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Renin Angiotensin System-mediated modulation of inflammation-induced Tissue Factor expression

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ABSTRACT

Background: An intricate cross-talk connects Tissue Factor (TF) to inflammation and is amplified by locally active angiotensin(ang)II, that stimulates TF expression. High glucose may activate pro-inflammatory pathways and increase glucose-mediated angII production.

Aim: To evaluate the effect of Renin Angiotensin System (RAS) blockade on TF expression in peripheral blood mononuclear cells (PBMCs) exposed to normal glucose (NG) and high glucose (HG), and stimulated by lipopolysaccharide (LPS), a well known pro-inflammatory agent.

Methods: PBMCs, from healthy donors were LPS-stimulated and exposed to NG and HG. TF mRNA levels, antigen expression and pro-coagulant activity were assessed by Real Time-PCR, ELISA and 1-stage clotting assay respectively, in absence or presence of pharmacological intervention.

Results: LPS stimulation increased TF expression in NG, an effect down-regulated by Aliskiren, a direct renin inhibitor, zofenopril, an angiotensin converting enzyme inhibitor, olmesartan, an angII type1 receptor (AT1R) blocker and Compound21, an highly selective angII type 2 receptor (AT2R) agonist. As compared with NG, HG amplified the LPS-stimulation of TF expression, an effect that Aliskiren, Zofenopril and Compound21 down-regulated in HG with a similar behavior to that in NG, while OLM activity was potentiated in HG.

Conclusions: Our data disclose tight connections between the apparently unrelated domains of innate immunity and metabolic and cardiovascular regulation, expanding the

restrictive view of angII as a peptide mainly involved in the control of blood pressure and water and salt homeostasis.

1. INTRODUCTION

1.1 Tissue Factor (TF).

Tissue factor (TF), also known as thromboplastin or CD142, is a glycosylated transmembrane protein consisting of a single polypeptide chain with a molecular weight of about 45 kDa [1]. The TF gene is located on chromosome 1 and consists of 6 exons [2]. After transcription of the TF gene F3, the primary TF transcript is processed on post-transcriptional level [3, 4], which consequently leads to generation of two naturally occurring isoforms: membrane-bound "full-length" (fl)TF and soluble "alternatively spliced" (as)TF [5, 6]. Due to alternative splicing the fifth exon of the primary TF transcript is excluded [6, 7] and translation of this messenger (m)RNA splice variant leads to the generation of asTF on protein level that lacks the transmembrane domain that is therefore soluble [6]. TF is the principal initiator of the clotting cascade and a major regulator of haemostasis and thrombosis [8]. The coagulation cascade is initiated as soon as TF comes into contact with circulating activated factor VII (FVIIa), resulting in the TF-FVIIa complex. The TF-VIIa complex activates factor IX, which in turn activates factor X; alternatively, factor X is directly converted to factor Xa by TF-FVIIa. In complex with factor Va and calcium (Ca^{2+}), Factor Xa catalyzes the conversion of prothrombin to thrombin, thereby leading to fibrin formation, platelet activation, and, ultimately, generation of a thrombus. Beside its primary function as initiator of the blood coagulation cascade [6, 9, 10], flTF plays an important role in a variety of other biological processes, such as migration and proliferation of vascular smooth muscle cells (VSMCs) [11],

protease-activated receptor (PAR-)2-mediated cell signaling and tumor progression [9, 12, 13].

In the vessel wall, TF is constitutively expressed in sub-endothelial cells such as VSMCs leading to rapid initiation of coagulation when the vessel is damaged [14]. TF is also present in different pools that are in contact with the circulating blood: associated with endothelial cells, white cells and platelet [15], associated with cell-derived microparticles (MP)[16] and present in a soluble form lacking the transmembrane domain [6]. In contrast with sub-endothelial cells, endothelial cells and monocytes, that show very little to no basal expression of TF under physiological conditions, express TF in response to various stimuli. Endothelial TF is induced by cytokines such as tumor necrosis factor-α (TNF-α)[17], interleukin(IL)-1β [18], or CD40 ligand [19], by biogenic amines such as serotonin [20] and by mediators such as thrombin, oxidized LDL, or vascular endothelial growth factor (VEGF) [21-23]. In monocytes TF expression can be induced by inflammatory *stimuli* such as C-reactive protein [24] or CD40 ligand [25], platelet-derived growth factor(PDGF)-BB, angiotensin(ang)II, oxidized LDL [26, 27] and endotoxin (lipopolysaccharide, LPS) [28, 29].

1.2 TF role in thrombosis.

TF expression by endothelial cells and monocytes following inflammatory stimuli contribute to the development of acute thrombotic events. TF activity is significantly higher in the lesions present in patients with unstable angina or myocardial infarction, compared to patients with a stable form of angina [30, 31]. The antigen levels of TF in plasma is associated with an increased risk of death from cardiovascular causes [32] and

with an increase in the thickness of the carotid intima-media, considered a marker of atherosclerosis [33]. Acute coronary patients have higher levels of TF bound to monocytes [34], and of circulating TF [35, 36] compared to patients with stable angina and normal subjects, suggesting a potential release of TF at the level of the coronary artery during the acute ischemia. Pathological studies have identified TF, present in the atherosclerotic plaque, as a candidate molecule responsible for the thrombogenicity associated with plaque rupture [37]. Monocytes infiltrate the intimal layer and then transform into macrophages and foam cells, which represents a hallmark of the inflammatory nature of atherosclerosis [38]. In this inflammatory environment, cytokines such as TNF-α and ILs are released and induce expression of TF. During the early stages of atherogenesis, enhanced TF expression is observed in monocytes [14]; at later stages, TF expression is also detected in foam cells, endothelial cells, and VSMCs [14, 39]. TF is present in the necrotic core of plaques as well, predominantly associated with microparticles derived from perishing foam cells, macrophages, or lymphocytes [39, 40]. Lipid-rich plaques with a thin cap, a large lipid core, extensive macrophage infiltration, and abundant TF expression are more prone to rupture than collagen-rich, fibrous plaques. Rupture of an atherosclerotic plaque exposes its highly procoagulant content to the circulating blood; thereby, TF-laden macrophages as well as TF-containing microparticles originating from the necrotic core initiate thrombus formation and related complications leading acute myocardial infarction (MI) but also to those that characterize the course of hypertension [41].

1.3 Bidirectional relation between Inflammation and TF.

Inflammation and coagulation play pivotal roles in the pathogenesis of vascular disease. Increasing evidence points to extensive cross-talk between these two systems, that closely

interact: coagulation considerably affects inflammatory activity and inflammation leads to activation of coagulation. Coagulation factors may activate PARs of which 4 types (PAR 1 to 4) have been identified, all belonging to the family of transmembrane domain, G-protein–coupled receptors expressed on mononuclear cells or endothelial cells [42], which may affect, for example, cytokine production or inflammatory cell apoptosis. Thrombin binds PARs 1, 3, and 4 and induces the production of several cytokines and growth factors, whereas PAR-2 can be activated by the TF-factor VIIa complex, resulting in a up-regulation of inflammatory responses in macrophages (production of reactive oxygen species and cell adhesion molecules) and affect neutrophil infiltration and pro-inflammatory cytokine expression (*e.g.* TNF-α, IL-1β) [43]. On the other hand activation of coagulation and fibrin deposition as a consequence of inflammation is well known and can be viewed as an essential part of the host defense of the body against, for example, infectious agents or non identical cells, in an effort to contain the invading entity and the consequent inflammatory response to a limited area. An exaggerated or insufficiently controlled response may, however, lead to a situation in which coagulation and thrombosis contribute to disease, as illustrated by the fact that thrombus formation on a ruptured atherosclerotic plaque, containing abundant inflammatory cells, is the pathological basis of acute arterial thrombotic events such as MI or unstable angina [44]. Expression of TF by inflammatory cells and probably due to sustained exposure to pro-inflammatory factors, such as IL-6, PDGF and monocyte chemoattractant protein (MCP)-1 in the unstable plaque may initiate activation of coagulation, and the thrombin generated will both activate platelets and result in the formation of a platelet-fibrin thrombus [45]. Blocking TF activity completely abrogates inflammation-induced coagulation activation in models of experimental endotoxemia or bacteremia, whereas antibodies that inhibit the contact system have no effect on thrombin formation [46, 47].

