ONLINE SUPPLEMENT:

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

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 4ºC 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[1] 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 bellow 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 Diplib, version 1.6 (http://www.diplib.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 overlayed 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 ± standard deviation, $21.1\pm13.4cm^2$), analyzed perimeter (mean ± standard deviation, 3.1±2.0cm) 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.
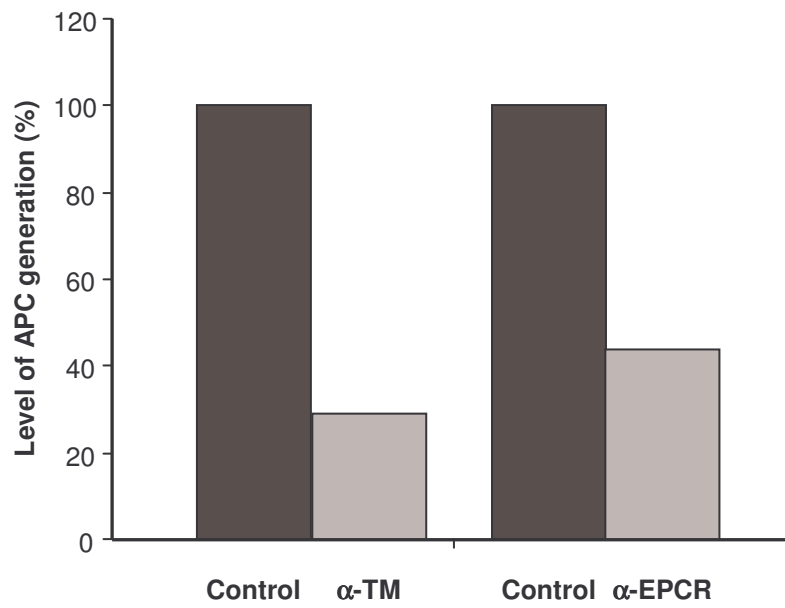
Figure S1. Thrombomodulin and EPCR dependence of APC generation. Thrombin-dependent generation of APC was measured *ex vivo* on the endocardial surface of atrial appendages samples. The protein C activation assay was performed in duplicate. Addition of specific blocking antibodies against thrombomodulin (MCA641) and EPCR (RCR-252) were used to demonstrate the thrombomodulin and EPCR dependence of the assay.

References

1. Laszik Z, Mitro A, Taylor FB Jr, Ferrell G, Esmon CT. Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. *Circulation*. 1997;96:3633-3640.