Microvascular COX-2/mPGES-1/EP-4 axis in human abdominal aortic aneurysm

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Running title: PGE2-pathway in AAA-associated angiogenesis

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Abreviations:
LD: Low Diameter
MD: Moderate Diameter
HD: High Diameter
AAA: abdominal aortic aneurysm
MVEC: Microvascular Endothelial Cell
AAc: Arachidonic Acid
NA: Normal Aorta
EC: Endothelial Cell
Abstract

We investigated the PGE$_2$-pathway in human abdominal aortic aneurysm (AAA) and its relationship with hypervascularization. We analyzed samples from patients undergoing AAA repair in comparison with those from healthy multiorgan donors. Patients were stratified according to maximum aorta diameter: low (LD, <55 mm), moderate (MD, 55-69.9 mm) and high diameter (HD, ≥70 mm). AAA was characterized by abundant microvessels in the media and adventitia with perivascular infiltration of CD45-positive cells. Like endothelial cell markers, COX-2 and m-PGES-1 transcripts were increased in AAA (4.4- and 1.4-fold, respectively). Both enzymes were localized in vascular cells and leukocytes, with maximal expression in the LD group, whereas leukocyte markers display a maximum in the MD-group, suggesting that the up-regulation of COX-2/mPGES-1 precedes maximal leukocyte infiltration. Plasma and in vitro tissue secreted levels of PGE$_2$-metabolites were higher in AAA than in controls [plasma-controls 19.9±2.2, plasma-AAA: 38.8±5.5 pg/mL; secretion-NA: 16.5±6.4; secretion-AAA 72.9±6.4 pg/mg; mean±SEM]. EP-2 and EP-4 were overexpressed in AAA, EP-4 being the only EP substantially expressed and colocalized with mPGES-1 in the microvasculature. Additionally, EP-4 mediated PGE$_2$-induced angiogenesis in vitro. We provide new data concerning mPGES-1 expression in human AAA. Our findings suggest the potential relevance of the COX-2/mPGES-1/EP-4 axis in the AAA-associated hypervascularization.

Supplementary Keywords: cyclooxygenase-pathway, Prostaglandin, angiogenesis
Introduction

Abdominal aortic aneurysm (AAA) is a late-age onset disorder that affects a high percentage of population in industrialized countries, and rupture of AAA is associated high mortality rates [1]. The etiology of AAA is complex with a relevant contribution of genetic factors [2]. Although much effort has been made to clarify the mechanism of AAA development, currently the only effective approach to prevent aneurysm rupture is surgical repair by conventional or endovascular techniques.

Evidences have established a relationship between atherosclerosis and AAA, both disorders characterized by an underlying chronic inflammation. However, there are marked differences between atherosclerotic lesions and AAA. Whereas atherosclerotic plaque is characterized by leukocyte infiltration at the lumen site and hyperproliferation of vascular smooth muscle cells (VSMC) causing neointimal hyperplasia, AAA is characterized by leukocyte infiltration into adventitia and depletion of VSMC in the media. Other relevant features of AAA are the wall tension strength breakdown caused by proteolytic enzymes progressively destructing elastic fibers [3] and hypervascularization of aortic tissue. It has been proposed that this vascularization might contribute to the development and rupture of aneurysms [4,5].

Prostaglandin (PG) E₂ has been recognized as a relevant mediator in AAA. Biosynthesis of PGE₂ begins with the formation of PGH₂, through the action of cyclooxygenase (COX) [6,7] on arachidonic acid (AAc) released by phospholipases from the membrane phosphoglycerides. PGH₂ is then isomerized to PGE₂ by PGE-synthases (PGES). The microsomal isoform of PGES (mPGES-1) is inducible by proinflammatory cytokines and seems to be the main isoenzyme involved in PGE₂ biosynthesis under inflammatory conditions [8-11]. COX-2/mPGES-1 is widely regarded as the major contributing enzymatic chain for PGE₂ biosynthesis under pathological conditions. COX-2-derived PGE₂ is involved
in the pathogenesis of AAA as data from animal models and human studies indicate [12-15]. Furthermore, deletion of mPGES-1 attenuates experimental AAA in mice [16].

PGE₂ exerts its cellular effects by binding to four distinct E-prostanoid receptors (EP1–4) that belong to the family of seven transmembrane G protein-coupled receptors. Each receptor is involved in different and often opposite biological effects of PGE₂. EP-2 and EP-4 are both Gs coupled receptors that upregulate intracellular cAMP levels, whereas EP-3 usually counteracts EP-2 and EP-4 mediated upregulation of cAMP by preferentially coupling to Gi proteins [17]. Recently, conflicting results have been reported on the role of EP-4 in AAA development in animal models [18-20]. These studies have been focused on the involvement of EPs in the activation of leukocytes (mainly macrophages) and VSMC and in the release of proteases during the immune-inflammatory process associated to AAA.