1.4 NF-kB and Induction of TF expression by pro-inflammatory stimuli.

Pro-inflammatory agents such as TNF-α and LPS are able to induce the synthesis of TF by the activation of nuclear transcription factor NF-kB [48]. NF-kB is a protein that controls networks of chemokine modulating, growth factor–modulating, translational control, and cellular survival genes [49]. NF-kB is a family of highly inducible DNA-binding proteins that consists of a group of dimeric complexes of members of the Rel protein family of which the p50-p65 eterodimer is the most common. In resting cells, NF-kB proteins are found mainly in the cytoplasm and form a complex with with a family of cytoplasmic inhibitors I-kBs, after cellular activation IκB is phosphorylated and degraded, allowing the transfer of NF-kB into the nucleus, where it binds to specific recognition sequences of target genes that code for pro-inflammatory and pro-atherogenic proteins [50]. The promoter of TF gene is partially under the control of NF-kB [28].

1.5 Angiotensin II as a modulator of TF expression.

Several studies have linked angiotensin(ang)II, the main effector of the renin-angiotensin aldosterone system (RAAS), to the synthesis of TF. A growing body of evidence gathered over the past several years has shown the wide ranging pro-thrombotic and pro-coagulant potential of angII including platelet sensitization, inhibition of fibrinolysis by PAI-1 stimulation and activation of TF expression [51]. Notably, TF modulation may occur locally by activation of a local RAS contained within the monocytes/macrophages complex in atherosclerotic plaques. AngII influences the expression of pro-inflammatory molecules

with mechanisms that overlap those typical of the pro-inflammatory cytokines, such as TNF-α [52, 53] activating NF-kB. The binding of angII to angII type 1 receptor (AT1R) induces the activation of NF-kB and the increase of synthesis of TF in monocytes [26, 54], as well as in aortic endothelial cells of rat [55], and in VSMCs [56].

Furthermore the inhibition of RAS in vitro induces a decrease of the expression of TF [26, 57-60].

1.6 Renin Angiotensin Aldosterone System.

The RAAS is a peptidergic system with endocrine characteristics which regulates the circulatory homeostasis. The system, present in most animal species, consists of several elements able to increase their circulating levels in response to the reduction in intravascular volume and to the decrease in renal perfusion pressure [61]. RAAS maintains blood pressure and body fluid volume homeostasis through arteriolar vasoconstriction, increased sympathetic activity and tubular Na^+ reabsorption and stimulation of aldosterone release from the adrenal cortex [62].

The substrate of the system, **angiotensinogen**, is an α-glycoprotein of high molecular weight, released from the liver [63] that is cleaved of the N-terminal portion in the circulation by the enzyme renin to form the decapeptide angiotensin(ang)I. **Renin** is synthesized by the cells juxtaglomerular (JG) of the kidney as a pre-pro-hormone, pro-renin, which is converted to renin by proteolytic removal of a peptide segment of 43-amino acids in its N-terminal portion. The renin secretion is stimulated by a fall in perfusion pressure or decrease in the transport of NaCl and by an increased activity of the sympathetic nervous system. Renin secretion and activity represents the rate-limiting step

of the RAAS [64-67]. The inactive decapeptide **angI** is hydrolyzed by **angiotensin-converting enzyme (ACE),** which removes the C-terminal dipeptide to form the octapeptide **angII** [Ang-(1-8)], a biologically active, potent vasoconstrictor. ACE is a membrane-bound exopeptidase and is localized on the plasma membranes of various cell types, including vascular endothelial cells, microvillar brush border epithelial cells (e.g., renal proximal tubule cells), and neuroepithelial cells [67]. AngII is the primary effector of a variety of RAAS-induced physiological and pathophysiological actions. At least 4 angiotensin receptor subtypes have been described. The **angII type 1 receptor (AT1R)** mediates most of the established physiological and pathophysiological effects of angII. These include actions on the cardiovascular system (vasoconstriction, increased blood pressure, increased cardiac contractility, vascular and cardiac hypertrophy), kidney (renal tubular sodium reabsorption, inhibition of renin release), sympathetic nervous system, and adrenal cortex (stimulation of **aldosterone** synthesis) [67, 68]. The AT1 receptor also mediates effects of angII on cell growth and proliferation, inflammatory responses, and oxidative stress [68, 69]. This receptor, which is typical of the G protein-coupled receptor superfamily containing 7 membrane-spanning sequences, is widely distributed on many cell types in angII target organs. The **angII type 2 receptor (AT2R)** is abundant during fetal life in the brain, kidney, and other sites, and its levels decrease markedly in the postnatal period. There is some evidence that, despite low levels of expression in the adult, the AT2Rs, whose activation tends to counteract the biological effects of AT1R-mediated responses, might mediate vasodilation and anti-proliferative, anti-inflammatory and apoptotic effects and inhibit growth and remodeling in the heart [68, 70-76]. In the kidney, it has been proposed that activation of AT2R may influence proximal tubule sodium reabsorption [67, 69, 77]. The **angII type 4 receptor (AT4R)** are thought to mediate the release of plasminogen activator inhibitor 1 by angII and by the N-terminal truncated

peptides (angIII and angIV), but the function of the **angII type 3 receptor (AT3R)** is unknown [67].

1.7 Intracellular Renin Angiotensin System.

One of the most significant advancements in the past two decades has been the discovery of local or tissue RASs [78, 79]. The intracellular RAS is characterized by the presence of its components inside the cell and synthesis of angII at an intracellular site. The primary nature of RAS components, such as the presence of signal peptides in AGT and renin, and the transmembrane nature of ACE, is generally thought not to be supportive of an intracellular system. However, the existence of differentially glycosylated isoforms of AGT [80, 81], alternatively spliced forms of renin [82, 83], intracellular and secreted forms of ACE [84], alternative angII-generating enzymes (such as cathepsins and chymase) [85, 86], and detection of these components intracellularly, under certain cellular conditions [84, 87], support the hypothesis of an intracellular RAS. In addition to its intracellular presence or synthesis, angII should be able to mediate biological effects from an intracellular location, to be functionally relevant. Local systems are regulated independently of the circulatory RAS but can also interact with the latter and have been demonstrated in heart [88, 89], in kidneys [90], in brain [91], in pancreas [92], and reproductive [78], lymphatic [78], and adipose tissues [93].

Growing evidence also showed the cytokine-like potential of locally-synthesized angII to act in a paracrine, autocrine and possibly intracrine manner to promote endothelial dysfunction and vascular inflammation, two main components of the atherogenic process

[94, 95]. Regarding this aspect, an intracellular RAS is present in monocytes, a cellular population capable of generating the whole set of RAS components, including renin, angiotensinogen, ACE and angII [96-99] and endowed with AT1Rs [100] and AT2Rs [101].

1.8 High glucose-induced intracellular synthesis of angII.

RAS activation plays a relevant role in diabetes [102], a prothrombotic state [103] to which elevated glucose, the disease hallmark, may contribute by activating pro-inflammatory pathways [104-106] and increasing the cellular content of RAS components [87, 107-109]. In particular, in a study on rat mesangial cells, high glucose resulted in a time-dependent increase in the expression of the genes encoding prorenin, AGT, ACE and cathepsin B. The increase in renin activity was accompanied by a several-fold increase in intracellular angII concentrations, which localized predominantly in the nucleus [87]. In human renal mesangial cells, angII levels increased only in cell lysates, and not in the medium, following high-glucose exposure [110]. In another study, intracellular angII synthesis was studied in Neonatal Rat Ventricular Myocytes (NRVMs), in response to elevated glucose levels. Receptor-mediated internalization of angII was prevented by an AT1 receptor blocker (candesartan) in the cellular medium. High glucose-induced intracellular angII synthesis was accompanied by increased intracellular retention and decreased extracellular levels of AGT and renin, consistent with intracellular angII generation. Additional experiments, using specific enzyme inhibitors, determined that renin and chymase, but not ACE, were responsible for high glucose-induced intracellular angII synthesis [111].

