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# Limited Ability to Activate Protein C Confers Left Atrial Endocardium A Thrombogenic Phenotype

## A Role in Cardioembolic Stroke?

Jorge Cerveró, BsC; Ramón Montes, PhD; Francisco España, PhD;  
Charles T. Esmon, PhD; José Hermida, MD, PhD

**Background and Purpose**—Atrial fibrillation is the most important risk factor for cardioembolic stroke. Thrombi form in the left atrial appendage rather than in the right. The causes of this different thrombogenicity are not well-understood. The goal herein was to compare the activation of the anticoagulant protein C and the thrombomodulin and endothelial protein C receptor/activated protein C receptor expression on the endocardium between right and left atria.

**Methods**—We harvested the atria of 6 monkeys (*Macaca fascicularis*) and quantified their ability to activate protein C *ex vivo* and we measured the thrombomodulin and endothelial protein C receptor expression by immunofluorescence.

**Results**—We found the ability to activate protein C decreased by half ( $P=0.028$ ) and there was lower expression of thrombomodulin in the left atrial endocardium than the right ( $52.5 \pm 19.9$  and  $72.1 \pm 18.8$  arbitrary intensity units, mean  $\pm$  standard deviation;  $P=0.028$ ). No differences were detected in endothelial protein C receptor expression.

**Conclusions**—Impaired protein C activation on the left atrial endocardium attributable to low thrombomodulin expression may explain its higher thrombogenicity and play a role in cardioembolic stroke. (*Stroke*. 2011;42:2622-2624.)

**Key Words:** anticoagulation ■ embolic stroke ■ embolism ■ endothelium ■ protein C anticoagulant system ■ thrombomodulin

Atrial fibrillation is the most commonly sustained cardiac arrhythmia and the most important risk factor for cardioembolic stroke.<sup>1</sup> The left atrium, particularly the left atrial appendage (LAA), is the most frequent location of the embolic thrombi even when the right atrium undergoes the same pathological process.<sup>2</sup> The blood stasis at the LAA and the right atrial appendage (RAA) is similar,<sup>3</sup> so this cannot explain the higher propensity of thrombi to be formed in the LAA. In the search for additional factors to explain the difference between the 2 atria in thrombogenicity, study of their endothelial phenotypes deserves consideration. Attention recently has focused on the functional consequences of endothelial heterogeneity among different vascular beds.<sup>4</sup> Endothelium has been classically considered a thromboresistent surface because of the presence of receptors involved in several antithrombotic pathways. Among these, thrombomodulin and the endothelial protein C/activated protein C receptor (EPCR) play a crucial role in the protein C anticoagulant pathway.<sup>5</sup> EPCR expression is high in large arteries and veins but is virtually absent in capillaries.<sup>6</sup> Thrombomodulin expres-

sion is particularly high in the pulmonary endothelium and is low in the brain endothelium.<sup>7</sup> Differences also have been reported within 1 vessel: in veins, both EPCR and thrombomodulin are expressed more in the valvular sinus endothelium as opposed to the vein luminal endothelium.<sup>8</sup> The association between protein C deficiency and thrombosis is well-documented.<sup>9</sup> For this reason, we propose the hypothesis that the left atrium has a lower ability to activate protein C than the right one.

## Materials and Methods

### Collection of Simian Atria

The appendages of the left and right atria of each animal (*Macaca fascicularis*) were harvested. Details of the study are provided in Supplemental Materials (<http://stroke.ahajournals.org>).

### Protein C Activation Assay on the Atrial Surfaces

The experiments were performed in the 2 hours after surgical removal of the atria. Two tissue slices 6 mm in diameter were cut from each atrial appendage using a circular biopsy punch (Stiefel) and were placed separately at the bottom of a 48-well plate (Corning). The experiment was performed as described<sup>10</sup> with minor