Main data regarding COX-2-alternative targets for the development of new anti-inflammatory drugs for AAA comes from studies in animal models [16,18-20]. In these models, AAA develops fast and its etiology differs substantially from human pathology. Despite the role of PGE₂ in neovascularization in cancer and other pathologies and that the relevance of angiogenesis in AAA is widely accepted, information concerning COX-2/mPGES-1 derived PGE₂ in the AAA, particularly in AAA-associated hypervascularization, is limited and restricted to COX-2-derived PGE₂ from macrophages [12,13,21,22]. Furthermore, the beneficial effects of COX-2 inhibitors and the deletion of mPGES-1 or EP-4 in experimental AAA is not fully understood in the context of this pathology in humans. Since PGE₂ modifiers have a possible therapeutic potential, the objective of the present study was to examine the elements involved in PGE₂ pathway in human AAA, particularly in microvasculature.
Materials and Methods

Tissue samples. The study was approved by the Hospital de la Santa Creu i Sant Pau (HSCSP) Ethics Committee, and informed consent was obtained from each patient. All procedures were reviewed by the Institutional Review Board at HSCSP. The aorta biopsies were obtained from patients undergoing open repair for AAA at our institution. Samples were obtained from remaining mid-infrarenal aortic wall after exclusion and prosthetic replacement of AAA. Normal aortas were obtained from healthy aorta of multiorgan donors and samples were also taken from the mid-portion of the infrarenal abdominal aorta at the time of organ harvest. When Luminal thrombus was present in AAA samples it was separated before the aorta biopsy was taken and aortic tissue was washed twice with cold phosphate buffered saline (PBS). A portion of each sample was placed in RNAlater solution (Qiagen GmbH, Hilden, Germany) and stored at 4°C for 24 hours before long-term storage at −80°C until further processing for RNA isolation. Another portion was fixed in formalin solution 10% (Sigma-Aldrich, Inc St Louis, MO) for 24 h and included in paraffin for immunohistochemical studies. Additional portions of aorta were placed in PBS to establish VSMC cultures and to obtain tissue secretomes.

Patients were stratified by the maximum transverse diameter defining three groups: low diameter (LD; <55 mm), moderate diameter (MD; 55-69.9 mm) and high diameter (HD, ≥70 mm). To determine the maximum aortic diameter, we use a transversal measurement to the true lumen center line at infrarenal level, based on Angio-CT with endovenous contrast, using Workstation AGFA IMPAX 6.4.0.4010 and OsiriX MD, FDA Cleared/CE IIa version, for primary diagnostic. Since surgical repair is not usually indicated in patients with AAA maximal diameter <55 mm, the aorta samples of the LD group were from patients with concomitant iliac artery aneurysm to be surgery repaired. Table 1 shows the characteristics of the individuals included in the study.
**Analysis of mRNA levels in the tissues and culture cells.** For total RNA extraction, tissues samples stored in RNAlater were homogenized in the FastPrep-24 homogenizer and Lysing Matrix D tubes (MP Biomedicals, Solon, OH) and RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Total RNA was extracted from cell cultures using Ultraspec (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer’s instructions. cDNA was prepared by reverse transcribing 1 µg RNA with High-Capacity cDNA Archive kit with random hexamers (Applied Biosystems, Foster City, CA). mRNA expression of the selected genes was studied by real-time PCR in an ABI Prism 7900HT using pre-designed validated assays (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA) and universal thermal cycling parameters. Relative expression was expressed as transcript/β-actin ratios.

**Analysis of PGE₂.** To analyze plasma levels, a sample of 10 mL of peripheral blood was collected from all participants in heparin-containing tubes. In the case of AAA patients, blood was collected before anesthesia in the operating room. It was centrifuged immediately and plasma aliquoted and frozen at -80°C until analysis. It is known that PGE₂ is rapidly transformed in vivo into 13,14-dihydro-15-oxo-PGE₂ that is unstable, and it undergoes a further transformation into 13,14-dihydro-15-oxo-PGA₂ [23,24]. To evaluate plasma levels of PGE₂ we used an enzyme immunoassay (EIA) kit that converts 13,14-dihydro-15-oxo metabolites of PGE₂ into a single stable derivative (Cayman Chemical, Ann Arbor, MI) following manufacturer’s instructions.