1.9 RAS Pharmacological inhibition.

Different clinical strategies have been developed for inhibiting the RAAS.

β-Blockers. In 1970, Laragh and colleagues have shown that in hypertensive patients, therapy with β-blockers (propanolol) reduced of about 75% plasma levels of renin as blocking the release of renin beta-1-mediated by the kidney. In addition to the suppression of renin release, there is evidence that the β-adrenergic receptor blockade may inhibit the conversion of intrarenal prorenina in renin [66, 112].

ACE Inhibitors (ACEIs). ACEIs block the action of ACE, and thus the conversion of angI to angII. The reduction of angII levels causes a dose-dependent reduction of cardiac preload and after-load, with a drop in systolic blood pressure and diastolic, while in normotensive and hypertensive patients without cardiac dysfunction, it causes little or no change in cardiac output and capillary pressure [113]. ACEIs also decrease renal vascular resistance, determine increase in renal blood flow and promote the excretion of water and sodium.

AT1R Blockers (ARBs). ARBs act by antagonizing the angII effects mediated by AT1R, reducing systemic blood pressure by decreasing systemic vascular resistance [114].

Direct Renin Inhibitors (DRIs). DRIs are the newest class of agents that block RAS and recently they have been approved for the treatment of hypertension. They block renin catalytic activity, the RAS initial step [115].

- **Aliskiren** Aliskiren, potent renin inhibitor, is the first nonpeptide to be approved by the FDA and EMEA for the treatment of hypertension. It is given either as a single agent and in combination with other antihypertensive agents. It is a highly lipophilic molecule that improves its oral bioavailability [115]. Subsequent studies

in normotensive subjects have demonstrated an efficient RAS blockade with Aliskiren [116, 117].

AT2R agonists. The AT2R activation tends to counteract the biological effects of AT1R-mediated responses including their pro-inflammatory potential [68, 70, 71, 73, 74, 118]. However, experimental verification of that hypothesis has been delayed by the absence of specific AT2R agonists, as previous studies addressing that issue [119] had only to rely upon pharmacological AT2R blockade, an approach encumbered by several conceptual and methodological pitfalls [76, 120].

- **Compound (C) 21** is a recently synthesized, highly selective AT2R agonist [121]. The specificity of the response of the compound C21 was evaluated in relation to those induced by angII in mechanisms in which it has a AT2R direct stimulation. C21 is able to induce neurite outgrowth *in vitro* with a similar effect to that angII mediated, an effect inhibited by the AT2R antagonist PD123,319. *In vivo* C21 increases in a concentration-dependent manner the alkaline duodenal secretion, an effect inhibited, also in this case, by the AT2R antagonist [121]. In rats in which was induced MI, C21-dependent AT2R stimulation decreases the synthesis of various pro-inflammatory cytokines such as IL-6, IL-2, as well as the inhibition, in the peri-infarct area, of caspase 3 and Fas-Ligand expression indicating an anti-apoptotic effect C21-mediated [122]. The anti-inflammatory effects modulated by C21 are confirmed also in endothelial cells and human fibroblasts, where it has been demonstrated that AT2R stimulation inhibits NF-kB, with consequent reduction of pro-inflammatory cytokines expression [123].

1.10 RAS Pharmacological inhibition and TF modulation.

Inhibition of the RAS downregulates TF expression in numerous experimental models. *In vitro* experiments with monocytes isolated from normal volunteers have shown that preincubation of LPS-stimulated monocytes with the ACE inhibitor, captopril, caused a significant reduction of TF activity, TF mRNA, and NF-kB translocation into the nuclei. Fosinopril and idrapril showed similar effects [59]. Blocking the RAS downstream, with the AT1R blocker Losartan, also inhibited TF activity in the same model [59] and prevented the upregulation of TF mRNA synthesis by rat aortic endothelial cells [55]. Similar results were obtained by He and colleagues, who showed that human peripheral blood monocytes cultured in the presence of angII express a procoagulant activity identical to TF, as well as TF mRNA, an effect inhibited by Losartan, an AT1R antagonist [38]. Del Fiorentino and colleagues have reported that blocking the RAS with the ACE inhibitor zofenopril, with the AT1R blockers olmesartan and with the direct renin inhibitor, aliskiren, inhibited TF synthesis and function in TNF-α stimulated human umbilical vein endothelial cells [58]. Valsartan-treated transgenic rats overexpressing the human renin and angiotensin genes showed inhibited NF-kB activation and TF synthesis as compared with placebo controls [124]. The role of RAS inhibition in TF synthesis was also investigated in humans. Three different AT1R blockers, losartan, irbesartan, and candesartan, or placebo, were administered to 122 hypertensive patients for 2 months. All treatments, as expected, caused a significant reduction of blood pressure, with no difference among them. TF activity was measured at the beginning and at the end of the study period; all treatments reduced TF activity with different potencies (candesartan>irbesartan>losartan) [125]. In coronary artery disease patients has observed a decreased circulating TF activity after treatment with enalapril, an ACE inhibitor [36].

2. AIMS

The aims of this work were twofold. First, to evaluate the effect of RAS inhibition, with aliskiren (ALI), a direct renin inhibitor (DRI), zofenopril (ZOF), an ACE inhibitor (ACEI), olmesartan (OLM), an AT1R blocker (ARB) and Compound(C)21, an highly selective AT2R agonist on TF expression in Peripheral Blood Mononuclear Cells (PBMCs) exposed to LPS, the principal glycolipid component of the outer membrane of Gram-negative bacteria and a well characterized inflammatory stimulus signalling through the redox-sensitive NF-kB pathway.

Second, to evaluate the effect of increasing glucose concentrations on TF expression in PBMCs exposed to LPS and in that experimental setting to investigate the modulating potential of RAS blockade on the antigenic and functional expression of LPS-induced TF.

3. MATERIALS AND METHODS

3.1 Cell isolation and culture.

Peripheral blood mononuclear cells (PBMCs) were obtained from unpooled buffy coats left over from blood bank draws taken from healthy donors, with the approval of the local ethics committee. According to local procedures, individuals with a history of hypertension, either on antihypertensive drugs or not, are excluded from blood donation. As detailed elsewhere [126], leukocytes were isolated from fresh buffy coats diluted 1:1 with sodium citrate 0.38% in saline solution, mixed gently with 0.5 volume of 2% Dextran T500 and left for 40 min for erythrocyte sedimentation. The leukocyte-rich supernatant was recovered and centrifuged for 10 min at 200xg. The pellet was resuspended in 30 ml of sodium citrate solution, layered over 15 ml of Ficoll-Hypaque and centrifuged for 30 min at 350xg at 20°C. The PBMC-rich ring was recovered, washed twice in sodium citrate 0.38% and resuspended in no glucose RPMI 1640 medium (Sigma Chemical, St Louis, Missouri, USA) supplemented with 100 U/ml penicillin-streptomycin. The final PBMC preparations typically contain 25–35% monocytes, 65–75% lymphocytes and less than 5% neutrophils [126].

After isolation, cells resuspended in polypropylene tubes ($3x10^6$ cells/ml) were exposed to experimental drugs or their appropriate vehicle in presence of different D-glucose concentrations (5.5 mM, heretofore referred to as Normal Glucose, NG, or 50 mM heretofore referred to as High Glucose, HG) or equivalent amounts of L-Glucose as a control for osmolarity 30 min prior to LPS (Escherichia coli 026:B6 LPS; Sigma

Chemical), 0.1 μg/mL, and left to incubate for 18 hs at 37°C in a 5% CO_2 atmosphere until assay.

PBMCs incubated without either LPS or drugs were also included to obtain baseline values. All reagents and solutions used for cell isolation and culture were prepared with endotoxin-free water and glassware was rendered endotoxin-free by exposure to high temperature. Drugs were kept in stock solution and diluted in serum-free RPMI at the appropriate concentrations immediately before use. Cell viability as assessed by dimethyl thiazolyl diphenyl tetrazolium (MTT) was verified (85% or more of viable cells) throughout all experimental phases.

