Induction of atrial endothelial senescence by angiotensin II and thrombin : role of oxidative stress and characterization of pro-thrombotic, pro-adhesive, proteolytic and pro-fibrotic phenotype

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Induction de la sénescence endothéliale auriculaire par l'angiotensine II et la thrombine : Rôle du stress oxydant et caractérisation du phénotype pro-thrombotique, pro-adhésif, protéolytique et pro-fibrotique

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DEDICATION

I would like to dedicate my thesis to my parents, ***Ch Ilyas Hasan*** and ***Batool Ilyas***, my husband ***Muhammad Umar*** and my siblings ***Ch Raza Hasan***, ***Sarah Hasan***, ***Fareeha Hasan*** and ***Maryam Nawal Hasan*** whose continuous support made this journey easier and possible.

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HASAN Hira

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List of Abbreviations

AF	Atrial fibrillation
AECs	Atrial endothelial cells
Ang II	Angiotensin II
AT1R	Angiotensin type 1 receptor
ACE	Angiotensin-converting enzyme
APD	Action potential duration
ANP	Atrial natriuretic peptide
CAD	Coronary artery disease
CABG	Coronary artery bypass graft
DTI	Direct thrombin inhibitor
ECG	Electrocardiogram
eNOS	Endothelial nitric oxide synthase
HF	Heart failure
ICAM-1	Intercellular adhesion molecule-1
MCP-1	Monocyte chemoattractant protein-1
NO	Nitric oxide
PVs	Pulmonay veins
PDGF	Platelet-derived growth factor
PAR	Protease-activated receptor
PAI-1	Plasminogen activator inhibitor-1
PKC	Protein kinase C
ROS	Reactive oxygen species
RAAS	Renin angiotensin aldosterone system
SASP	Senescence-associated secretory phenotype
TGF-β1	Tranforming growth factor-β1

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

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RESUME

RESUME (Version French)

La fibrillation auriculaire (FA) représente un enjeu mondial de santé publique. Les estimations suggèrent que son incidence devrait doubler d'ici 20 ans. Bien que les progrès accomplis dans le diagnostic et le traitement de la FA soient considérables, la pathologie reste associée à une morbidité et une mortalité importantes notamment en raison de son rôle dans la survenue des accidents vasculaires cérébraux d'origine ischmiques. Il est généralement reconnu que la fibrose est un processus central qui contribue au remodelage pathologique du massif auriculaire, conduisant au développement et au maintien de la FA. Cependant, les mécanismes sous-jacents de la fibrose et du rtemodelage tissulaire dans la FA restent mal connus. La prévalence de la FA est liée à l'âge. Le raccourcissement télomérique, caractéristique du vieillissement, est corrélé positivement à l'incidence de la FA indiquant qu'il s'agit d'un facteur de risque majeur de développer la pathologie.

La sénescence est une réponse cellulaire caractérisée par un arrêt de croissance associé à l'acquisition d'un phénotype pro inflammatoire. Elle joue un rôle dans le développement normal, le maintient de l'homéostasie tissulaire et limite la progression tumorale. Cependant, la sénescence est également considérée comme une cause majeure de maladie liée à l'âge. La sénescence peut être induite par différents stimuli, notamment un dysfonctionnement télomérique, suite à une exposition à des rayonnements ionisants, les espèces réactives de l'oxygène (ROS), de fortes concentrations en glucose ou l'exposition des cellules à des cytokines pro inflammatoires. Il est bien établi que l'arrêt du cycle cellulaire est médié par p21 et p16, deux inhibiteurs de la kinase dépendante des cyclines (CDK). De plus, les dommages persistants de l'ADN sont rapportés pour être à la base du phénotype caractéristique inflammatoire et tumorigène des cellules sénescentes. On parle du phénotype sécrétoire associé à la sénescence (SASP). Ce SASP, largement dépendant de la signalisation NF-κB, est caractérisé par l'exposition de molécules d'adhérence, l'activation de métalloprotéinases (MMP) ainsi que la sécrétion de nombreuses cytokines. Il a également été montré que la sénescence des cellules endothéliales (CE) pouvait se produire dans différents contextes pathologiques *in vivo*. En effet, des niveaux élevés de sénescence vasculaire ont été observés dans l'arc aortique de rats spontanément hypertendus et dans l'aorte de rats diabétiques. Des études sur des cellules en culture ont montré que la sénescence des CE était associée à une diminution de l'expression de la monoxyde d'azote synthase endothéliale (eNOS), à l'induction d'un état pro inflammatoire et à des dommages de l'ADN. En outre, la surexpression du suppresseur

de tumeur p53 est responsable d'un dysfonctionnement endothélial et d'une biodisponibilité réduite du monoxyde d'azote (NO) dans des aortes de rat ex vivo et des CE en culture.

Le stress oxydatif a été suggéré comme un contributeur majeur au développement de la dysfonction endothéliale et à l'hyper contractilité artérielle liée au vieillissement. Ceci en réduisant la biodisponibilité de facteurs vasodilatateurs comme le NO et le facteur hyperpolarisant dépendant de l'endothélium, mais également par l'induction de réponses contractiles dépendantes de l'endothélium. Le stress oxydatif implique plusieurs sources de ROS, notamment la nicotinamide adénine dinucléotide phosphate (NADPH) oxydase, les mitochondries, les eNOS non couplés et les cyclooxygénases. En outre, des études antérieures ont montré que l'apocynine, un inhibiteur de la NADPH oxydase, restaurait les relaxations dépendantes de l'endothélium altérées au cours du vieillissement et ce, aussi bien dans les micro-vaisseaux humains, que dans les modèles animaux. La vitamine C également, par son action antioxydante, permet de restaurer l'augmentation de flux sanguin induite par l'acétylcholine, réduite chez les sujets âgés.

De nombreux travaux suggèrent que le système rénine angiotensine (RAS) est acteur majeur du dysfonctionnement endothélial lié au vieillissement. En effet, l'enzyme de conversion de l'angiotensine (ACE) et les récepteurs AT1R de l'angiotensine II (Ang II) sont augmentés dans paroi artérielle d'individus âgés. De plus, les traitements par les inhibiteurs de l'ACE ou les antagonistes des récepteurs de l'Ang II préviennent le dysfonctionnement endothélial lié au vieillissement. En outre, des études antérieures ont démontré un rôle clé de l'Ang II dans la pathogenèse de la FA. Le récepteur AT1R est connu pour activer les protéines kinases activées par les mitogènes (MAPK), qui favorisent le remodelage auriculaire en induisant la prolifération des fibroblastes, l'hypertrophie cellulaire et l'apoptose.

On sait depuis longtemps que la FA a été associée à l'activation de facteurs de coagulation locaux et circulants. Cette hypercoagulabilité augmente considérablement le risque de formation de caillots et d'accident vasculaire cérébral chez les patients atteints de FA. Cependant, le rôle potentiel de cette hypercoagulabilité dans le remodelage du tissu auriculaire et plus spécifiquement le rôle de la thrombine ou du facteur Xa (FXa) sont inconnus. Outre ses effets hémostatiques, il a également été démontré que la thrombine induisait des effets cellulaires par l'activation des récepteurs activés par les protéases (PAR).

Les récepteurs PAR constituent une famille de récepteurs couplés aux protéines G qui s'activent par clivage protéolytique du domaine N-terminal, révélant un nouveau ligand captif qui se lie de

manière intramoléculaire pour induire une transduction de signal intracellulaire. Quatre membres de la famille PAR sont identifiés, PAR-1 à 4. PAR-1 étant principalement activé par la thrombine, alors que PAR-2 est principalement activé par la trypsine et les protéases analogues. De nombreux types de cellules sont activés par l'action de la thrombine sur les PAR, notamment les plaquettes, les cellules musculaires lisses (CML) vasculaires, les lymphocytes et les CE, liant ainsi la coagulation à l'inflammation. Les récepteurs PAR-1 et PAR-2 sont exprimés dans le cœur. PAR-1 est exprimé par les myocytes, les fibroblastes, les CE et les CML. Bien que PAR-2 soit également exprimé par les myocytes, les CE et les CML, son expression par les fibroblastes n'a pas été confirmée.

La thrombine entraine l'expression de P-sélectine à la surface des plaquettes et des CE, de molécules d'adhésion telles que ICAM-1, VCAM-1 et la E-sélectine à la surface des fibroblastes, des CML et des CE ainsi que l'expression de diverses cytokines telles que l'interleukine-6 et la chimiokine MCP-1. Ces mécanismes conduisent au recrutement de leucocytes dans la paroi vasculaire, à leur diapédèse, et contribue aux processus inflammatoires et fibrotiques. De plus, la thrombine induit l'apoptose des CE par l'activation de NF-κB et des caspases, et augmente la perméabilité vasculaire en modifiant la structure et les adhérences endothéliales. Finalement, lla démonstration récente que certains anticoagulants oraux directs, comme le dabigatran, puissent limiter la progression du remodelage auriculaire suggère un rôle direct de la thrombine dans ce processus.

Des effets directs du FXa ont également été rapportés. Le FXa augmente l'expression des récepteurs PAR et de médiateurs inflammatoires dans des sections d'oreillette humaines. D'autres travaux ont établi que la tachyarythmie par elle même, était capable d'augmenter l'expression du récepteur PAR-1. D'une façon générale, il est probable que l'activation des récepteurs PAR contribuent au remodelage structural des oreillettes, caractérisées notamment par une fibrose et une dilatation. Ces modifications de substrat jouent un rôle majeur dans la perpétuation de la fibrillation auriculaire. Par ailleurs, la réaction inflammatoire, la fibrose tissulaire et l'hypertrophie cellulaire contribuent de manière significative à la perte de conductivité électrique entre les myocytes et par conséquent majorent les perturbations de conduction dans les oreillettes pathologiques. Par conséquent, au vu de l'efficacité des inhibiteurs spécifiques de la coagulation dans la prévention des modifications cellulaires arythmogènes, le rôle des récepteurs PAR et/ou de l'hypercoagulabilité dans le développement de la FA pourrait être déterminants.

Cette étude vise à caractériser les changements phénotypiques associés à la sénescence des CE auriculaires et à déchiffrer le lien entre vieillissement et thrombogénicité. En outre, nous avons évalué la contribution de facteurs de la cascade de coagulation, tels que la thrombine, dans l'induction de la sénescence prématurée des CE auriculaires et l'acquisition d'un profil pro-thrombotique et pro-fibrotique.

Pour mener à bien cette étude, un modèle original de culture primaire de CE auriculaires a été mis au point. Les cultures ont été obtenues après digestion à la collagénase d'oreillettes de porcs. La sénescence endothéliale a été évaluée par la mesure de l'activité bêta-galactosidase (SA-β-gal) par cytométrie en flux, par l'expression de protéine par Western blot, par la mesure de l'agrégation plaquettaire, par la mesure d'acteurs du remodelage de la matrice extracellulaire par zymographie (MMP matricielles) et par mesure du stress oxydatif (sonde dihydroéthidium). Une sénescence réplicative a été induite par le passage des CE auriculaires de P1 à P4 et une sénescence prématurée par l'exposition à un inhibiteur de la eNOS (L-NAME), le peroxyde d'hydrogène, la thrombine ou l'Ang II.

La sénescence des CE auriculaires est caractérisée par une augmentation de l'activité de la SA-β-gal, une augmentation d'un régulateur de la sénescence cellulaire, la protéine p53, et d'inhibiteurs clés de la CDK, p21 et p16. L'exposition des CE auriculaires à la thrombine entraîne une augmentation concentration-dépendante de l'activité de la SA-β-gal, à un niveau similaire à celui induit par l'Ang II et le peroxyde d'hydrogène. La réponse pro-sénescence à la thrombine a également été associée à une expression accrue de p16, p53 et p21. De plus, le phénotype des CE auriculaires sénescentes était caractérisé par: (i) une thrombogénicité cellulaire accrue via une augmentation de l'expression du facteur tissulaire, une diminution de la eNOS et un potentiel antiagrégant plaquettaire réduit, (ii) une augmentation des protéines d'adhésion cellulaire comme ICAM-1, (iii) une protéolyse matricielle et un remodelage pro fibrosant attestée par l'expression accrue des MMP-2 et 9 et du TGF-β1, et (iv) l'activation du SRA local par l'expression accrue des récepteurs AT1R et de l'ACE. Le losartan, un antagoniste des récepteurs AT1R comme le Perindoprilat, un inhibiteur de l'ACE, empêchent la sénescence des CE auriculaires. Tout comme l'Ang II, la thrombine provoque un stress oxydatif et cet effet est bloqué par la N-acétylcystéine, un antioxydant, par l'inhibiteur de la NADPH oxydase le VAS-2870, par l'inhibiteur de la cyclooxygénase, l'indométacine et par les inhibiteurs de la chaîne respiratoire mitochondriale (roténone, myxothiazol et KCN), ainsi que par le losartan et le périndoprilat. De plus, nous avons

également des données préliminaires suggérant un effet similaire du facteur Xa sur l'induction de la sénescence et l'augmentation du stress oxydatif dans les CE auriculaires.

Ainsi, à partir de cette étude, nous pouvons conclure que la sénescence de l'endothélium auriculaire favorise la thrombogénicité, l'inflammation, le remodelage matriciel et la régulation positive du SRA local. Les présents résultats indiquent en outre que la thrombine est un puissant inducteur de sénescence prématurée des CE auriculaires caractérisée par une altération de la voie protectrice du NO et par l'induction de réponses pro-inflammatoires et pro-fibrotiques. Ils mettent en évidence l'implication du SRA local et suggèrent qu’un ciblage de la voie Ang II / AT1R pourrait constituer une stratégie thérapeutique prometteuse pour limiter les effets délétères du vieillissement endothélial auriculaire.

RESUME (English version)

Atrial fibrillation (AF) has become a serious epidemic health problem across the world, and the incidence is expected to double within the next 20 years. Although there is considerable progression in the diagnosis and treatment of AF, it is associated with increased morbidity and mortality. It is generally known that atrial fibrosis contributes to atrial structural remodeling, leading to the development and maintenance of AF. However, the underlying mechanisms of fibrosis in AF remain unclear. Whilst numerous epidemiological studies have demonstrated the close link between AF and ageing, the description of precise mechanisms is still lacking. Among Pioneering study has demonstrated that short telomere length, a hallmark of aging and senescence, was associated with the incidence of AF suggesting that senescence per se could pave the way to AF onset. Senescence is a cellular response characterized by a stable growth arrest and other phenotypic alterations that include the acquisition of a proinflammatory secretome. Senescence plays a role in normal development, maintains tissue homeostasis, and limits tumor progression. However, senescence has also been implicated as a major cause of age-related disease. Senescence can be induced by a plethora of stimuli, including ionizing radiation telomere dysfunction, ROS, high glucose concentrations or inflammatory cytokines. It has been established that the underlying cell cycle arrest is mediated by p21 and p16, two cyclin-dependent kinase inhibitors, and that persistent DNA damage signaling drives the hallmark - inflammatory and tumorigenic - phenotype of senescent cells, termed the senescence-associated secretory phenotype (SASP). This SASP, which prominently involves NF-κB signaling, comprises adhesion molecules, metalloproteinases, and many cytokines. Endothelial cell senescence has also been shown to occur *in vivo* in several types of pathological arteries. Indeed, high levels of vascular senescence have been observed in the aortic arch of spontaneously hypertensive rats and in the aorta of diabetic rats. Studies with cultured cells have indicated that endothelial cell senescence is associated with the down-regulation of endothelial nitric oxide synthase (eNOS), the induction of a proinflammatory state, and DNA damages. In addition, it was previously established that the overexpression of endothelial p53, a mediator of endothelial senescence, induced endothelial dysfunction and decreased nitric oxide (NO) bioavailability in rat aortic sections and the down-regulation of eNOS in cultured endothelial cells.

Oxidative stress has been suggested to be a major contributor to the development of aging-related endothelial dysfunction by reducing the bioavailability of both the endothelial NO and the endothelium-dependent hyperpolarization response, and possibly also by induction of endothelium-dependent contractile responses. Indeed, a high level of oxidative stress is observed throughout the aged arterial wall, which has been suggested to involve several sources of ROS including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondria, uncoupled eNOS, and cyclooxygenases. The major relevance of this pathway was emphasized by previous studies demonstrating (i) that the NADPH oxidase inhibitor apocynin improved aging-related blunted endothelium-dependent relaxations in human microvessels, mice aortas, and in the rat mesenteric artery, a(ii) that the antioxidant vitamin C enhanced the blunted acetylcholine-induced forearm blood flow in old subjects.

Several lines of evidence have suggested that the angiotensin system is a major contributor to the aging-related endothelial dysfunction. Indeed, both angiotensin-converting enzyme (ACE) and AT1 receptors are upregulated within the old arterial wall, and treatments with either an ACE inhibitor or an AT1 receptor antagonist prevented aging-related endothelial dysfunction. Also, previous studies have demonstrated a key role of Ang II in the pathogenesis of AF. Ang II type 1 receptor (AT1) activation is known to induce the activation of mitogen-activated protein kinases, (MAPK) which, in turn, favors atrial remodeling through fibroblast proliferation, cellular hypertrophy and apoptosis.

It is known for many decades that AF has been associated with the activation of local and circulating coagulation factors (hypercoagulability). This AF-related hypercoagulability significantly enhances the risk of clot formation and stroke in patients with AF. However, little has been described about the potential role of this AF-related hypercoagulability in atrial tissue remodeling and predominantly the role of thrombin or factor Xa. Apart from its haemostatic effects, thrombin has also been shown to induce cellular effects that have been mediated by protease-activated receptors (PARs).

PARs constitute a family of G protein-coupled receptors that become activated by proteolytic cleavage of the N-terminal domain, revealing a new tethered ligand that binds intramolecularly to activate the receptor and to induce intracellular signal transduction. Four members of the PAR

family are identified, PAR-1 to 4. PAR-1 being mainly activated by thrombin, whereas PAR-2 being primarily activated by trypsin and trypsin-like proteases. Numerous cell types are activated by thrombin, including platelets, vascular smooth muscle cells (VSMCs), lymphocytes and endothelial cells (ECs) through the PARs activation, thus linking coagulation with inflammation. Both PAR-1 and PAR-2 are found in the heart. PAR-1 is chiefly expressed by myocytes, fibroblasts, endothelial cells, and SMCs. Although PAR-2 is also expressed by myocytes, endothelial cells, and SMCs, its expression by fibroblasts has not been confirmed.

Thrombin activates proinflammatory signaling pathways, which lead to the expression of adhesion molecules and P-selectin on the membrane of platelets and ECs, as well as expression of various cytokines such as interleukin -6 and chemokines MCP-1 and adhesion molecules such as ICAM-1, VCAM-1 and E-selectin from fibroblasts, VSMCs and ECs, leading to leukocyte recruitment to the vessel wall and contributing to inflammatory and fibrotic processes. Moreover, thrombin induces ECs apoptosis through the activation of nuclear factor κB (NF-κB) and caspases, regulates prosctacyclin and NO production leading to ECs shape change and to the enhancement of barrier permeability. Thrombin contributes in formation of left atrial remodeling and it has been known that direct oral anticoagulants, such as the direct thrombin inhibitor dabigatran, can prevent its progression.

Moreover, direct signaling effects of factor Xa (FXa) have also been noted. FXa increased the expression of PARs and inflammatory molecules in human atrial tissue slices. The cellular effects of this stimulation have been likely to contribute to structural remodeling in fibrillating and dilated atria. Inflammatory changes, tissue fibrosis, and cellular hypertrophy significantly contribute to loss of electrical conductivity between myocytes leading to conduction disturbances in fibrillating and dilated atria. Overall, the role of PAR activation and hypercoagulability in the development of AF might be important for the notion that specific coagulation inhibitors may prevent not only structural tissue remodeling but also arrhythmogenic cellular changes favouring AF maintenance.

Thus, this study aims to characterize phenotypical changes associated with atrial endothelial cells (AEC) senescence and to depict the link between ageing and thrombogenicity. In addition, we also

evaluate the possibility that coagulation cascade-derived factors such as thrombin could induce premature AEC senescence leading to the acquisition of a pro-thrombotic and pro-fibrotic profile.

To conduct the study, an original model of primary cell culture of atrial endothelial cells was established in the laboratory. In this model, atrial endothelial cells were obtained after collagenase digestion of porcine atria freshly sacrificed and cultured (primary cultures). Endothelial senescence was assessed by senescence-associated beta-galactosidase activity (SA-β-gal), using flow cytometry, protein expression by Western blot analysis, platelet aggregation using an aggregometer, extracellular matrix remodeling by gel zymography and oxidative stress using the redox-sensitive probe dihydroethidium. Replicative senescence was induced by passaging AECs from passage P1 to P4, and premature endothelial cell senescence by exposing AECs at passage P1 to L-NAME, an endothelial NO synthase (eNOS) inhibitor, H_2O_2, thrombin and angiotensin II.

AEC senescence was characterized by an increase in SA-β-gal activity and an up-regulation of p53, a key regulator of cellular senescence, and of p21 and p16, key cyclin-dependent kinase inhibitors. Exposure of AECs to thrombin caused concentration-dependent increased in SA-β-gal activity to a similar level as that induced by the pro-senescence inducers Ang II and hydrogen peroxide. The pro-senescence response to thrombin was also associated with an increased expression of p16, p53 and p21. Moreover, senescent AECs phenotype was characterized by (i) cell thrombogenicity through an up-regulation of tissue factor expression, eNOS down-regulation and reduced NO-mediated inhibition of platelet aggregation, (ii) cell adhesion through up-regulation of ICAM-1, (iii) proteolysis and fibrosis remodeling through MMP-2, 9 and TGF-ß1 expression, and (iv) up-regulation of the local Ang II system through enhanced AT1 receptors (AT1R) and angiotensin-converting enzyme (ACE) expression. Losartan, an AT1 receptor antagonist, and Perindoprilat, an ACE inhibitor, prevented atrial endothelial cell senescence. Thrombin induced oxidative stress in the same extent to Ang II and this effect was prevented by the antioxidant N-acetylcysteine, the NADPH oxidase inhibitor VAS-2870, the cyclooxygenase inhibitor indomethacin and by inhibitors of the mitochondrial respiratory chain (rotenone, myxothiazol and KCN), and also by the AT1R antagonist losartan and perindoprilat. Moreover, preliminary findings may suggested that factor Xa, another marker of hypercoagulability may contribute to the induction of senescence and increased oxidative in atrial endothelial cells (data now shown).

Altogether, the present data demonstrates the existence of a new paradigm linking atrial endothelial senescence to thrombogenicity, inflammation, matrix remodeling and the up-regulation of the local Ang II system. The present findings further indicate that thrombin is a potent inducer of premature senescence in atrial endothelial cells leading to an endothelial dysfunction with the down-regulation of the protective NO pathway and the induction of pro-infiltrative and pro-fibrotic responses. They further suggest the involvement of the local angiotensin system and that targeting the Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial ageing and subsequent atrial tissue remodeling.

SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

H. Hasan, M. Abbas, C. Auger, S.H. Park, B. Marchandot, P. Ohlmann, M.A. Farooq, E. Belcastro, F. Toti, V. Schini-Kerth, O. Morel, L. Jesel-Morel **"***Atrial endothelial cells senescence promotes thrombogenicity, inflammation and extra-cellular matrix remodeling: Role of the Ang II / AT1 receptor/ oxidative stress pathway***"** – ***in prepaparation***

H. Hasan, S.H. Park, C. Auger, K. Matsushita, B. Marchandot, G. Kauffenstein, P. Ohlmann H.H. Lee, A. W. Qureshi, F. Toti, V. Schini-Kerth, O. Morel, L. Jesel-Morel *''Thrombin induces oxidative stress and atrial endothelial cells senescence : Impact on pro-thrombotic, pro-inflammatory, pro-fibrotic and pro-remodeling patterns''* – ***in preparation***

S.H. Park, E. Belcastro, **H. Hasan** et al., *"Angiotensin II induced oxidative stress-mediated upregulation of sodium-glucose cotransporters 1 and 2 (SGLTs) expression in cultured coronary artery endothelial cell****"- in preparation.***

L. Jesel-Morel, M. Abbas, M. kindo, **H. Hasan**, Z. Niazi, C. Auger, S. Park, P. Ohlmann, J. Mazzucotelli, V. Schini-Kerth, O. Morel, F. Toti, "*Impact of atrial fibrillation progression on human atrial senescence burden as determined by p53 and p16 expression*"- ***submitted to the Archives of Cardiovascular Diseases.***

H.H. Lee*, K. Sharma***, H. Hasan,** D.S. Kong, M.H. Oak*, ''Particulate matter 10 induces endothelial senescence by the activation of the redox-sensitive local angiotensin system''-**in preparation**), * equal contribution.

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ORAL COMMUNICATIONS

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POSTER PRESENTATIONS

H. Hasan, M. Abbas, C. Auger, E. Belcastro, M.A. Farooq, S.H. Park, P. Ohlmann, F. Toti, V. Schini-Kerth, O. Morel, L. Jesel-Morel **"***Atrial endothelial cells senescence promotes thrombogenicity, inflammation and extracellular matrix remodeling: role of the local Ang II / AT1 receptor pathway***" *** Poster Presentation at Printemps de la Cardiologie Recherche Fondamentale & Clinique, 4-6 April, Montpellier.***

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*thrombogenicity, inflammation and extracellular matrix remodeling: role of the local Ang II / AT1 receptor pathway***" *Poster presentation at Journée du Campus D'Illkirch 2018 (JCI), 8 & 9 May 2018 Illkirch.***

S.H. Park, E. Belcastro, **H. Hasan**, C. Auger, V. Schini Kerth *"Angiotensin II induced oxidative stress-mediated upregulation of sodium-glucose cotransporters 1 and 2 (SGLTs) expression in cultured coronary artery endothelial cell"* ***Poster presentation at WCP 2018, July 1- 6 2018, Kyoto, Japan.***

ATRIAL FIBRILLATION AND RISK FACTORS

1 ATRIAL FIBRILLATION

1.1 Introduction and definition

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia observed by the clinician and constitutes the most important cause of embolic stroke. AF is characterized by a rapid and high rate (400–600 beats/minute) of asynchronous atrial cell depolarization of the atria without discrete P waves on the surface electrocardiogram (ECG) causing a loss of atrial contractile function and irregular ventricular rates (Calvo et al., 2018; Wijffels et al., 1995). It affects more than 33 million people worldwide (Chugh et al., 2014) and is also the number one cause of hospitalization for arrhythmias (Heijman et al., 2014; Miyasaka et al., 2006). Prevalence increases with advancing age and so are its associated comorbidities, like heart failure (Sardar et al., 2016). The pathophysiology of AF is complex, involving dynamic interactions among several factors, including substrate, triggers, and perpetuators, and the therapeutic approaches/strategies aims at targeting each steps of the disease progression from atrial tissue remodeling, initiation of the abnormal electrical rhythm, perpetuation of arrhythmia, thrombus formation and stroke.

1.2 Classification of atrial fibrillation

AF is classified as first detected or diagnosed AF which is independent of its duration, and presence or absence of any symptoms; paroxysmal AF constituting episodes lasting less than 24–48 h that terminate spontaneously but may also last up to7 days; persistent AF that is sustained beyond 7 days requiring termination by either direct electrical cardioversion or pharmacological intervention; long-lasting persistent AF consisting of episodes lasting longer than one year mainly present the cases where a rhythm control strategy is recommended mostly to consider catheter ablation of AF; permanent AF in which the presence of the AF is accepted by the patient and physician, and no further attempts will be made either to restore or maintain sinus rhythm; lone AF consisting of patients below age 60 with clinically detectable structural cardiovascular disease, which could be paroxysmal, persistent, permanent; AF burden which can be defined as the proportion of time patients are in AF (Calkins et al., 2017; Calvo et al., 2018; Kirchhof et al., 2016a; Shenasa et al., 2014).