Secretomes were obtained from approximately 150 mg of NA and AAA aorta fragments by incubating the tissues in 1 mL serum-free DMEM (Biological Industries, Kibbutz Beit Haemek, Israel) in a cell incubator for 48 hours. Medium was then recovered and kept at -80°C until PGE₂ metabolites, 6-oxo-PGF₁α, TxB₂ and leukotriene (LT) B₄ released were analyzed by EIA (Cayman Chemical) following manufacturer’s instructions.
Immunohistochemistry. Immunohistochemical studies were performed using a mouse monoclonal antibody anti-COX-2 (ref 160112 clone CX229, diluted 1:50), rabbit polyclonal antibodies against EP-2 (ref 101750, diluted 1:1000), EP-3 (ref 101760, diluted 1:400) and EP-4 (ref 101775, diluted 1:100) all from Cayman Chemical, and a rabbit polyclonal antibody against mPGES-1 (ref HPA045064 prestige antibodies, diluted 1:50) from Sigma. Blanks were performed using the corresponding blocking peptides all from Cayman. Monoclonal antibodies (ref M0616, diluted 1:35; and ref IR751, without further dilution) from Dako were used for von Willebrand Factor (vWF, EC marker) and CD45 (leukocyte marker) immunostaining. Three-micrometer sections of paraffin-embedded tissue samples were stained in a Dako Autostainer Link 48 using the Dako EnVision Flex Kit. Diaminobenzidine was used as chromogen. Immunostainings used for comparative purposes were processed simultaneously.

Microvascular endothelial cells (MVEC) culture. MVEC were isolated from human adult foreskins using a previously described technique [25,26]. In brief, foreskins obtained from adult circumcisions were placed in PBS supplemented with penicillin 200 units/mL, streptomycin 200 μg/mL and amphotericin B 0.5 μg/mL (all from Biological Industries). Foreskins were cut into 3 mm squares and placed in PBS containing 0.3% trypsin and 1% EDTA at 37ºC for 30 minutes. Segments were then washed several times with PBS, placed in a Petri dish in M199 containing 10% FBS, and individually compressed with the side of a scalpel blade to express microvascular fragments. The microvascular segments were passed through a 150 μm stainless steel mesh and collected by centrifugation at 300xg for 15 minutes. MVEC were seeded on a gelatin-coated cell culture flask and cultured in medium MCDB 131 with 20% FBS; L-glutamine 2 mmol/L, penicillin 200 units/mL, streptomycin 200 μg/mL, EGF 20 ng/mL and bFGF 5 ng/mL (all from Biological Industries). When cells reached confluence, they were purified with Dynabeads CD31 (Dynabeads, Invitrogen Dynal ASA, Oslo, Norway) following the manufacturer’s instructions. Flow cytometry and positive staining for CD31, platelet endothelial cell adhesion molecule-1
(PECAM-1) confirmed the purity of the cell population. MVEC in passage 3 were used for mRNA determinations and in passages 5-7 for angiogenesis assays.

**Aortic VSMC culture.** Aortic human VSMC cultures were established by an explant procedure from multi-organic donor aortas as previously described [9,10]. The artery was longitudinally split and the endothelium was removed by gently scraping. The tissue was minced and seeded onto the culture surface in a small volume of medium DMEM containing 10 % foetal bovine serum (FBS, Biological Industries). VSMC were characterized by α-actin positive staining.

**Immunofluorescence staining of mPGES-1 and EP-4 in MVEC.** MVEC (passage 3) grown in Millicell EZslide (Millipore Corporation, Billerica, MA) were fixed in methanol:acetone 1:1 at -20°C for 20 minutes. For double immuno-staining, cells were incubated with a mouse monoclonal antibody anti mPGES-1 (ref 10004350, diluted 1:100) and a rabbit polyclonal antibody anti EP4 (ref 101775, diluted 1:100, both from Cayman) simultaneously for 60 minutes. This was followed by Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 donkey anti-rabbit IgG (Invitrogen) incubation for one hour. Nuclei were counterstained with Hoechst 33342 (Sigma) 1 μg/mL in PBS for 10 minutes. The slips were mounted in ProLong Gold antifade reagent (Molecular Probes, Life Technologies Co, Eugene, OR) and photographed through an Olympus BX50 microscope.

**In-vitro Angiogenesis Assay.** In vitro angiogenesis assays were performed with MVEC as described [27,28]. Briefly, 12.5 x 10³ cells were seeded onto Matrigel (BD Matrigel Basement Membrane Matrix, BD Biosciences, Bedford, MA) in 96 wells plates and exposed to treatments as required. For IL-1β assays, cells were pre-incubated for one hour with the assay medium with or without 50 U/mL of human recombinant IL-1β (Roche Diagnostics GmbH) before being seeded into Matrigel. After 4 hours of treatment, photographs were
taken with an Olympus Digital camera mounted on an Olympus IMT-2 inverted microscope using a 10x/0.40 objective, and the number of closed polygons in the endothelial cell mesh was counted.