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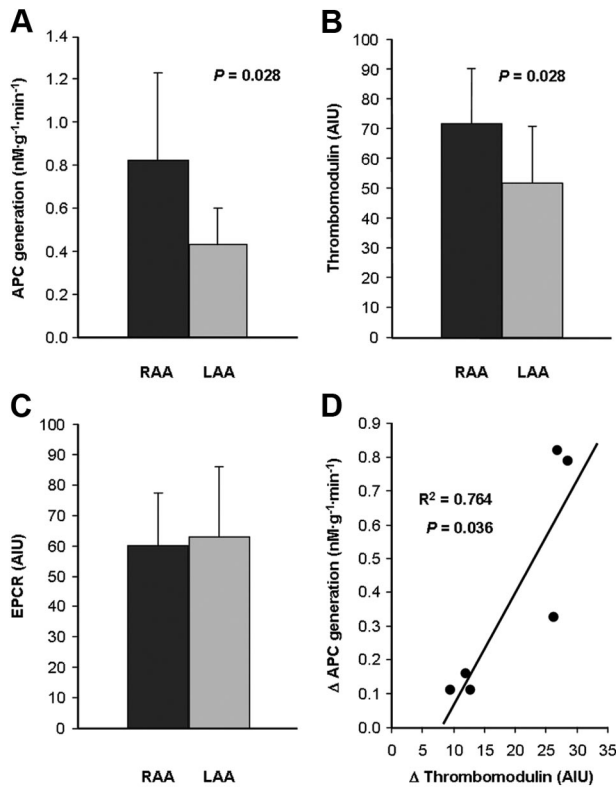
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**Figure 1.** The protein C system in the endocardium of the right atrial appendage (RAA) and left atrial appendage (LAA). **A**, Thrombin-dependent generation of activated protein C (APC) was measured ex vivo on the endocardial surface of RAA and LAA samples of macaque origin ( $n=6$ ). The protein C activation assay was performed in duplicate. The mean  $\pm$ SD of 6 independent experiments is represented. Thrombomodulin (**B**) and endothelial protein C/activated protein C receptor (EPCR) (**C**) expression on the endocardial surface of the RAA and LAA. The mean  $\pm$ SD of the tissue expression is represented in arbitrary intensity units (AIU). **D**, The  $R^2$  Spearman correlation coefficient between the difference in the thrombomodulin expression (RAA–LAA) and the difference in the APC generation (RAA–LAA).  $\Delta$ , RAA–LAA.

modifications, which are described in Supplemental Materials. Activation was thrombomodulin-dependent and EPCR-dependent (Supplemental Figure I, <http://stroke.ahajournals.org>).

### Immunofluorescence Analysis of the Atrial Expression of Thrombomodulin and EPCR

Images were analyzed as described in Supplemental Materials.

### Statistical Analysis

Data handling is described in Supplemental Materials.

## Results

In 6 out of 6 cases, activation of protein C was higher on the RAA endocardium than on the LAA one. On average, generation of activated protein C (APC) was twice as high on the right atrial surface (Figure 1A). APC not only plays a major anticoagulant role but also is also involved in a variety of cytoprotective mechanisms in response to inflammatory stimuli.<sup>11</sup> It is well-known that inflammation plays a role in the initiation and maintenance of atrial fibrillation as well as in thrombosis.<sup>11,12</sup> Thus, the right atrium would be better-prepared to prevent the formation

of thrombi arising as a consequence of the atrial fibrillation. The fact that changes in APC circulating levels have been shown to influence the thrombotic risk reinforces this notion.<sup>13</sup> Furthermore, low protein C levels recently have been associated with ischemic stroke, particularly the cardioembolic type, in the Atherosclerosis Risk in Communities Study.<sup>14</sup>