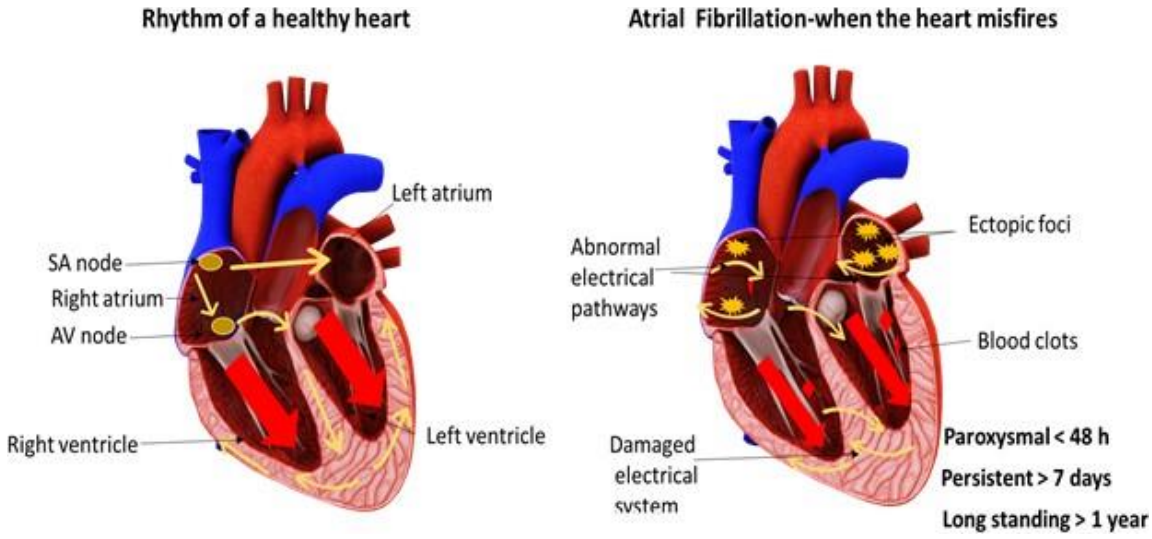

Figure 1: Rhythm of healthy heart and fibrillating heart (Shenasa et al., 2014)

1.3 Epidemiology and impact for patients

1.3.1 Incidence and prevalence of atrial fibrillation

The ratio of men and women with AF worldwide, in 2010, is about 1.5:1 (Chugh et al., 2014). This represents, at the global level in 2010, an estimated number of 20.9 million men and 12.6 million women with AF. Developed countries show greater number of frequency and presence of AF (Colilla et al., 2013). It is estimated that about 25% i.e. 1 in every 4 middle-aged adults in Europe and US will suffer from AF (Go et al., 2001a; Heeringa et al., 2006; Lloyd-Jones et al., 2004). The risk of AF becomes twofold every 10 years of life. The growing frequency of AF is highlighted by the anticipated numbers of AF patients – by 2030, the number of AF patients will be between 14-17 million with 120,000 – 215,000 new AF patients every year (Colilla et al., 2013; Krijthe et al., 2013; Zoni-Berisso et al., 2014). The higher occurrence of AF is observed especially in older individuals and in patients with certain health conditions such as hypertension, heart failure, coronary artery disease (CAD), valvular heart disease, obesity, diabetes mellitus, or chronic kidney disease (CKD) (Ball et al., 2013a; Chiang et al., 2012; Kannel et al., 1998; McManus et al., 2012; Nguyen et al., 2013; Oldgren et al., 2014; Zoni-Berisso et al., 2014). Generally, the estimated numbers approximately show that 3% of adults aged 20 years or older suffer from AF (Bjorck et al., 2013; Haim et al., 2015). Better diagnosis of AF has contributed towards AF prevalence. Better

detection of silent AF (Kishore et al., 2014; Sanna et al., 2014; Wang et al., 2003) coupled with increasing age and health conditions susceptible for AF (Schnabel et al., 2015) have all contributed towards the higher incidence and prevalence of atrial fibrillation.

1.3.2 Morbidity, mortality, and health care burden of atrial fibrillation

AF is independently responsible for higher risk of death in women as opposed to men. Studies suggest that AF, as an existing condition, doubles the mortality risk for women and results in a 1.5-fold increase in mortality risk for men (Andersson et al., 2013; Benjamin et al., 1998; Stewart et al., 2002) (Table 1). Certain conditions can be treated to mitigate the risk of death, for instance, anticoagulation can reduce the likelihood of death by stroke but the current evidence suggests that cardiovascular deaths due to heart failure and sudden death are still frequent even in AF patients under anticoagulant therapy (Kotecha et al., 2014). AF is linked to higher occurrence of heart failure and stroke i.e. it results in increased morbidity (Krahn et al., 1995; Stewart et al., 2002; Wolf et al., 1991). Recent studies indicate the diagnoses of AF in 20-30% of patients of ischaemic stroke either prior to, during or after the first occurrence (Grond et al., 2013; Henriksson et al., 2012; Kishore et al., 2014). 10-40% of AF patients are hospitalized every year (Kirchhof et al., 2014; Kotecha et al., 2014; Steinberg et al., 2014). Poor lifestyle resulting in decreased quality of life (Marzona et al., 2012; Thrall et al., 2006) and depression (von Eisenhart Rothe et al., 2015) are common in AF patients. The cognitive impairment and white matter lesions in the brain (Ball et al., 2013b; Knecht et al., 2008; Ott et al., 1997) are also very frequently observed in such patients. AF has and will continue to significantly result in increased healthcare costs unless it is prevented and treated effectively. The direct costs associated with AF-related treatments and complications are approximately 1% of total healthcare expenditure in the UK and between 6-26 billion US dollars in United States for year 2008 (Kim et al., 2011; Stewart et al., 2004).

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Event	Association with AF
Death	Increased mortality, especially cardiovascular mortality due to sudden death, heart failure or stroke.
Stroke	20–30% of all strokes are due to AF. A growing number of patients with stroke are diagnosed with 'silent', paroxysmal AF.
Hospitalizations	10–40% of AF patients are hospitalized every year.
Quality of life	Quality of life is impaired in AF patients independent of other cardiovascular conditions.
Left ventricular dysfunction and heart failure	Left ventricular dysfunction is found in 20–30% of all AF patients. AF causes or aggravates LV dysfunction in many AF patients, while others have completely preserved LV function despite long-standing AF.
Cognitive decline and vascular dementia	Cognitive decline and vascular dementia can develop even in anticoagulated AF patients. Brain white matter lesions are more common in AF patients than in patients without AF.

AF = atrial fibrillation; LV = left ventricular.

Table 1: Morbidity and mortality linked with AF (Kirchhof et al., 2016a)

1.4. Atrial fibrillation risk factors

1.4.1 Ageing

AF is strongly age dependent, and it affects approximately 1%, 4% and 15% at 50, 65 and 80 years, respectively (Andrade et al., 2014). Aging plays a critical role in the genesis of AF and also increases the risks of cardiac dysfunction and stroke in AF patients. Clinical and laboratory evidence indicates that aging is significant in the creation of atrial electrical and structural remodeling that leads to increased susceptibility to AF occurrence. Aging is commonly associated with cardiovascular comorbidities, oxidative stress, calcium dysregulation, atrial myopathy with apoptosis, and fibrosis, which all contribute to the genesis of AF (Lin et al., 2018). Mounting evidence suggests that extracellular matrix (ECM) and perivascular fibrosis were increased progressively with age, leading to cardiac remodeling and dysfunction in elderly individuals (Horn and Trafford, 2016; Sahin et al., 2011). Besides, telomere attrition affects mitochondrial function, thus promoting aging (Sahin et al., 2011), and short telomere length is considered to be a hallmark of aging (Lopez-Otin et al., 2013). Recently, Carlquist et al found that AF subjects had shorter

telomeres compared without a history of AF subjects (Carlquist et al., 2016). Such finding suggests that aging, and/or replicative senescence, may contribute to the development and maintenance of AF (Xie et al., 2017). Koura and colleagues (Koura et al., 2002) demonstrated that with aging, the amount of interstitial fibrosis and fatty infiltrates increases, predisposing the atrial muscle to electrical impulse conduction disturbances. These disturbances, such as the so-called zigzag electrical impulse propagation aberrancy, are considered to be "trigger" events that lead to AF initiation and maintenance. At the cellular level, several anomalies may also contribute to age related AF initiation. For example, atrial myocytes in an aged atrium exhibit a prolonged action potential duration (APD). Moreover, evidences were provided that a larger APD heterogeneity exist across the atrium (Xu et al., 2013).

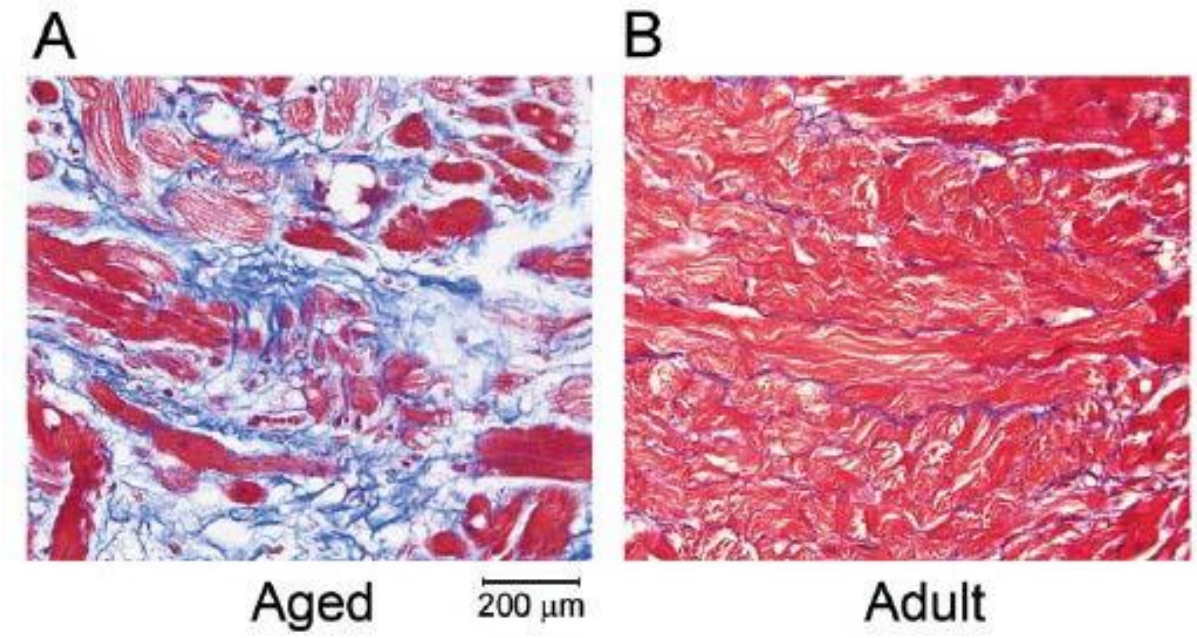

Figure 2: Histological analysis of a rabbit's adult and aged (age >2 years) left atrium (LA) after Masson trichome staining. (**A**) showing marked fibrosis associated with cell loss and cardiomyocytes hypertrophy accompanied with increased fiber diameter and large-volume nuclei in the aged rabbit LA when compared with adult rabbit LA (**B**) with normal cardiomyocytes (Lin et al., 2018; Tsai et al., 2014).

1.4.2 Hypertension

Hypertension has been shown an independent risk factor for incident AF. The Framingham cohort displayed an independent increased risk of AF by factors of 1.5 in men and 1.4 in women related

to hypertension (Benjamin et al., 1994; Rogers et al., 2018). Hypertension increases sympathetic output which may lead to increased left atrial pressure and volume, as well as renin–angiotensin–aldosterone system (RAAS) activation, thereby leading to atrial fibrosis, structural and electrical atrial remodeling, and promotion of AF (Brandes et al., 2018; Lau et al., 2012). Long-term longitudinal studies from Framingham Heart Study1 and Women's Health Study revealed both high systolic and diastolic BP increase the risk of developing AF (Tedrow et al., 2010). Moreover, in spontaneously hypertensive rats, the inducibility of atrial tachycardia was increased, accompanied by a rise in atrial fibrosis (Choisy et al., 2007). In a sheep model of long-standing elevated blood pressure induced by prenatal corticosteroid exposure multiple proarrhythmic abnormalities were seen: increased AF stability, reduced conduction velocities, (Hong and Glover, 2018; Shenasa et al., 2014) and increased fibrosis with myocyte hypertrophy and myolysis (Kistler et al.,2006).

The first evidence that optimal treatment of hypertension may prevent AF and improve outcomes came from intervention trials in hypertensive patients. In the Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study, which compared the use of ARB losartan with the beta-blocker atenolol, losartan prevented more cardiovascular morbidity and death than atenolol for a similar reduction in BP (Dahlof et al., 2002). A *post-hoc* analysis from this trial showed that the greatest reduction (40 %) in risk of incident AF occurred in patients who achieved optimal systolic BP levels of <130 mmHg, compared to those with systolic BP ≥142 mmHg. Moreover, incident AF occurred less frequently in patients treated with losartan than in those treated with atenolol, although there was no significant difference in BP reduction (Wachtell et al., 2005). A Danish nationwide nested case-control study also found less new-onset AF in patients with hypertension treated with ARBs or ACE inhibitors compared to beta-blockers or diuretics (Marott et al., 2014). These findings suggest that inhibition of the renin–angiotensin system itself might have a beneficial effect on the reduction of incident AF besides BP control (Brandes et al., 2018)

1.4.3 Heart failure and coronary artery disease

AF may be caused by any cardiac condition with, however, a predominance of heart failure (HF) and coronary artery disease (CAD) (Benjamin et al., 1994; Benjamin et al., 1998; Kannel et al., 1983; Kannel and Benjamin, 2008; Krahn et al., 1995; Roy et al., 2009). HF represents major AF risk factor as HF patients are associated with approximately 5-fold increased risk of AF onset

(Kannel et al., 1998). The risk of AF increases with the severity of HF clinical symptoms (Jais et al., 2000; Maisel and Stevenson, 2003; Tsang et al., 2002). Atrial fibrosis is markedly increased in the setting of HF, similar to that observed with aging and hypertension-related AF. Moreover, in HF too, the formation of atrial interstitial fibrosis plays a strong determinant of the occurrence of AF (Cha et al., 2004; Shinagawa et al., 2002; Tanaka et al., 2007). Specifically, the spatial distribution of atrial fibrosis could be an indicator of AF electrophysiologic mechanisms—reentry or spontaneous focal discharges—and of the exact locations of AF electrical sources. Therefore, understanding of fibrosis or scar distribution could be an asset in the performance of tailored AF ablation procedures (Trayanova, 2014). Thus, HF-related fibrosis formation has been one of the main targets of so-called upstream therapies, such as inhibitors of the renin-angiotensin-aldosterone system (Savelieva et al., 2011).

Acute and chronic CAD has emerged as a substantial risk factor of AF onset (Miyasaka et al., 2006) and perpetuation (Goldberg et al., 2002; Kannel et al., 1983; Wong et al., 2000). Although AF after ventricular myocardial infarction might be also triggered by an increase in intra-atrial pressure in the context of acute ventricular dysfunction, (Moller et al., 2003; Tsang et al., 2001) various works have shown that isolated atrial infarction is common. It was documented that the pathophysiologic role of atrial ischemia/infarction in AF onset has been greatly underestimated. Understanding of the pathophysiology linking CAD and AF has benefited from experimental studies. These works have highlighted several atrial ischemia/infarction related electrophysiologic changes. Spontaneous discharges have been significantly more numerous in cells bordering the infarcted region. Atrial ischemia/infarction had also been shown to reduce atrial refractory periods, to increase AF inducibility and adversely modulate regional electrical impulse propagation, and finally lead to an acceleration of atrial drivers (Anumonwo and Kalifa, 2016).

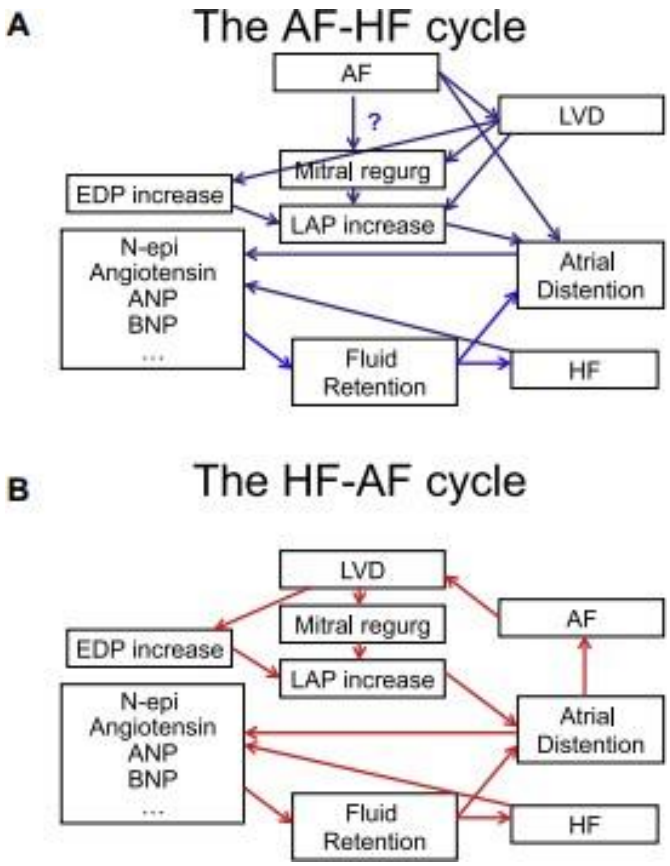

Figure 3: Panel (**A**) depicts the association between AF and HF cycle whereas, panel (**B**) HF-AF cycle. (ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; EDP, end diastolic pressure; LAP, left atrial pressure; LVD, left ventricular dilation) (Shenasa et al., 2014).

1.4.4 Diabetes

The Veterans Health Administration Hospitals study showed that the prevalence of AF in patients with diabetes mellitus was 14.9%, this being pointedly higher than that of hypertension. Thus, diabetes mellitus represents a strong and independent risk factor for the occurrence of AF, with an odds ratio of 2.13 (Pb.0001) (Lin et al., 2013; Movahed et al., 2005). The proposed mechanisms linking diabetes mellitus and AF include autonomic remodeling, structural remodeling, electrical remodeling, and insulin resistance. With respect to structural remodeling, in a diabetes mellitus rat model Kato et al. has demonstrated fibrosis in the atria with formation of anchoring points for reentry circuits and changes in the forward propagation of fibrillatory wavelets, thus resulting in atrial fractionated potentials and conduction delay (Kato et al., 2006). The atrial tissue collected

from diabetes mellitus patients which were then biopsied during coronary artery bypass graft surgery, displayed mitochondrial dysfunction causing oxidative stress which could also be involved in the formation of hyperglycemia-associated AF substrates, which ultimate lead to atrial interstitial fibrosis (Anderson et al., 2009). It is documented that the advanced glycation end products (AGEs) and AGE receptors (RAGEs) (both constituting the AGERAGE system) enable the interstitial collagen deposition in atrial myocardium of diabetes mellitus rats by encouraging up-regulation of the expression of connective tissue growth factors, and, as a consequence, result in myocardial structural remodeling (Koektuerk et al., 2016). Interestingly, in the atrial myocardium of rats with induced diabetes mellitus, the expression of Cx43 was found to be increased whereas its phosphorylation was decreased, thus leading to disorders of intercellular electrical coupling and consequent atrial arrhythmia (Mitasikova et al., 2009).

1.4.5 Thyroid Dysfunction

In the setting of hyperthyroidism, AF has been considered as one of the most frequent rhythm disturbance with its occurrence ranging from 2% to 20% (Klein and Danzi, 2007). When compared with a population with normal thyroid function and a 2.3% prevalence of AF, the incidence of AF in overt hyperthyroidism has been 13.8%. Shimizu et al. demonstrated that in a cohort of patients with hyperthyroidism studied for age distribution, AF incidence increased stepwise in each decade, climaxing at about 15% in patients N70 years, thus showing that hyperthyroidism-related AF has been more common with advancing age (Auer et al., 2001; Shimizu et al., 2002). Various potential mechanisms of AF in hyperthyroidism are proposed constituting elevation of left atrial pressure as a result of increased left ventricular mass and impaired ventricular relaxation, enhanced atrial ectopic activity, and ischemia secondary to raised resting heart rate (Bielecka-Dabrowa et al., 2009; Fazio et al., 2004; Sgarbi et al., 2003). Interestingly, it is recently documented that both hypothyroidism and hyperthyroidism cause increased AF vulnerability in a rat thyroidectomy model (Zhang et al., 2013). In fact, hypothyroidism and hyperthyroidism, while inducing opposite electrophysiological changes in heart rates and atrial effective refractory period, both pointedly increase AF susceptibility (Weltman et al., 2015) .

1.4.6 Alcohol

The connection of episodic heavy alcohol (ethanol) use with the onset of AF is termed as “holiday heart syndrome”. However, more recently, it is proposed that even habitual heavy alcohol consumption could be linked with a risk of AF (Balbao et al., 2009). Related to alcohol consumption and AF, Kodama et al. showed a meta-analysis of studies to summarize the estimated risk of AF associated to alcohol. It was found that the pooled estimate for AF for highest vs. lowest alcohol intake in individual investigations was 1.51 and a positive relationship between AF risk and heavy alcohol intake had been consistently found in all stratified analyses (Kodama et al., 2011). Over the last few years, a number of mechanisms by which alcohol consumption could be linked to the development of AF have been suggested: a direct toxic effect on cardiac myocytes, a hyperadrenergic state which has been reached during both drinking and withdrawal of alcohol, an impaired vagal tone, an increase in intra-atrial conduction time (which is also testified by a P-wave prolongation) (Corradi, 2014b).

1.4.7 Pericardial fat and obesity

A considerable portion of the epicardial surface in large mammals is normally covered by adipose tissue, and fat cells (adipocytes) may be participating in myocyte-adipocyte cross talk significant in the normal function of the myocardium. Obesity considerably increases plasma levels of free fatty acids as well as overall visceral and epicardial adiposity in the studies comprising humans and animal models. With obesity, extensive fatty infiltration leads to elevated levels of biofactors. These biofactors have been potentiated by paracrine and vasocrine signaling pathways and overload the myocardium resulting in deterioration of the myocardial function and also lead to abnormal impulse initiation mechanisms and myocyte atrophy. In obese patients, it is also documented that steatosis of the myocardium well correlates with epicardial fat behaves as an independent contributing factor to myocardial dysfunction. Additionally, experimental studies in isolated myocytes reveal that excess epicardial adiposity, or its biofactors, lead to abnormality in myocardial electrical excitation (Anumonwo and Kalifa, 2014; Shenasa et al., 2014).

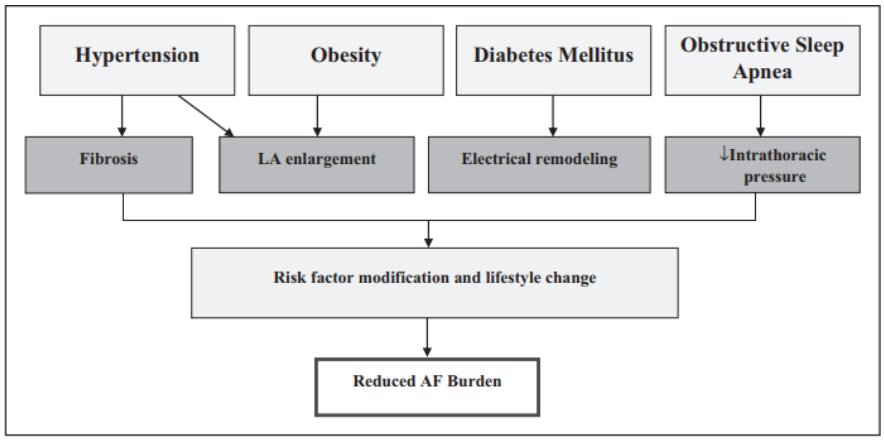

Figure 4: Management of AF-related risk factors by life style modification (Hong and Glover, 2018).

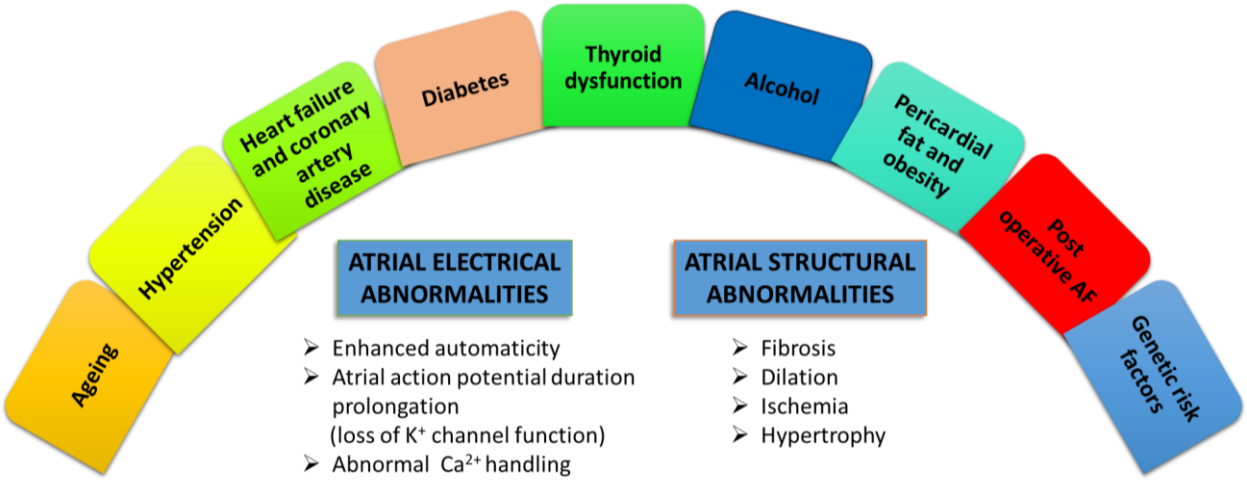

Figure 5: Risk factors and proposed mechanisms associated with AF (Fabritz et al., 2015).

1.4.8 Post-operative atrial fibrillation

The most common arrhythmia after cardiac surgery is AF occurring in approximately 20–50% of patients depending on the type of surgery performed. AF occurs in 30–40% of patients post coronary artery bypass graft (CABG) and up to 60–70% of patients with combined CABG and valve surgery. Majorly, post-op AF converts to sinus rhythm spontaneously in the first 24–48 h, but if it takes longer than 48 h it increases the risk of stroke and prolongs hospitalization and associated expenses. Post-operative AF affects both early and late mortality after isolated CABG. Most of the complications being related to stroke thus, post-operative surveillance and long-term management with antiarrhythmic agents and antithrombotic management have been warranted. Preoperative treatments with beta-blockers have been shown to effectively decrease the risk of AF. Colchicine as well as statins has been effective in prevention of this arrhythmia (Deftereos et al., 2013; Omae and Kanmura, 2012; Shenasa et al., 2014).

1.4.9 Genetic risk factors

Genetic predisposition also cannot be ignored (Kirchhof et al., 2016b) A considerable portion of AF occurring in younger ages has been known to be associated with a genetic predisposition than the accompanying disease. Some studies conducted report that more than 30% of AF have a common genetic variation. Among the various known genetic factors, the most important variants have been located close to the paired-like homeodomain transcription factor 2 gene on chromosome 4q25 (Gudbjartsson et al., 2007). This genetic variation has been known to be associated with up to a 7-fold increase the incidence of AF. In addition to the genetic variants that are likely to cause AF itself, gene mutations that contribute to the atrial remodeling process and electrophysiological changes described above may also mediate the occurrence of AF (Cha, 2018).

1.4.10 Other risk factors

Chronic kidney disease and smoking are accepted as independent AF risk factors but their respective importance is still debated. An example of a controversial risk factor is exercise. Although moderate physical activity may decrease AF incidence, a cumulative life practice of more than 1500 h is associated with 3-fold AF risk. Pathophysiologic mechanisms are still unclear, but the role of an increased vagal tone seems to be accepted (Anumonwo and Kalifa, 2014).

ATRIAL FIBRILLATION PATHOPHYSIOLOGY

2. ATRIAL FIRILLATION MECHANISM AND PATHOPHYSIOLOGY

The lack of reliable experimental models resembling this complex arrhythmia presents one of the major problems in understanding the mechanism leading to AF. However, an increased awareness of the role of "atrial remodeling" over the past 10 to 15 years has significantly increased our understanding of AF pathophysiology. Atrial remodeling constitutes any persistent change in atrial structure or function. Many forms of atrial remodeling promote the occurrence or maintenance of AF by acting on the fundamental arrhythmia mechanisms (Nattel et al., 2008) as illustrated in Figure below. AF requires both a trigger and a susceptible substrate (Dobrev and Nattel, 2010; Iwasaki et al., 2011b; Schotten et al., 2011). The trigger for initiation and maintenance of AF is mostly related to an enhanced electrical activity of the pulmonary vein cardiomyocyte sleeves, while non-pulmonary vein sources also become more important as AF continues to persistent form. Thereafter, AF is often sustained by a primary "driver" mechanism, which may be either focal ectopic sources or rapid local re-entry in a vulnerable substrate. Re-entry also involves both a substrate (a modified atrium or a portion of it) and a trigger (often an ectopic beat) (Nattel et al., 2008). The excitation seems to propagate through the susceptible substrate with a circular or spiral wavefront, mentioned as a rotor, thereby sustaining the AF and the alteration of the structure of the atrium (substrate).

Atrial remodeling has the ability to enhance the probability of ectopic or reentrant activity through a multitude of potential mechanisms (Khaji and Kowey, 2017; Nattel et al., 2008)

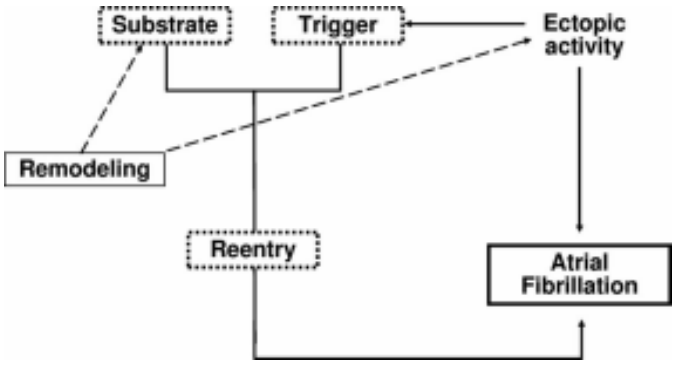

Figure 6: Schematic illustration of atrial fibrillation (Nattel et al., 2008).