Statistical analysis. Sigma-Stat software was used for statistical analysis. When data fit a normal distribution, statistical significance between more than two groups was assessed using one-way ANOVA and the Student-Newman-Keuls test; Student t test was used to compare two groups. When normality failed we used the Mann-Whitney Rank Sum Test to compare two groups and Kruskal-Wallis One Way Analysis of Variance on Ranks for Multiple Comparisons (Dunn's Method). To determine association between variables, data were Log10 transformed in order to normalize their distribution, Pearson Product Moment Correlation method was then used. A "p" value below 0.05 was considered significant.

Results
Evaluation of hypervascularization and leukocyte infiltration in AAA samples. Table 1 summarizes the characteristics of patients and donors included in this study. We used vWF immunostaining to assess the distribution of microvessels in aortic samples. In normal aortas (NA) microvessels originated in the adventitia distributed regularly, traversed media and finished in the intima (many of them after bifurcation), but round microvessels in the media were scarce (Figure 1). Microvessels were abundant in the adventitia and in the media of AAA samples (Figure 1) and the intima was often absent. While infiltrated leukocytes (determined by CD45 immunostaining) were scarce in NA, they were common in AAA. Observation of 20 AAA samples revealed infiltrating leukocytes in the media in all of them. Adventitia was also extensively vascularized with a concomitant accumulation of leukocytes. Leukocyte infiltrate was systematically located in perivascular areas of microvessels (Figure 1).
We determined mRNA levels of two endothelial cell (EC) markers, vWF and endothelial nitric oxide synthase (eNOS) as an index of vascularization. In addition, we also analyzed expression levels of the VEGF-receptor-2 (VEGFR2) a gene highly expressed in EC. Expression levels of these genes did not fit a normal distribution. Results in Figure 2A show that mRNA expression levels of the three genes were significantly enhanced in AAA. To further evaluate levels of these markers at different stages of AAA development we stratified AAA samples as a function of the maximum aorta diameter. Figure 2B shows the median of the EC marker expression for each AAA diameter group. We observed that expression of all three markers was maximal in the LD group and decreased thereafter. We also analyzed the expression levels of two leukocyte markers (undetectable in vascular cells in culture, data not shown): CD45 and five-lipoxygenase activating protein (FLAP). Additionally, we analyzed the expression of MMP-9, a metalloproteinase highly expressed by leukocytes. All of them were highly expressed in AAA samples (Figure 2C) and exhibited a maximum in the MD group (Figure 2D).

Expression of the enzymes involved in PGE$_2$ biosynthesis. Results in Figure 3A show that the expression of COX isoenzymes and mPGES-1 was significantly higher in AAA than in NA samples. Immunohistochemistry showed that COX-2 expression was weak but ubiquitous in NA samples, mainly in adventitial microvessels and in medial VSMC (Figure 3B). In AAA samples we found COX-2 immunostained MVEC in the adventitia and media layers, VSMC in the media and also infiltrating leukocytes, immunostaining being markedly increased in AAA (Figure 3B). Localization of mPGES-1 was similar to that of COX-2, NA and AAA displaying apparent similar immunostaining intensity (Figure 3B).

In an attempt to approach the relative contribution of the endothelium to PGE$_2$ production in AAA, we determined the statistical association of PGE$_2$ with other cell-characteristic eicosanoid secreted by AAA samples considered as independent variables. The best
association was found between PGE$_2$ and PGI$_2$ (in terms of its stable metabolite 6-oxo-PGF$_{1a}$; $R=0.890$, $p=9.95\cdot10^{-12}$, $n=32$). The statistical association between PGE$_2$ and TxA$_2$ (in terms of TxB$_2$) was weaker ($R=0.492$, $p=0.007$), and no association between PGE$_2$ and LTB$_4$ was observed.

*Expression levels of PGE$_2$ biosynthetic pathway enzymes in AAA stratified by aorta diameter.* Since PGE$_2$ is rapidly transformed *in vivo*, we analyzed plasma levels of its 13,14-dihydro-15-oxo-metabolites. Circulating levels of PGE$_2$-metabolites were significantly elevated in AAA patients compared to normal controls (Figure 4A). The characteristics of patients and controls included are shown in Table 1. In figure 4A, we also show production of PGE$_2$ by NA and AAA samples after 48 h of incubation. AAA samples produced about 5-fold more PGE$_2$ than NA in terms of the median. In Figure 4B, transcript levels of COX-1, COX-2 and mPGES-1 are depicted as a function of AAA maximum diameter, showing that all of them displayed a maximum in the LD group.

*Co-expression of EP-4 and mPGES-1 in aorta microvessels.* In human aorta samples, EP-1 was scarcely expressed and no significant differences were found between NA and AAA. EP-2 and EP-4 were significantly increased in AAA while EP-3 was substantially decreased (Figure 5). EP-4 immunostaining was not detected in the VSMC of NA or AAA samples (not shown), but in both samples it co-localized with mPGES-1 in MVEC (Figure 6). Additionally, EP-4 and mPGES-1 were also detected in the areas of leukocyte infiltration in AAA (Figure 6). In contrast, none of the other receptors were appreciably expressed in microvessels (not shown). We found few EP-2-positive cells scattered in the media in both NA and AAA, but it was particularly relevant in infiltrating leukocytes in AAA. EP-3 positive cells, that were abundant in the media of NA, decreased in the media of AAA samples according to the
depletion of VSMC in AAA. Nevertheless, many EP-3 immunostained cells were found in the areas of infiltrating leukocytes (not shown).