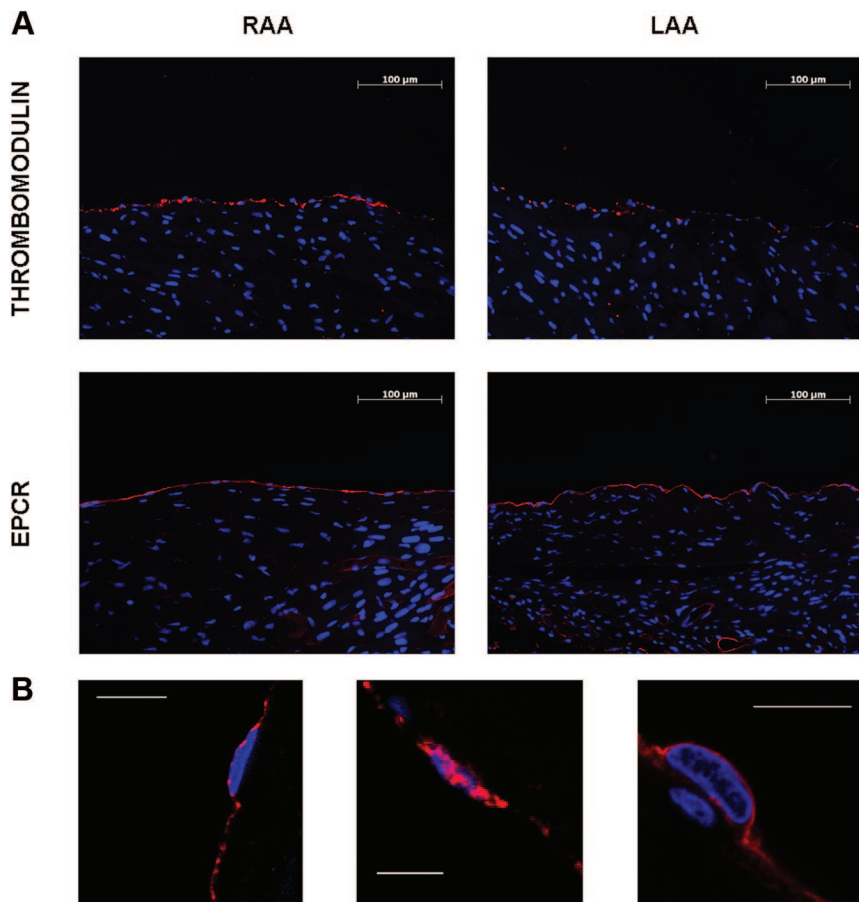
In the search for an explanation for our results, we focused our attention on the 2 endothelial receptors involved in protein C activation. Thrombin must bind thrombomodulin for activation to proceed efficiently, but the cofactor role played by EPCR is also relevant.<sup>5</sup> We therefore decided to study the expression of thrombomodulin and EPCR on the RAA and LAA endocardium of the same animals used for the protein C activation assays. As expected, thrombomodulin and EPCR were located on the cell surface as shown by confocal images (Figure 2A, B). The von Willebrand factor labeling served to ensure specific endothelial staining (Figure 2B). Again, in 6 out of 6 animals thrombomodulin expression was higher in the RAA endocardium. The mean intensity values were  $72.1 \pm 18.8$  and  $52.5 \pm 19.9$  (mean  $\pm$ SD) for the RAA and LAA endocardium, respectively (Figure 1B, 2A). EPCR expression did not differ between RAA and LAA (Figure 1C, 2A). The presence of thrombomodulin on the endothelial surface is crucial for APC generation. Therefore, these data correspond with the finding that the RAA endocardium displays a higher capacity to activate protein C than its left counterpart. The strong positive correlation found between the RAA versus LAA difference in APC generation capacity and the RAA versus LAA difference in thrombomodulin expression ( $R^2=0.764$ ) further substantiates the notion that the higher expression of TM is responsible for the higher ability of RAA to generate APC (Figure 1D). The biological mechanisms that lead to the reduced thrombomodulin expression in the LAA are presently unknown, although they could involve differences in vascular beds expression, shedding, internalization, or exposition to different local regulating factors.

## Discussion

The main drawback of this study is that we analyzed nonhuman primate samples and, therefore, application of these results to humans is not immediate. However, because the majority of the findings obtained in other studies dealing with the protein C system using nonhuman primates have been proven to apply to humans, we consider it reasonable to apply our results to the human setting.<sup>6,15</sup>

## Conclusions

In conclusion, for the first time to our knowledge the ability of the left atrial endocardium and right atrial endocardium to activate protein C has been compared. The right atrial endocardium exhibits a more thromboresistant phenotype, at least regarding this anticoagulant mechanism, which would help to explain why thrombi originate in the left atrium. Increasing thrombomodulin expression on the endocardium of the left atrium could provide a new approach in preventing cardioembolic stroke associated with atrial fibrillation.