3.2 TF pro-coagulant activity (PCA).

PCA was assessed by a one-stage clotting time test in PBMCs disrupted by three freeze–thaw cycles, as previously described [126]. In brief, disrupted cells (100 ml) were mixed with 100 ml of normal human plasma at 37°C, adding 100 ml of 25 mmol/l $CaCl_2$ at 37°C. Time to clot formation was recorded and values converted to arbitrary units (AU) by comparison with a human brain TF calibration curve covering clotting times from 20 to 600 s, corresponding to 1000 and 0 AU, respectively. Experiments were run in triplicate and averaged. As contaminating platelets (typically less than 1 platelet/PBMC) may contribute to PCA [127], we performed the clotting assay in PBMC-free preparations in preliminary experiments containing a comparable number of platelets. PCA under these conditions was undetectable.

3.3 TF antigen (ag).

Cells were disrupted by three repeated freeze–thaw cycles and debris pelleted by centrifugation at 100xg for 1 h at 4°C and supernatants used for ELISA (Imubind TF kit Sekisui Diagnostics, West Malling, United Kingdom). Briefly, 100 ml of the TF standards or the supernatant samples were incubated overnight at 4°C in microwells precoated with a murine antihuman TF capture antibody. The captured TF was detected using a biotinylated antibody fragment. Then, binding of streptavidin-conjugated horseradish peroxidase (HRP) completed the formation of the antibody–enzyme detection complex, and the addition of tetramethylbenzidine substrate and its subsequent reaction with the HRP created a blue coloured solution. The sensitivity of the method was increased by the addition of 0.5 mol/l sulfuric acid stop solution, which yielded a yellow colour. Solution absorbances were measured in a microplate reader at 450 nm. TF ag levels were expressed in pg/ml using a reference curve created by the TF standards. Within and between assay variability was 3.5 and 5.5%, respectively.

3.4 TF mRNA.

Total RNA was extracted from PBMCs using the Rneasy mini kit (Qiagen). RNA concentration and purity were determined by optical density measurement via Nanodrop (Thermo Fisher Scientific, Wilmington, Delaware USA). A mixture of 0.5 ng total RNA per sample was retro-transcribed with random primer-oligodT into complementary DNA

(cDNA) using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany). The retro-transcription cycle was performed at 25°C for 5 min, 42°C for 30 min and 95°C for 3 min. RealTime-PCR was carried out in a iQ5 Real Time PCR System and SsoAdvanced Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA) was employed on the basis of the manufacturer's instructions with a final reaction of 20 µl. RealTime-PCR was performed under the following conditions: 95°C, 30s; 40 cycles 95°C, 5s, 60°C, 15s;a final melting protocol with ramping from 65°C to 95°C with 0,5°C increments of 5sec was performed. The primers sequence for realtime-PCR were: TF, sense 5'-TTGGCAAGGACTTAATTTATACAC-3', antisense 5'-CTGTTCGGGAGGGAATCAC-3'; GAPDH, sense: 5'-CCCTTCATTGACCTCAACTACATG-3' and antisense: 5'-TGGGATTTCCATTGATGACAAGC-3' (Sigma-Aldrich, St. Louis, MO). All samples were analysed in duplicate and averaged. The relative expression of the target gene was normalized to the level of GAPDH in the same cdna.

3.5 Experimental Design.

3.5.1 Effect of RAS Blockers on LPS-induced TF ag expression and PCA.

Aliskiren (ALI), a direct renin inhibitor (DRI)[128], zofenopril (ZOF), an angiotensin converting enzyme inhibitor (ACEI)[129], olmesartan (OLM)[130], an angII type 1 receptor blocker (ARB) or vehicle were added at log-increasing (10^{-11}–10^{-6} M) steps to PBMCs LPS-stimulated (0.1 µg/mL) under NG conditions to delineate the profile of the respective inhibitory concentration-response curves.

In additional series, PCA was characterized by adding an antihuman TF antibody (30 μg/mL, 30 minutes), which neutralizes the procoagulant activity of both TF and TF/FVIIa complexes (affinity purified inhibitory goat anti-human-TF IgG, epitope-specific for amino acid sequence 1-25, American Diagnostica), to suspensions of LPS-stimulated PBMCs under either HG or NG conditions.

3.5.2 Effect of AT2R agonism and antagonism on LPS-induced TF expression.

To establish the effect of C21, a specific AT2R agonist [121], on TF ag and PCA and delineate its concentration–response profile, LPS-stimulated PBMCs were exposed at log-increasing drug concentrations (10^{-8}–10^{-5} M). To assess the effect of C21 on TF transcription, levels were quantified in LPS-stimulated PBMCs in absence or presence of maximally effective drug concentrations (10^{-5} M). To validate the underlying assumption of AT2R agonism by C21, additional experiments were run in LPS-stimulated PBMCs preincubated with maximally effective concentrations of PD123,319 or OLM (10^{-6} M for both).

To evaluate, also, the effect of AT2R blockade *per se* on TF ag and PCA and delineate its concentration–response profile, LPS-stimulated PBMCs were exposed at log-increasing concentrations PD123,319 (di(trifluoroacetate) salt hydrate, Sigma, Milan, Italy, 10^{-11}–10^{-6} M), an AT2R antagonist [131].

3.5.3 Effect of Glucose on TF expression.

To evaluate the effect of glucose on TF expression, LPS-stimulated PBMCs were exposed to four increasing concentrations of D-Glucose (5.5, heretofore referred to as normal glucose, NG, 16.5, 26 and 50 mM) using L-Glucose as a control for osmolarity changes.

The effect of 50 mM D-glucose (the concentration achieving the maximum effect, heretofore referred to as HG) on TF expression was then compared to NG in PBMCs activated by LPS.

3.5.4 Effect of AT2R agonism and AT1R blockade on LPS-induced TF expression in PBMCs exposed to HG.

To assess the effect of AT2R agonism and AT1R blockade on LPS-induced TF expression under HG conditions, C21 or OLM were added at log-increasing (10^{-8}-10^{-5} M) steps to LPS-stimulated PBMCs with NG as a control.

3.5.5 Effect of AT2R agonism in AT2R-blocked PBMCs exposed to HG.

To evaluate the C21 AT2R specificity, C21 was added at log-increasing (10^{-8}-10^{-5} M) steps to LPS-stimulated PBMCs exposed to NG or HG, after a pre-incubation of 30 minutes with PD123,319 (10^{-6} M, , Sigma Milan, Italy), a specific AT2R antagonist.

3.5.6 Effect of angII on LPS-induced TF PCA under NG and HG conditions.

To assess the assumption of the involvement of locally expressed angII in our system, angII was tested in PBMCs exposed to NG or HG.

3.5.7 Effect of NF-kB inhibition on LPS-induced TF PCA under NG and HG conditions.

To test the involvement of NF-kB in our system, BAY 11-7082 (10-5 M Sigma, Milan, Italy), a well characterized inhibitor of NF-kB [132], the key controller of TF gene expression [8], was added in LPS-stimulated PBMCs exposed to NG and HG.

3.5 Statistics

Mann Whitney test for unpaired data and Wilcoxon test for paired data were used on absolute data or percent changes from control conditions. Data were reported as means±SD unless otherwise reported. A two-tailed p-level <0.05 was the threshold for statistical significance.

4. RESULTS

LPS stimulation (0.1 μg/mL) increased TF PCA (from 0.0054±0.0033 to 0.93±0.36 AU, n=33, p<0.001), TF ag expression (from 32±24 to 1486±388 pg/mL, n=19, p<0.001) and TF mRNA (from 0.005±0.0027 to 0.65±0.5 normalized fold expression, n=8, p<0.001)(Fig.1 panels a,b,c respectively).