EP-4-activation-mediated in vitro angiogenesis. To test the hypothesis that PGE$_2$-induced angiogenesis was mediated by EP-4, we performed experiments in MVEC. EP-4 was practically the only EP expressed in MVEC in culture (analyzed by RT-PCR; not shown). Both EP-4 and mPGES-1 were coexpressed in MVEC in culture (Figure 7A). Exogenously added PGE$_2$ and Cay10598 (a specific EP-4 receptor agonist) similarly induced angiogenesis, an effect that was abolished by the EP-4 antagonist AH23848 (Figure 7B). It is well known that IL-1$\beta$ induces AAc mobilization in addition to COX-2 and mPGES-1 expression. Therefore, to explore whether the endogenous production of PGE$_2$ by MVEC promoted angiogenesis, we stimulated MVEC with human recombinant IL-1$\beta$. IL-1$\beta$-induced angiogenesis was also suppressed by AH23848 (Figure 7B).

Discussion

This is the first study that analyzes the expression of the PGE$_2$ pathway as a whole in human AAA. The main objectives were: first, to investigate the expression of mPGES-1 in human AAA which has not been previously reported; second, to tentatively approach the contribution of microvascular endothelium to the bioavailability of PGE$_2$ in AAA; and third, to highlight the potential contribution of MVEC-derived PGE$_2$, to the AAA-associated hypervascularization.

vWF immunostaining of aorta biopsies showed notable differences between NA and AAA. NA was characterized by a regular distribution of microvessels that cross the vessel wall from the adventitia to the intima. In contrast, AAA exhibited many vessels parallel to the VSMC fibers in addition to adventitia-to-intima ones. The highest density of infiltrating
leukocytes in the media was in perivascular areas of these latter microvessels. The enhanced expression of EC markers in AAA, was consistent with our immunochemistry observations and confirm previous reports showing higher vascularization of AAA [4,5]. After stratification by the aortic diameter the maximal transcript levels of EC-markers were found in the LD group whereas markers used to estimate leukocyte infiltration exhibited a maximum level in the MD group. These results are in agreement with the previous report by McMillan et al. [29] who found the highest MMP-9 expression in moderate-diameter AAA. Assuming that maximum diameter is an approach to the evolutionary stage of the aneurismatic lesion, collectively our observations allow to hypothesize that angiogenesis precedes the maximum inflammatory response during AAA development. A similar concept was proposed by Herron et al. [30] regarding levels of proteinases in AAA.

We found that not only the inducible COX-2 but also the constitutive COX-1 were elevated in AAA. Two main factors may influence relative levels of a particular transcript in heterogeneous cell samples; one is an effective up- or down-regulation of its expression in one or more cell types present in the sample, and the other is the alteration of the proportion of cell types expressing that transcript. It is well-known that COX-1 is ubiquitously expressed [6,7]. Therefore, the increase of COX-1 in AAA was probably due to the enhanced proportion of cells with high COX-1 expression, such as macrophages. Consistently with this notion, an excellent statistical correlation was observed between COX-1 and CD68 in AAA samples (R=0.654, p=2.53·10⁻⁷). Our results regarding localization of COX-2 showed that it was highly expressed in vascular cells and infiltrating leukocytes in AAA but weakly expressed in NA. These data strongly suggest that in AAA COX-2 was effectively up-regulated in vascular cells included MVEC. We found that, even significant, the increase of mPGES-1 expression in AAA was not as high as that of COX-2 (1.4-fold versus 4.3-fold, respectively). This suggests a dissimilar regulation of these two enzymes in AAA, as we previously described [8,9]. A factor that could explain the modest increase of mPGES-1 found in AAA is the breakdown of VSMC in AAA. Indeed, VSMC abundantly express mPGES-1 [9,10] and we
localized mPGES-1 immunoreactivity in all vascular cells including VSMC in both NA and AAA. The increase in the expression of PGE$_2$ biosynthetic machinery was also consistent with the higher \textit{in vitro} production of PGE$_2$ by the AAA tissues, and with the higher levels of PGE$_2$ metabolites detected in plasma of these patients. Nevertheless, not the entire increase in plasma levels of PGE$_2$ may be coming from the AAA, itself, as AAA should be considered a systemic disease of the vasculature \cite{31} and other vascular territories may contribute to the circulating PGE$_2$ levels.