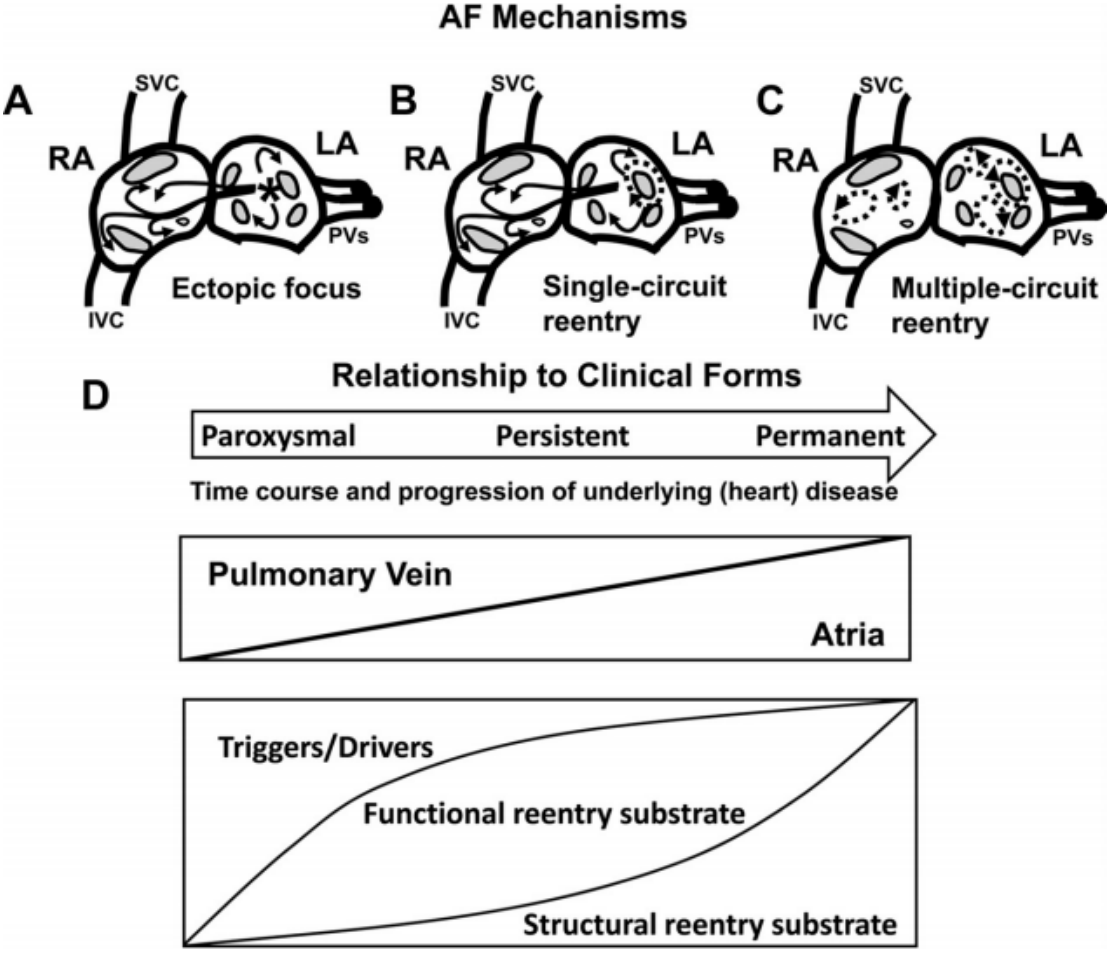

Figure 7: AF mechanisms and relationship to clinical forms (A) represents local ectopic firing, (B) represents single circuit reentry, (C) represents multiple-circuit reentry. (D) represents various AF clinical forms in relation to mechanisms. Paroxysmal forms involve local triggers/drivers mainly from pulmonary veins (PVs). Reentry substrates (initially functional and then structural) become prominent as AF continues towards permanent. Where, RA: right atrium; SVC: superior vena cava; LA: left atrium; IVC: inferior vena cava (Nattel et al., 2008).

2.1 Electrophysiological remodeling

Sustained AF with atrial rhythms as high as 350 to 600 bpm, in turn, results in electrophysiological remodeling, which consists of mainly the outward K^+ current (I_{to}), the ultra-rapid delayed rectifier K^+ current (I_{Kur}), and L-type Ca^{2+} current ($I_{Ca,L}$), and, in parallel, upsurges in the inward rectifier K^+ current (I_{K1}), the agonist-independent form of the acetylcholine-dependent K^+ current ($I_{K,ACh}$),

and the slow component of the delayed rectifier K+ current (I_{Ks}). The consequence of these various alterations in currents leads to shortening of the action potential and effective refractory period (ERP), and thus maintenance of AF (Nattel et al., 2008). Significantly, this electrophysiological remodeling may also be accompanied with abnormal Ca^{2+} handling and enhanced propensity of potentially proarrhythmic Ca^{2+} release events from sarcoplasmic reticulum during diastole, which has the potential to compromise atrial contractility and show an exasperating role in the initiation and maintenance of ectopic (triggered) activity (Beavers et al., 2013; Chelu et al., 2009; Dobrev et al., 2011; Hove-Madsen et al., 2004; Neef et al., 2010; Voigt et al., 2014; Voigt et al., 2012). Electrophysiological remodeling is vague when the heart is in sinus rhythm, and is less recurrent in paroxysmal AF, mainly due to reversibility during AF-free intervals (Dobrev and Nattel, 2010; Voigt et al., 2014; Voigt et al., 2013). Remodeling can occur within h, days, or weeks of the onset of arrhythmia. It is linked with a higher incidence of delayed after depolarizations (DADs) and triggered activity (Voigt et al., 2014).

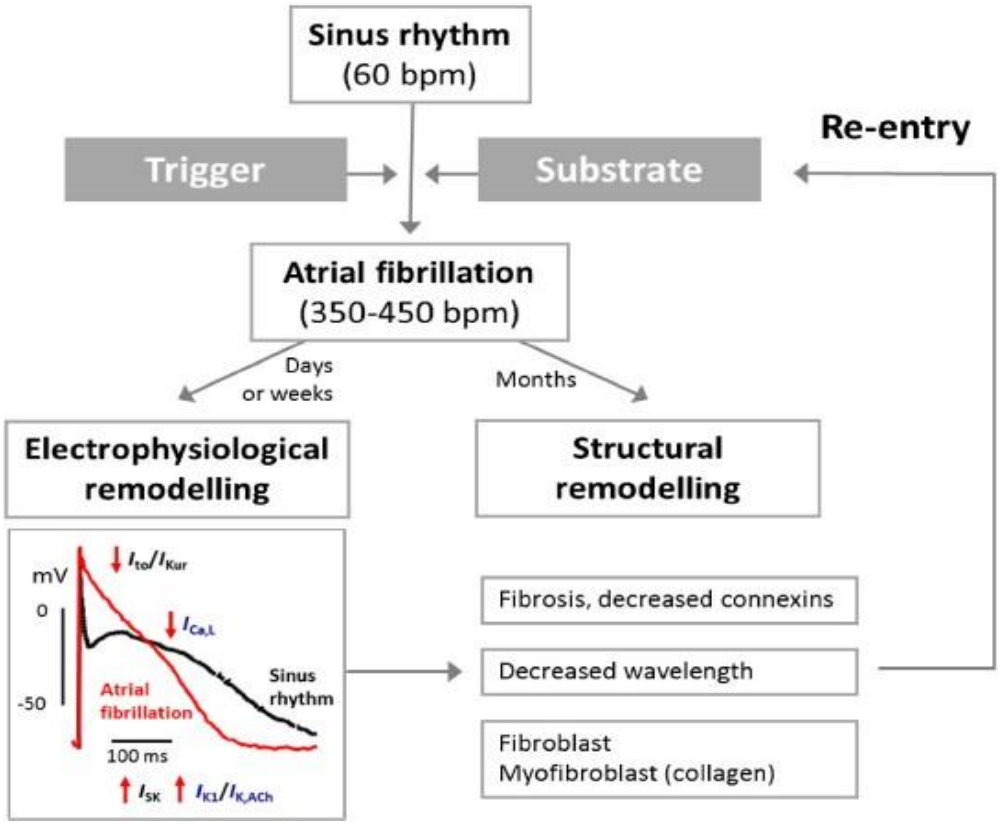

Figure 8: AF mechanism (Ferrari et al., 2016).

2.2 Atrial stretch and mechanical remodeling

Several studies have documented that the size of the atrium is an important determinant of AF occurrence. The Framingham study, which prospectively followed up adults with routine M-mode echocardiograms, revealed that left atrial size is an independent risk factor for the subsequent development of AF with a hazard ratio of 1.39 for every 5-mm incremental increase in left atrial size. Likewise, in the Cardiovascular Health Study, a left atrial diameter > 5 cm had been associated with a relative risk of 4.05 (1.95–8.35) for the development of AF (Corradi, 2014a). From a pathophysiologic point of view, atrial stretch is shown to result in a wide range of electrophysiologic changes, constituting prolongation of late repolarization while early repolarization has been shortened, increased excitability, and changes in the nature of AF electrical sources. Using optical mapping techniques, it was previously established that atrial stretch increases the frequency or reentries (rotors) and the spatiotemporal stability of AF waves. In patients with HF or mitral valve disease, it is not uncommon to have restoration of sinus rhythm when the size of the atria diminishes after surgical repair of the valve. Moreover, once AF has been initiated, the hemodynamic status of the patient worsens because of the loss of atrial contraction, creating a vicious circle that greatly favors AF maintenance (Anumonwo and Kalifa, 2014). The mechanical (contractile) remodeling is started as fast as the electronic one, usually within 48 h after AF onset. The decreased release of calcium ions, secondary to down regulation of the channels responsible, and the loss of sarcomeres (myolysis) leads to contractile remodeling. The loss of mechanical atrial activity results in atrial dilatation and formation of thrombi and, also leads to AF progression through the formation of a larger space for fibrillatory wave perpetuation. Due to loss of sarcomeres, the recovery of contractile activity after the conversion to sinus rhythm becomes more difficult than reverse electrical remodeling (Vizzardi et al., 2014).

2.3 Structural remodeling and fibrosis

By contrast to electrophysiological remodeling, structural remodeling happens on a larger timescale over months or even years, and seems to be connected with age, hypertension, and numerous comorbid cardiac diseases. This is the basis for early and aggressive management of allied situations, such as hypertension, heart failure, and coronary artery disease, which may precede AF (Nattel et al., 2014; Van Gelder et al., 2011). The intimate molecular mechanisms are not fully comprehended, though interstitial fibrosis, mainly through the cardiomyocyte–

myofibroblast interaction, has been frequently suggested. Indeed, AF favours the differentiation of fibroblasts into myofibroblasts, which secrete more collagen than fibroblasts, express some cardiac channels, including I_{Kur}, and exert a paracrine activity on cardiomyocytes. This interaction has been critical to both electrophysiological and structural remodeling, including maintenance of the re-entrant substrate (Heijman et al., 2014).

Reactive interstitial fibrosis separates muscle bundles, whereas reparative fibrosis replaces dead cardiomyocytes, interfering with electric continuity and slowing conduction. Fibroblasts can couple electrically to cardiomyocytes and, when increased in number, promote reentry and/or ectopic activity (Akoum et al., 2011; Yue et al., 2011). AF itself may enhance structural remodeling generating a long term positive feedback loop that leads to the development of permanent forms (Iwasaki et al., 2011b).

Marrouche et al., showed that the magnitude of atrial fibrosis correlates with increased risk of AF as well as progression from paroxysmal to persistent and permanent. Thus, categorized the magnitude of atrial fibrosis to Utah 1–4. Utah 1 has 0–5% fibrosis, Utah II >5–20%, Utah III >20–35% and Utah IV >35% (Mittal et al., 2011; Shenasa et al., 2014)

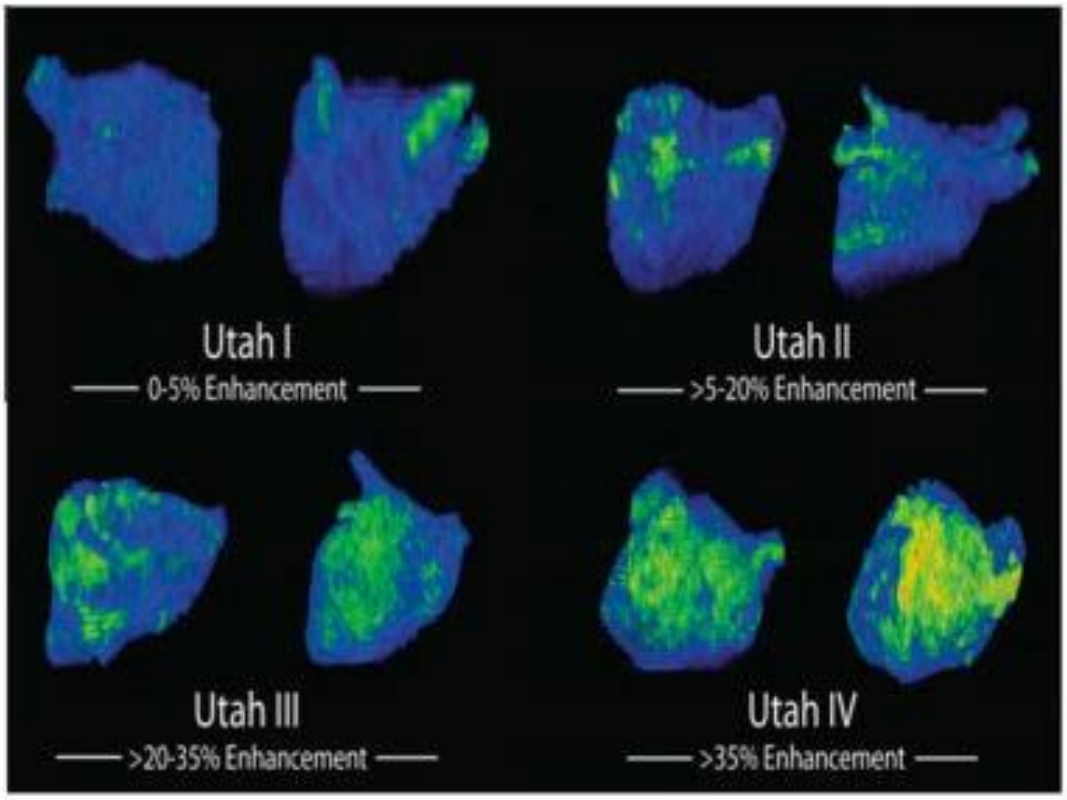

Figure 9: The link between types of AF and magnitude of atrial fibrosis which is observed by delayed-enhanced magnetic resonance imaging (MRI) (Shenasa et al., 2014).

Atrial interstitial fibrosis may be the consequence of non-specific scar-like reparative methods subsequent to cardiomyocyte necrosis or, more interestingly, be secondary to reactive fibro-proliferative signaling pathways. Numerous secreted factors are found to be profibrotic and potential mediators of structural remodeling. In addition to their individual effects, they often act synergistically. Angiotensin II and transforming growth factor-1 (TGF-1) are well-established profibrotic molecules, and recent evidence points to significant roles for platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), the ANP and the highly promising galectin-3 (Corradi et al., 2008; Nattel et al., 2008).

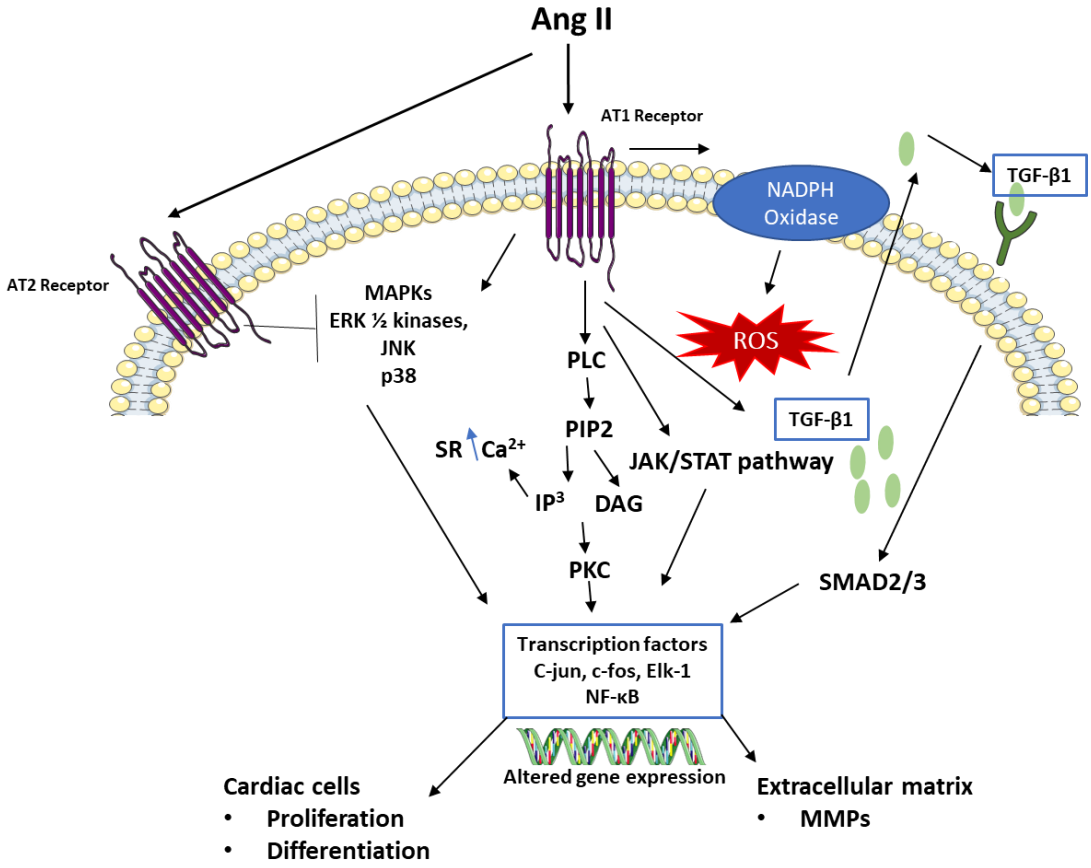

Figure 10: Profibrotic and proremodeling responses of angiotensin II. Where, Ang II, angiotensin II; DAG, diacylglycerol; ERK 1/2, extracellular signal-related kinase 1/2; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK 1/2, mitogen-activated/ERK kinase 1/2; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PKC, protein kinase C; PIP2, phosphatidylinositol bisphosphate; PLC, phospholipase C; SMAD, SMA- and MAD-related proteins; STAT, signal transducers and activators of transcription; TAK1, TGF-β1–activated kinase 1; TF, transcription factor; TGF-βR, TGF-β receptor and IP3, inositol 1,4,5-trisphosphate (Nattel et al., 2008).

2.3.1 Angiotensin II

Angiotensin II (Ang II), through its Ang II type 1 receptors (AT1R) has the capacity of encouraging, each time, cardiomyocyte hypertrophy (Sadoshima and Izumo, 1996), endothelial changes (Usui et al., 2000), vasoconstriction, apoptotic cardiomyocyte death, and myocardial fibrosis (Corradi, 2014a). Ang II facilitates cardiac fibrosis in a variety of cardiac pathologies, including hypertensive heart disease, CHF, myocardial infarction, and cardiomyopathy. Marked atrial dilation with focal fibrosis and AF was seen in transgenic mice with cardiac-restricted ACE overexpression (Xiao et al., 2004). Angiotensin II (Ang II), has been also known to promote aging and cellular senescence (Shan, Bai et al. 2008). Ang II mainly acts by binding to 2 discrete receptor subtypes, angiotensin type I (AT1R) and type II (AT2R) receptors both of which have opposing actions. AT1R signaling through the Shc/Grb2/SOS adapter-protein complex triggers the small GTPase protein Ras, which instigates mitogen-activated protein kinase phosphorylation cascades that are centrally involved in remodeling (Hunyady and Catt, 2006) The mitogen-activated protein kinases ERK (extracellular signal related kinase)-1 and -2, p38, and JNK (c-Jun N-terminal kinase) activate transcription factors (Elk-1, c-jun, and c-fos) that regulate gene expression. Moreover, AT1R activation also activates phospholipase C. Phospholipase C, in turn, breaks down membrane phosphoinositol bisphosphate (PIP2) into diacylglycerol and inositol 1,4,5-trisphosphate (IP3). Diacylglycerol stimulates protein kinase C, and IP3 causes intracellular Ca^{2+} release, both of which endorse remodeling. Signal transduction also happens through the JAK/STAT pathway,

galvanizing transcription factors such as activator protein-1 and nuclear factor-κB. AT2R instigation inhibits mitogen-activated protein kinases (Hunyady and Catt, 2006) via dephosphorylating actions of phosphotyrosine phosphatase and protein phosphatase 2A and results in antiproliferative and survival-promoting effects that are opposite to AT1R-mediated changes.

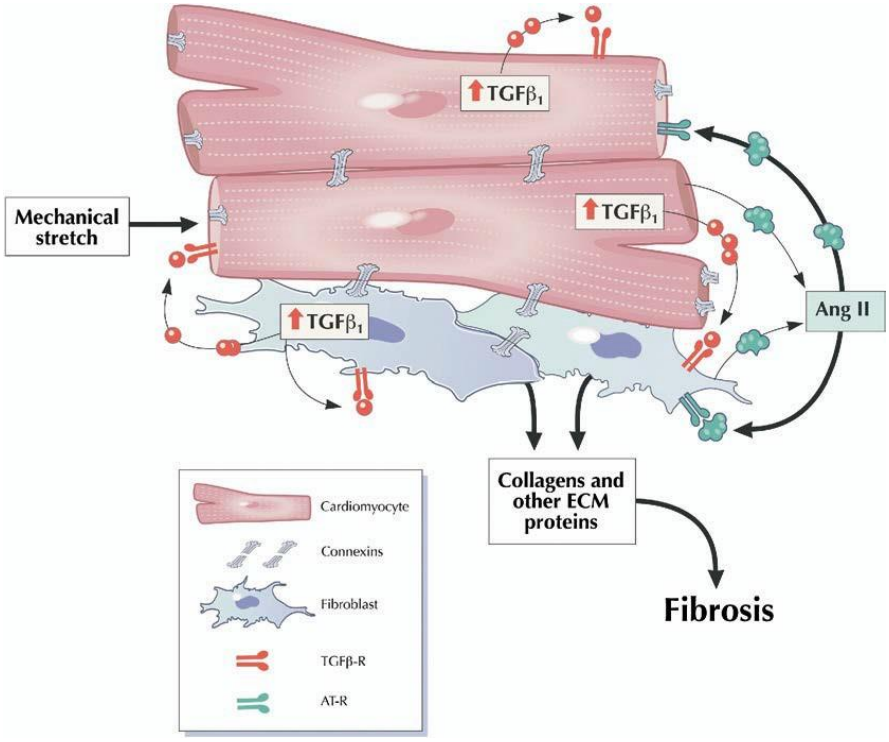

Figure 11: Cardiomyocyte-fibroblast crosstalk. Humoral and mechanical stimuli are amplified by various autocrine and paracrine mechanisms which lead to tissue fibrosis. Where, Ang II: angiotensin II; AT-R: angiotensin receptor; ECM : extracellular matrix; TGF transforming growth factor; TGF-βR: trasforming growth factor beta receptor (Burstein and Nattel, 2008).

2.3.2 Transforming growth factor-β1 (TGF-β1)

TGF-β1 is secreted by both cardiomyocytes and fibroblasts and acts as a primary downstream mediator of Ang II effects in both autocrine (influencing the cell that produces Ang II/ TGF-β1) and paracrine (influencing adjacent cells) manners. Ang II induces TGF-β1synthesis, which potently stimulates fibroblast activity. In turn, TGF-β1 reciprocally enhances the production of Ang II and additional profibrotic factors to create positive feedback (Rosenkranz, 2004) TGF-β1 acts primarily through the SMAD protein (homolog of the Drosophila protein "mothers against decapentaplegic," or MAD, and the Caenorhabditis. elegans protein, SMA) pathway to stimulate fibroblast activation and collagen deposition (Attisano and Wrana, 2002). Cardiac overexpression

of constitutively active TGF-β1 causes selective atrial fibrosis, atrial conduction heterogeneity, and AF promotion (Zhang et al., 2014). Mechanical stretch can itself induce in fibroblast Ang II and TGF-β1 expression, therefore greatly influencing the atrial structural remodeling and its propensity to arrhythmic disorders (Schotten et al., 2003).

2.3.3 Platelet-derived growth factor (PDGF)

PDGF rouses fibroblast proliferation and differentiation. Occupation of PDGF receptors causes them to dimerize, which stimulates a tyrosine kinase that forms part of the PDGF receptor molecule. This tyrosine kinase phosphorylates intracellular domains of the PDGF receptor (autophosphorylation). Autophosphorylation activates PDGF receptors, resulting in signaling via mitogen-activated protein kinase, JAK/ STAT, and phospholipase C pathways shared with TGF-1 and Ang II. PDGF seems to underlie atrium-selective fibroblast hyperresponsiveness, which may explain why atria are much more susceptible to fibrotic remodeling than ventricles (Burstein et al., 2008).

2.3.4 Connective tissue growth factor (CTGF)

CTGF is a member of the CCN (Cyr61, CTGF, NOV) (Wu et al., 2016) protein family and a major downstream effector of TGF-β1 fibrosis promotion. Areas with active myocardial remodeling show coordinate CTGF expression with TGF-1 (Chuva de Sousa Lopes et al., 2004) CTGF is upregulated by both Ang II and TGF-β1, (Chen et al., 2000) and it directly activates fibroblasts (Ahmed et al., 2004).

2.4 Coagulation cascade components and AF

It is known for many decades that AF has been associated with the activation of local and circulating coagulation factors (hypercoagulability). This AF-related hypercoagulability significantly enhances the risk of clot formation and stroke in patients with AF (Watson et al., 2009). However, little has been known about the potential role of this AF-related hypercoagulability in atrial tissue remodeling and predominantly the role of thrombin or factor Xa. Apart from its haemostatic effects, thrombin also shows cellular effects that have been mediated by protease-activated receptors (PARs) (Papadaki et al., 2017).

PARs constitute family of G protein-coupled receptors that become activated by proteolytic cleavage of the N-terminal domain, revealing a new tethered ligand that binds intramolecularly to activate the receptor and to induce intracellular signal transduction. Four members of the PAR family are identified, PAR-1 to -4. PAR-1 being mainly activated by thrombin, whereas PAR-2 being primarily activated by trypsin and trypsin-like proteases. Numerous cell types are activated by thrombin, including platelets, vascular smooth muscle cells (VSMCs), lymphocytes and endothelial cells (ECs) through the PARs activation, thus linking coagulation with inflammation (Papadaki et al., 2017). Both PAR1and PAR2 are found in the heart. PAR1 is chiefly expressed by myocytes, fibroblasts, endothelial cells, and SMCs (Antoniak et al., 2011). Although PAR2 is also expressed by myocytes, endothelial cells, and SMCs, its expression by fibroblasts has not been confirmed.

Thrombin activates proinflammatory signaling, which lead to the expression of adhesion molecules and P-selectin on the membrane of platelets and ECs, as well as expression of various cytokines such as interleukin -6 and chemokines MCP-1 and adhesion molecules such as ICAM-1, VCAM-1 and E-selectin from fibroblasts, VSMCs and ECs, leading to leukocyte recruitment to the vessel wall and contributing to inflammatory and fibrotic processes. Moreover, thrombin induces ECs apoptosis through the activation of nuclear factor κB (NF-κB) and caspases, regulates prosctacyclin and nitric oxide production leading to ECs shape change and to the enhancement of barrier permeability (Papadaki et al., 2017). Thrombin contributes in formation of left atrial remodeling and it has been known that direct oral anticoagulants, such as direct thrombin inhibitors (DTIs), can prevent its progression (Jumeau et al., 2016a).

An arrhythmogenic potential has been suggested for thrombin in ventricular myocytes in addition to effects related to tissue remodeling (Spronk et al., 2014). The action potential duration is prolonged by thrombin. Also, thrombin increased ventricular arrhythmias in reperfusion experiments performed in adult rat hearts (Spronk et al., 2014). In addition, thrombin enhanced arrhythmogenesis in rabbit pulmonary vein preparations, by reducing the spontaneous beating rate and inducing delayed after depolarizations and burst firing (Chang et al., 2012). Besides an arrhythmogenic potential, thrombin also caused an increased release of atrial natriuretic peptide from adult rat atrial cardiomyocytes (Klapper et al., 1996) as well as from rat ventricular myocytes (Spronk et al., 2014). Thrombin and PAR1 being detected in human autopsied hearts, and their expression levels were higher in left atrial tissue compared with left ventricular tissue (Ito et al.,

2013). Thrombin-activated PAR1 also induced arrhythmogenic effects in atrial preparations and increased sodium influx by increasing the persistent sodium current in human atrial cardiomyocytes (Pinet et al., 2008).

Moreover, direct signaling effects of factor Xa (FXa) have also been noted. FXa persuaded the expression of PARs and inflammatory molecules in human atrial tissue slices (Bukowska et al., 2013) and tachyarrhythmia alone also increased the expression of PAR1. The cellular effects of this stimulation have been likely to contribute to structural remodeling in fibrillating and dilated atria (Sabri et al., 2000). Inflammatory changes, tissue fibrosis, and cellular hypertrophy significantly contribute to loss of electrical conductivity between myocytes and thus conduction disturbances in fibrillating and dilated atria. Overall, the pivotal role of PAR activation and hypercoagulability in the development of AF was underlined by the recent demonstration that specific coagulation inhibitors targeting thrombin or Xa may prevent arrhythmogenic cellular changes or tissue remodeling (Jumeau et al., 2016b).