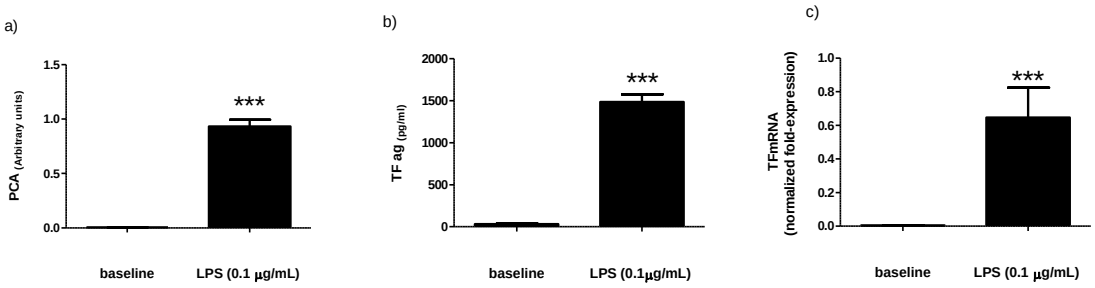

Fig.1. LPS stimulation (0.1 μg/mL) increased TF PCA (n=33, panel a), TF ag expression (n=19, panel b) and TF mRNA (n=8, panel c). *** p<0.001

To LPS-stimulated PBMCs was added an anti-human TF antibody (30 μg/mL), which neutralizes the TF PCA (from 0.93±0.36 to 0,019±0,0095 AU, n=5, p<0.001) (Fig.2).

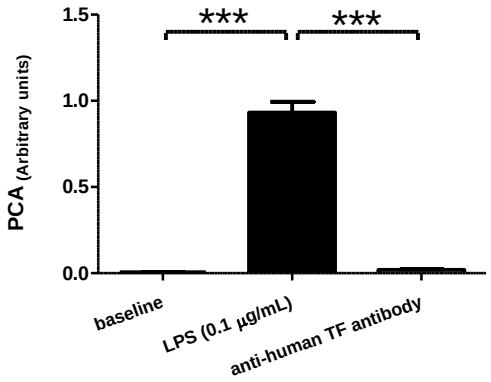

Fig. 2. The anti-human TF antibody (30 μg/mL) neutralizes the TF PCA LPS-stimulated (n=5). *** p<0.001

4.1 Effect of RAS Blockers on LPS-induced TF expression.

ALI, ZOF and OLM downregulated LPS-activated TFAg expression and PCA to a comparable extent as regards maximum efficacy under NG conditions (TFAg: ALI:−44±6%, n=6, ZOF:−40±19%, n=6, OLM:−37±5%, n=5; PCA: ALI:−44±22%, n=11, ZOF:−52±15%, n=6, OLM:−48±18%, n=5, $p<0.001$ vs vehicle for all). However, inhibition by ALI achieved its peak at 10^{-11} M and decreased at higher drug concentrations, a pattern opposite to that of both ZOF and OLM (Fig. 3, left and right panels).

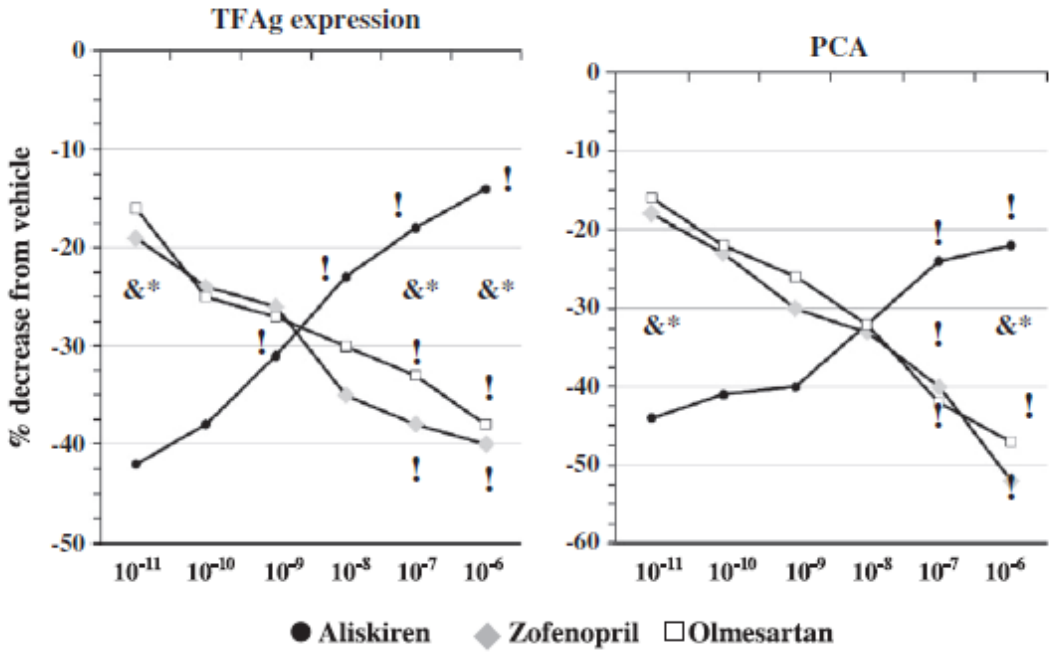

Fig. 3. Decreasing inhibition of LPS-stimulated TFAg (left panel, n=6) and PCA (right panel, n=11) by ascending concentration of aliskiren vs the opposite behaviour of zofenopril and olmesartan. For the sake of clarity, figures report only mean values. * $p<0.001$ Aliskiren vs Zofenopril; & $p<0.001$ Aliskiren vs Olmesartan; ! $p<0.05$ or less vs 10^{-11} M values.

4.2 Effect of AT2R agonism and antagonism on LPS-induced TF expression.

C21 downregulated in a concentration-dependent manner TFag and PCA, an effect antagonized by PD123,319 and left unchanged by OLM (Fig. 4, left and right).

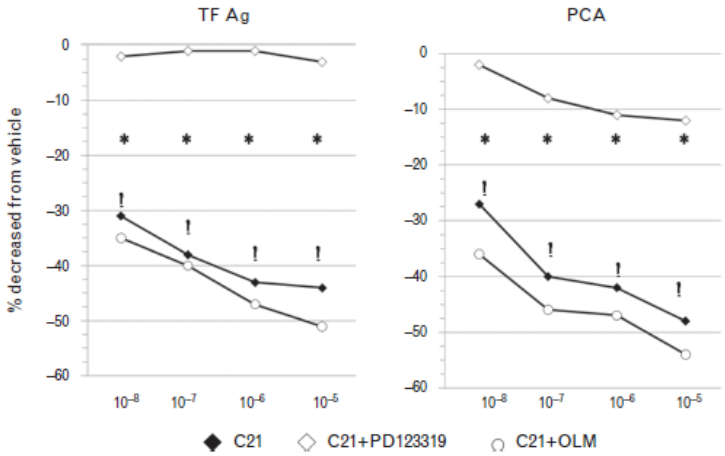

Fig.4. C21 downregulates lipopolysaccharide-stimulated tissue factor antigen (left, n=13) and PCA (right, n=14), an effect antagonized by PD123,319 (10^{-6} M, n=6 and 14, respectively) and left unchanged by OLM (10^{-6} M, n=7 and 10, respectively). For the sake of clarity, only mean values are reported. ! $p<0.001$ C21 vs. vehicle; * $p<0.001$ C21 vs. C21+PD123,319.

C21 blunted LPS-induced TF mRNA (from 0.82±0.57 to 0.24±0.13 normalized fold expression, n=5 each, $p<0.001$), a 3.5-fold inhibition (Fig. 5).

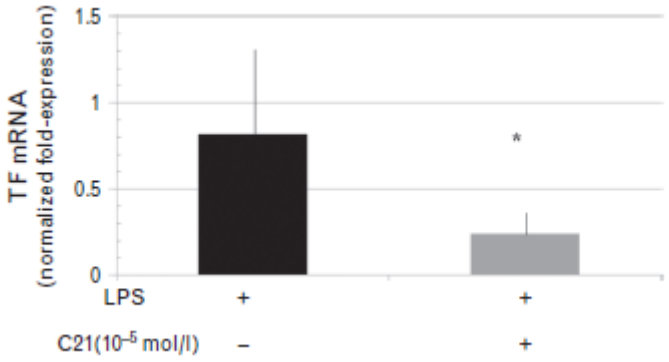

Fig.5. C21 (10^{-5} M) inhibits lipopolysaccharide-induced tissue factor mRNA stimulation. Mean±SD, n=5 each; * $p<0.001$.