Several reported biological activities of PGE$_2$ account for its involvement in AAA physiopathology among others, its ability to induce the expression of metalloproteinases (MMPs) \cite{32} and to inhibit the production of extracellular matrix components such as fibronectin and collagens type I and III \cite{33}. The presence of mPGES-1 in a particular cell is necessary for PGE$_2$ biosynthesis \cite{8-11,34,35}. In inflammatory diseases it is generally assumed that PGE$_2$ comes from invading leukocytes, mainly macrophages, and from tumor cells in neoplasias. Nevertheless, we have recently shown that stroma cells present in tumor environment could be a more relevant source of PGE$_2$ than tumor cells themselves \cite{28}. Since COX-2 and mPGES-1 were expressed in both, vascular cells and infiltrating cells, macrophages could also be a relevant source of PGE$_2$ in the AAA. Indeed, macrophage COX-2-derived PGE$_2$ has been found to be relevant in the pathogenesis and rupture of AAA \cite{12,13,21,22}. We can not rule out, however, the contribution of macrophage-associated COX-1 to the PGE$_2$ pool, since we found that COX-1 is increased in AAA probably due to recruited macrophages as mentioned above.

The contributions of the different cell types present in aneurismatic tissue to the PGE$_2$ pool is not possible to evaluate directly without seriously modify tissue samples. As an attempt to approach the contribution of MVEC in the PGE$_2$ produced by AAA samples, we explored the statistical association of PGE$_2$ production with other eicosanoids typically released by vascular cells or by leukocytes, considering its production as independent variables. A
statistic association between two variables could indicate either a causal relationship between them, or that the variation of these parameters has a common cause. Prostaglandin I-synthase is expressed in vascular cells but not in leukocytes, thromboxane A-synthase is mainly expressed in macrophages and platelets, while 5-lipoxygenase and its counterpart FLAP are expressed in leukocytes (mainly polymorphonuclear). We observed an excellent statistical association between the secretion of PGE\(_2\) and PG\(_{12}\), a modest association between PGE\(_2\) and TxA\(_2\) and a lack of association between PGE\(_2\) and LTB\(_4\). The influence of PG\(_{12}\) or TxA\(_2\) on PGE\(_2\) levels is unlikely; rather a common cause should underlie these correlations. Therefore, the biosynthesis of PGE\(_2\) in AAA would be associated with cells producing PG\(_{12}\) (vascular cells), and in a lesser extent with those producing TxA\(_2\) (macrophages), suggesting a relevant contribution of MVEC to PGE\(_2\) biosynthesis in these tissues. Nevertheless, this circumstantial evidence should be corroborated by further studies.

Since EP-receptors are critical in the biological action of PGE\(_2\) we explored those receptors in AAA. The role of EP-3 in angiogenesis is not fully understood [36-39], but EP-2 and EP-4 have been reported to be involved in the release of angiogenic factors and/or angiogenesis in different experimental models and pathologies [40-42]. We observed that EP-4 was practically the only PGE-receptor detected in MVEC in culture and was mostly expressed in aorta microvessels and some infiltrating leukocytes. In addition, exogenous and endogenous PGE\(_2\) induced angiogenesis \textit{in vitro} via EP-4 activation. Co-expression of COX-2, mPGES-1 and EP-4 in MVEC is consistent with a role of MVEC-derived PGE\(_2\) in AAA-associated hypervascularization and MVEC-derived PGE\(_2\) could be biologically effective in the AAA-associated angiogenesis in an autocrine manner. Hypervascularization per se could be a determinant factor in reducing mechanical strength, since it turns media layer fluffy and besides favors leukocyte-mediated matrix degradation.
In summary, we provide new evidence concerning the expression of mPGES-1 and the contribution that MVEC could have in the biosynthesis of PGE$_2$ in human AAA. Our data allow us to speculate that the COX-2/mPGES-1/EP-4 axis in MVEC is relevant for the PGE$_2$-mediated hypervascularization of AAA, from the early stages of human AAA development. Our data are also consistent with reports showing that suppression of either COX-2, mPGES-1 or EP-4 expression reduces AAA development in animal models [15,16,19,20], and reinforce the potential of mPGES-1 and EP-4 as alternative targets for therapy in AAA patients.
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Figure legends

**Figure 1.** Representative immunohistochemical images of vWF and CD45 in aorta samples. Upper panel shows normal aorta (NA) immunostained with anti-vWF. Middle panels show serial sections of an AAA sample immunostained for vWF and CD45. Bottom panels are serial sections of an AAA sample immunostained for vWF and CD45, showing leukocyte perivascular accumulation. Arrows indicate some immunostained cells. Size bars: 500 μm. Red bars highlight specific immunostaining. In middle panels black-tipped arrows are used to orient the position of the vessel by indicating to where intima and adventitia are.

