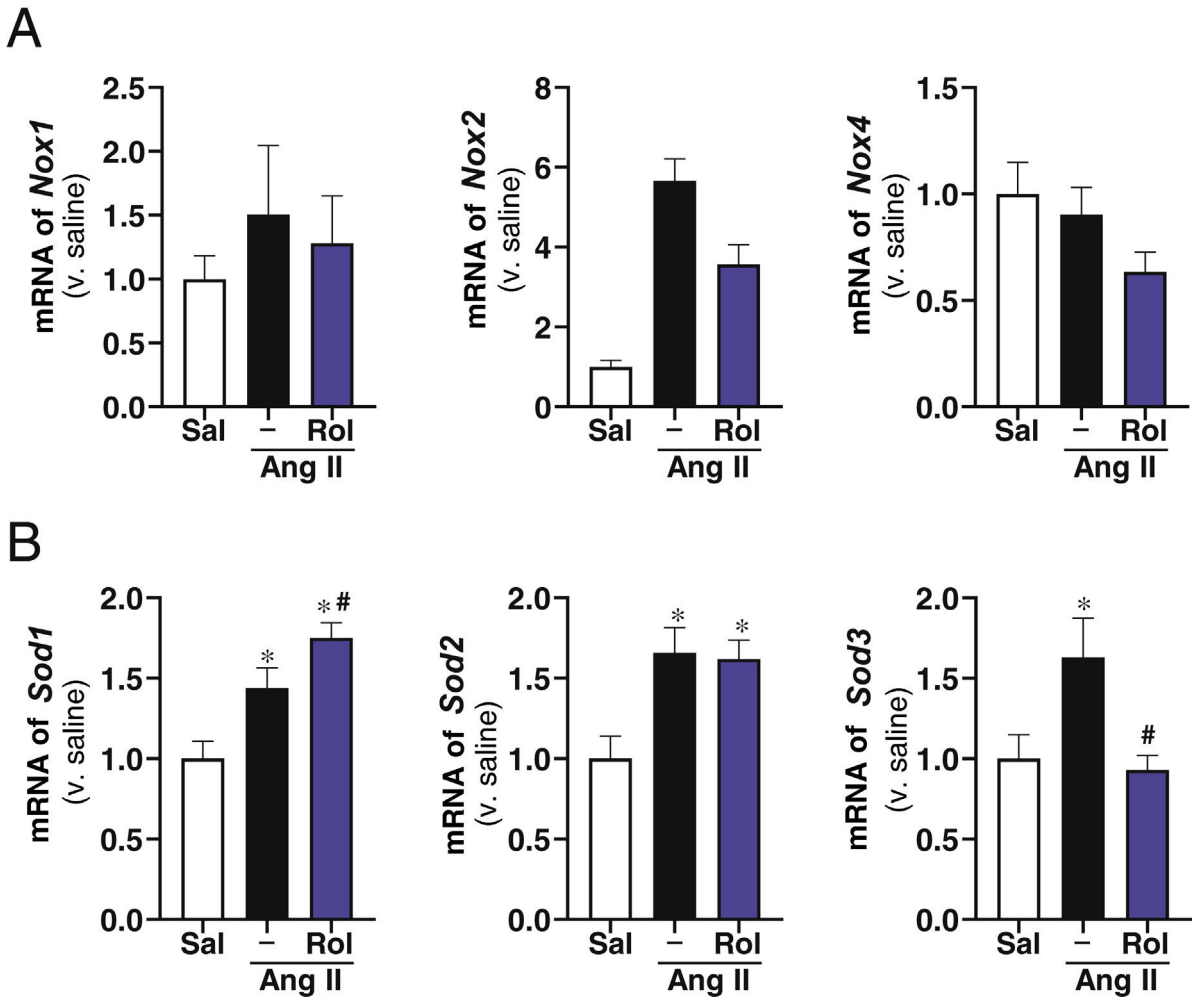

Figure 4 Effect of rolipram treatment on the expression of enzymes involved in redox homeostasis in Ang II-infused ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were infused with saline (Sal) or Ang II (1,000 ng/kg/min) for 28 days and treated or not with rolipram (Rol; 3 mg/kg/day; i.p.). (A) Expression levels for *Nox1*, *Nox2*, and *Nox4* assessed by RT-PCR in the abdominal aorta of each experimental group are shown (B) mRNA level for *Sod1*, *Sod2*, and *Sod3*. Results are shown as mean ± SEM. *p < .05 vs. saline. #p < .05 vs. Ang II alone (saline, n = 10; Ang II, n = 8; rolipram, n = 12).