PD123,319 *per se* did not affect LPS-induced TF PCA and antigen expression (Fig. 6, left and right).

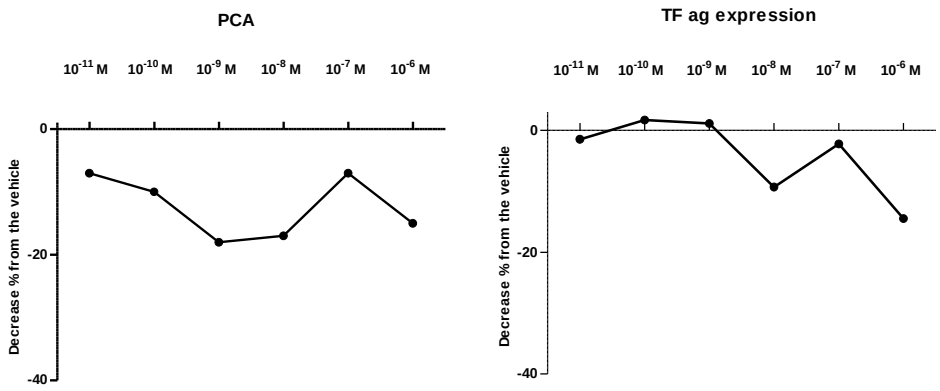

Fig.6. PD123,319 effect on lipopolysaccharide-stimulated TF PCA (right, n=11) and ag expression (left, n=7). For the sake of clarity, only mean values are reported.

4.3 Effect of Glucose on TF expression.

Increasing D-glucose up to a 50 mM concentration augmented LPS-induced PCA without evidence of a plateau. Equimolar L-glucose concentrations were neutral (Fig. 7).

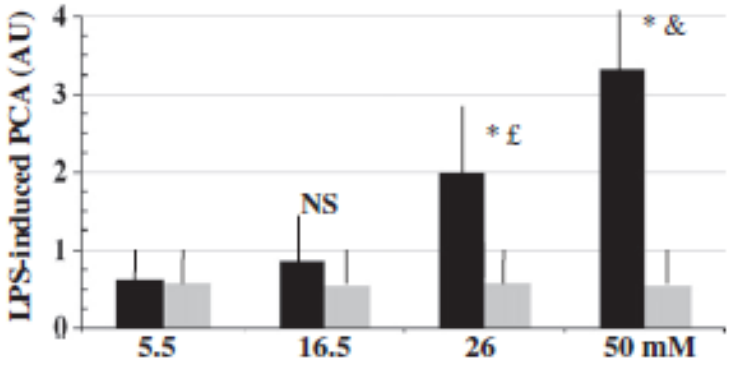

Fig. 7. Increasing D-Glucose concentrations (black bars) augment LPS (100 ng/mL)-induced PCA as opposed to the ineffectiveness of L-glucose (gray bars). Mean±SD, n=11; * $p<0.001$ vs 5.5 nM, & $p<0.001$ vs 26 nM, £ $p<0.001$ vs 16.5 nM, NS=Not significant vs 5.5 nM.

As compared with NG, HG amplified the stimulatory effect of LPS also on TF ag (from 1486±388 to 2652±622 pg/mL, n=19, p<0.001) and TF mRNA (from 0.65±0.5 to 0.85±0.53 normalized fold expression, n=8, p<0.01) (Fig. 8, panels a and b, respectively).

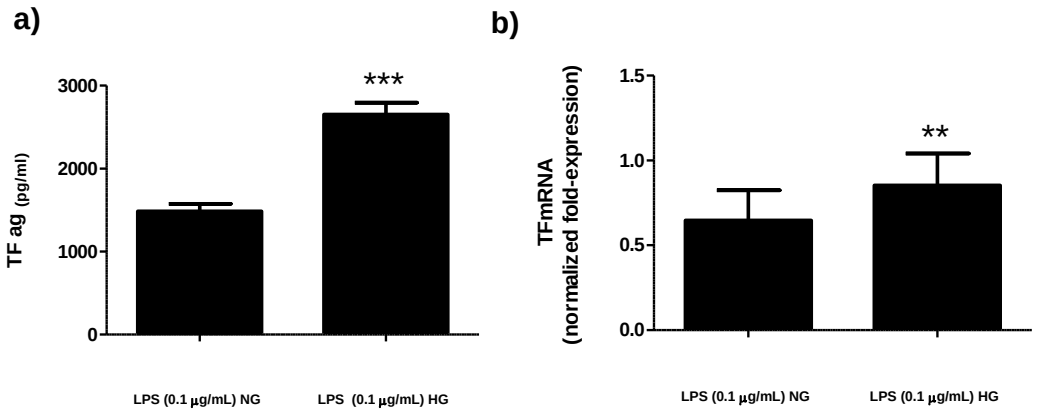

Fig.8. HG amplified the stimulatory effect of LPS on TF ag (n=19, panel a) and TF mRNA (n=8, panel b) *** p<0.001, ** p<0.01

4.4 Effect of AT2R agonism and AT1R blockade on LPS-induced TF expression in PBMCs exposed to HG.

C21 down-regulated in a concentration dependent manner LPS-induced TF PCA without differences between NG and HG (Fig.9, panel a) while the concentration dependent inhibitory effect of OLM was greater in HG than in NG (Fig.9, panel b). Similar data were obtained evaluating TF ag and TF mRNA (Fig.10, panel a and panel b respectively).

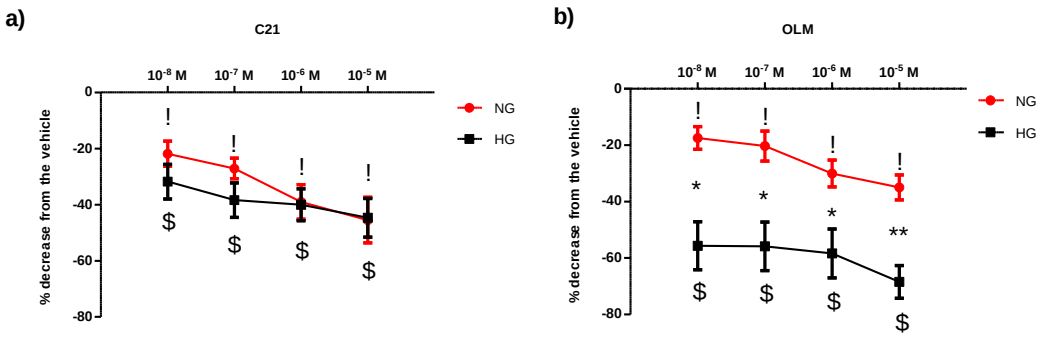

Fig. 9. C21 downregulated in a concentration dependent manner LPS-induced TF PCA in NG maintaining unchanged its effect in HG (panel a, n=12). Figures report mean±SD, *** p<0.001, ** p<0.01 vs. vehicle in NG, ! p<0.001 vs. vehicle in HG. OLM downregulated in a concentration dependent manner LPS-induced TF PCA in NG potentiating its effect in HG (panel b, n=12). ** p<0.01, * p<0.05 OLM in HG vs. OLM in NG, ! p<0.001 vs. vehicle in NG, $ p<0.001 vs. vehicle in HG.