**Figure 2.** A, Expression levels of EC markers in NA (eNOS and VEGFR2, n=17; vWF, n=25) and AAA samples (vWF, n=86; eNOS and VEGFR2, n=60); points without error bars indicate individual values and points at right are the mean±SEM; * p<0.05, *** p<0.001 when compared with NA samples. B, Open points are the relative median values of mRNA levels of EC markers normalized to the highest media value in the stratified patient groups as a function the aorta diameter (NA, n=25; LD: <55 mm, n=14; MD: 55-69.9 mm, n=37; and HD: ≥70 mm, n=35), closed points are the mean±SEM of the three EC markers (mean EC-M); * p<0.05 compared with normal aorta and # p<0.05 when compared with the other patient groups. C, mRNA levels of leukocyte markers in NA (n=25) and AAA samples (n=86); FLAP, five lipoxygenase activating protein. D, Open points are the relative median values of mRNA levels of leukocyte characteristic proteins normalized to the highest media value of the stratified patient groups; closed points are the mean±SEM of the three leukocyte markers (Mean L-M); * p<0.05 compared with normal aorta and # p<0.05 when compared with the other patient groups.

**Figure 3.** A, Expression levels of COX iso-enzymes and mPGES-1 in NA (n=25) and AAA samples (n=86); points without error bars indicate individual values and points at right are the mean±SEM; * p<0.05, *** p<0.001 when compared with NA samples. B, Representative
examples of immunohistochemistry for COX-2 and mPGES-1 in NA and AAA samples. Arrows indicate some immunostained cells in microvessels (ad, adventitia; med, media). Size bars: 100 μm.

**Figure 4.** A, Plasma levels of PGE$_2$ metabolites (PGE$_2$M) in Controls (n=39) and AAA (n=39) and production of PGE$_2$M by NA (n=15) and AAA samples (n=32); points without error bars indicate individual values and points at right are the mean±SEM. B, Relative median values of transcript levels of COX-1, COX-2 and mPGES-1 (NA, n=25; LD: <55 mm, n=14; MD: 55-69.9 mm, n=37; and HD: ≥70 mm, n=35) normalized to the highest median value of the stratified patient groups.* p<0.05, ** p<0.01, when compared with NA samples.

**Figure 5.** Transcript levels of PGE-receptors (EP) in NA (n=25) and AAA samples (n=86); points without error bars indicate individual values and points at right are the mean±SEM; *** p<0.001 when compared with NA samples.

**Figure 6.** Representative images of serial sections showing co-expression of EP-4 and mPGES-1 in microvessels of a NA and AAA sample; arrows indicate some immunostained cells in microvessels and in leukocytes (Size bars: 50 μm).