**Figure 2.** Representative immunofluorescence and confocal images of the right atrial appendage (RAA) and left atrial appendage (LAA) expression of thrombomodulin and endothelial protein C/activated protein C receptor (EPCR). **A**, Representative captures of thrombomodulin and EPCR on the endocardium of the RAA and LAA. Thrombomodulin and EPCR are stained red. Blue 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. **B**, Confocal microscopy captures of thrombomodulin (left), von Willebrand factor (middle), and EPCR (right), all red, in representative endothelial cells. The von Willebrand factor staining was used as an endothelial-positive control. Blue Topro-3 was used to stain the nuclei. The scale bars indicate 10 μm.

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### Disclosure

None.

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**ONLINE SUPPLEMENT:**

**“Limited ability to activate protein C confers left atrial endocardium a thrombogenic phenotype. A role in cardioembolic stroke?”**

## Supplemental Methods

### Collection of simian atria

The appendages of the left and right atria of each of six male monkeys (*Macaca fascicularis*) enrolled in a Parkinson's study were harvested. The study had been approved by the Animal Experimentation Committee of the University of Navarra. Parkinson's disease was induced in four out of six monkeys by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). No side effects on the endothelial function have been described for MPTP. Animals were sacrificed at least fourteen weeks after the last MPTP dosage. The other two animals were not MPTP exposed. The mean age at the time of sacrifice was 55 months (range = 51-60). The animals were anesthetized with an intravenous single dose of 15mg/Kg ketamine and 0.75mg/Kg xylazine and gently perfused with sterile phosphate buffered saline (PBS) (Invitrogen, San Diego, CA, USA) immediately before the cardioectomy. The atria were collected separately. The appendages were divided into two portions, which were embedded in PBS or fixed in 4% paraformaldehyde for four hours at 4°C for subsequent protein C activation assay or immunofluorescence staining respectively.

### Protein C activation assay on the atrial surfaces

100 nM protein C and 0.02 U/mL thrombin (Enzyme Research Laboratories, Swansea, UK) were added to each well. 60 minutes afterwards, lepirudin (Schering AG, Berlin, Germany) was added to inhibit thrombin and the chromogenic substrate S-2366 (Chromogenix, Milan, Italy) was subsequently used at 0.4 mM to kinetically monitor APC generation in an absorbance microplate reader (iEMS-Reader, Labsystems, Finland). Dry weight of the slices was determined for normalization purposes and all the measurements were performed in duplicate. Known amounts of APC (Xigris, Eli Lilly, Indianapolis, IN, USA) were used to build a standard curve and convert absorbance units into concentrations of generated APC.

Additional controls to determine the thrombomodulin-EPCR dependence of APC generation were performed. Atrial samples were incubated with either anti-thrombomodulin M0617 (DAKO, Carpinteria, CA, USA) or anti-EPCR RCR-252, gently provided by Dr. K. Fukudome (Saga Medical School, Japan). Both antibodies decreased APC generation (Figure S1).

### Immunofluorescence analysis of the atrial expression of thrombomodulin and EPCR

Three µm thick tissue sections were cut from each atrial appendage. The following processing steps were performed: deparaffinization, rehydration, microwave antigen retrieval with 10 mM sodium citrate dihydrate and endogenous peroxidase inhibition. Tissue sections were then blocked in preimmune rabbit or goat serum. The following antibodies were: anti-thrombomodulin monoclonal antibody (mAb) MCA641 (AbD Serotec, Oxford, United Kingdom), anti-EPCR mAb 1489<sup>1</sup> and anti-von Willebrand factor polyclonal antibody A0082 (DAKO). Appropriate biotinylated secondary antibodies were used for later signal amplification with *Tyramide Signal Amplification Cyanine3 kit* (Perkin Elmer, Waltham, MA, USA). Nuclei were contrasted with DAPI (Vector Laboratories, Burlingame, CA, USA). Negative controls were performed for each primary antibody to check non-specific staining, and no immunofluorescence labeling was detected. We demonstrated the integrity of the endothelial lining by staining serial slices for EPCR, as a specific endothelial marker. As additional controls