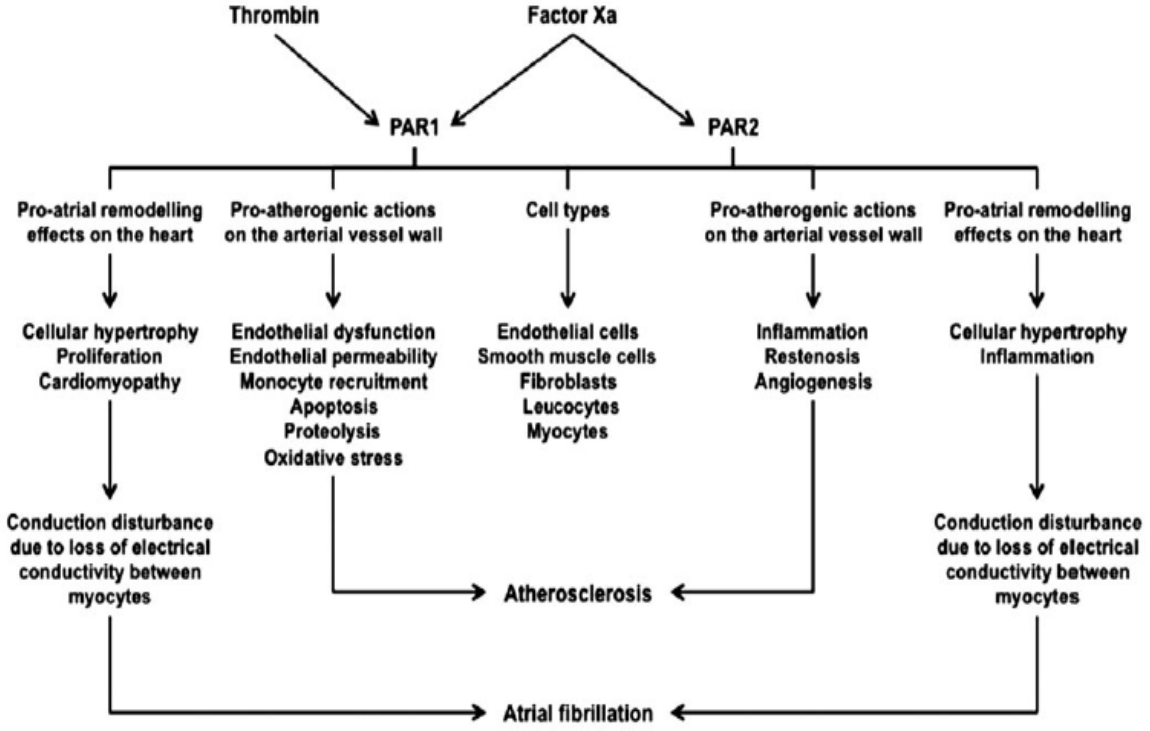

Figure 12: Cellular locations of PAR1 and PAR2 and the effetcs observed by thrombin- or factor Xa-mediated PAR acivation on the heart and vasculature, which leads to atherosclerosis and AF (Spronk et al., 2014).

2.5 Neural/Autonomic Remodeling

Autonomic nervous system factors play an important role in AF. Vagal discharge increases acetylcholine-dependent K current (I_{KACh}), decreasing APD and stabilizing reentrant rotors. Adrenoceptor activation enhances diastolic Ca^{2+} leak and encourages DAD-related ectopic firing by hyperphosphorylating RyR2s. Atrial sympathetic hyperinnervation ensues in persistent AF patients and tachycardia-remodeled dogs. Autonomic neural remodeling adds to positive feedback loops that enhance AF persistence and recurrence. Suppression of autonomic signaling may contribute to the efficacy of PV-directed ablation procedures for AF, particularly in certain patient subsets; in experimental AF models, model-specific autonomic ganglion ablation effects depend on autonomic innervation changes (Chou and Chen, 2009; Dobrev et al., 2011; Iwasaki et al., 2011a; Nishida et al., 2011).

2.5 Anatomic Factors

2.6.1 Roles of specific structures

Haïssaguerre et al. observed that PVs are a major source of ectopic beats with 94% of the triggers initiating AF originate within one or more PVs and may interact with the surrounding left atrial substrate through discrete or wide fascicles and, can frequently initiate paroxysms of AF (triggered AF episodes). In fact, irregular atrial sleeves of cardiomyocytes with potential spontaneous electrical activity extend over the veno-atrial junction into the PV wall and have electrical activity (Corradi, 2014b). The sleeves (whose size is up to 25 mm in length) mainly consist of circularly or spirally oriented bundles of cardiomyocytes that interconnect with each other in a continuous pattern with some gaps throughout. Several mechanisms have been associated with PV arrythmogenicity. Experimentally, triggered activity and irregular high-frequency rhythms have been observed following ryanodine infusion, atrial stretching, rapid atrial pacing and congestive heart failure, but, seemingly, not in normal PV cardiomyocytes. In this situation, the anatomical and electrical isolation of PVs has become a foundation of ablation techniques (Allessie et al., 2010; Haissaguerre et al., 1998; Verma, 2011). However, the success of this PV isolation is limited in some patients with paroxysmal AF and, especially, in the great majority of those subjects with persistent/permanent AF, very likely because of more extensive atrial remodeling additionally involving extra-PV locations (Chen et al., 1999; Oral et al., 2002). The most frequent sites of non-

PV atrial triggers include the posterior wall of the left atrium, the superior vena cava, the coronary sinus, the ligament of Marshall, and the region adjacent to the atrioventricular valve annuli. Furthermore, the atrial ganglionated plexi may play a significant role in the pathogenesis of AF (Corradi et al., 2013).

2.6.2 Regional Ion Current Differences

The LA has most important role in AF initiation and maintenance, mainly for paroxysmal AF. Reentrant rotors are usually faster in the LA than in the right atrium, causing them more likely to be drivers, mainly because of larger K currents that reduce APD. PV cardiomyocytes have shorter APDs due to larger delayed rectifier K currents and smaller I_{CaL}, along with reduced resting potentials because of smaller I_{K1} (Iwasaki et al., 2011a)

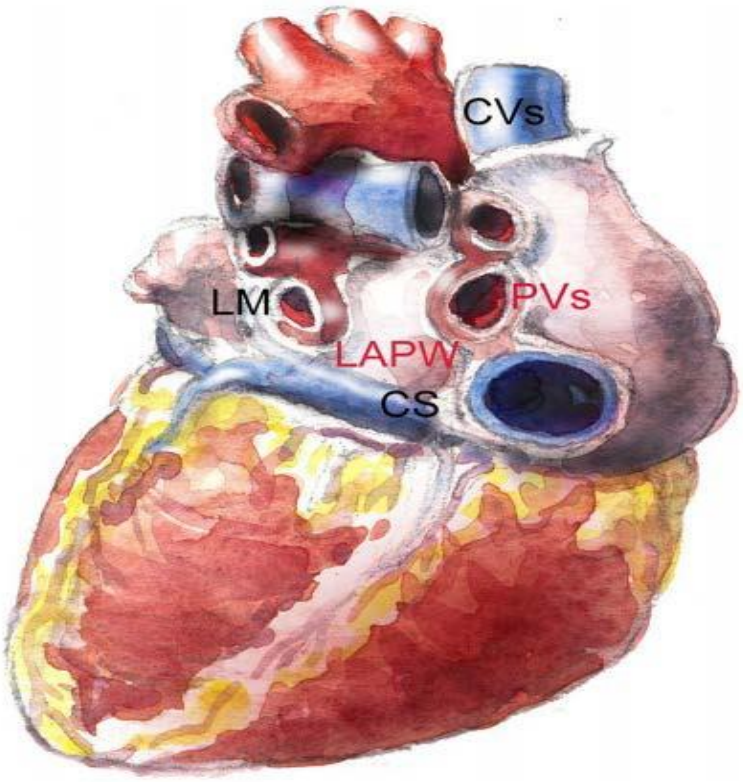

Figure 13: AF trigger points. Red abbreviations showing the most and black abbreviations showing the less AF common trigger points. Where, CS: coronary sinus; CVs: canal veins; LAPW: left atrial posterior wall; LM: ligament of Marshall; PVs: pulmonary veins (Corradi, 2014a).

2.7 Atrial fibrillation and inflammation

Wondering whether inflammation is the cause or consequence of AF is probably the "chicken and egg" conundrum. Based on the available literature, very likely, both hypotheses are true. Inflammation constitutes a noteworthy trigger for the arrhythmia and, at the same time, AF produces an inflammatory environment. In addition, many studies provide convincing evidence that inflammation plays an important role in the pro-thrombotic state associated with AF. The mechanism linking these two phenomena involve activated inflammatory cells (i.e., monocytes, macrophages, and lymphocytes) which trigger endothelial dysfunction, platelet activation, and increase fibrinogen production (Guo et al., 2012).

Several inflammatory markers—such as C-reactive protein (CRP), tumor necrosis factor alpha (TNFα), interleukin 2, interleukin 6 (IL-6), interleukin 8 (IL-8), and monocyte chemoattractant protein 1 (MCP1) are linked with AF (e.g. in post-operative AF) and its outcome (Ozaydin, 2010). Marcus et al. found increased CRP levels in the left atrium than in the corresponding coronary sinus and concluded that trans-cardiac cytokine gradients in AF ascend by sequestration of inflammatory cytokines in the heart (Guo et al., 2012). Also, the presence of inflammation in the heart or systemic circulation not only predicts the onset of AF and recurrence in the general population, but also in patients after cardiac surgery, cardioversion, and catheter ablation. Mediators of the inflammatory response can change atrial electrophysiology and structural substrates, thereby resulting in enhanced vulnerability to AF. Inflammation also modulates calcium homeostasis and connexins, which are linked with triggers of AF and heterogeneous atrial conduction. Myolysis, cardiomyocyte apoptosis, and the activation of fibrotic pathways via fibroblasts, transforming growth factor-β and matrix metalloproteases have also been controlled by inflammatory pathways, which can all cause structural remodeling of the atria. The progression of thromboembolism, a detrimental complication of AF, is also associated with inflammatory activity. Thus, understanding the complex pathophysiological processes and dynamic changes of AF-associated inflammation might help to improve specific anti-inflammatory strategies for the prevention of AF (Hu et al., 2015).

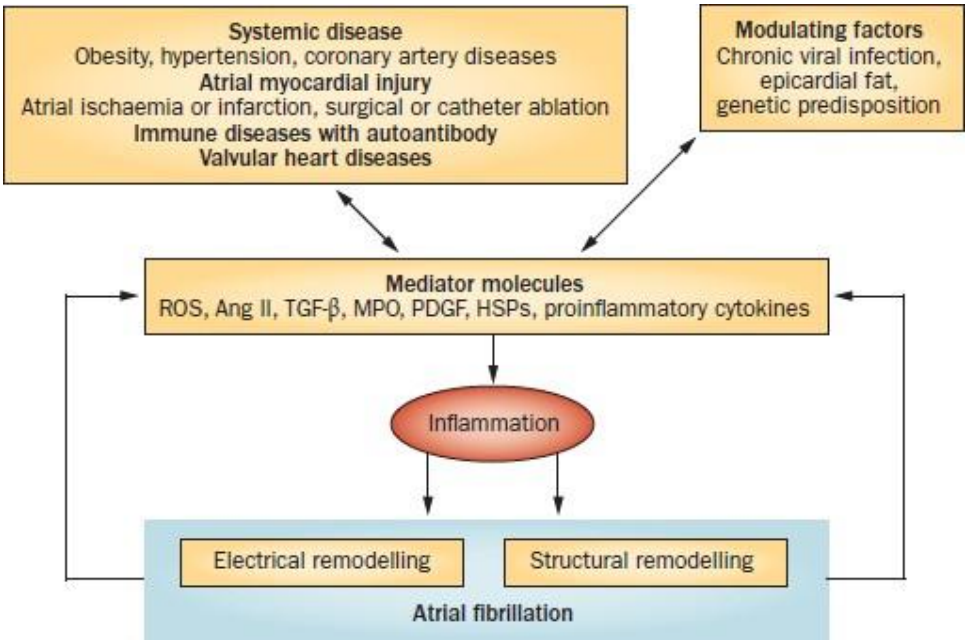

Figure 14: Sources of inflammation in AF patients. Activated inflammatory pathways significantly alter the remodeling of the atria. Where, Ang II: angiotensin II; HSP: heat shock protein; MPO: myeloperoxidase; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; TGF-β: transforming growth factor β (Hu et al., 2015).

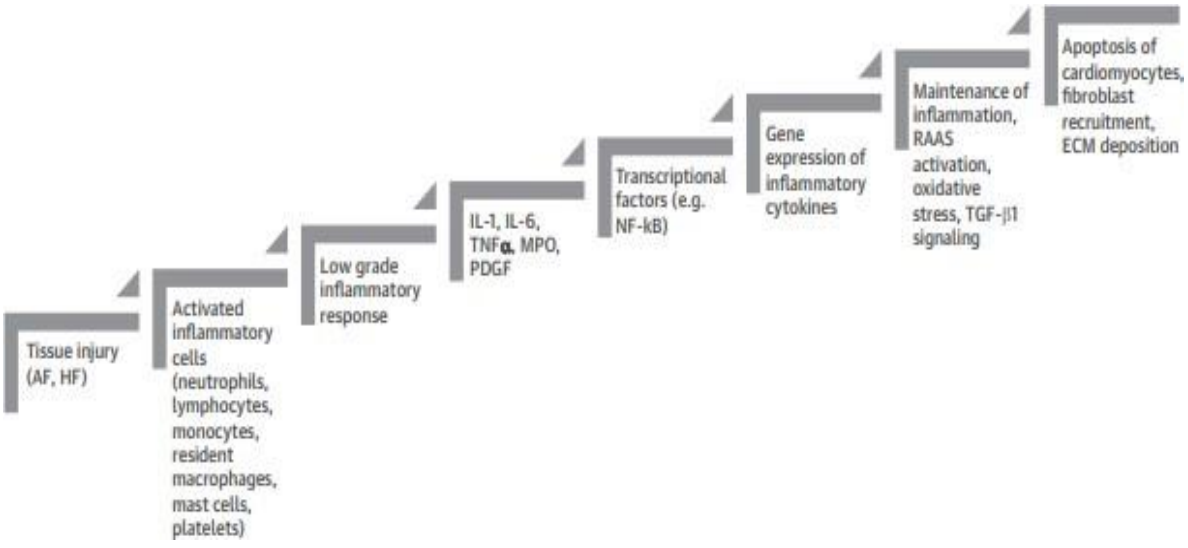

Figure 15: Association of inflammatory pathways in cardiac fibrosis. Where, IL: interleukin; MPO: myeloperoxidase; NF-κB: nuclear factor kappa B; PDGF: platelet-derived growth factor,

RAAS: renin-angiotensin-aldosterone system; TGF-β1: transforming growth factor β1; TNF: tumor necrosis factor (Dzeshka et al., 2015).

2.8 Atrial fibrillation and oxidative stress

Several studies conducted show a positive correlation between oxidative stress and AF induction and maintenance (Corradi et al., 2008; Li et al., 2010; Youn et al., 2013b). Increased levels of reactive oxygen species (ROS) such as superoxide anions and H_2O_2 have been found to be associated with AF in the myocardium (Kim et al., 2005; Kim et al., 2008b; Youn et al., 2013a; Zhang et al., 2012). The oxidized GSSG/reduced glutathione and oxidized cysteine/reduced cysteine ratios are increased in the blood of patients with AF (Neuman et al., 2007). Amplified ROS levels result in damage to proteins, lipids, and DNA, and aggravate inflammation. In addition, ROS have also been implicated in cardiac structural and electrical remodeling. In fact, it is shown that hydroxyl radical (OH^-) and peroxynitrate ($ONOO^-$) facilitate oxidative damage of myofibrils in AF (Babusikova et al., 2004; Mihm et al., 2001). With regard to atrial electrical remodeling, this has been found to be linked with intracellular calcium overload. Carnes et al., in a dog model, showed that AF induced by rapid pacing decreased myocardial tissue ascorbate levels and upregulated protein nitration (Carnes et al., 2001). Coronary artery bypass surgery, which is often intricated by post-operative AF, is accompanied with an increase in oxidized glutathione and lipid peroxidation (Basu et al., 2000; Wolin and Gupte, 2005). In patients with persistent AF and mitral valve disease, higher myocardial tissue levels of the inducible oxidative stress marker heme oxygenase 1 (HO-1) were documented compared to controls. Interestingly, HO-1 was more articulated where the structural remodeling peaked (left atrial posterior wall vs left atrial appendage). In comparison with controls, both HO-1 overexpression and greater 3-nitrotyrosine levels were noted in the left and right atrial free walls of individuals with idiopathic persistent AF (Corradi, 2014b).

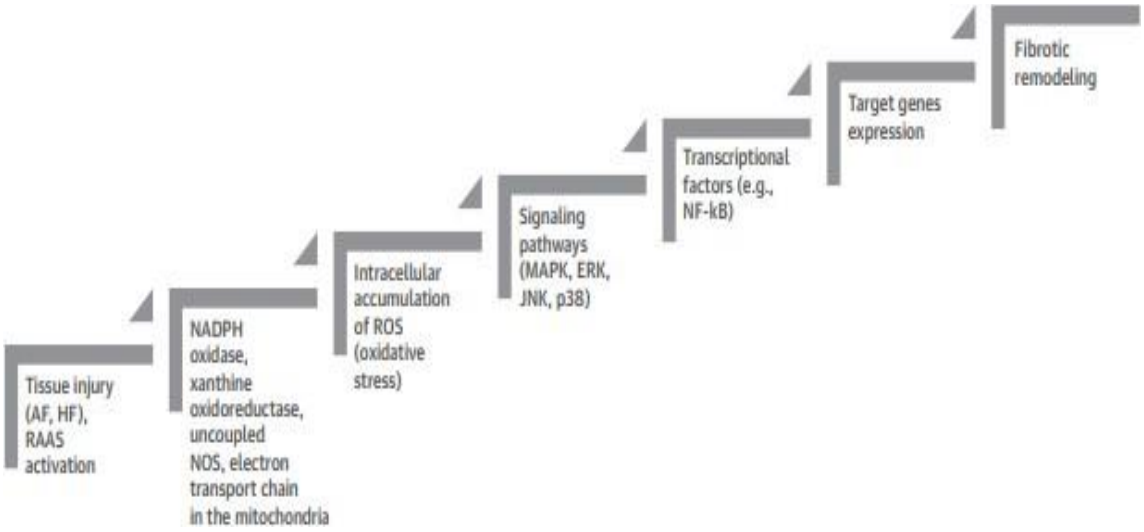

Figure 16: Oxidative stress in cardiac fibrosis. Where, ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; NADPH: reduced nicotinamide-adenine dinucleotide phosphate; NOS: nitric oxide synthase; p38: p38 mitogen activated protein kinase; ROS: reactive oxygen species (Dzeshka et al., 2015).

2.9 Atrial fibrillation, stroke and microparticles

AF has been found in about 25% of patients admitted with ischemic stroke as it increases the risk of stroke by 3–6 fold. The most devastating complication of AF is stroke which causes death, neurological deficits, longer hospitalization and disability. Many studies have documented the relationship between AF and stroke. The Stroke Prevention in Atrial Fibrillation Study (SPAF III) data revealed that up to 45% of the patients enrolled had ECG documentation of AF. Stroke can occur as the first manifestation of AF. Based on the STROKESTOP trial it is suggested that patients with silent AF should be screened for the risk of stroke (Friberg et al., 2013). Several stroke risk prediction scores have been documented. The most widely accepted predictor is CHADS2 (Congestive Heart Failure (CHF), hypertension, age, diabetes, stroke/transient ischemic attack) however, recently CHADS2-VASC score has been suggested which additionally involves age >65 years, vascular disease and female sex. CHADS2 has been validated by several studies and CHADS2-VASC is more popular in Europe. There is a stepwise approach increase in risk of stroke as the CHADS2 or CHADS2-VASC score increases (Mason et al., 2012) .

Being, small membrane vesicles, microparticles (MPs) that are found to shed from virtually all cells in response to stress have been extensively described in various atherothrombotic diseases.

Also, many recent findings propose a predominant role of circulating MPs in the setting of hypercoagulable state which is associated with supraventricular tachyarrhythmia (Jesel et al., 2013; Wang et al., 2018), thus suggesting a critical association with atrial fibrillaton.

$CHADS_2$		
C	Congestive HF	1 point
H	Hypertension	1 point
A	Age ⩾75 yrs	1 point
D	Diabetes	1 point
S_2	Stroke	2 points
$CHADS_2$-VAS_c		
C	Congestive HF	1 point
H	Hypertension	1 point
A	Age ⩾75 yrs	2 points
D	Diabetes	1 point
S_2	Stroke	2 points
V	Vascular disease	1 points
A	Age ⩾65 yrs	1 points
S_c	Sex category, female	1 points

Table 2: Risk scheme for $CHADS_2$ and $CHADS_2$-VAS_2 (Shenasa et al., 2014)

2.10 Management of Atrial fibrillation

Atrial fibrillation management constitutes symptoms management and prevention of complications requiring treatment of associated cardiac or endocrine disease, control of cardiac rhythm and ventricular rate, and antithrombotic therapy (Kirchhof, Benussi et al. 2016, Andrade, Verma et al. 2018).

2.10.1 Rate control therapy in atrial fibrillation

Rate control being an integral part of the AF management, has been often sufficient to improve AF-related symptoms. Pharmacological rate control has been usually attained by medications that decrease the number of impulses conducting into the ventricles thus increasing the degree of block at AV node level. Most commonly used agents are β-blockers (e.g., metoprolol, bisoprolol or nebivolol), non-dihyrdopyridine calcium channels blockers (e.g., diltiazem or verapamil) or digitalis compounds (Andrade, Verma et al. 2018). Sometimes, when pharmacological interventions are inefficient or not tolerated in AF subset patients then, the option is a non-specific and palliative last

resort strategy consisting of AV node ablation and pacemaker implantation (Dobrev and Nattel 2010, Ravens 2010).

2.10.2 Rhythm control therapy in atrial fibrillation

Rhythm control drugs target to restore a normal sinus rhythm, process called cardioversion. The classic antiarrhythmic Na+ current inhibitors, class I drugs decrease excitability whereas, K+ channel blocking, class III drugs suppress re-entry circuits by increasing action potential duration and repolarization. Amiodarone, being most frequently used antiarrhythmic drug to achieve and maintain normal sinus rhythm also possess heart rate lowering effect thus concomitantly can be used for rate control. However, in some cases the use of amiodarone is limited due to its severe adverse effects mainly pulmonary toxicity, skin discoloration, thyroid toxicity, corneal deposits, optic neuropathy, and sinus bradycardia (Schmidt, Kisselbach et al. 2011). Although many clinicians are of the view that preserving sinus rhythm results in improved outcomes in AF patients, however, all trials that compared rhythm control and rate control to rate control alone (alongwith appropriate anticoagulation) resulted in neutral outcomes (Kirchhof, Benussi et al. 2016). Thus, in clinical practice, the decision between rate or rhythm control relies on various patient-specific factors constituting duration and frequency of AF episodes, underlying structural or endocrine disease, and the consequences of previous treatment regimes (Andrade, Verma et al. 2018)

2.10.3 New antiarrhythmic drugs

Dronedarone was approved as new antiarrhythmic drug based on the results of a Placebo Controlled, Double-Blind, Parallel Arm Trial to Assess the Efficacy of Dronedarone 400 mg bid for the Prevention of Cardiovascular Hospitalization or Death from any Cause in Patients with Atrial Fibrillation/Atrial Flutter (ATHENA trial) (Hohnloser, Crijns et al. 2009) that shows both rhythm and rate control by dronedarone in AF patients, with less side effects compared with amiodarone. Thus, showing decreased frequency of hospitalization resulting from cardiovascular events or death in high-risk AF patients. Another, antiarrhythmic drug vernakalant granted market approval after a Phase III Superiority Study of Vernakalant vs Amiodarone in Subjects with Recent Onset Atrial Fibrillation (AVRO) trial for the conversion of recent-onset AF to sinus rhythm (Dorian, Pinter et al. 2007, Dobrev, Hamad et al. 2010). The trial showed dominance of vernakalant over amiodarone for cardioversion within 90 minutes (Camm, Capucci et al. 2011).

2.10.4 Catheter ablation of atrial fibrillation

Catheter ablation has been predominantly achieved by isolation of the pulmonary veins, sometimes necessitate whole isolation to achieve full effectiveness and supplementary ablation in the posterior left atrial wall. When it is performed in well-equipped centres by sufficiently trained teams appeared to be more effective than antiarrhythmic drug therapy in maintaining sinus rhythm (Camm, Kirchhof et al. 2010).

2.10.5 Prevention of thromboembolic events in atrial fibrillation

With Atrial fibrillation there is an increased risk of stroke, and it has been shown by various studies that oral anticoagulation consisting vitamin K antagonists (VKA) are more efficacious with respect to aspirin regarding stroke prevention (Hart, Benavente et al. 1999, Gage, Waterman et al. 2001, Gage, van Walraven et al. 2004, Fuster, Ryden et al. 2006). Antithrombotic therapy is advised on the basis of systemic embolism and patient-specific evaluation of risk factors for stroke (Lip, Nieuwlaat et al. 2010, Fuster, Ryden et al. 2011) In patients with a CHADS2 score of 0–1 additional risk stratification using the CHADS2 -VASC scheme has been recommended. Direct thrombin inhibitors (e.g., dabigatran) and factor Xa inhibitors (e.g., rivaroxaban, apixaban) are new oral anticoagulant drugs that are effective, safe and convenient to use (Connolly, Ezekowitz et al. 2009, Lopes, Alexander et al. 2010). In addition, the results of the Stroke Prevention using the Oral Direct Factor Xa Inhibitor Rivaroxaban Compared with Warfarin in Patients with Nonvalvular Atrial Fibrillation (ROCKET AF) trial (2010) showed that the factor Xa inhibitor rivaroxaban was as effective as warfarin in preventing stroke in AF patients and did not increase their risk of bleeding.

2.10.6 Interventional approaches to stroke prevention

Almost 90% of thromboembolisms linked with AF have been due to thrombi originating from the left atrial appendage (LAA). Therefore, occlusion of the LAA deliver a non-pharmacological substitute for curtailing the risk of stroke in AF patients (Kanderian, Gillinov et al. 2008, Dawson, Asopa et al. 2010).

2.10.7 Upstream therapy

Upstream therapy targeted against myocardial remodeling accompanied with hypertension and heart failure may hinder AF development (primary prevention) and can also decrease recurrence rates and

progression to permanent AF (secondary prevention) (Savelieva, Kakouros et al. 2011). Angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and statins has been considered for primary and secondary prevention in patients with concomitant structural heart disease and in cases of recurrent AF despite antiarrhythmic drug therapy respectively (Fuster, Ryden et al. 2006, Camm, Kirchhof et al. 2010). However, evidences from different trials conducted for upstream therapy for prevention of atrial remodeling still remains controversial (Savelieva and Camm 2007, Disertori, Barlera et al. 2012)

ENDOCARDIAL ENDOTHELIAL CELLS

3 ENDOCARDIAL ENDOTHELIAL CELLS

3.1 Introduction

Cardiac endothelial cells comprise microvascular endothelial cells (MVECs) and endocardial endothelial cells (EECs), whereas vascular endothelial cells line the interior surface of blood vessels. Endocardial endothelium line the complex cavitary surface of the heart wall that continues over the surface of the valve and extends on to form the lining of large blood vessels. By creating a natural biological barrier (blood-heart barrier) between the circulating blood in heart cavities and cardiomyocytes, endocardial endothelium creates a complex but finely tuned balance of interactions between these units (Brutsaert, 2003). Endocardial endothelial cells constitute endocrine and sensory role, thus playing an important physiological role on cardiomyocytes, terminal network of Purkinje fibers and subendocardial nerve plexus (SNP) (Verma and Anderson, 2002).

The existence of an intact endocardial endothelium significantly modify the contractility of the heart, hence, the change in the contraction curve can be observed if there is selective damage or dysfunction of endocardial endothelium. Furchgott and Zawadski in 1980 first described the impact of vascular endothelium on the vascular smooth muscle contractility (Furchgott and Zawadzki, 1980) and later it was observed for the endocardial endothelium as well (Brutsaert et al., 1988; Shen et al., 2013; Smiljic et al., 2010). By increasing the sensitivity of myofilament to Ca^{2+} ions through the release of endothelial mediators the intact endocardial endothelium improves the contractility of the heart muscle (Shen et al., 2013).

Endocardial and myocardial capillary endothelium release many autocrine and paracrine signaling substances such as nitric oxide (NO), endothelin (ET-1), prostaglandins (PGI_2, PGF_2, PGE_2) and angiotensin II (Ang II) thus, having an affect on the contractility of cardiomyocytes. Also, in the regulation of cardiac inotropic state, the important role of other endothelial mediators, such as fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), neuregulin-1 (NRG-1) and angiopoietin has also been documented (Brutsaert, 2003; Noireaud and Andriantsitohaina, 2014b).

The development of endocardial endothelium and the endothelium of blood vessels ensues simultaneously during embryonic growth. Endocardial endothelium exhibit an important role for the proper formation of trabecular myocardium and in heart development. It is necessary for the trans-differentiation of myocytes into the heart conduction system cells and Purkinje's fibers.

In addition, they are also involved in endocardial-mesenchymal conversion and the development of endocardial cushions, which later on give rise to several imperative structures within the heart, including the valves, the membranous portion of the interventricular septum, and the atrial septum (Brutsaert, 2003).