We also found that rolipram reduces inflammatory infiltrate and attenuates vascular oxidative stress. Multiple studies have demonstrated the marked redox imbalance in the vascular wall of AAA patients and the association of oxidative stress with AAA pathogenesis.[26,27] The main source of reactive oxygen species (ROS) in the cardiovascular system is the NOX family of enzymes. In the aneurysmal lesions of our experimental model, we detected specific and significant induction of *Nox2*, a response that was attenuated in the presence of rolipram. Since deletion of *Nox2* is able to limit the development of AAA in experimental models,[28] this effect could be implicated in the benefit exerted by rolipram on the development of AAA. For their part, Sod enzymes are one of the main antioxidant defence mechanisms. Studies in experimental models of AAA have reported the early induction of Sod activity[29] and the expression of *Sod2*,[30] which has been proposed as a compensatory response to increased ROS in the aneurysm.[31] Similarly, we determined that the expression of the three Sod enzymes analysed (*Sod1*, *Sod2*, *and Sod3*) increased in aneurysmal lesions in the Ang II infused ApoE$^{-/-}$ mouse. Of note, rolipram exacerbated the increased mRNA level of *Sod1* (cytoplasmic isoform) in the abdominal aorta, which may contribute to its protective effect against AAA formation. This drug did not alter the mRNA level of *Sod2* (mitochondrial isoform) in response to Ang II; however, rolipram attenuated the induction of *Sod3* (extracellular isoform) detected in aneurysmal lesions, a response that would have little ultimate impact because rolipram nevertheless reduced the increase in vascular oxidative stress in our experimental model.[16]

The development of AAA is associated with the alteration of numerous signal transduction pathways, including the induction by phosphorylation of ERK1/2[32,33] and AKT[34–36] that play a key role in the activation of MMPs during AAA development.[33,34] Unlike AKT, rolipram was able to limit induction of ERK1/2 in our experimental aneurysm model, in line with previous studies in other pathological settings that have shown the efficacy of this drug in attenuating induction of ERK1/2 and thus limiting apoptosis and fibrosis, processes involved in the pathophysiology of AAA.[37,38]

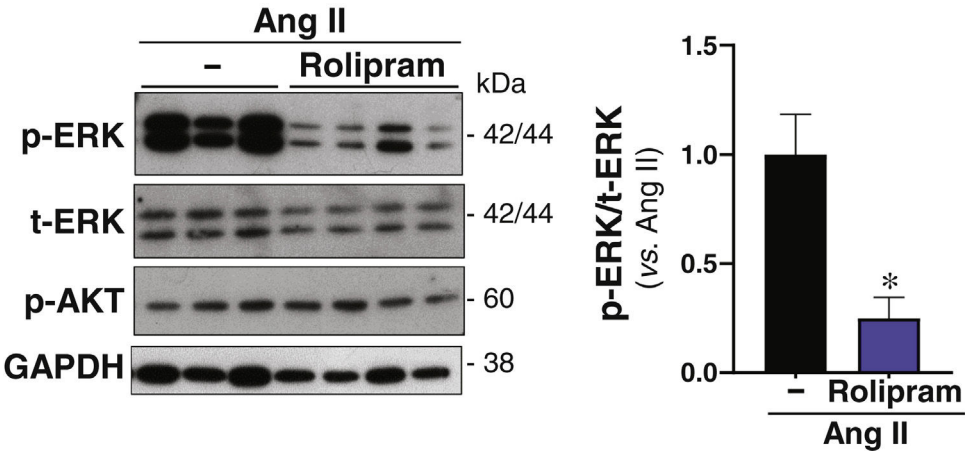

Figure 5 Effect of rolipram treatment on ERK1/2 and AKT activation in Ang II-infused ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were infused with Ang II (1,000 ng/kg/min) for 28 days and treated or not with rolipram (Rol; 3 mg/kg/day; i.p.). Representative images from Western blot analysis are shown for levels of the phosphorylated form of ERK1/2 (p-ERK1/2), total ERK (t-ERK), and the phosphorylated form of AKT (p-AKT). GAPDH levels are shown as loading control. The histogram corresponds to the relative quantification of the level of AKT phosphorylation in each experimental group. Results are shown as mean ± SEM (saline, n = 4; Ang II, n = 6; rolipram, n = 4). *p < .05 vs. Ang II alone.