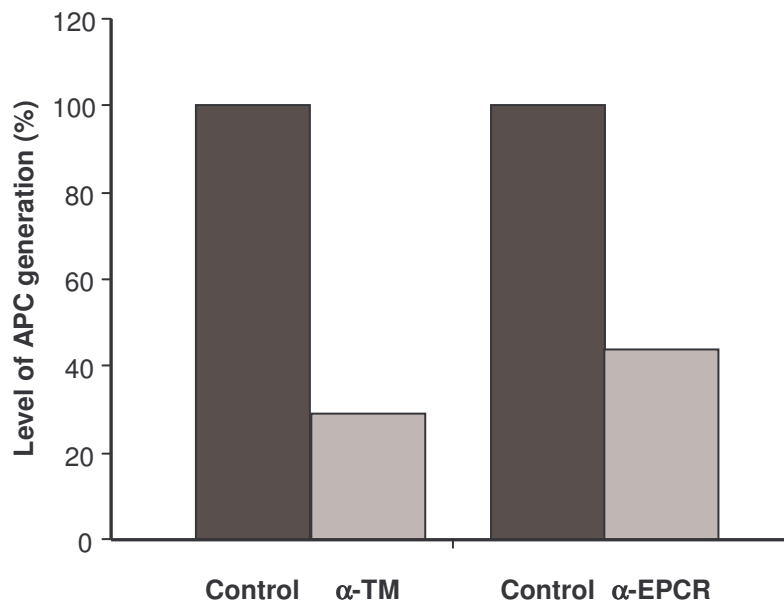
we stained several slices with hematoxiline-eosine and, again, we demonstrated the integrity of the endothelial lining.

The microscope used was a Zeiss Axio Imager M1 automated fluorescence microscope (Zeiss, Obercochen, Germany) equipped with an AxioCamm MRm camera, and a Plan-Neofluar 10X/0.3 NA objective lens (Zeiss). All images were captured at 24-bit depth using Axiovision software (Zeiss) The images were acquired as mosaics, combining several single camera shots, in order to cover all the atrial appendage area. The images were acquired using the appropriate excitation and emission filters. To allow proper quantitative analysis, the detector gain was adjusted for each pair of atria, slightly below the level of saturation.

Uncompressed, 24 bit TIFF color images were analyzed using in house developed software. The software was developed using Matlab 7.1.0.83 R14 (SP3) and the image processing library Dilib, version 1.6 (<http://www.dilib.org/>), on a system running Red Hat Linux Enterprise 5.5 O.S. The segmentation algorithm first detected the image borders using Laplace filtering followed by the second Gaussian derivate along the gradient. Then, all negative objects were deleted, the borders smoothed and overlaid on the original image, to allow the user to check and confirm the segmentation. Once confirmed, the results were stored in an excel file. The parameters measured were the analyzed area (mean  $\pm$  standard deviation,  $21.1 \pm 13.4 \text{cm}^2$ ), analyzed perimeter (mean  $\pm$  standard deviation,  $3.1 \pm 2.0 \text{cm}$ ) and mean intensity (range 0-256). This software was developed by the Imaging Laboratory of our institution and is available upon request. Selected areas of interest were imaged with a Zeiss LSM 510 confocal microscope (Zeiss) using a Plan-Apochromat 63X/1.40AN Oil objective lens (Zeiss).

### **Statistical analysis**

The Wilcoxon matched-pairs signed ranks test was used to compare both the APC generation and the expression of receptors between RAA and LAA. The differences between both atria (RAA-LAA) in APC generation and in thrombomodulin expression were calculated for each animal and the Spearman's rank correlation coefficient was determined.



**Figure S1. Thrombomodulin and EPCR dependence of APC generation.** Thrombin-dependent generation of APC was measured *ex vivo* on the endocardial surface of atrial appendages samples. The protein C activation assay was performed in duplicate. Addition of specific blocking antibodies against thrombomodulin (MCA641) and EPCR (RCR-252) were used to demonstrate the thrombomodulin and EPCR dependence of the assay.



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