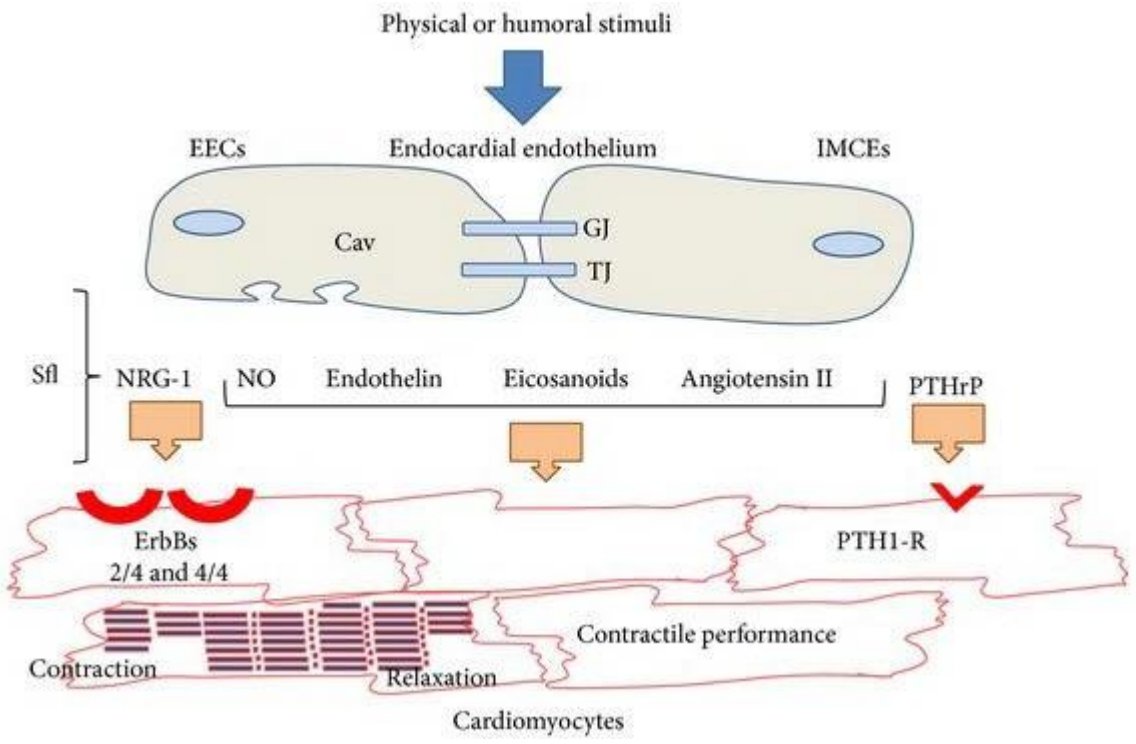

Figure 17: Paracrine relations between cardiac endothelial cells and cardiomyocytes. Endocardial endothelial cells (EECs) and intramyocardial capillary endothelial cells (IMCEs) react to physical and humoral stimuli by releasing various mediators such as nitric oxide (NO), endothelin 1, eicosanoid, and angiotensin II. These endothelial cells also release neuregulin-1 (NRG-1) which primarily acts on ErbB receptors found on myocardial cardiomyocytes. IMCEs also secrete parathyroid hormone-related peptide (PTHrP) which acts on PTH1 receptor (PTH1-R) present on cardiomyocytes. Gap junctions (GJ) facilitate cell-to-cell coupling for rapid intercellular inter-relationship of functional demands. Tight junctions (TJ, constituting zonula adherens) regulate the transendocardial endothelial-permeability via intercellular clefts. A large number of endothelial signaling molecules localize to caveolae (Cav). Sfl: subendocardial fibroelastic layer (or extracellular matrix including sympathetic nerve fascicles) (Noireaud and Andriantsitohaina, 2014b).

3.2 Structural characteristics of endocardial endothelial cells

The functional morphological characteristics of endocardial endothelium cells and myocardial capillary endothelium are not same as well as their embryonic origin. Thus, the impact of EECs and MVECs on myocardial contractility, rhythm and remodeling is different. Also, the distribution of receptors for these two endothelial types is not same. In addition, EECs and MVECs have diverse cytoskeletal features, such as the existence of contractile bundles of actin filaments (stress fibers) and vimentin and microtubule filaments. MVECs have more actin filaments as a consequence of greater shear stress exposure. Endocardial cells possess well established organelles, importantly Golgi apparatus, and in comparison to microvascular endothelium can synthesize more endothelial mediators such as prostacyclin, nitric oxide (NO).

Moreover, it has been found that EECs are slightly larger compared to endothelial cells of all other components of the circulatory system (Mebazaa et al., 1995) and the specificity of EECs has been categorized by the existence of particular cellular connections and intracellular spaces when compared to vascular or microvascular endothelial cells (Dejana and Del Maschio, 1995; Mebazaa et al., 1995). Various complex structural gap-junctions regulate the transendothelial permeability and facilitate quick passage of charged ions (primarily Ca^{2+}), secondary messenger molecules and small metabolites.

The existence of plenty of gap junctions in endocardial endothelium, which are not so abundant in other endothelial structures, enable a functional connection and the behavior of endocardial endothelium as a single operating syncytium. The transcellular ion transport from blood to the cardiomyocytal interstitium occurs via passive diffusion through ion channels (inward rectifier K^+ channels, Ca^{2+}-activated K^+ channels, voltage-gated K^+ channels, volume activated Cl^- channels, stretch-activated cation channels) and via active transport (Na^+/K^+-ATPase) (Kuruvilla and Kartha, 2003). The secondary messengers pass many gap junctions after the activation of individual EECs to the neighboring endocardial endothelial cells, thus amplifying their sensory capacity.

3.3 Physiological role of endocardial endothelium

Endothelial cells being metabolically active exert important paracrine, endocrine and autocrine functions including tissue growth and remodeling, immune responses, cell adhesion, angiogenesis,

hemostasis and vascular permeability. Endothelium-derived factors with vasodilatory and antiproliferative effects mainly include endothelium-derived hyperpolarization (EDH), nitric oxide (NO) and prosacylin (PGI_2), while angiotensin II, reactive oxygen species (ROS) and endothelin-1 possess vasoconstrictive effects. Endothelial cells also secrete antithrombotic (NO and PGI_2 both inhibit platelet aggregation) and prothrombotic molecules such as von Willebrand factor, which promotes platelet aggregation, and plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrinolysis (Feletou, 2011; Sena et al., 2013).

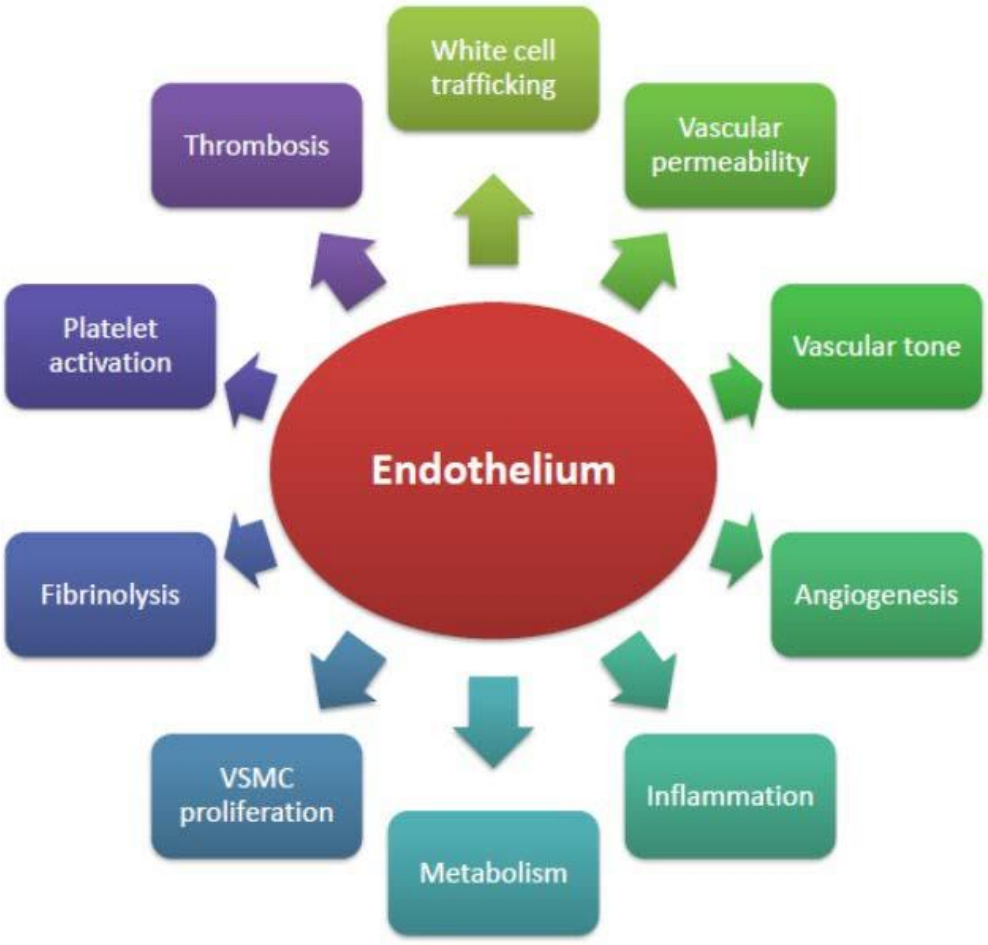

Figure 18: Various functions of endothelium (Sena et al., 2013).

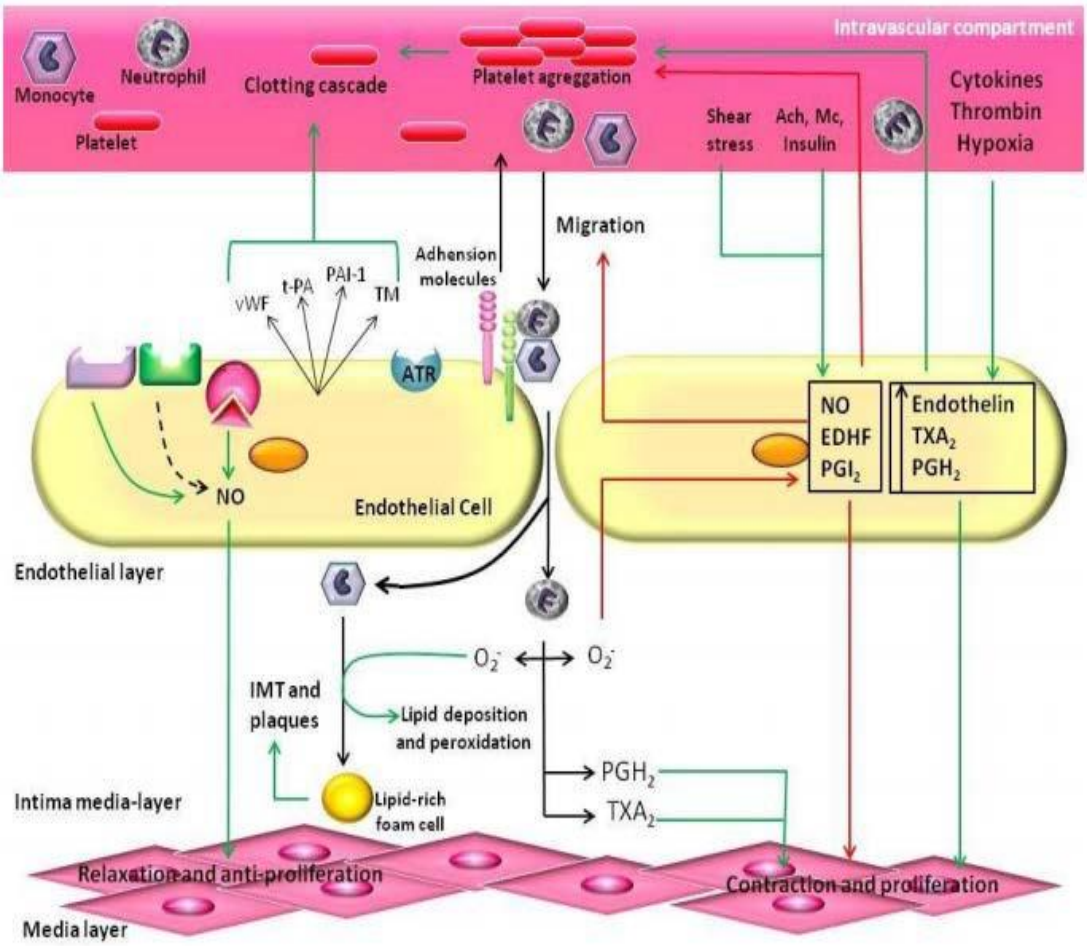

Figure 19: Endothelial cells are responsible for various physiological functions, including: 1) modulation of vascular tone through release of vasodilators and vasoconstrictors; 2) regulation of blood fluidity and coagulation via release of factors that regulate platelet activity, the clotting cascade, and the fibrinolytic system; and 3) control of inflammatory processes through expression of cytokines and adhesion molecules. Where, Ach: acetylcholine; ATR: angiotensin II receptor; BK: bradykinin; EDHF: endothelium-derived hyperpolarisation factor; NO - nitric oxide; PAI-1: plasminogen activator inhibitor-1; PGH_2: prostaglandin H2; PGI_2: prostacyclin; $O^{2\bullet-}$ - superoxide anions; t-PA: tissue plasminogen activator; TM: thrombomodulin, TxA_2: thromboxane A_2; vWF: von Willebrand factor (Sena et al., 2013).

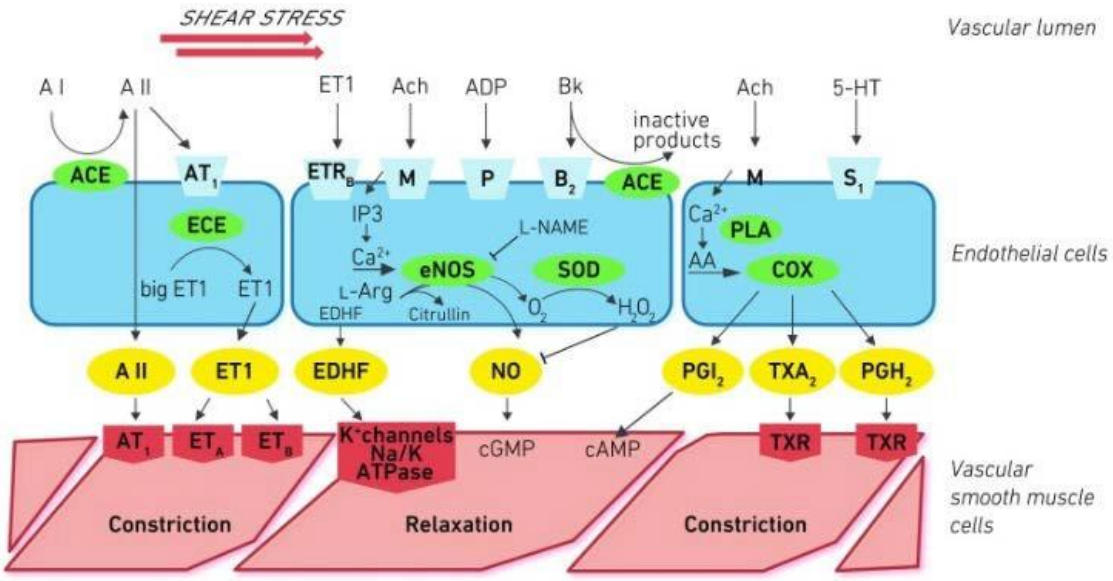

Figure 20: Relaxing/dilating and constrictive signals send by endothelial cells to the smooth muscle cells. Three central pathways have been showed: The constituent endothelial nitric oxide synthase (eNOS, NOS III) being regulated by endocrine and paracrine effects such as endothelin-1 (ETR, ET-1) and acetylcholine (ACh*)* as well as shear stress through pertussis toxin-sensitive $G_{q/i}$ pathways, calcium, and calmodulin. Nitric oxide (NO) signals relaxation, but uncoupling can lead to increased oxidative stress (H_2 O_2). The endothelial cells also cause hyperpolarization of the cell membrane of smooth muscle cells (endothelium-dependent hyperpolarization factor (EDHF)). Cyclooxygenase 1 (COX) releases eicosanoids that constitute in the case of prostacyclin (*PGI* $_2$) relaxing results through cyclic AMP or constrictive effects predominantly for thromboxane A_2 (TXA_2). Angiotensin II (Ang II*)* show direct (by angiotensin receptor 1 (AT1R*)*) or indirect constrictive effects through ET-1 (Barthelmes et al., 2017).

3.3.1 Nitric oxide

Cardiac endothelial cells produce and secrete many mediators which effect cardiac growth, metabolism, contractility and rhythm, mainly nitric oxide (NO), whose synthesis has been catalyzed by endothelial, neural and induced nitric oxide synthase (NOS) (Andries et al., 1998). Endothelium constitutive nitric oxide synthase (eNOS) has been found in the coronary endothelium, myocardial capillary endothelium, endocardial endothelium and in small amount in cardiomyocytes (Balligand et al., 1995) whereas, neuronal NOS (nNOS) has been found in cardiac myocytes; nerve fibers in the atrial tissue; in a subpopulation

of intracardiac ganglia and and in the perivascular nerve fibers of the ventricular myocardium (Zhang, 2016). On the other hand, inducible NOS (iNOS) is functional only under stress conditions and release of cytokines (Balligand et al., 2009).

The cyclical changes in the heart during systole and diastole directly impacts the activity of eNOS and NO synthesis. The cyclical release of NO mainly in the subendocardial regions of the heart depicts endocardial endothelium as its major source (Balligand et al., 2009; Smiljic et al., 2014a). The endothelial-borne reactive oxygen species (ROS), such as superoxide anions, can abruptly quench NO synthesized by the endothelial cells, without altering the expression of eNOS (Paolocci et al., 2001). The myocardial tissue oxygen consumption is decreased by NO both in physiological and pathophysiological conditions (Trochu et al., 2000) which indicates its potential cardioprotective effect. Also NO can reversibly compete with oxygen for a common binding site on cytochrome-c oxidase which impedes electron transfer to oxygen. Nitric oxide produced by myocardial capillary endothelium and endocardial endothelium plays an important role in local myocardial metabolism (Jones and Bolli, 2006). In addition, NO also inhibits platelet aggregation and prevents binding of neutrophils to endothelium thus playing a fundamental role in cardiovascular disease protection.

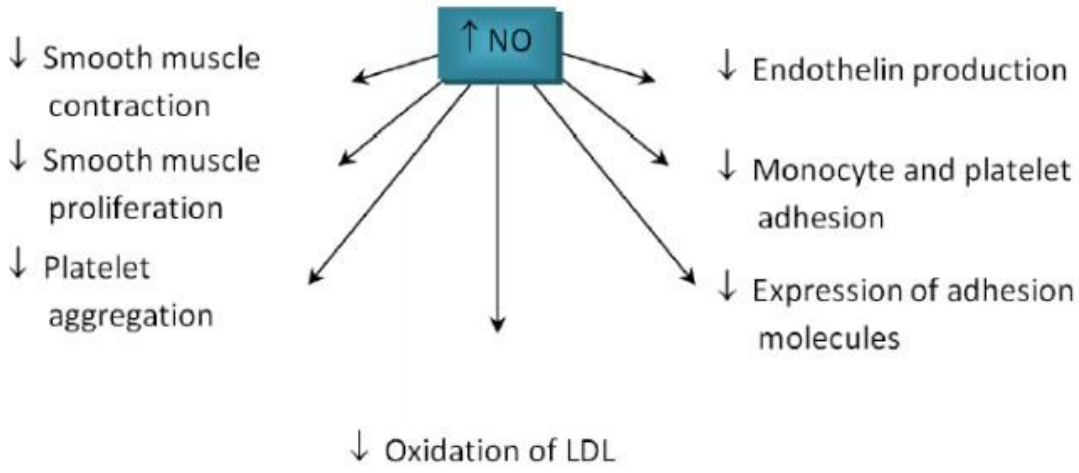

Figure 21: Atheroprotective characteristics of nitric oxide generated by endothelial nitric oxide synthase (M. Vanhoutte, 2018; Sena et al., 2013).

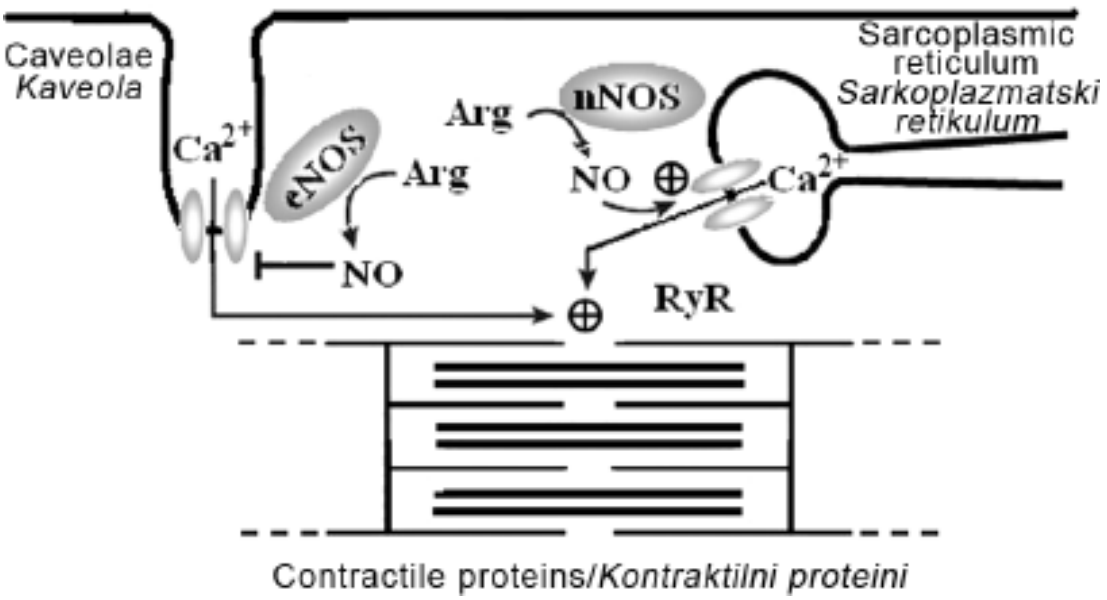

Figure 22: Depicts biosynthesis of nitric oxide and opposing roles of eNOS and nNOS in modulating heart contraction (Smiljic et al., 2014a).

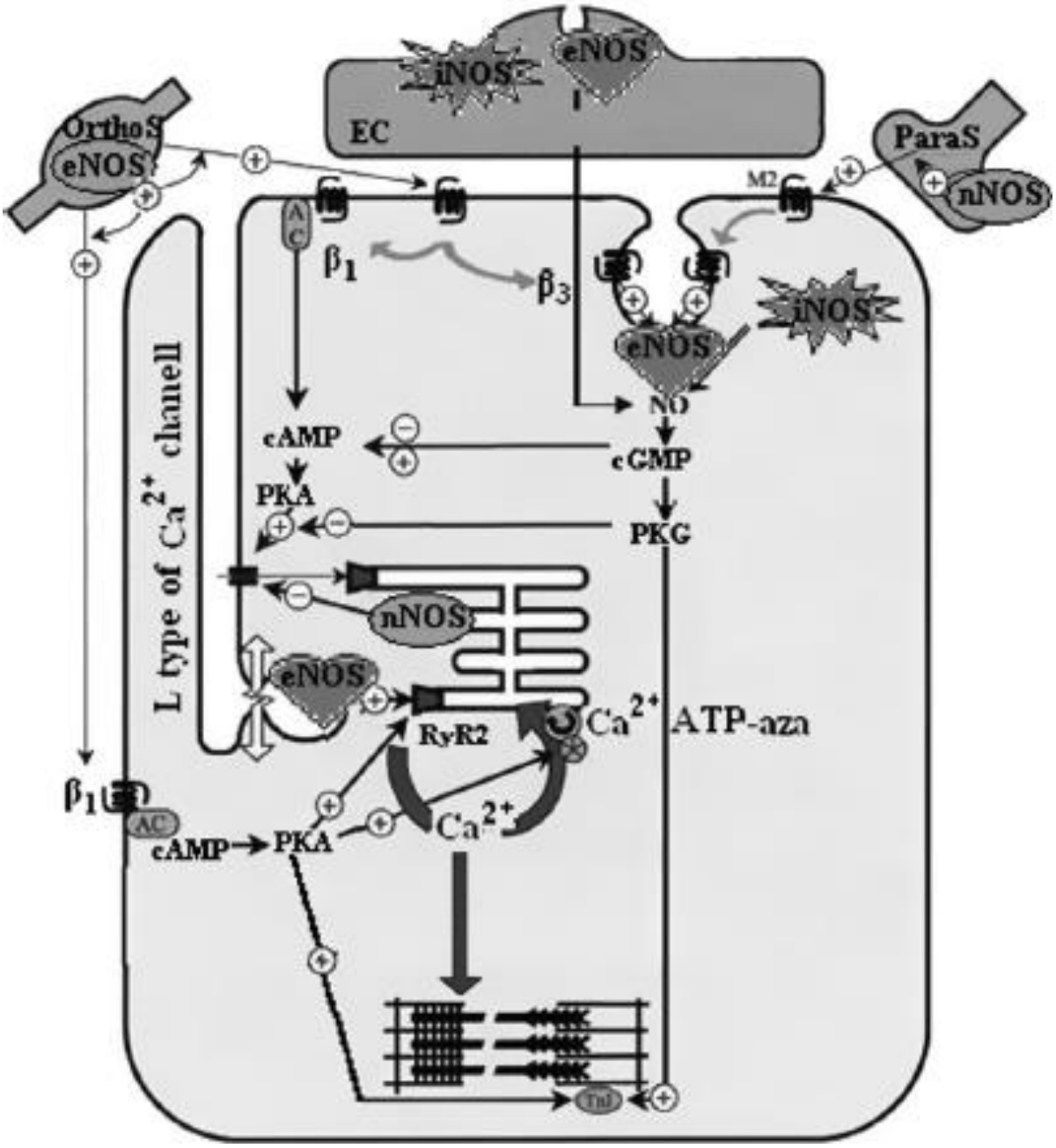

Figure 23: Autocrine and paracrine nitric oxide modulation of cardiomyocyte (Smiljic et al., 2014a).

3.3.2 Vasostatin-1 and endothelin-1

By regulating the contractility of the heart catecholamines and neurohormones play an important role in heart remodeling. Chronically elevated catecholamine (CA) levels are known to harm the heart (Chien et al., 1991; Goldspink et al., 2004; Samuels, 2007). Chromogranin A (CgA) being

an important soluble protein, is co-stored in and co-released with CAs from secretory vesicles found in adrenal medulla chromaffin cells (Mahapatra, 2008). CgA gives rise to several bioactive peptides via a posttranslational proteolytic processing mechanism. One of these peptides, vasostatin (VS), is a novel cardiac modulator and a stabilizer of adrenergic tone, which plays an important role in cardio-circulatory homeostasis and exhibit cardio-depressive and anti-adrenergic effects on isolated and perfused hearts in eel, frog and rat (Cerra et al., 2006; Corti et al., 2004; Gallo et al., 2007). Studies have shown that the anti-adrenergic effect prompted by VS-1 is due to the Ca^{2+}-independent, PI3K-dependent endothelial release of NO and not due to direct interaction with cardiac cells. In addition, VS-1 also exhibits a protective effect in ischemic hearts via an adenosine/nitric oxide signaling mechanism (Cappello et al., 2007). This strongly suggests that these fragments demonstrate important role in the autocrine/ paracrine regulation of cardiac function. Thus, a molecular signaling pathway analysis indicated that the beneficial effects of VS-1 on cardiac remodeling may be mediated via the enhanced activation of the eNOS-cGMP-PKG pathway (Wang et al., 2016). This strategy was implied to inhibit hypertrophy, fibrosis, and ventricular remodeling whilst recouping the cardiac function in the experimental heart models of rats that were injected with isoprenaline (Krenek et al., 2009; Wang et al., 2016).

EECs are a major source of endothelin-1 (ET-1) with cardiomyocytes its principal target. ET-1 exhibit potent positive inotropic action exerted by amplified sensitivity of myofilaments to Ca^{2+} (Jacques et al., 2000). A positive inotropic outcome is the consequence of the activation of protein kinase C (PKC) and protein kinase A (PKA) (Chu et al., 2003). When there is no stimulation for ET-1 synthesis and secretion, it exerts an autocrine effect by binding itself to ETB receptors on endocardial endothelial cells. Small amounts of ET-1 play an important defensive role in adult heart by instigating the release of nitrogen oxides and PGI_2 (Castrillo, 2016)

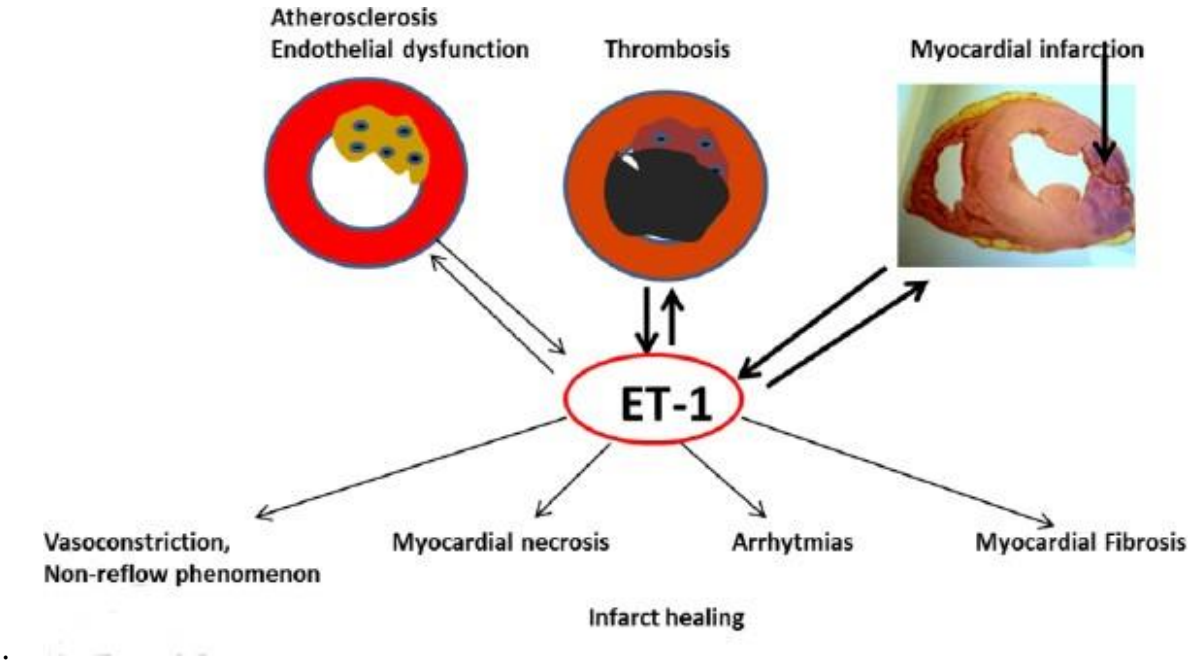

Figure 24: Depicts the possible role of endothelin-1 in the pathophysiology of myocardial infarction, heart failure and coronary artery disease (Castrillo, 2016).