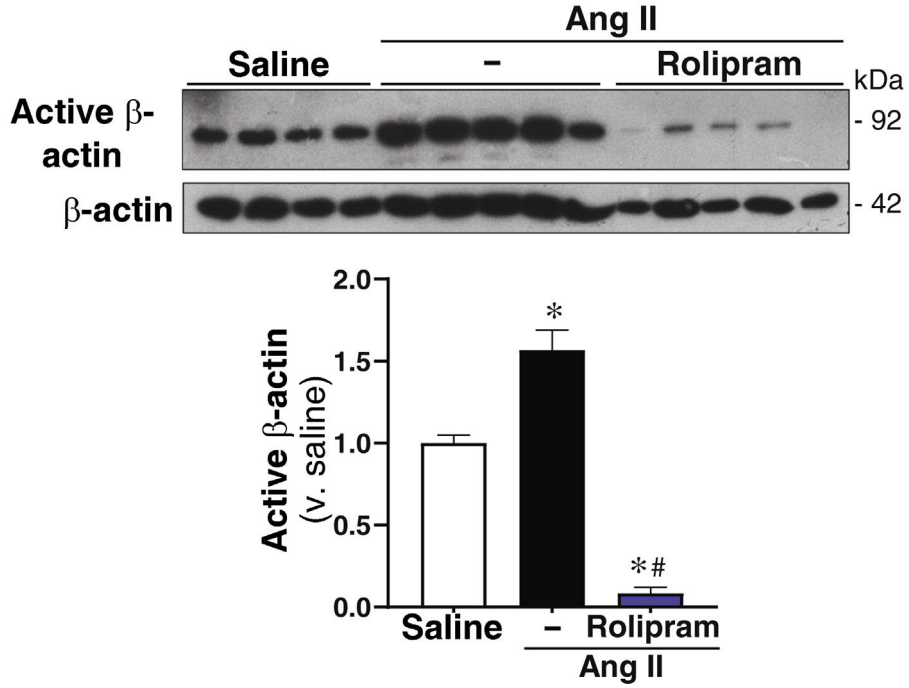

Figure 6 Rolipram attenuates canonical Wnt pathway activation in Ang II-infused ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were infused with saline or Ang II (1,000 ng/kg/min) for 28 days and treated or not with rolipram (Rol; 3 mg/kg/day; i.p.). Representative images of Western blot analysis corresponding to the levels of active β-catenin in each experimental group are shown. Levels of β-actin are shown as loading control. The histogram corresponds to the quantification of the level of active β-catenin in each experimental group. Results are shown as mean ± SEM. p < .001: * vs. saline; # vs. Ang II alone. (saline, n = 4; Ang II, n = 5; rolipram, n = 5).

In recent decades, Wnt signalling has attracted the attention of the scientific community due to its high therapeutic potential. In adults, this pathway is reactivated in several pathological scenarios, including cardiovascular disease, and, in relation to the cardiovascular system, excessive activation of Wnt signalling is considered to have detrimental consequences.[39] Our previous studies demonstrated that activation of the Wnt pathway is characteristic of aneurysmal disease.[21] Specifically, we observed that in human and experimental AAA there is an alteration of multiple components of Wnt-signalling associated with activation of the Wnt/β-catenin pathway.[21] Interestingly, in Ang II-infused animals, rolipram was able to significantly reduce the level of active β-catenin, an effect similar to that described for

other specific PDE4 inhibitors in models of acute myeloid leukaemia.[40] Inhibition of this pathway could be related to the suppression of osteoclastogenic macrophage activation, a phenomenon that would contribute to the development of AAA,[41] thus suggesting its contribution to the beneficial response exerted by rolipram.

In conclusion, this paper characterises the molecular mechanisms modulated by rolipram in aneurysmal disease, determining the impact of this drug on two of the enzyme systems that contribute to redox homeostasis in the vasculature (NOX and Sod isoforms) and on key signalling pathways in aneurysmal disease such as ERK1/2 and the Wnt pathway. These mechanisms could contribute to the beneficial effect on AAA of inhibiting PDE4 activity.

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Conflict of interests

Alicia Santamaría-Orleans is an employee of Laboratorios Ordesa S.L.

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