**Figure 7.** A, Immunofluorescent staining of MVEC in culture for EP-4 and mPGES-1 (upper panels), and double staining for EP-4 and mPGES-1 (bottom panels). B, Effect of EP-4 activation on *in vitro* angiogenesis MVEC were seeded onto Matrigel in 96 wells plates and exposed to 10 nmol/L of PGE$_2$ or Cay10598 (an EP-4 agonist). Two hundred nmol/L AH23848 were used to block EP-4. For IL-1β assays, cells were pre-incubated or not for 1 hour with human recombinant IL-1β (50 U/mL) in the assay medium before being seeded in Matrigel. After 4 hours of treatment, photographs were taken and the number of closed polygons in the endothelial cell mesh was counted. Bars are the mean±SEM (expressed as
relative to the corresponding controls). Inside the bars: number of independent experiments performed in triplicate. Representative photographs are also shown (bottom panels). * p<0.05, when compared with controls.
Table 1.- Clinical characteristics of individuals with AAA and NA included in the study. Demographics and risk factors.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>mRNA levels</th>
<th>Plasma PGE&lt;sub&gt;2&lt;/sub&gt;M</th>
<th>Secretion*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAA</td>
<td>NA</td>
<td>AAA Controls</td>
</tr>
<tr>
<td>Number</td>
<td>86</td>
<td>25</td>
<td>39</td>
</tr>
<tr>
<td>Aortic diameter (mm)</td>
<td>66.7±13.5</td>
<td>-</td>
<td>63.3±9.8</td>
</tr>
<tr>
<td>LD (&lt;55 mm)</td>
<td>49.7±6.4</td>
<td>-</td>
<td>52.8±1.2</td>
</tr>
<tr>
<td>MD (55-69.9 mm)</td>
<td>60.1±3.2</td>
<td>-</td>
<td>59.9±3.6</td>
</tr>
<tr>
<td>HD (≥70 mm)</td>
<td>80.8±7.5</td>
<td>-</td>
<td>76.5±6.7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>70.9±6.6</td>
<td>56.3±16.0</td>
<td>71.2±6.7</td>
</tr>
<tr>
<td>Male</td>
<td>84 (97.7%)</td>
<td>16 (64%)</td>
<td>35 (89.7%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>19 (22.1%)</td>
<td>2 (8%)  a</td>
<td>10 (25.6%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>55 (64%)</td>
<td>2 (8%)  a</td>
<td>34 (87.2%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>55 (64%)</td>
<td>2 (8%)  a</td>
<td>30 (76.9%)</td>
</tr>
<tr>
<td>Smoking habit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>7 (8.1%)</td>
<td>21 (84%)  a</td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>26 (30.2%)</td>
<td>3 (12%)  a</td>
<td>9 (23.1%)</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>53 (61.6%)</td>
<td>1 (4%)  a</td>
<td>27 (69.2%)</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>22 (25.6%)</td>
<td>0 a</td>
<td>14 (35.9%)</td>
</tr>
<tr>
<td>Angor pectoris</td>
<td>3 (3.5%)</td>
<td>0 a</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>6 (7%)</td>
<td>0 a</td>
<td>6 (15.4%)</td>
</tr>
<tr>
<td>Coronary intervention/CABG</td>
<td>13 (15.1%)</td>
<td>0 a</td>
<td>7 (17.9%)</td>
</tr>
<tr>
<td>Chronic renal insufficiency</td>
<td>36 (41.9%)</td>
<td>0 a</td>
<td>13 (33.3%)</td>
</tr>
<tr>
<td>Dialysis</td>
<td>0 (0%)</td>
<td>0 a</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>44 (51.1%)</td>
<td>0 a</td>
<td>21 (53.8%)</td>
</tr>
<tr>
<td>Absence pulses</td>
<td>29 (33.7%)</td>
<td>0 a</td>
<td>14 (35.9%)</td>
</tr>
<tr>
<td>Intermittent claudication</td>
<td>15 (17.4%)</td>
<td>0 a</td>
<td>7 (17.9%)</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>7 (8.1%)</td>
<td>1 (4%)  a</td>
<td>4 (10.3%)</td>
</tr>
<tr>
<td>Cerebral vascular attack</td>
<td>5 (5.8%)</td>
<td>1 (4%)  a</td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Transient ischemic attack</td>
<td>2 (2.3%)</td>
<td>0 a</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>COPD</td>
<td>27 (31.4%)</td>
<td>0 a</td>
<td>7 (17.9%)</td>
</tr>
<tr>
<td>Antiplatelet users</td>
<td>39 (45.9%)</td>
<td>1 (4%)  a</td>
<td>19 (50%)</td>
</tr>
<tr>
<td>Statins users</td>
<td>53 (61.6%)</td>
<td>0 a</td>
<td>30 (76.9%)</td>
</tr>
<tr>
<td>IECAs users</td>
<td>20 (24.1%)</td>
<td>0 a</td>
<td>13 (33.3%)</td>
</tr>
<tr>
<td>NSAD users</td>
<td>6 (7%)</td>
<td>0 a</td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Corticoid users</td>
<td>6 (7%)</td>
<td>0 a</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>Immuno-suppressors users</td>
<td>4 (4.7%)</td>
<td>0 a</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

(a) In some cases due to the nature of NA samples some of the clinical characteristics are not always recorded and infra-evaluation of them is probable.

Nominal variables are presented as number and as percentage (%) and continuous variables as mean±SD. Abbreviations: Aortic diameter: aneurysm maximum transverse diameter in mm. Chronic renal insufficiency: estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m². Ex-smokers: quit smoking <1 year. CABG: coronary artery bypass grafting. COPD: chronic obstructive pulmonary disease. Secretion*: PGE₂ secretion by NA and AAA samples.
Figure 1
Figure 3

mRNA Log_{10}, relative to β-actin

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
<th>COX-2</th>
<th>mPGES-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>med</td>
<td>NA</td>
<td>med</td>
<td>ad</td>
</tr>
<tr>
<td>ad</td>
<td>NA</td>
<td>med</td>
<td>ad</td>
</tr>
<tr>
<td>AAA</td>
<td>NA</td>
<td>med</td>
<td>ad</td>
</tr>
<tr>
<td>med</td>
<td>NA</td>
<td>med</td>
<td>ad</td>
</tr>
</tbody>
</table>

**A**

**B**

Downloaded from www.jlr.org on October 22, 2017.
Figure 4
Figure 5

mRNA
Log$_{10}$ relative to β-actin


***

NA

***
In vitro angiogenesis normalized for the control.

**Figure 7**

**A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EP-4</th>
<th>mPGES-1</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PGE₂</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AH23848</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cay10598</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**B**

Bar graph showing in vitro angiogenesis normalized for the control. The graph includes data points for the following treatments:

- Control
- PGE₂
- AH23848
- Cay10598
- IL-1β

Significance is indicated by * (p < 0.05).

Figure 7