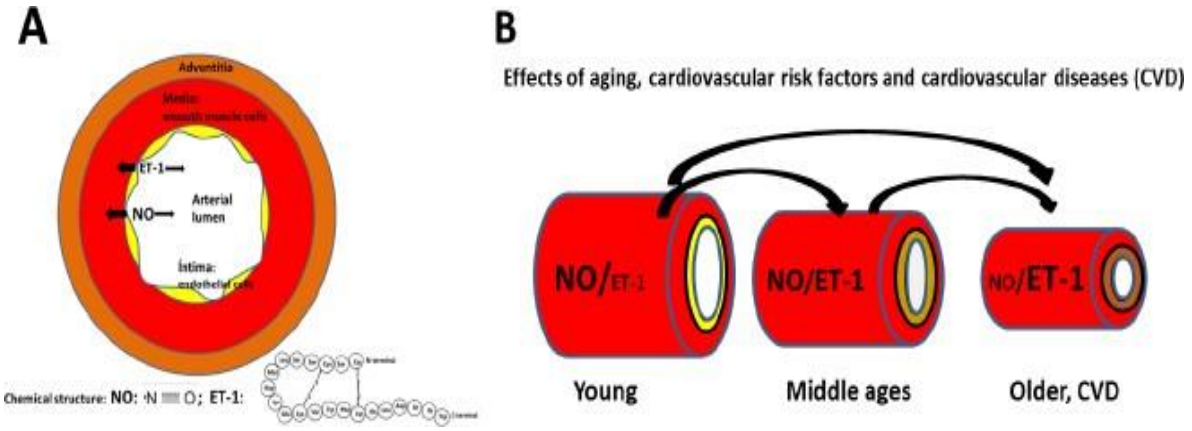

Figure 25: (**A**) show transverse section of healthy artery depicting the various layers of artery wall, release of endothelin 1 (ET-1) and nitric oxide (NO) by endothelial cells (**B**) showing alterations in the respective role for NO and ET-1 in the modulation of vascular tone after aging, various cardiovascular diseases and risk factors (Castrillo, 2016).

A large number of nonendothelial cells in the heart, including cardiomyocytes, can also produce ET-1 in response to myocardial stretch, Ang II, and adrenaline (Morimoto et al., 2000). By increasing myocardial oxygen consumption it enhances the inotropic effect, while at the same time

through its powerful coronary vasoconstrictive effect it also decreases the oxygen supply (Smiljic et al., 2014b; Smiljic et al., 2018).

3.3.3 Prostaglandins

In response to various humoral, chemical, immunological, and mechanical stimuli. cardiac endothelial cells can synthesize and release prostaglandins. Cyclooxygenase that show a key regulatory role in prostaglandin synthesis (PGE_2, $PGF_{2\alpha}$, PGI_2) is expressed in all endothelial cells in the heart and is believed to have cytoprotective effects. PGI_2 activates adenylate cyclase, causing increased production of cAMP resulting in vasodilation and it is also potent antiproliferative agent, reduces oxidative stress and prevents cellular adhesion to the vascular wall (Sena et al., 2013). PGH_2 is found to be the precursor of the prostaglandin E_2, F_2 and thromboxane A_2 (TxA_2) thus playing an important role in platelet activation and aggregation (Vanhoutte and Tang, 2008). Moreover, in the endocardial zone the activity of COX-1 is twice as high compared with the myocardium. However, the positive inotropic effect of prostaglandin (PGE_2 and $PGF_{2\alpha}$) could be completely abolished after the removal of EE in the atria of the heart. The positive inotropic effect is because of the release of prostaglandins from EECs upon the stimulation of muscarinic M3 receptors (Tanaka et al., 2001).

3.3.4 Angiotensin II

The effect of Ang II on cardiac growth and contractile performance is the result of locally produced Ang II. Ang II is synthesized locally through ACE and an ACE-independent kinase pathway, both of which are expressed predominantly in coronary vascular and cardiac endothelial cells (Dostal and Baker, 1999). Ang II generally exerts a positive inotropic effect, which may not always be the case as different effects are obtained in different conditions. Through its G-protein-coupled Ang II type 1 receptor (AT1R), Ang II activates various intracellular protein kinases, such as receptor or non-receptor tyrosine kinases, which constitute epidermal-growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), c-Src, PYK2, FAK, JAK2. Also, Ang II activates serine/threonine kinases such as mitogen-activated protein kinase (MAPK) family, p70 S6 kinase, Akt/protein kinase B and various protein kinase C isoforms. Ang II also induces the generation of intracellular ROS, which show perilous roles in activation and modulation of above signal transduction. Recent studies document that endothelial Ang II signaling negatively regulates

the NO signaling pathway and thereby induces endothelial dysfunction (Gomolak and Didion, 2014; Nakashima et al., 2006; Watanabe et al., 2005). Many studies are conducted which describe the interactions of the cardiac synthesis, release and activity of Ang II with bradykinin-NO and PGI_2 pathways, as well as with ET-1. Ang II and ET-1 (Meulemans et al., 1990), for example, elicit a synergistic effect on the heart while their receptors on cardiomyocytes are also coupled, through similar G proteins, so that their intracellular signaling pathways may be similar (Castrillo, 2016).

3.3.5 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are reactive intermediates of molecular oxygen being physiologically formed in cells as byproducts of cellular metabolism, or as toxic molecules involved in bacterial killing and host defense. At physiological concentrations, ROS mainly act as important second messengers that transduce intracellular signals, which are involved in various biological processes (Mittal et al., 2014; Sena and Chandel, 2012; Thomas et al., 2008). However, when an aberrant production of ROS surpasses the buffering ability of the antioxidant defense systems or when antioxidant enzymes are flawed, oxidative stress occurs. In addition, several studies have documented that oxidative stress shows fundamental role in mediating the production and secretion of cytokines (Bulua et al., 2011; Zhou et al., 2011), thus relating ROS with inflammation and endothelial activation and dysfunction. ROS is generated by a variety of sources within the cell. The free radical superoxide anion ($O2^{\bullet-}$) is the first to be generated and is responsible for the formation of other reactive species of physiological relevance such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet-}$), and peroxynitrite ($ONOO^-$). Superoxide anions produced through the partial reduction of molecular oxygen to $O2^{\bullet-}$ by the mitochondrial electron transport chain (ETC), as well as by NADPH oxidase, uncoupled eNOS and xanthine oxidase. Reduction in NO bioavailability by increased NO degradation by superoxide anions, marks the onset of endothelial dysfunction. Specifically, superoxide anions react with NO and leads to the formation of peroxynitrite $ONOO^-$ (Landmesser et al., 2003; Wolin et al., 2010). In turn, peroxynitrite leads to protein nitration and results in dysfunction and death of endothelial cells. In addition, it is well known that cardiac myocytes have a high density of mitochondria. Therefore, the consumption of oxygen in cardiac myocytes is relatively higher and also any alterations in mitochondrial respiratory chain complexes provide an important contribution to the generation of

the oxidative stress-related chemical species (He and Zuo, 2015; Incalza et al., 2018). Moreover, Kuroda *et al.* (Kuroda et al., 2010) found that $Nox4^{-/-}$ mice displayed augmented cardiac hypertrophy whereas cardiac function was worsened by Nox4 overexpression.

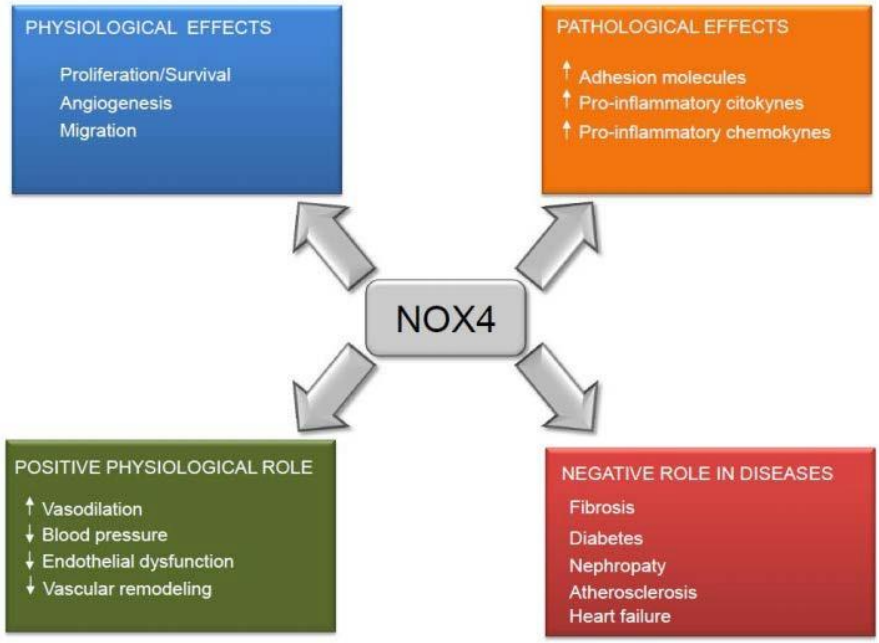

Figure 26: Dual actions of endothelial NOX4. The NOX enzymes release ROS as their only function. At normal physiological conditions, superoxide anion production and subsequently H_2O_2 production enhance angiogenesis, migration, proliferation and survival of endothelial cells through ROS mediated p38 MAPK and Akt activation, however, under excessive oxidative stress, the NOX4-generated ROS result in an increase in the levels of pro-inflammatory cytokines and chemokines, as well as of adhesion molecules, thus resulting in an endothelial pro-thrombogenic phenotype. NOX4 is elevated in various diseases, such as pulmonary fibrosis, diabetes, diabetic nephropathy, atherosclerosis, and heart failure. On contrary, NOX4-generated ROS can result in intracellular signals that inhibit vascular injury and promote vasodilation, reduction in blood pressure and vascular remodeling (Incalza et al., 2018).

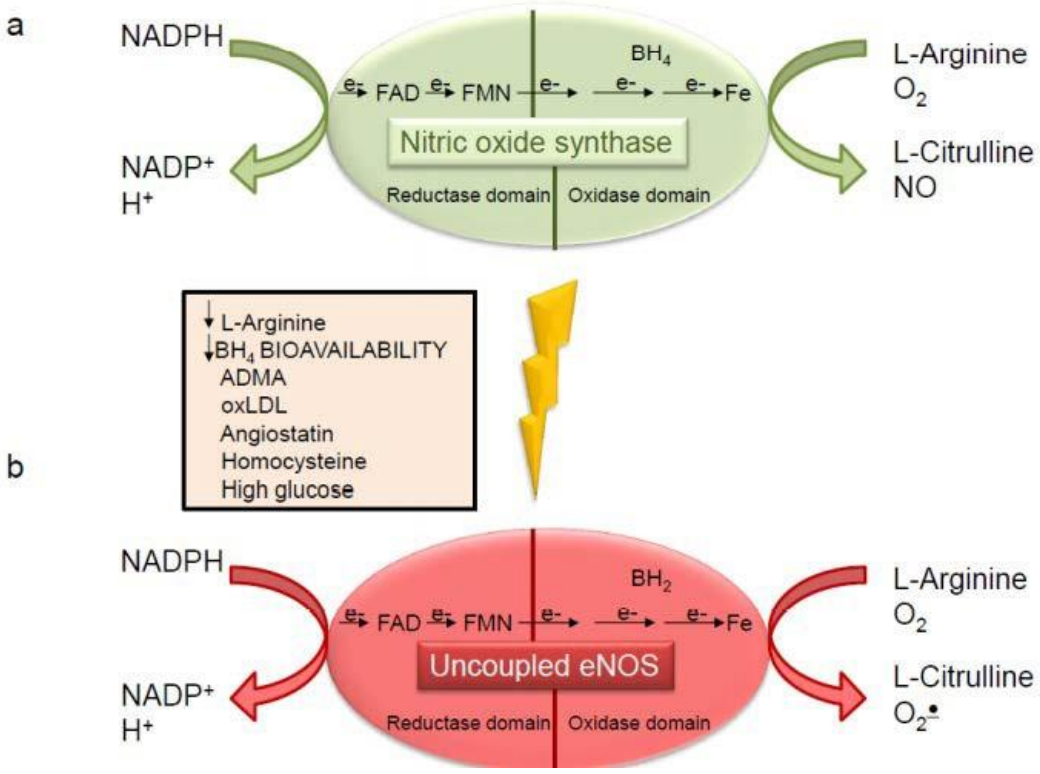

Figure 27: eNOS uncoupling. (**a**) In the presence of suitable concentration of substrate and co-factors, eNOS catalyzes the reduction and incorporation of O_2 into the guanidine group of L-arginine to form NO and L-citrulline. Electrons being transferred from NADPH, bound to the C-terminal reductase domain, to the heme iron and cofactor tetrahydrobiopterin in the N-terminal oxygenase domain. (**b**) At conditions associated with limitation of L-arginine or tetrahydrobiopterin, oxidation of tetrahydrobiopterin to dihydrobiopterin, or in the presence of the competitive inhibitor ADMA, eNOS form superoxide anions instead of NO. Numerous stimuli that mimic the metabolic alterations underlying endothelial dysfunction have the ability to induce eNOS uncoupling. Where, BH4: tetrahydrobiopterin; BH_2: dihydrobiopterin; ADMA: asymmetric dimethylarginine; oxLDL: oxidized LDL (Incalza et al., 2018).

3.3.6 Peptide growth factors

An increasing significance is given to peptide growth factors that play an important role in cell proliferation and angiogenesis and mainly include platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor and vascular endothelial growth factor. VEGF has an important role in endorsing arteriogenesis and the conversion of endocardial endothelial cells into

coronary endothelial cells, predominantly after acute myocardial infarction. Thus, EECs are a source of endothelial cells in the development of arteriogenesis in pathological conditions (Miquerol et al., 2015a).

Neuregulin-1 has been mainly synthesized in endocardial endothelial cells and myocardial capillary endothelium. ErbB3 receptors have been distributed in endothelial cells and ErbB2/ErbB4 receptor complex in cardiomyocytes. Soluble NRG-1 promotes a substantial upsurge in embryonic cardiac myocyte proliferation, as well as an improved survival and inhibition of apoptosis of cultured cardiomyocytes and can also persuade hypertrophic growth in both neonatal and adult ventricular cardiomyocytes (Smiljić et al., 2016). NRG-1 plays an important role in endothelial-myocardial signaling for normal cardiac function (Noireaud and Andriantsitohaina, 2014a; Parodi and Kuhn, 2014). Just like neuregulin-1, IGF-1, FGF-1, FGF-2, urocortin, VEGF, TGF-β1 and cardiotrophin-1 are all correlated with the inhibition of apoptosis in the heart.

3.4 Endocardial endothelial dysfunction

Intact endocardial endothelium is important for the embryonic development of the heart, the optimal rhythm and contractility as well as the remodeling of the heart. Impaired communication between the EE cells and cardiomyocytes and, endocardial endothelial dysfunction results in the development of heart and blood vessels diseases. The outcome of endothelial dysfunction is the declining of the endothelial barrier regulation and the electrolyte imbalance of subendocardial interstitium. In addition, endothelial dysfunction has been associated with alteration of synthesis of endothelial mediators which primary influence cardiomyocyte performances and also the response of cardiomyocytes to circulating mediators or hormones.

Healthy Endothelium	Dysfunctional Endothelium
• Vasodilatory (↑ NO, PGI_2) • ↓ Oxidative stress , low uric acid • Anti-coagulant (↓ PAI-1, vWF, P-selectin) • Anti-inflammatory (↓ sICAM, sVCAM, E-selectin, CRP, TNF-α, IL-6, MCP-1) • ↑ Repair (EPCs), ↓ Damage (CECs, MPs)	• Impaired vasodilation (↓ NO, PGI_2) • ↑ Oxidative stress , uric acid • Pro-coagulant (↑ PAI-1, vWF, P-selectin) • Pro-inflammatory (↑ sICAM, sVCAM, E-selectin, CRP, TNF-α, IL-6, MCP-1) • ↓ Repair (EPCs), ↑ Damage (CECs, MPs)

Table 3: Differences between a healthy and a dysfunctional endothelium.

Where, CECs: circulating endothelial cells; CRP: C-reactive protein; EMPs: endothelial microparticles; EPCs: endothelial progenitor cells; IL-6: interleukin-6; MPs: microparticules; NO, nitric oxide; PAI-1: plasminogen activator inhibitor 1; PGI_2: prostacyclin; ROS: reactive oxygen species; sICAM: soluble intercellular adhesion molecule; sVCAM: soluble vascular cell adhesion molecule; TNF-α: tumor necrosis factor alpha; vWF: von Willebrand factor (Sena et al., 2013).

3.5 Endocardial endothelium dysfunction and heart diseases

Numerous diseases of the cardiovascular system can not only be a consequence but also the cause of endocardial endothelial dysfunction. In the preservation of homeostasis of the heart and blood vessels, the endothelium maintains a balance between anti-thrombotic factors (NO, prostacyclin, plasminogen activator, protein kinase C, tissue factor inhibitor) and pro thrombotic factors (ET-1, oxidant radicals, plasminogen-activator inhibitor 1, thromboxane A_2, fibrinogen, tissue factor). Selective damage to the endocardial endothelium and subendocardium occurs in arrhythmia, atrial fibrillation, ischemia/reperfusion injury, cardiac hypertrophy and heart failure (Schoner et al., 2015). Typical lesions of endocardial and microvascular endothelium are also been described

in sepsis, myocardial infarction, inflammation, thrombosis, and in hypertensive patients (Gray et al., 2010).

In sepsis, endocardial endothelium shows a proinflammatory phenotype. Higher levels of the proinflammatory transcription factor NF-kB, endorse the adhesion of polymorphonuclear cells to EECs, and their migration into the subendocardial space and the interstitial space of the heart. Polymorphonuclear cells adhered to EECs leads to oxidative stress in EECs and cardiomyocytes, which have an effect on the contractility of the heart (Potz et al., 2016).

Under physiological conditions, the endothelium hinders the formation of thrombus. In cardiac insufficiency, the ventricular endocardial endothelium displays prothrombotic characteristics. This causes frequent thromboembolic complications in patients with HF. However, endothelial dysfunction in HF due to increased release of the sympathetic mediators and vWF can be modified by the use of galanin as many studies suggested that neuropeptide galanin promotes an anti-thrombotic phenotype on endocardial endothelial cells (Tyrrell et al., 2017). The preserved function of EECs has been necessary in the revascularization of the areas damaged by a myocardial infarction. In the infarcted area, EECs have been identified as a source of endothelial cells used to encourage vascularization. The plasticity of endocardial endothelial cells plays an important role in the revascularization of ischemic heart tissue (Miquerol et al., 2015b). EE dysfunction or the dysregulation of the transforming growth factor in EECs can lead to their transition into mesenchymal cells. This occurs in endocardial fibroelastosis, a form of fibrosis where a de novo subendocardial layer is being formed that encapsulates the cardiomyocytes and stops heart growth (Xu et al., 2015).

Fully removing or partially damaging EECs directly have an effect on contractile cardiac performance and results in lower contractility of cardiomyocytes. The inotropic effect of EE has been achieved through the synthesis and release of endothelial mediators, the sensory ability to detect changes in blood plasma and the quality of blood-heart barrier to control trans-endothelial transport. Thus, EECs plays an important role in Na^+ transport. Increased Na^+ plasma concentrations and high levels of aldosterone lead to an increased entry of Na^+ into EECs and the transition to subendocardial space, endothelial glycocalcax and glycosaminoglycan network. Increased levels of Na^+ in EECs modify their characteristics and lead to a decrease in NO synthesis (Bonilla et al., 2012). At the same time, higher Na^+ levels in the subendocardial zones lead to an accumulation of fluids.

Endothelial dysfunction can be evaluated by determining the biomarkers of endothelial dysfunction (vWF, soluble thrombomodulin, CRP, cytokines, vascular cell adhesion molecule-1 [VCAM-1], intracellular adhesion molecule-1 [ICAM-1], selectins P and E, asymmetric dimethyl arginine, circulating endothelial cells and microparticles). Along with markers of plaque destabilization and/or markers of ischemia or myocardial necrosis, they may offer additional prognostic information

3.6 Endocardial endothelial dysfunction and atrial fibrillation

Atrial fibrillation most common trigger being the automatic ectopic activity of left atrial cardiomyocytes with altered electrophysiological properties is due to the influence of congenital and/or acquired conditions and diseases (Go et al., 2001b). Series of morphological and functional changes constitute remodeling of the left atrium that occur as an adaptive response to factors that lead to atrial fibrillation. The endothelial cells promote fibroblast accumulation through an endothelial-mesenchymal transition in the atrium of patients with atrial fibrillation. Experiments with immunofluorescence multi-labeling identified that heat shock protein 47, prolyl-4-hydroxylase, and procollagen type 1 co-localized with snail and S100 calcium-binding proteinA4 (S100A4) within the endothelial cells of the left atrium, indicating the mesenchymal phenotype to produce collagen (Kato et al., 2017; Ter Maaten et al., 2016) .

Adaptive changes in left atrium normally depend on the duration of arrhythmias and the presence of other comorbidities such as heart failure, myocardial ischemia due to coronary insufficiency and proinflammatory conditions. Moreover, changes in the electrophysiological properties of cardiomyocytes favour situations enabling the reoccurrence and the formation of permanent forms of atrial fibrillation. With the changes in the structure and the function of the myocardium, there have been simultaneous alterations in the left atrial endocardium. Both dilation and hypocontractility may predispose to thrombosis (Nattel et al., 2008). Diseases and conditions that favour structural changes in myocardium and endocardium results in increased volume [dilatation] and reshaping of the left atrium and its auricle and reduced contractile ability of the myocardium, also, cause the loss of anticoagulant characteristics of the left atrial endocardium (Shirani and Alaeddini, 2000).

Structural changes in the atrial endocardial endothelium in AF have been manifested as endothelial cells edema and fibrinous transformation. At the same time, small areas of endothelial denudation can also be found with the formation of platelet aggregates, especially in the left atrium appendage, which can be observed as precursors for thrombosis. This results in rough and wrinkled appearance of endothelium (Masawa et al., 1993). Changes in the structure of atrial endocardial endothelium arose functional changes being characterized by synthesis and secretion of different mediators.

Endothelial dysfunction in AF is characterized by a reduced synthesis of mediators with anticoagulant, antithrombotic, anti-inflammatory and anti-proliferative effects such as NO, prostacyclin and tissue plasminogen activator and the increased production of procoagulant factors mainly von Willebrand factor, tissue factor, plasminogen activator inhibitor and microparticles. Also, there is an increased expression of adhesion molecules, the release of chemoattractants, growth factors and reactive oxygen species (Polovina et al., 2015) . The more intense expression of vWF by left atrial appendage (LAA) tissue being a significant predictor of postoperative AF. Other findings points towards a possible role of endothelial damage/dysfunction [as reflected by VWF changes] in the pathogenesis of postoperative AF (Kaireviciute et al., 2011). Impaired protein C activation on the left atrial endocardium attributable to low thrombomodulin expression may explicate its higher thrombogenicity and has a significant role in cardioembolic stroke (Cervero et al., 2011). Other study has described a reduced NOS activity in AF together with an increased release of von Willebrand factor enhanced infiltration of proinflammatory markers in endocardium and myocardium, accompanied by the presence of products of oxidative modification and markers of hypoxic damage within atrial tissue (Cai et al., 2002) . EE cells of the left atrium synthesize and release NO that has an important role in the regulation of platelet activity, the inhibition of adhesion and procoagulant molecules on the surface of endothelial cells, and the modulation of inflammation and oxidative stress (Bonilla et al., 2012; Fleming and Busse, 2003). At physiological conditions, NO production in the left atrium is significantly higher than its production in any other part of the cardiovascular system. Systemic vascular endothelium produces NO under conditions of laminar blood flow. Turbulent blood flow reduces the activity of eNOS and the production of NO (Meulemans et al., 1990) . The entire cardiac output crosses through the left atrium, and the atrial endocardium represents an endocrine organ whose NO synthesis, through the formation of nitroso-thiol compounds, provides circulating NO donors in the systemic circulation (Cai et al., 2002; Matsushita et al., 2003). Thus, the atrial

endocardial dysfunction with a reduced NO synthesis may have an adverse effect on the function of systemic blood vessels.

In patients with atrial fibrillation, there can be a systemic endothelial dysfunction that combines endocardial and vascular endothelial dysfunction resulting in increased hemodynamic load of the left atrium and increased synthesis and release of natriuretic peptides, Ang II, aldosterone and growth factors from the atrial myocardium (Ellinor et al., 2005; Nattel et al., 2008). These mediators can induce, along with paracrine effects, the adverse effects on distant tissues and organs, thus promoting the development of cardiovascular diseases.

ENDOTHELIAL SENESCENCE

4 ENDOTHELIAL SENESCENCE

4.1 Cellular senescence

Endothelial senescence drew great interest in the cardiovascular field after seeing its important role in the development of endothelial dysfunction (Erusalimsky, 2009; Herrera et al., 2010). Cellular senescence was mentioned more than 40 years ago by Leonard Hayflick as a phenomenon that restricted the proliferation of normal human cells in culture which after around 50 to 70 population doublings being irreversibly arrested in G1 phase of the cell cycle show unresponsiveness to proliferative mitogenic stimuli (Hayflick, 1965; Sosinska et al., 2016). There are mounting evidences, which indicate that normal healthy endothelial cells gradually enter into a senescent replicative state in which they do not react to mitogenic stimuli although they remain metabolically active (Erusalimsky and Kurz, 2005, 2006). The long-term existence of senescent cells in tissues with age either due to the elevated rate of senescent cell formation or failure of aging immune system or decreased clearance (Burton and Krizhanovsky, 2014) has the potential to endorse age-related diseases and could contribute to explain the decline of organ function with aging (Salama et al., 2014).The ambivalence of senescence lies in the early benefit of tumor proliferation where it is being recognized as a potent tumor suppressor (Vijg et al., 2008) and the late deleterious impact associated to aging, this duality being known as antagonistic pleiotropy.

Various stimuli constituting similar pathways can induce senescence (van Deursen, 2014) by telomere shortening (replicative senescence) or independent of telomere length that permanently induce cell senescence (pre-mature senescence like phenotype). Oncogene-induced senescence involves activation of oncogenes such as RAS (Burton and Krizhanovsky, 2014; Serrano et al., 1997) and RAF involving DNA damage and has been associate with increased ROS (Bartkova et al., 2006)

In addition, the activation of the ERK pathway could also initiate senescence by increasing the degradation of proteins required for cell cycle progression (Deschenes-Simard et al., 2013). Also, other pathways could stimulate endothelium senescence, independently of the DNA damage, probably through p38 and NFKB pathways activation.

4.2 Biomarkers and features of senescence

4.2.1 Morphological characteristics

Senescent cells possess various characteristic morphological and biochemical features as a consequence of their arrest in the G1 phase. Generally, senescent cells increase in size (Kurz et al., 2000) and depending on the senescence trigger as well are usually large, flat, and multinucleated or rather refractile. Enhanced mTOR-dependent protein synthesis signaling has been suggested for better understanding of these changes (Fingar et al., 2002; Mamane et al., 2006). Cells which undergo H-RASv12 induced senescence, or stress-induced senescence or DNA damage-induced senescence showed common phenotypic characteristics such as the acquisition of a flat cell morphology (Astle et al., 2011) whilst, senescent cells resulting from BRAFE600 expression or the silencing of p400, attain a more spindle-shaped morphology (Kuilman et al., 2010b; Zhang et al., 2015).

4.2.2 Cell cycle arrest

The growth arrest is principally constant and cannot be reversed by acknowledged physiological stimuli (Campisi and d'Adda di Fagagna, 2007). However, multiple ways has been described to reverse the arrest, allowing cells to re-enter the cell cycle. The inactivation of p53 pathway allows senescence reversal while some of the interleukin revoke the arrest (Blagosklonny, 2011). This reversibility relies on p16 expression prior entering senescence. The tumor suppressor's p16 and p21 are mediators of cell cycle arrest and senescence. Since, for the induction or maintenance of the senescence program neither p16 and p21 is strictly required, their predictive value is limited when used individually (d'Adda di Fagagna, 2008).

4.2.3 Induction of senescence associated -beta-galactosidase activity (SA-β-gal)

SA-β-gal is the most widely used senescence biomarker (Debacq-Chainiaux et al., 2009). The increase of β-gal activity in senescent cells is related to an expansion of the lysosomal compartment, leading to an increase in β-galactosidase activity which is detectable at pH 6.0 that is different from the acidic β-galactosidase activity found in normal cells at pH 4.0 (Dimri et al., 1995; Lee et al., 2006; Yang and Hu, 2005).