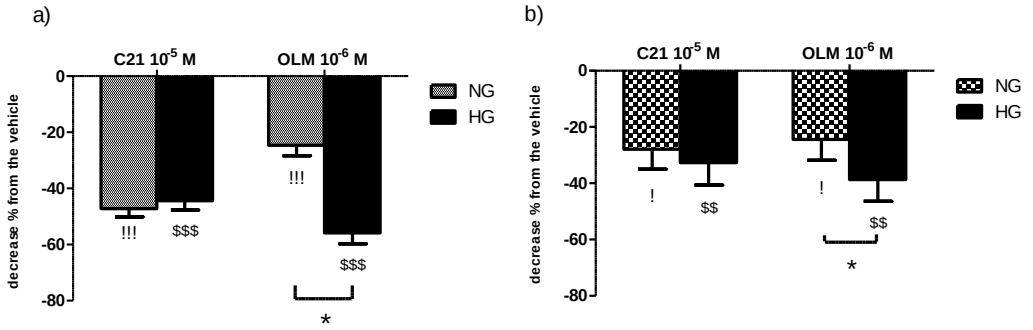

Fig.10. C21 and OLM downregulated LPS-induced TF ag (panel a, n=7) and TF mRNA (panel b, n=8) in NG. OLM inhibitory effect on TF ag and TF mRNA was potenziated in HG (panel a and panel b respectively). Figures report mean±SD. ! p<0.05, !!! p<0.001 vs. vehicle in NG; $$ p<0.01, $$$ p<0.001 vs. vehicle in HG; * p<0.05, *** p<0.001 OLM in HG vs. OLM in NG

4.5 Effect of AT2R agonism in AT2R-blocked PBMCs exposed to HG.

The pre-treatment with PD123,319 (10^{-6} M) inhibited C21 downregulation of LPS-induced TF PCA in NG and in HG (Fig.11) demonstrating AT2R specificity of these effects also under HG conditions. These results has also been confirmed with TF ag (Fig.12).

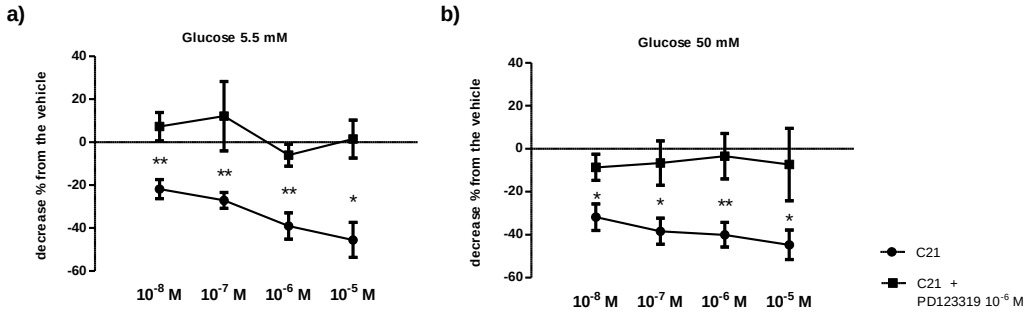

Fig.11. The pre-treatment with PD123,319 (10^{-6} M) inhibited C21 effect in NG and in HG (left panel and right panel, respectively)(n=6) Figures report mean±SD. ** p<0.01, * p<0.05 vs. C21+ PD123,319.

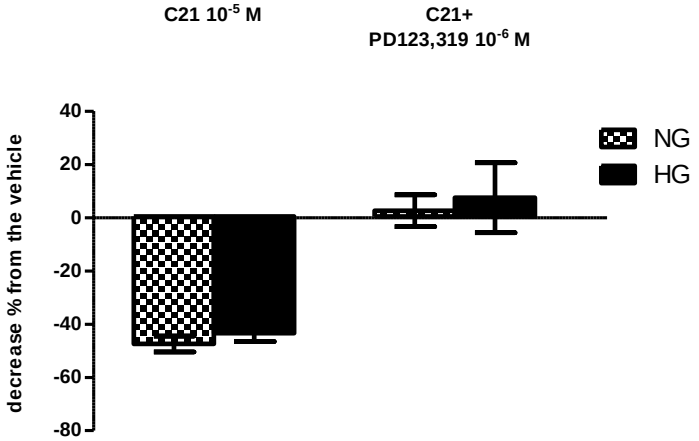

Fig.12. The pre-treatment with PD123,319 (10^{-6} M) inhibited C21 effect in NG and in HG (left panel and right panel, respectively)(n=6) Figures report mean±SD. ** p<0.01, * p<0.05 vs. C21+ PD123,319. (NG: 3±15%, n=6, p<0.001 vs C21 alone; HG: 9±30%, n=6, p<0.001 vs C21 alone)

4.6 Effect of angII on LPS-induced TF PCA under NG and HG conditions.

AngII (10^{-6} M) stimulated TF PCA in NG (from 0,005±0,002 to 0,01±0,006 AU, n=14, p<0.01) and in HG (from 0,011± 0,007 to 0,02± 0,01 AU, n=10, p<0.05), an effect inhibited by OLM (10^{-6} M)(NG: 10^{-6} M -38±17%, n=4, p<0.01; HG: 10^{-6} M -59±3%, n=4, p<0.001)(Fig.13).

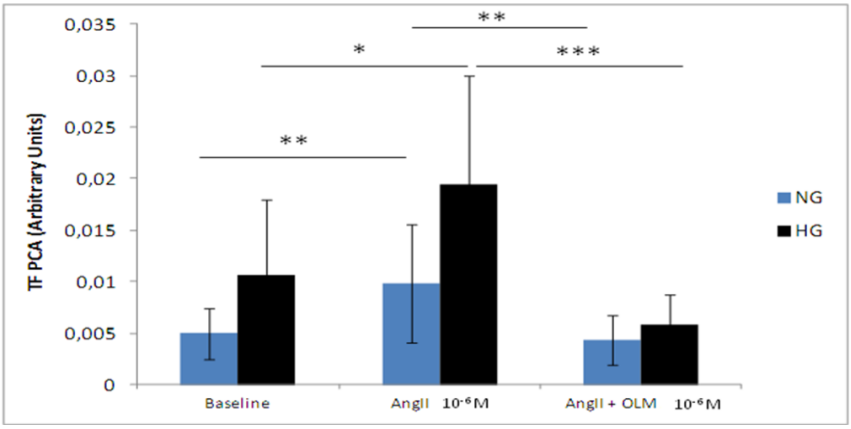

Fig.13. AngII (10^{-6} M) stimulated TF PCA in NG (n=14) and in HG (n=10), an effect inhibited by OLM (10^{-6} M) in NG and in HG (n=4). Figures report mean±SD. *** p<0.001, ** p<0.01, * p<0.05

4.7 Effect of NF-kB inhibition on LPS-induced TF PCA under NG and HG conditions.

BAY 11-7082, a NF-kB inhibitor, abolished at a 10^{-5} M concentration the LPS-stimulated PCA in PBMCs exposed to NG or HG (NG: -95±3%, n=8, p<0.001; HG: -99±0.3%, n=8, p<0.001).

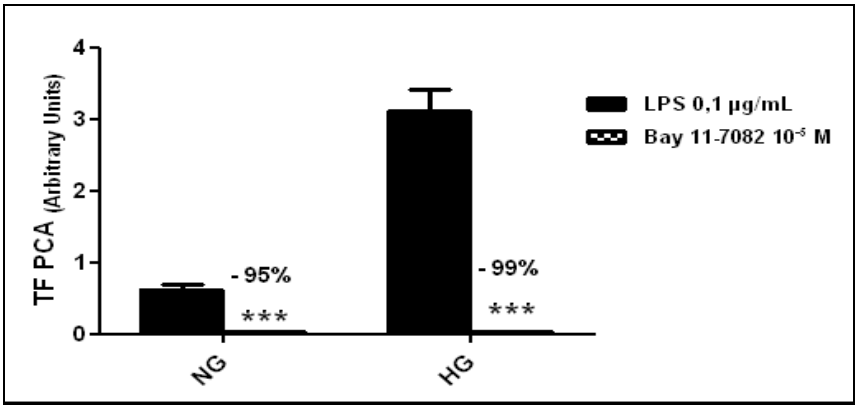

Fig. 14. BAY 11-7082, a NF-kB inhibitor (10^{-5} M) abolished the LPS-stimulated PCA in PBMCs exposed to NG and HG (n=8). Figures report mean±SD. *** p<0.001

5. DISCUSSION

Our results highlight the ability of the RAS and in particular of its main effector, angII, to modulate the expression of TF in monocytes a cellular population capable of generating the whole set of RAS components: angII [99], renin [96], angiotensinogen [98], ACE [97], angII receptors [100, 101]. The antagonism of LPS-stimulated TF expression by a highly heterogeneous group of drugs such as ALI, ZOF, OLM sharing angII blockade as the only common pharmacological denominator, confirms the involvement of the RAS in the TLR-4 signalling pathway reported by others in different experimental conditions [133]. In addition, our data provide original evidence suggestive of endotoxin-mediated activation of the upstream and rate-limiting step of the system since TF inhibition by ALI, a direct renin inhibitor, was maximal at concentrations (10^{-11} M) coincident with those inhibiting the interaction of human renin with its substrate angiotensinogen (6×10^{-12} M)[128]. The peculiar inhibitory concentration-response relationship of ALI, characterized by fading efficacy as a function of increasing drug concentrations, a trend opposite to that elicited by ZOF and OLM opens obvious but unanswered questions about mechanisms although unrestrained renin production as a reaction to a disrupted angII-mediated negative feed-back by ALI may plausibly facilitate its binding to newly identified (pro)renin receptor((P)RR)s [134] and promote the synthesis of counteracting pro-thrombotic mediators independent of the classical pathways operated by angII [135].