4.2.4 Senescence-associated heterochromatin foci (SAHF)

At the nuclear level, senescent cells exhibit condensed heterochromatin (transcriptionally silent) foci known as senescence-associated heterochromatin foci (SAHF) (Narita et al., 2006) that are

detectable as clusters after DAPI labelling of the DNA and are distinct from the homogeneous staining in non-senescent cells. These DNA SAHF are found to be enriched in methylated lysine 9 of histone H3 (a modification catalysed by the histone methyltransferase suv39h1) (Agger et al., 2008) whereas histone H3-lysine 9 acetylation and lysine-4 methylation which are euchromatin (transcriptionally active) foci are excluded from SAHF. SAHF has been associated with the downregulation of genes regulated by the E2F transcription factor, such as cyclins, and to the occurrence of different pathways involving p16 or p53 proteins (Kuilman et al., 2010a).

4.2.5 Secreted factors in senescence

Large amount of proteins are secreted by senescent cells secrete constituting metalloproteinases, (MMPs), plasminogen activator inhibitor 1 (PAI-1) (Young and Narita, 2010), growth factors, proteases, cytokines, chemokines and many others which have strong autocrine and paracrine actions (Campisi, 2013). Cells undergoing either replicative or premature senescence show noticeable alterations in their secretome. This term encompasses the senescence-associated secretory phenotype (SASP), that has been controlled by microRNA, the cytokine receptor CXCR2, IL-1 receptor signaling, the transcription factors NF-κB and C/EBP-B, and the JAK/STAT signaling pathway (Jurk et al., 2014). The secretome consists of various proinflammatory proteins and growth factors, the composition of which subject to cell lineages or sources of triggers (Young and Narita, 2010). Activation of inflammatory transcriptome involves NF-κB and C/EBP-B transcription factors.

Because senescent cells increasingly accumulate with progressive aging, it can be concluded that SASP display an important role in the time course of age-related diseases (Ovadya and Krizhanovsky, 2014). Also, it is possible that numerous inflammatory cytokines may have an indispensable part in the establishment and maintenance of the senescence arrest such as signaling through IL-8 (CXCR2) and IL-6 receptors was shown to be mandatory to induce cell senescence in response to oncogenic BRAF or replicative exhaustion. It had also been suggested that senescent cells may favour cancer by stimulating the proliferation of incipient tumour cells that exist in their microenvironment (Dumont et al., 2000; Kuilman et al., 2010b).

4.3 Mechanism of senescence

4.3.1 Replicative senescence

When the length of one or more telomeres attain a specific minimal threshold, usually characterized with the single-strand overhang erosion, the exposed telomeric DNA ends have been recognized as double-strand breaks (DSBs) by the DNA damage response (DDR) (Kuilman et al., 2010b). The telomeres of vertebrate chromosome are distinct structures constituting of extended arrays of repetitive tandem hexameric units-TTAGGG (Nergadze et al., 2004). The presence of the telomeric repeats at the chromosome ends is important to guarantee proper functioning of telomeres and for endurance of chromosome integrity and stability (Blackburn, 2001). Telomere specific proteins shield chromosome from degradation or chromosomal end-to-end fusion during DNA-healing process (O'Sullivan and Karlseder, 2010).

Telomeres have been subjected to abrasion due to the fact that DNA polymerase fails to completely replicate the lagging strands this so called "end replication problem" which leads to telomere shortening (de Magalhães and Passos, 2018; Storer et al., 2013). Together with the propagation of human cells in culture, telomeres are subjected to progressively shortened, ultimately causing cells to reach their "hayflick limit". This phenomenon being termed as replicative (cellular) senescence. In addition, replicative senescence has also been linked to Rb tumor suppressor including p16, a cyclin-dependent kinase inhibitor. Indeed, both p53 and p16 activation seems to be mandatory for senescence induction in a multiplicity of human cell strains. It is possible that the relative contribution of these Rb proteins depend on the cell types; Depending of cell strains, onset of senescence could be deferred following p16 inactivation alone, following p53 inactivation or requiring both p53 and p16 inactivation (Kuilman et al., 2010b)

4.3.2 Premature senescence

It has also been known that senescence can also be induced in the absence of any noticeable telomere loss or dysfunction, by an assortment of conditions. Telomere extension by telomerase overexpression in human fibroblasts has been documented not to protect against senescence observed after exposure to UV, or H_2O_2, confirming the existence of a senescence mechanism independent of telomerase action (Gorbunova et al., 2002). This type of senescence has been described as premature senescence independent of telomere shortening (de Magalhães and Passos, 2018).

Premature senescence like phenotype can be induced in response to numerous conditions. Mainly recognized as stress-induced premature senescence, which constitute oxidative stress or DNA

damage, and oncogene-induced senescence (OIS) involving persistent mitogenic stimulation or exposure to radiation (Erusalimsky, 2009).

4.3.3 Molecular machinery of cellular senescence

In mammalian cells, the Rb and its family members have fundamental roles in the initiation of cellular senescence and the onset of cell cycle arrest (Ben-Porath and Weinberg, 2005; Johnson and Walker, 1999; Maddika et al., 2007). There are two different classes of CDKI, the KIP/CIP family CDKI (p21, p27, and p47) (Cerqueira et al., 2014) known to inhibit a wide range of CDK, and the INK4 family CDKI (p16, p15, p18 and p19), that binds and inactivates CDK4 and CDK6 (Liggett and Sidransky, 1998). At physiological conditions, proliferating cells displayed a low expression of CDKI. In response to a variety of oncogenic stimuli responsible for DNA damage, the expression level of CDKI (p21 and p16) genes have been significantly up-regulated both in a p53-dependent and in p53-independent manner (Maddika et al., 2007; Rodier and Campisi, 2011). The activation of this pathway in turn inactivates all CDK, prevents Rb family protein phosphorylation and leads to cell cycle arrest (Kilbey et al., 2008; Sherr and Roberts, 1999). It had been also demonstrated that the activation of p16-RB pathway induces elevation of intracellular levels of ROS, thereby triggering activation of PKC. PKC is an important downstream mediator of the ROS signaling pathway that leads to cytokinetic block, which in turn promotes further ROS production and thereby maintain ROS-PKC activated in senescent cells (Bihani et al., 2007). p53 is a tumor suppressor known as “guardian of the genome”. The importance of this tumor suppressor has been emphasized by the demonstration that p53 has been mutated or lost in a vast majority of the human cancers (Smith et al., 1995). Physiological p53 activity prevents from cancer and protects from aging, however unrestrained and excessive p53 activation still protects from cancer, but is known to be detrimental to healthy aging (Rufini et al., 2013). Several studies pointed that the excessive expression of p53 activity compromise healthy aging (Gottlieb and Vousden, 2010). Induction of p53 has been supposed to be essential for the induction of senescence mainly following its activation by DDR. One of the most important p53-target genes is its downstream effector CDKN1 A/p21, both overexpressed during replicative senescence (Thakur et al., 2010). In addition, p53 triggers expression of the AMP-activated protein kinase (AMPK), activating mTOR which acts to promote cell and tissue aging (Sengupta et al., 2010; Zoncu et al., 2011).

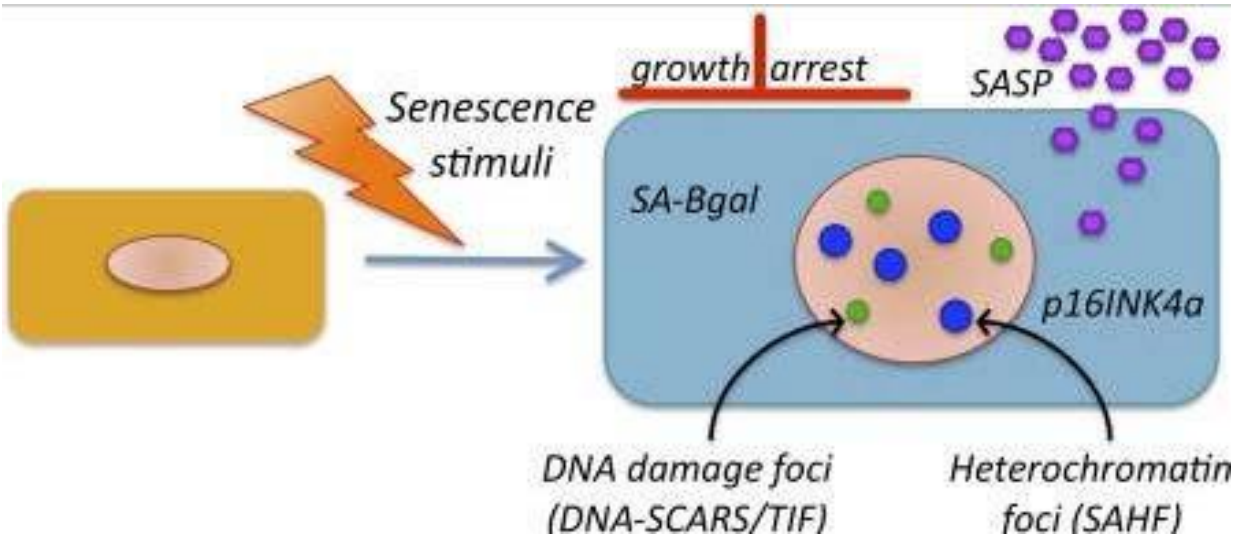

Figure 28: Characteristics of senescent cells**.** Senescent cells vary from other nondividing (quiescent, terminally differentiated) cells in many ways, although no single feature of the senescent phenotype has been exclusively specific. Features of senescent cells consists of essentially irreversible growth arrest; expression of SA-βGal and p16INK4a; profound release of various growth factors, cytokines, proteases, and other proteins (SASP); and nuclear foci containing DDR proteins (DNA-SCARS/TIF) or heterochromatin (SAHF). The pink circles in the nonsenescent cell (left) and senescent cell (right) represent the nucleus (Rodier and Campisi, 2011)

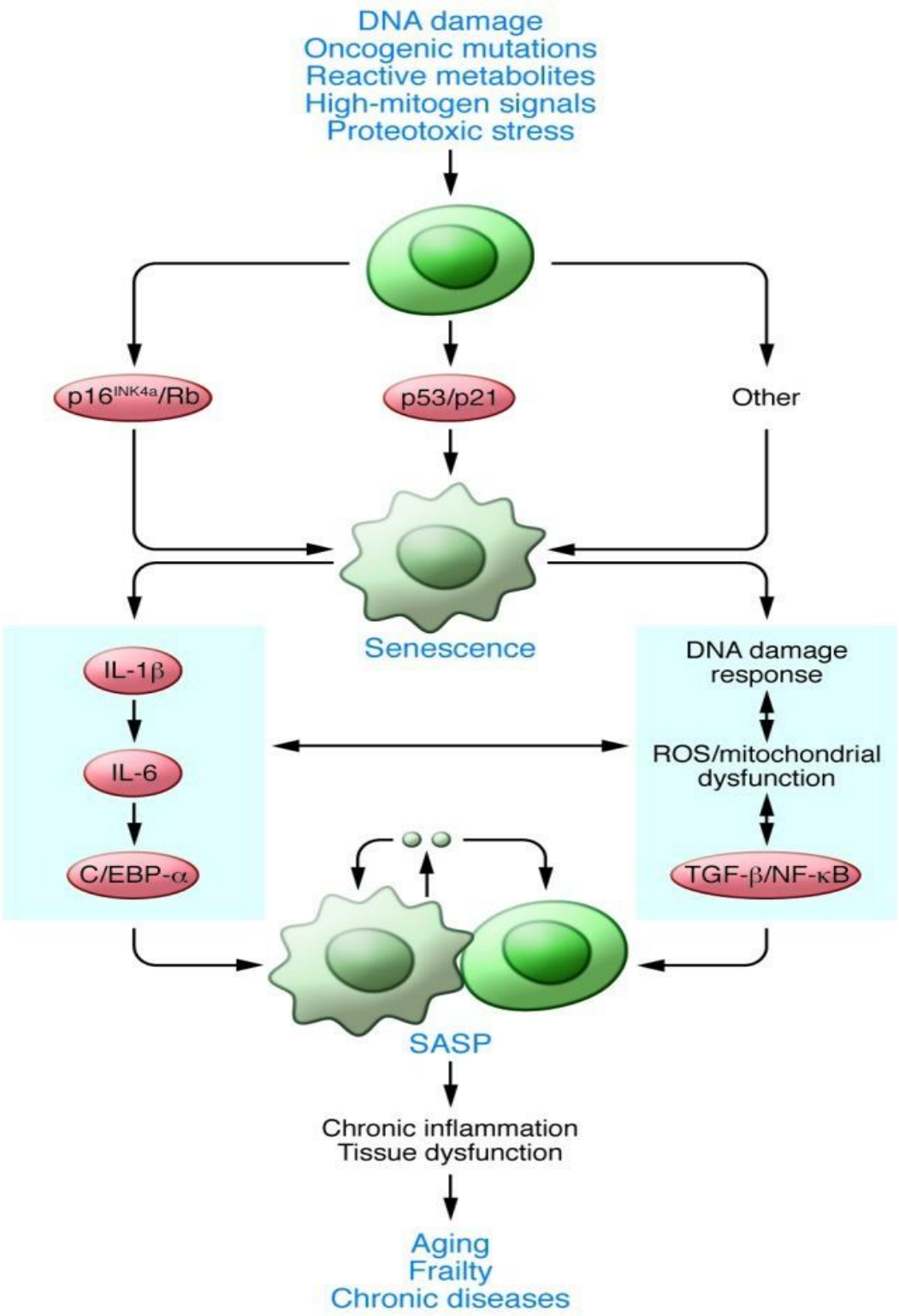

Figure 29: Various inducers have the ability alone or in combination to move the cells into the senescent cell fate via pathways involving $p16^{INK4a}$/Rb, p53/p21, and likely other pathways. Stimuli normally consists of DNA damage (e.g., telomere shortening and single- and double-strand

breaks); oncogenic mutations (e.g., Ras, Myc, B-Raf); reactive metabolites (e.g., ROS, ceramides, fatty acids, high glucose); increase levels of mitogen and nutrient signals that enhance mTOR activity; and proteotoxic stress (e.g., protein aggregation and unfolded proteins). These lead to widespread alterations in gene expression and chromatin remodeling (heterochromatin formation) that consist of senescence-associated growth arrest, the SASP, and alterations in morphology. Accordingly, cellular senescence can be regarded as a cell fate reminiscent of differentiation, replication, or apoptosis (external and internal inducers, transcription factor cascades, gene expression changes and chromatin remodeling, leading to changes in function). Many intracellular autocrine loops emphasize development of irreversible replicative arrest, heterochromatin formation, and initiation of the SASP requiring days to weeks. Moreover, senescence also plays an important role in tissue dysfunction and chronic disease predisposition in addition to removing cells from the progenitor/stem cell pool, through the SASP and associated chronic sterile inflammation and degradation of the extracellular matrix (Tchkonia et al., 2013b).

4.3.4 Reactive oxygen species

Senescent cells activate downstream signaling pathways that trigger the production and release of ROS thereby creating a microenvironment characterized by increased oxidative stress (Unterluggauer et al., 2003). In human diploid fibroblasts, it has been shown that p16INK4a expression causes increased ROS generation via PKCδ activation (Takahashi et al., 2006). Telomere attrition has the ability to increase ROS production by induction of p21CIP1/WAF1 expression in human lung and mouse embryonic fibroblasts and in intestinal crypts of Terc−/− G4 mice (Passos et al., 2010). Passos and colleagues suggest a positive feedback loop between p21CIP1/WAF1 and reactive oxygen production, which is necessary for the induction and maintenance of senescence (Passos et al., 2010). The majority of ROS production during the induction of senescence is supposed to be originated from the mitochondria (Stowe and Camara 2009). On the contrary, suppression of oxidative stress or hypoxia preserves telomere length and prolongs lifespan of cells (Bhayadia et al., 2016; Minamino and Komuro, 2007).

Previous studies have also shown that NADPH oxidase subunits Nox1 and Nox4 have been involved in the excessive formation of ROS in senescent human endothelial cells (Schilder et al., 2009). In addition, an upregulation of NADPH oxidase subunits p47phox, Nox2, and Nox4 was observed in $JunD^{-/-}$ mice displaying premature vascular senescence associated with an impairment

of the $O2^{\bullet-}$/NO balance (Paneni et al., 2013). Furthermore, Nox4 gene interference using small-hairpin RNA was able to delay replicative senescence in human umbilical vein endothelial cells (Lener et al., 2009). Previous studies have also shown that COX-2 contributed to the establishment and maintenance of senescence of human fibroblasts (Martien et al., 2013), and a low dose of aspirin delayed the onset of senescence in circulating endothelial progenitor cells (Hu et al., 2008).

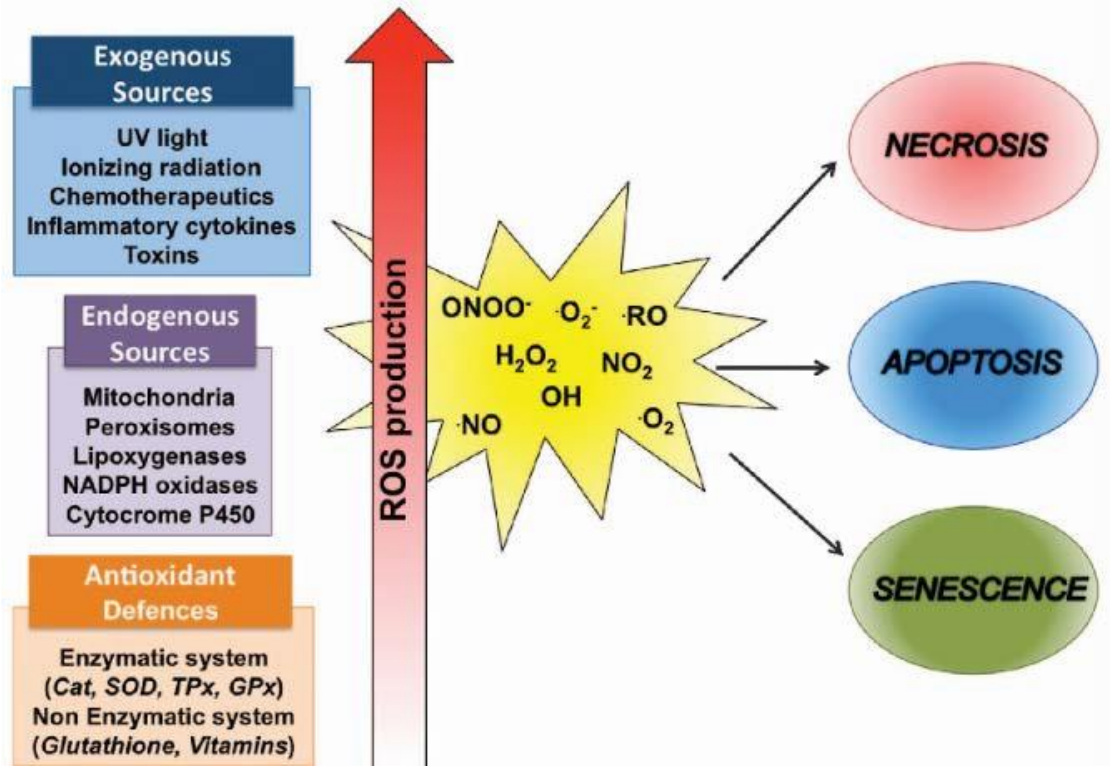

Figure 30: ROS can possess both endogenous and exogenous sources. The moderate levels of ROS are maintained by ROS production and release from specialized scavenging enzymes (Rufini et al., 2013).

Figure 31: Level of H_2O_2 present predict the physiological outcomes with large amounts of mROS are associated with damage to proteins, lipids, and nucleic acids and small amounts of mROS mainly act as signaling molecules to overcome the stress. Morever, even very small amounts of mROS are shown to be necessary for normal cell homeostasis. Thus, mROS have not been categorically harmful (Sena and Chandel, 2012).

4.4 Senescence and endothelial dysfunction

Endothelial function is impaired in aging and cellular senescence thus, endothelial cell senescence has been suggested to contribute to aging-related endothelial dysfunction, which plays a key role in the initiation and/or progression of various cardiovascular diseases (Herrera et al., 2010). It has been demonstrated that strong association exists between the presence of stress-induced cellular senescence and impaired endothelial function as depicted in mouse aortas showing an increased expression of senescence markers p16INK4a and p19ARF (Serrano et al., 1996; Wei et al., 2001). Also, the increased number of p21CIP1/WAF1-positive endothelial cells in aged aortas is consistent with the previous observations, which stated that p21CIP1WAF1 had been induced by oxidative stress in endothelial cells via ataxia telangiectasia mutated protein (Zhan et al., 2010). In addition, an increase in DNA damage foci containing activated H2AX (γH2AX) was found in endothelial cells, which has been regarded as a reliable indicator of senescence (Braun et al., 2012; Lawless et al., 2010).

Moreover, one of the hallmarks of endothelial dysfunction is the inability of the endothelium to induce an appropriate vasodilatory response due to insufficient NO bioavailability (Deanfield et al., 2007). Thus, the studies conducted with cultured cells have demonstrated that endothelial cell

senescence is associated with the downregulation of endothelial nitric oxide synthase eNOS, the induction of a proinflammatory state, and DNA damages (Botden et al., 2012). In addition, several lines of evidence suggest that the blunted NO formation has been an early key event, which triggers the signal transduction cascade leading ultimately to endothelial senescence (Bhayadia et al., 2016; Kansui et al., 2002; Khemais-Benkhiat et al., 2016a). Indeed, treatment of young endothelial cells with the eNOS inhibitor, L-NAME, promoted a pronounced premature induction of endothelial senescence as assessed by SA-β-gal activity. Moreover, both the NO donor DETA-NO and transfection of eNOS into human umbilical vein endothelial cells has been shown to decrease the number of senescent endothelial cells (Hayashi et al., 2006). NO is being proposed to delay endothelial senescence by increasing telomerase activity thus preventing the alteration and the shortening of length of telomeres (Vasa et al., 2000). Also, NO may also retard the induction of senescence by preventing the downregulation of eNOS expression as the eNOS inhibitor L-NAME showed a pronounced decrease of the eNOS protein level in young cells. Moreover, NO may also contribute to postpone endothelial senescence by limiting the level of oxidative stress, a potent inducer of senescence, in endothelial cells

Recent findings also suggest a predominant role of p53 in the mechanism underlying the downregulation of eNOS. Previous work has established that the overexpression of endothelial p53 induced endothelial dysfunction and decreased nitric oxide (NO) bioavailability in rat aortic sections and the downregulation of eNOS in cultured endothelial cells (Kumar et al., 2011), Indeed, p53 is shown to downregulate KLF2 expression, a positive transcriptional regulator of eNOS expression (Atkins and Jain, 2007), either directly by transcriptional repression (Kumar et al., 2011) or indirectly by upregulation of p66shc, a negative transcriptional regulator of KLF2 (Kim et al., 2008a).

4.5 Local angiotensin system and senescence

The local angiotensin system has a determinant role in the induction of both replicative and premature endothelial senescent as indicated by the fact that both the ACE inhibitor and the AT1 receptor blocker concentration-dependently reduced SA-β-gal activity. Activation of the local angiotensin system has been shown to contribute to the increased level of oxidative stress observed in pathological arteries such as in old arteries and in arteries from hypertensive animals and humans mostly by an AT1R-mediated activation of NADPH oxidase (Doughan et al., 2008;

Harrison et al., 2003; Khemais-Benkhiat et al., 2016a). It was found that Ang II–induced ROS-mediated DNA damage leads to accelerated biological aging of hVSMCs via acute SIPS, which is known to be telomere independent, and accelerated replicative senescence which has been associated with accelerated telomere attrition (Herbert et al., 2008; Imanishi et al., 2005). Also Ang II predominantly decreased the expression level of Bcl-2, in part via the activation of extracellular signal-regulated kinase (ERK). Moreover, findings also proposed that Ang II can induce senescence via the mitogen-activated protein kinase (MAPK) signal pathway (Abbas et al., 2017; Shan et al., 2008).

4.6 Atrial fibrillation and senescence

Mounting evidence proposed that extracellular matrix (ECM) and perivascular fibrosis had been increased progressively with age, resulting in cardiac remodeling and dysfunction in elderly individuals (Horn and Trafford, 2016). Moreover, telomere attrition affects mitochondrial function, thus promoting aging (Sahin et al., 2011), and short telomere length has been considered to be a hallmark of aging (Lopez-Otin et al., 2013). In addition, Carlquist et al recently found that AF subjects had shorter telomeres in comparison with SR subjects (Carlquist et al., 2016). These evidences propose that aging, also called replicative senescence play an important role in the development and maintenance of AF. In a previous study, increased expression of senescence markers, $p21_{CIP1/WAF1}$ and $p16_{INK4}$ and SA-β-gal was evidenced in AF patients with valvular heart diseases. In this work, cardiac fibroblasts (CFs) were evidenced as predominant cells lineage that experience senescence. Altogether, this study establishes a link between premature senescence of CFs and the progression of AF. Moreover, Xie et al. also found that an increase in atrial fibrosis as assessed by sirius red staining has been associated with both paroxysmal AF (PaAF) and persistent AF (PeAF). In addition, atrial fibrosis is characterized by excessive deposition of ECM, comprising of Col I and Col III (Cao et al., 2013; Horn and Trafford, 2016; Wu et al., 2015; Xie et al., 2017). It was also showed that $p16_{INK4a}$ and SA-β-gal were positively correlated with atrial fibrosis in LAAs from patients with valvular diseases. Altogether the data suggested the possibility of the association of premature senescence with AF development with advanced atrial fibrosis (Xie et al., 2017).

4.7 Senescence, coagulation cascade components and atrial fibrillation

Uncontrolled coagulation play an important role in the pathophysiology of several chronic inflammatory diseases. In these conditions, senescent cells have often been observed and have been involved in the generation of inflammation. The coincidence of hyper-coagulation, cell senescence, and inflammation proposes the existence of a common underlying mechanism (Sanada et al., 2016). Anti-coagulation therapy targeting cardiovascular diseases has made great advances in recent years (Maan et al., 2012). Treatment strategies directly targeting activated coagulation FXa have been established for AF and deep vein thrombosis (Konstantinides and Torbicki, 2014; Levy et al., 2014). Recent evidence suggests that activated coagulation factor Xa plays a role in the processes beyond blood coagulation (Esmon, 2014; Walenga et al., 2003). These non-hematologic functions have been mainly mediated by protease-activated receptors, PARs, (Sparkenbaugh et al., 2014) and increase tissue inflammation and remodeling (Esmon, 2014; Hara et al., 2015). Senescent cells have been frequently observed and appeared to be involved in the progression of chronic inflammation by releasing inflammatory cytokines, senescence-associated secretory phenotype (SASP) (Tchkonia et al., 2013a). When human umbilical vein endothelial cells were stimulated with FXa for 14 days it was found that continuous FXa stimulation decreased EC proliferation, up-regulated the senescence markers such as p53, p16INK4a, and senescence-associated β -galactosidase (SA-β gal)-positive cells, through up-regulation of insulin-like growth factor binding protein 5 (IGFBP-5) and early growth response 1 (EGR-1). Inhibition of FXa by a direct FXa inhibitor, rivaroxaban, or IGFBP-5 by siRNA decreased FXa-induced cell senescence, restoring cell proliferation (Sanada et al., 2016). Moreover, in an ischemic hind limb mouse model, FXa inhibited neovascularization by endothelial progenitor cell. However, rivaroxaban significantly restored FXa-induced impaired angiogenesis. These data indicated that FXa induced EC senescence which has been associated with inflammatory burden and overall remodeling, thus paving the way for atrial fibrillation perpetuation (Sanada et al., 2016).

AIMS OF THE STUDY

AIMS OF THE STUDY

Ageing is a main determinant of atrial remodeling that paves the way to atrial fibrillation (AF) and enhanced thrombogenicity. Preclinical studies on endothelial atrial cells are conspicuously lacking. As a result, the initial cellular triggers for clotting, inflammation and proteolysis in atrial fibrillation remain speculative. Endothelial cell senescence and dysfunction are the hallmark of cardiovascular diseases. Cellular senescence is a stress and damage response resulting in an irreversible cell cycle arrest and the appearance of distinct morphological and functional changes associated with impairment of cellular homeostasis. Senescent endothelial cells also secrete pro-inflammatory and pro-remodeling factors, which, in turn may alter tissue environment. In addition, previous studies have demonstrated a key role of Ang II in the pathogenesis of AF. Ang II type 1 receptor (AT1R) activation is known to induce the activation of mitogen-activated protein kinases (MAPK) which in turn favors atrial remodeling through fibroblast proliferation, inflammation, oxidative stress cellular hypertrophy and apoptosis.

It is known for many decades that AF has been associated with the activation of local and circulating coagulation factors (hypercoagulability). The AF-related hypercoagulability significantly enhances the risk of clot formation and stroke in patients with AF. However, little has been described about the potential role of AF-related hypercoagulability in atrial tissue remodeling and, in particular, the role of two major coagulation serine proteases, thrombin and factor Xa. Apart from its haemostatic effects, thrombin was also demonstrated to modulate cellular effects that are predominantly mediated by protease-activated receptors (PARs).

Thus, this study aims

- To develop an original model of primary cultures of atrial endothelial cells from pig hearts;
- To characterize phenotypical changes associated with atrial endothelial cells (AECs) senescence (replicative and premature) and to depict the link between ageing and thrombogenicity;
- To evaluate the impact of coagulation cascade-derived factors such as thrombin in the induction of premature AEC senescence possibly leading to the acquisition of a pro-inflammatory, pro-thrombotic and pro-fibrotic profile;
- To study the role of reactive oxygen species (ROS) in the induction of AECs senescence;

- To study the effect of the local angiotensin system, a major inducer of senescence, on the induction of AECs senescence.