Moreover, we document for the first time the inhibitory potential of AT2R stimulation on inflammation-mediated pro-coagulant response by down-regulating TF expression. In particular TF mRNA down-regulation by C21 likely reflects NF-kB inhibition, a crucial controlling step in gene transcription in response to inflammatory agonists [8], consistent

with previous reports obtained in different experimental systems with C21 itself [123] and other AT2R agonists [118]. Inhibition by C21 of IL-6 production in TNF-α stimulated human and murine dermal fibroblasts [123] and primary rat astrocytes exposed to LPS [136] and reduced expression of inflammatory mediators in experimental models of myocardial and renal damage [122, 137, 138] as well as the additional evidence reviewed in detail elsewhere [76, 136] strengthen our conclusions. An obvious question raised by these results considers the reasons for the discrepancy between the inhibition induced by AT2R stimulation and the missing response to AT2R antagonism, this latter an already reported behaviour [119] for which several explanations might be envisaged. In fact, despite their predominance in foetal life, AT2R number decrease after birth and AT1Rs overcome the quantitative distribution of the other subset in adulthood by several fold [120]. Therefore, studies with the AT2R antagonist PD123,319 are easily bound to produce negative or equivocal results, as it is difficult to antagonise biological effects whose baseline functional expression is negligible [120]. As a matter of fact, unchanged levels of AT2R mRNA in inflamed human glomerular endothelial cells [119] suggest that the effect of C21 might represent the result of a pharmacological manipulation of physiologically silent binding sites exposed to an agonist attaining concentrations at the receptor site far exceeding those achieved by angII, the endogenous ligand [76, 136]. Other possible, not alternative explanations for the neutral effect of the AT2R antagonism may relate to the difficulty of eliciting some additional stimulation in an already stimulated system of which RAS activation is one of the several and interacting signalling pathways.

Another relevant outcome of this study was the TF sensitivity of LPS-primed PBMCs to augmented ambient D-glucose, an effect independent of osmolarity changes and requiring cellular uptake and subsequent metabolism of the molecule, given the inefficacy of

equimolar concentrations of the impermeant L-isomer. RAS activation plays a relevant role in diabetes [102], a prothrombotic state [103] to which elevated glucose, the disease hallmark, may contribute by activating pro-inflammatory pathways [104-106] and increase glucose-mediated angII production reported by other authors in isolated cell systems exposed to HG [87, 107, 108]. The HG-dependent activation of inflammation/coagulation-related protein expression is due, at least partly, via increased oxidative stress [104]. Although not documented in our setting, the subsequent chain of intracellular events were likely to include, according to the available evidence, increased generation of reactive oxygen species (ROS) resulting from activation of NADPH oxidase and mitochondrial metabolism [106] acting as signalling molecules for NF-kB [104], a redox-sensitive transcription factor critical for the expression of genes encoding TF-stimulating pro-inflammatory cytokine [105] and TF itself [8]. In accord with these data we observed that BAY 11-7082, a well characterized NF-kB inhibitor [132], completely abolished the pro-coagulant response to endotoxin, in normal and high glucose. The data are also coherent with previous reports of HG-induced overexpression of Toll-like receptor(TLR)4 [139] and CD14 [140], the LPS-receptor complex that, by recruiting adaptor proteins to the cytoplasmic Toll/interleukin-1 receptor domain, initiates a series of intracellular events leading to NF-kB activation [141]. In that complex chain of events RAS and TLR-4 are interconnected signalling pathways [57, 133, 142] making RAS activation an active part of the wide spectrum of biological responses finalized to defend the host from invading bacteria [141] and in which activation of the clotting cascade is a prominent part [44].

In the above delineated experimental context, C21, an AT2R agonist, modulated the expression of TF in NG [60] and its efficacy was unchanged by exposure to HG quite in

contrast with the enhanced response to OLM, an AT1R antagonist, an expected behavior explained by several, not alternative nor exhaustive mechanisms including increased glucose-mediated angII production [87, 108, 111], upregulated AT1R [143-146] or AT1R activation as a consequence of other mechanisms unrelated to receptor density or affinity [147].

In conclusion, our results open insofar unexplored and potentially relevant facets to our understanding of the complex links connecting angII to inflammation ad coagulation and are useful to delineate more precisely the determinants of the atherothrombotic risk in diabetic patients [103]. Downregulation of responses finalized to defend the host from invading bacteria by blockade of a biological system pivotal to the onset and evolution of diabetes [102] and cardiovascular disease [100] discloses tight connections between the apparently unrelated domains of innate immunity and metabolic and cardiovascular regulation expanding the restrictive view of angII as a peptide mainly involved in the control of blood pressure and water and salt homeostasis.

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ABBREVIATIONS

ACE **A**ngiotensin **C**onverting **E**nzyme

ACEI **A**ngiotensin **C**onverting **E**nzyme **I**nhibitor

AGT **An**gio**t**ensinogeno

ALI Aliskiren

ANOVA **AN**alysis **O**f **VA**riance

ARBS **A**ngiotensin II **R**eceptor **B**locker**s**

AS TF **A**lternatively **S**pliced **T**issue **F**actor

AT1R **A**ngiotensin **II T**ype **1 R**eceptor

AT2R **A**ngiotensin **II T**ype **2 R**eceptor

AT3R **A**ngiotensin **II T**ype **3 R**eceptor

ATR4 **A**ngiotensin **II T**ype **4 R**eceptor

DRI **D**irect **R**enin **I**nhibitor

ELISA **E**nzyme **L**inked **I**mmuno-**S**orbent **A**ssay

FL TF **F**ull-**L**ength **T**issue **F**actor

HG **H**igh **G**lucose

ICAM-1 **I**nter**c**ellular **A**dhesion **M**olecule 1

I-kB **I**nhibitor of Nuclear Factor **κ**appa **B**

IL **I**nterleukin

JG **J**uxtaglomerular **C**ells

LPS **L**ipo**p**oli**s**accaride

MCP-1 **M**onocyte **C**hemoattractant **P**rotein – 1

MI **M**yocardial **I**nfarction

MP **M**icroparticles

NF-kB **N**uclear **F**actor **kB**

NG **N**ormal **G**lucose

NO **N**itric **O**xide

NRVMs **N**eonatal **R**at **V**entricular **M**yocytes

OLM **O**lmesartan

PAR-1 **P**rotease **A**ctivated **R**eceptor **1**

PAR-2 **P**rotease **A**ctivated **R**eceptor **2**

PDGF **P**latelet **D**erived **G**rowth **F**actor

RAAS **R**enin **A**ngiotensin **A**ldosterone **S**ystem

RAS **R**enin **A**ngiotensin **S**ystem

SD **S**tandard Deviation

TF ag **T**issue **F**actor **A**ntigen

TF PCA **T**issue **F**actor **P**ro-**c**oagulant **a**ctivity

TF **T**issue **F**actor

TGF-β **T**ransforming **G**rowth **F**actor **β**eta

TNF-α **T**umor **N**ecrosis **F**actor-**α**lpha

VCAM-1 **V**ascular **C**ell **A**dhesion **M**olecule **1**

VEGF **V**ascular **E**ndothelial **G**rowth **F**actor

VSMCs **V**ascular **S**mooth **M**uscle **C**ells

ZOF Zofenopril