RESULTS

Atrial endothelial cells senescence promotes thrombogenicity, inflammation and extra-cellular matrix remodeling: Role of the Ang II/AT1 receptor/oxidative stress pathway

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Abstract

Background: Ageing is a main determinant of atrial remodeling that paves the way to atrial fibrillation and enhanced thrombogenicity. However, the initial cellular events promoting proteolysis, inflammation and clotting in atrial fibrillation still remain speculative. The aim of this study was to establish a cell culture model of atrial endothelial cells (AECs), and to characterize the phenotypical changes associated with AECs ageing and, in particular, the role of the angiotensin II (Ang II) /AT1 receptor pathway, a major pro-senescence, pro-fibrotic and pro-thrombotic factor in the cardiovascular system.

Methods and results: Porcine left AECs were isolated and cultured. Senescence, as assessed by senescence-associated β-galactosidase activity (SA-β-gal), was induced either by passaging cells from passage P1 to P4 (replicative senescence) or following exposure of cells at P1 to L-NAME (an eNOS inhibitor), H_2O_2 or Ang II (premature senescence). Both replicative and premature senescence were associated with an up-regulation of p53, p21 and p16, key regulators of the cell cycle. Senescent AECs phenotype was characterized by (i) cell thrombogenicity through tissue factor up-regulation, shedding of procoagulant microparticles, eNOS down-regulation and reduced NO-mediated inhibition of platelet aggregation, (ii) cell adhesion and inflammation through up-regulation of ICAM-1 and cyclooxygenase-2, (iii) proteolysis and fibrosis remodeling through MMP-2, 9 and TGF-ß1 expression, and (iv) up-regulation of the local Ang II system through enhanced angiotensin-converting enzyme (ACE) and AT1 receptors expression, and oxidative stress. Perindoprilat (an ACE inhibitor) and Losartan (an AT1 receptor antagonist) prevented AECs senescence.

Conclusions: The present findings indicate that atrial endothelial senescence promotes thrombogenicity, inflammation, and matrix remodeling involving an up-regulation of the local Ang II system. They further suggest that the Ang II/AT1 receptor pathway may be a promising therapeutic target to delay atrial endothelial phenotypic changes associated with ageing.

Keywords: senescence, thrombosis, atrial fibrillation, endothelium, microparticles

Introduction

Ageing is a main determinant of atrial remodeling that contributes to atrial fibrillation (AF) initiation and perpetuation and enhanced thrombogenicity. Because one of the main consequences of AF is thrombotic stroke, the hypothesis that AF *per se* is a pro-thrombotic disease has been raised [1]. Evidences in favor of this paradigm include increased levels of plasma markers of a pro-thrombotic state such as von Willebrand factor, fibrinogen, D-dimer and pro-coagulant microparticles (MPs) in the plasma of AF patients [1, 2]. Although the link between ageing and thrombogenicity has been extensively studied in epidemiological studies, little is known about phenotypical changes associated with AECs ageing that could contribute to clot formation. Moreover, preclinical studies on AECs are conspicuously lacking and have been hampered mostly by the inability to isolate and culture endothelial cells from the atrium. Therefore, the initial cellular triggers for clotting, inflammation and proteolysis induction in AF still remain mainly speculative.

Endothelial cell senescence and dysfunction are hallmarks of cardiovascular diseases [3, 4]. Cellular senescence is a stress and damage response resulting in an irreversible cell cycle arrest and the appearance of distinct morphological and functional changes associated with impairment of cell homeostasis. Indeed, senescent endothelial cells characterized by an increased senescence-associated ß-galactosidase (SA-ß-gal) activity, have been shown to secrete pro-inflammatory and pro-remodelling factors, which, in turn, may impact the tissue environment [3, 4]. They are also characterized by an increased level of oxidative stress and a down-regulation of endothelial nitric oxide (NO) synthase expression resulting in a reduced bioavailability of the major vasoprotective factor NO [5]. The senescence-inducing signals engage predominantly the p53/p21 and p16/retinoblastoma protein tumour suppressor pathways, as the final effectors of the senescence program.

In the setting of AF, inflammation, oxidative stress, angiotensin II (Ang II), low or turbulent shear stress, increased mechanical stretch, hypoxia have all been involved in atrial remodeling. Moreover, rhythm disturbance-induced low and oscillatory shear stress resulted in blunted NO formation[6] and induction of endothelial-derived MPs release [7] and endothelial

senescence via a p53-dependent pathway [8]. Previous studies have also indicated a key role of Ang II in the pathogenesis of AF[9]. Indeed, the activation of the Ang II type 1 receptor (AT1R) leads to mitogen-activated protein kinases (MAPK) activation, which, in turn, promotes atrial remodeling by stimulating proliferation of fibroblasts, cellular hypertrophy and apoptosis. The importance of Ang II in atrial remodeling was further emphasized by studies demonstrating an enhanced Ang II production during tachypacing in canine hearts and arrhythmogenic atrial structural remodeling mediated by p38 MAPK phosphorylation[10]. Since ageing is a main determinant of AF, we have established a model of replicative and premature senescence in primary cultures of porcine left atrium endothelial cells (AECs), and characterized their phenotypic changes. Due to the fact that Ang II is a potent inducer of endothelial senescence, oxidative stress, MPs release and thrombogenicity [11, 12], we have examined the possibility that the local Ang II/AT1R pathway promotes premature atrial endothelial senescence.

Materials and methods

Chemicals

Unless indicated, all chemicals and solvents were from Sigma-Aldrich (Sigma-Aldrich SARL, St Quentin Fallavier, France). Losartan was obtained from Merck Research Laboratories (NJ, U.S.A) and perindoprilat was obtained from Servier (Paris, France).

Isolation and culture of atrial endothelial cells

AECs were isolated from left atria after collection of porcine hearts from the local slaughterhouse (SOCOPA, Holtzheim, France). Heart-lung blocks were dissected to isolate the left atrium. At the level of mitral valve, the left ventricle was removed and ligation of pulmonary veins was done. Left atrium was cleaned and flushed with phosphate-buffered saline (PBS) without calcium to discard remaining blood. To prepare primary cultures, AECs were isolated by collagenase treatment (type 1, Worthington, 1 mg/ml for 40 min at 37 °C) and cultured in culture dishes containing MCDB131 medium (Invitrogen) supplemented with penicillin (100 UI/ml), streptomycin (100 UI/ml), fungizone (250 µg/ml), L-glutamine (2 mM, all

from Lonza, St Quentin en Yvelines, France) and 15% fetal calf serum and grown for 3-4 days (passage 0). The medium was changed every 48 h. To induce premature senescence, AECs were incubated at passage 1 either with H_2O_2 (100 µM) or L-NAME (1 mM) for 48 h. For Ang II induced premature senescence, cells were exposed to serum-free medium for 2 h before the addition of Ang II (100 nM) for 24 h in serum-free medium. In some experiments, cells were exposed to losartan or perindoprilat for 30 min before the addition of either H_2O_2 (100 µM) or L-NAME (1 mM). To induce replicative senescence, AECs were detached with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Life Technologies SAS) and further passaged at a ratio of 1:3 at regular intervals. For platelet aggregation experiments, AECs were cultured on Cytodex-3 beads, which were hydrated and sterilized according to the instructions supplied by the manufacturer (GE Healthcare Life Sciences). In some experiments, AECs at passage 1 (P1) grown on Cytodex-3 beads were incubated in the absence or presence of L-NAME (1 mM) for 30 min before their addition to platelet suspensions as reported previously [13].

Determination of SA-β-gal activity by flow cytometry

SA-β-galactosidase activity was determined by flow cytometry using the fluorogenic substrate C_{12}FDG (5-dodecanoylaminofluorescein Di-β-D-galactopyranoside, Invitrogen, Life Technology, SAS) as described previously [14]. AECs were pretreated with 300 µM chloroquine for 1 h to induce lysosomal alkalinization. C_{12}FDG (33 µM) was then added to the incubation medium (without phenol red) for 1 h. At the end of the incubation period, AECs were washed with ice-cold PBS, resuspended following trypsinization and analyzed using a flow cytometer (FACScan, BD Bioscience, CA, USA). Data were acquired and analyzed using the Cellquest software (Becton Dickinson). Light scatter parameters were used to eliminate dead cells and subcellular debris. The C_{12}-fluorescein signal was measured on the FL1 detector, and the proportion of ECs with SA-β-gal activity was estimated using the median fluorescence intensity of the population. Autofluorescence assessed in parallel in AECs not exposed to C_{12}FDG was negligible.

Western blot analysis

AECs were washed with PBS and then lysed in extraction buffer (composition in mM: NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), Tris/HCl 20 (pH 7.5; QBiogene), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen). Equal amounts of proteins were separated on denaturing SDS (10-12%) polyacrylamide gel. Separated proteins were transferred electrophoretically onto nitrocellulose membrane (GE Healthcare Life Sciences). Blots were blocked at room temperature with 5% bovine serum albumin in PBS plus 0.1% Tween 20 (Sigma). For detection of proteins, membranes were incubated with the respective primary antibody: mouse monoclonal anti-eNOS (diluted 1:2500; BD Transduction Laboratories; cat. nº #610297), rabbit polyclonal anti-p53 (diluted 1:1.000; Santa Cruz; cat. nº SC-6243), mouse monoclonal anti-p21 (diluted 1:1000; Santa Cruz; cat. nº SC-817), rabbit polyclonal anti-p16 (diluted 1:1000; Delta Biolabs; cat. nº DB018), mouse monoclonal anti-tissue factor (TF, diluted 1:5000; Sekisui Diagnostics, LLC; cat. nº 4509), rabbit polyclonal anti-VCAM-1 (diluted 1:5000; Abcam; cat. nº ab134047), mouse monoclonal anti-ICAM-1 (diluted 1:1000; Abcam; cat. nº ab171123) mouse monoclonal anti-transforming growth factor-β1 (TGF-β1, diluted 1:2500; Abcam; cat. nº ab27969), rabbit polyclonal anti-cyclooxygenase-1 (COX-1, diluted 1:1.000; Abcam; cat. nº ab109025), rabbit polyclonal anti-COX-2 (diluted 1:1000; Abcam; cat. nº ab15191), rabbit polyclonal anti-angiotensin-converting enzyme (ACE, diluted 1:1000; Abbiotec; cat. nº 250450), rabbit polyclonal anti-angiotensin type 1 receptor (AT1R, diluted 1:1000; Abcam cat. nº ab124505), mouse monoclonal anti-MMP2 (diluted 1:1000; Abcam; cat. nº ab86607), rabbit polyclonal anti-MMP9 (diluted 1:1000; Abcam; cat. nº ab38898) or mouse monoclonal anti-β-tubulin (diluted 1:20000; Sigma-Aldrich; cat. nº T7816) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin G, dilution of 1:5000; Cell Signaling Technology; cat. nº #7074, #7076, respectively) at room temperature for 60 min. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunocomplexes were detected by chemiluminescence reaction (ECL; Amersham, Les Ulis, France) followed by densitometric analysis using the software Image J.

Determination of oxidative stress

P1 and P3 AECs were seeded in Millicell EZ SLIDE 8-well glass slides for 24 h, then exposed to serum-free MCDB 131 (Invitrogen) for 6 h. The redox-sensitive fluorescent dye dihydroethidium (DHE) was used to evaluate the formation of reactive oxygen species (ROS) [15]. AECs were incubated with DHE (5 µM) for 20 min at 37 °C in a light protected manner. To determine the cellular sources of ROS, cells were exposed either to N-acetyl cysteine (NAC, an antioxidant, 1 mM, 3 h), VAS-2870 (VAS, a NADPH oxidase inhibitor, 5 µM, 30 min), or indomethacin (INDO, a cyclooxygenase inhibitor, 30 µM, 30 min). AECs were then washed and mounted with fluorescent mounting medium (DAKO, S3023) and examined under confocal microscope (Leica SP2 UV DM Irbe). Images were analyzed using Image J software.

Platelet aggregation

Washed human platelet suspensions kindly provided by the Etablissement Français du Sang - Alsace (Strasbourg), were prepared as previously described [13]. Suspensions of washed platelets (450 µl, 3.10^8 platelets/µl) were incubated for 2 min in a Chronolog 490 aggregometer (Stago BNL) with continuous stirring at 1000 rpm before addition of a submaximal concentration of U46619 (0.07 µM, a thromboxane A_2 analog) and fibrinogen (1.6 mg/ml). A volume of 10 to 20 µl of beads covered with ECs was added to platelet suspensions 2 min before the addition of U46619.

MPs isolation and measurement

Microparticles (MPs) were collected from conditioned medium of senescent cells by sequential centrifugation under sterile conditions as described previously [16]. Briefly, cells and cellular debris were discarded by a 2-steps 800 *g* centrifugation for 15 min at room temperature, and the supernatant was further centrifuged at 13 000 *g* and 4 °C for 60 min. The final suspension was kept in Hanks balanced solution (HBSS) less than 30 days at 4 °C.

MPs measurement was performed by prothrombinase assay after capture onto Annexin-5 as

previously described using microplate spectrophotometric reader set in kinetic mode, at 405 nm [17]. This capture-based assay allows extensive washing of captured MPs, taking advantage of the high affinity of Annexin-5 for phosphatidylserine (Phtdser) exposed at MPs surface. MPs concentration was referred to as Phtdser equivalent, by reference to a standard curve made with synthetic vesicles of known amounts of PhtdSer [17]. In some experiments, MPs-free supernatant was centrifuged during 4 h at 25 000 *g* to discard exosomes and assayed as a control of truly soluble content of the conditioned medium.

Determination of MMPs activity by zymography

MMP-2 and MMP-9 activities in conditioned medium of cultured AECs were analyzed by substrate-gel electrophoresis (zymography) with the use of SDS-PAGE (8%) containing 0.1% gelatin, as described previously [18]. Then, gels were washed in denaturing buffer for 30 min and incubation was done in developing buffer for 24 h at 37˚C. Thereafter, gels were stained in 30 % Coomassie blue for 30 min and then destained with destaining buffer. Gelatinolytic activity appeared as bands with blue background. Images were taken and the bands were analyzed using Image J software.

Statistical Analyses

Data are presented as mean ± SEM of n different experiments. Mean values were compared using Student's paired *t* test or an analysis of variance followed by the post-hoc Bonferroni test to identify significant differences between treatments using GraphPad Prism (v5.0). The difference was considered to be significant when the *P* value was less than 0.05.

Results

Replicative passaging of atrial endothelial cells and eNOS inhibition promotes senescence

Replicative passaging of AECs caused a progressive appearance of endothelial cells with increased levels of SA-β-gal activity (Figure 1A). Likewise, L-NAME, an eNOS inhibitor, and

H_2O_2, a strong inducer of premature endothelial senescence [11] promoted also AECs senescence (Figure 1B). The induction of senescence was also witnessed by a strong up-regulation of p53, a key regulator in cellular senescence, and of down-stream p21 and p16, key cyclin-dependent kinase inhibitors, in AECs at P4 (Figures 2A-C), and in L-NAME- and H_2O_2-treated AECs at P1 (Figures 2D-F). We have verified that the induction of replicative senescence did not heighten the level of apoptosis as reported previously in porcine aortic endothelial cells.[19]

Atrial endothelial senescence, endothelial dysfunction, inflammation and thrombogenicity

Because endothelial senescence is usually characterized by endothelial dysfunction and a reduced formation of NO [20, 21], the expression level of eNOS was assessed in AECs. A significant down-regulation of the expression level of eNOS was observed by Western Blot analysis in AECs at P4 compared to P1 (Figure 3A). Given the key role of NO to prevent very effectively platelet aggregation, we have investigated the potential of AECs to inhibit platelet aggregation. In contrast to AECs at P1, senescent AECs at P3, and L-NAME-treated AECs at P1 showed a reduced platelet anti-aggregatory effect suggesting a blunted formation of NO (Figure 3B). In addition, senescence shifted the endothelial phenotype to a pro-inflammatory and pro-coagulant status in AECs at P4 as evidenced by an increased expression level of intracellular adhesion molecule-1 (ICAM-1) and tissue factor (TF), and about a 3-fold enhancement of pro-coagulant MPs shedding (Figures 4A-C). In addition, H_2O_2 and L-NAME treatments of AECs at P1 also prompted an increased expression level of ICAM-1 and TF, and the shedding of MPs (Figures 4D-F).

Atrial endothelial senescence is associated with characteristic pro-adhesive and tissue remodeling patterns

To underscore the impact of atrial senescence on cell adhesion and remodeling, the expression level of TGF-ß1 and MMPs, pivotal mediators in the control of tissue remodeling

during cardiac fibrosis, were investigated in AECs at P1 and P4, and also in AECs at P1 exposed to either L-NAME or H_2O_2. Both replicative senescence and premature senescence in response to H_2O_2 and L-NAME were associated with an increased expression level of TGF-ß1, MMP-2 and MMP-9 (Figures 5A-F).

Redox-sensitive NADPH oxidase- and COX-mediated induction of replicative and premature AECs senescence

Since NO is well-known to reduce oxidative stress, a potent inducer of endothelial senescence[22 23], the role of oxidative stress in the induction of AECs senescence was determined. The level of oxidative stress as assessed using the redox-sensitive probe DHE, was increased in AECs at P3 compared to those at P1 (Figure 6A). Next, pharmacological tools were used to characterize the sources of ROS in senescent AECs. Exposure of AECs at P3 to either the antioxidant N-acetylcysteine, or an inhibitor of NADPH oxidase (VAS-2870) or COX (indomethacin) significantly reduced the level of oxidative stress and the SA-ß-gal activity, indicating that NADPH oxidase and cyclooxygenase pathways contribute to the redox-sensitive induction of replicative senescence (Figures 6A,B). Similarly, N-acetylcysteine, VAS-2870 and indomethacin also prevented the L-NAME-induced induction of senescence in AECs at P1 suggesting that eNOS-derived NO has a pivotal role to counteract the induction of AECs senescence (Figure 6C).

The local redox-sensitive angiotensin system contributes to replicative and premature endothelial senescence

Because oxidative stress has been shown to upregulate the expression of both ACE and ATR1 in endothelial and vascular smooth muscle cells [24, 25 26], the role of the local angiotensin system in the induction of both replicative and premature AECs senescence was determined. Exposure of endothelial cells to either the ACE inhibitor perindoprilat or the AT1R blocker losartan decreased SA-β-gal activity in AECs at P3 (Figure 7A) and in L-NAME-treated AECs at P1 (Figure 7B). In addition, a significant upregulation of both ACE and AT1R protein levels

was observed in senescent AECs at P4 (Figures 7C,E) and in premature senescent L-NAME- and H_2O_2-treated AECs at P1 (Figures 7D,F).

Ang II-induced AECs senescence promotes pro-inflammatory, pro-coagulant and pro-fibrotic responses, oxidative stress and the up-regulation of the local angiotensin system

Since Ang II has been reported to be a strong inducer of senescence and MPs shedding through enhanced oxidative stress in cultured murine aortic ECs [11], we examined the possibility of a redox-sensitive induction of the local angiotensin system contributes to Ang II-induced senescence in AECs at P1 after a 24-h incubation period. As shown in Figure 8, exposure of AECs with 100 nM Ang II significantly increased SA-ß-gal activity (Figure 8A) and up-regulated the expression level of p53, p21 and p16 indicating a pro-senescent effect (Figures 8B-D). In addition, the premature senescence was associated with a decreased expression level of eNOS (Figure 8E) whereas those of VCAM-1 and TF were significantly increased (Figures 8F, G). Premature Ang II-induced senescence was also associated with the induction of a pro-fibrotic phenotype as indicated by an enhanced expression level of TGF-ß1, active MMP-2 and active MMP-9 (Figures 8H-J). Moreover, Ang II stimulated the formation of ROS in AECs at P1 (Figure 8K) and triggered the up-regulation of ACE and AT1R protein expression levels (Figures 8L, M) [27].

Discussion

The present findings indicate that both replicative and premature atrial endothelial senescence promote thrombogenicity, inflammation, proteolysis, and fibrosis that are mediated by the local Ang II system. They further suggest that targeting the Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial phenotypical alterations associated with senescence.

Atrial endothelial senescence is associated with endothelial dysfunction and a pro-thrombotic pattern

Although the potential role of senescence in endothelial dysfunction has lately attracted a lot of interest using cultured ECs and experimental models of atherosclerosis[3], its impact during arrhythmogenic remodeling associated with AF remains unknown. Both premature endothelial senescence, as observed in response to Ang II or H_2O_2, and replicative endothelial senescence are characterized by oxidative stress and a pronounced down-regulation of eNOS expression and the endothelial formation of NO[4, 5]. The present findings further extent these previous reports by showing that replicative senescence promotes, besides blunted NO formation, a pronounced pro-thrombotic pattern in senescent AECs as indicated by their reduced ability to prevent platelet aggregation, the upregulation of the expression level of TF and the shedding of procoagulant MPs. We and others have recently demonstrated that senescent ECs-derived MPs act as effective mediators propagating a pro-senescent and pro-thrombotic message to neighboring healthy ECs and, hence, they will contribute to promote locally thrombus formation [11]. Besides representing a surrogate marker of vascular and endothelial dysfunction, circulating MPs, evidenced at elevated levels in AF [2, 28] or within hours following radiofrequency or cryoablation procedures [29, 30] might also contribute to endothelial dysfunction and thrombotic propensity of atrial cells. Because procoagulant MPs circulate at high levels during AF or during ablation procedures associated with thromboembolic complications, MPs might behave as potent effectors of stroke events during AF. Indeed, circulating levels of endothelial MPs were related to infarct size and clinical outcome in patients with acute ischaemic stroke [31].

If the link between ageing and endothelial dysfunction appears robustly established, the relationship between ageing and the propensity to favor arterial thrombosis independently of other relevant risk factors remains controversial. In a recent investigation using organ chamber experiments, arterial thrombosis as induced by photochemical injury was not enhanced in old mice and was not associated with changes of the arterial expression of TF protein and activity, and of platelet activation [32]. By contrast, another report showed that H_2O_2, a potent inducer of

senescence, promoted ageing-related platelet hyper-reactivity and thrombosis in mice [33]. Moreover, alteration in atrial hemodynamics caused by AF rhythm disturbances promoted a prothrombotic endothelial pattern through enhanced TF expression [34].

Atrial endothelial senescence is associated with a pro-adhesive pattern

Besides the progressive establishment of a pro-thrombotic profile, pro-adhesive and pro-inflammatory proteins are another characteristic features of senescent cells (senescent-associated secretory phenotype) including an enhanced expression of ICAM-1 and secretion of various chemokines by senescent ECs [4]. The relevance of the inflammatory response has recently been suggested by the colocalization of enhanced macrophages infiltration and thrombus formation within atrial tissues of AF patients [35] and by the enrichment of monocyte-platelet aggregates or CD11b expression in AF patients with proven thrombus formation [36]. Other recent insights came from studies showing that (i) the extent of peri-atrial epicardial fat, a marker of AF burden and outcome after AF ablation, is associated with enhanced expression of ICAM-1 and vWF levels [37] and that (ii) inflammation of epicardial fat predicts the occurrence of paroxysmal AF[38]. In AF, several reports have underlined a possible nexus between the pro-thrombotic state and inflammation. For instance, recent data from a large cohort of patients with AF and receiving an anticoagulant treatment, have indicated that biomarkers of inflammation were significantly associated with an increased risk of mortality [39].

The present findings indicate that the induction of AECs senescence is associated with the up-regulation of the adhesion molecule ICAM-1. Moreover, Toll-like receptor 4 activation, besides promoting the induction of senescence, has also been shown to stimulate the expression of VCAM-1 in ECs thereby promoting atrial thrombogenesis [40]. Enhanced expression of ICAM-1 on the endothelial cell surface will promote the binding of inflammatory cells that express CD11b/CD18 integrins (Mac1), and constitute a prerequisite for subsequent leukocyte extravasation [41]. Moreover, Ang II has been shown to be a potent stimulus for polynuclear neutrophils activation and CD11b/CD18 expression [42] that could contribute to enhance

transmigration of inflammatory cells subsequent to an up-regulation of cytoadhesins at the surface of senescent ECs. In line with this view, endothelial senescence was demonstrated to alter tight junction integrity thereby promoting inflammatory cells transendothelial migration [43]. The major role of leukocyte infiltration in atrial remodeling and AF vulnerability has been observed following the invalidation of the CD11 gene, which protected mice from AF vulnerability [42]. These data are consistent with the recent demonstration of a strong relationship between the enlargement of the left atrium and the occurrence of inflammatory cells infiltration in AF patients[44]. Altogether, these data suggest that the pro-adhesive pattern associated with premature atrial endothelial senescence may favor leukocyte infiltration and atrial remodeling.

Atrial endothelial senescence induces enhanced MMPs expression: relevance to fibrosis

Several observational studies have pointed out the relationship between MMPs and TIMPs (tissue inhibitors of matrix metalloproteinases) expression as measured in atrial tissues or in the peripheral blood and the occurrence of AF [45]. The human MMPs belong to a class of pro-inflammatory and pro-angiogenic factors that control tissue remodeling through the tuning of extracellular matrix degradation, and the release of TGF-β that triggers a pro-fibrotic pathway. Atrial fibrosis can lead to atrial remodeling that contributes to AF persistence. In aging mice, recent studies have depicted an enhanced metalloproteinase-9 (MMP-9) expression concomitant with the development of diastolic dysfunction, as a surrogate marker of cardiac fibrosis [46]. Furthermore, the deletion of MMP-9 attenuated the age-related decline in diastolic function, in part, by reducing the TGF-ß pro-fibrotic signal [47]. Recent findings have also underscored that TGF-β1 increased p16 expression and senescence through the production of mitochondrial ROS and most likely subsequent to a reduced level of various antioxidant mechanisms [48]. Moreover, Xie J et al. observed that premature senescence is associated with atrial fibrosis in valvular AF as indicated by the analysis of left atrial appendages of patients before valve surgery.[49] In the present study, the enhanced release of collagenases MMP-2

and 9 observed in senescent AECs is consistent with previous findings obtained in bovine aortic ECs [50]. Altogether, these data emphasize the view that AECs senescence might contribute to cardiac tissue remodeling through MMPs-mediated extracellular matrix degradation and the TGF-ß pathway.

The Ang II/ AT1 receptor pathway regulates atrial endothelial senescence

Several reports have pointed out the paramount role of Ang II in atrial remodeling, fibrosis, and in the development of an arrhythmogenic substrate [51]. In contrast, its impact on atrial endothelial cell function remains poorly studied. The fact that Ang II is a potent inducer of vascular endothelial senescence [12] prompted investigations whether the local angiotensin system contributes to AECs senescence. The present findings indicate that Ang II, at concentrations that could be reached in pathophysiological issues, is a strong inducer of AECs premature senescence to a similar level as that induced by H_2O_2, a well-known inducer of senescence, and L-NAME, an eNOS inhibitor. The importance of the Ang II/AT1R pathway in the induction of premature senescence is further emphasized by the demonstration that both losartan, an AT1R inhibitor and perindoprilat, an ACE inhibitor, abolished the induction of premature and replicative senescence. Altogether, these findings highlight a pivotal role of the local angiotensin system in the induction of endothelial senescence and its pro-adhesive, pro-thrombotic and pro-fibrotic phenotypes.

Conclusion

These findings provide compelling evidences indicating that atrial endothelial senescence promotes thrombogenicity through enhanced TF expression, blunted NO-mediated inhibition of platelet aggregation and procoagulant MPs shedding. Senescence also promoted the induction of an inflammatory response, proteolysis, fibrosis and the up-regulation of the local Ang II system. They further suggested that targeting the Ang II/AT1R pathway might be a

promising therapeutic strategy to delay atrial endothelial phenotypical alterations associated with ageing.

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Disclosures

None

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Figure legends

Figure 1. Sequential passaging of AECs induces replicative senescence, and L-NAME and H_2O_2 induce premature senescence. (A) Senescence assessed by SA-β-gal activity in serially passaged AECs from P1 to P4. (B) AECs at P1 incubated with either H_2O_2 (100 μM) or L-NAME (1 mM) for 48 h before the determination of SA-β-gal activity. Results are shown as mean ± SEM of n= 3-4 different experiments. *P < 0.05 versus respective control, $^{\#}P$ versus P2.

Figure 2. Replicative and premature senescence are associated with an upregulation of major cell cycle regulatory proteins: p53, p21 and p16. Passaging of AECs from P1 to P4 and exposure of AECs at P1 to either L-NAME (1 mM) or H_2O_2 (100 μM) for 48 h increased the protein expression level of (A, D) p53, (B, E) p21 and (C, F) p16 as assessed by Western blot analysis. Results are presented as representative immunoblots (upper panels), and corresponding cumulative data (lower panels) and are shown as ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control.

Figure 3. Replicative AECs senescence induces eNOS down-regulation and reduces the ability of AECs to inhibit platelet aggregation. (A) eNOS expression level in healthy P1 and senescent AECs at P4 as assessed by Western blots analysis. (B) AECs at P1 incubated with or without L-NAME (1 mM, 30 min), and ACEs at P3 were grown on microcarrier beads before their addition to platelet suspensions 2 min before a submaximal aggregation with U46619. Results are shown as mean ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control, $^{\#}P$ < 0.05 L-NAME-treated AECs at P1 versus respective control.

Figure 4. Replicative and premature senescence are associated with the induction of pro-inflammatory and pro-coagulant phenotypes in AECs. Passaging of AECs from P1 to P4 and exposure of AECs at P1 to either L-NAME (1 mM) or H_2O_2 (100 μM) for 48 h increased the protein expression level of (A, D) intracellular cell adhesion molecule ICAM-1, (B, E) tissue

factor (TF) as assessed by Western blot analysis, and (**C, F**) enhancement of pro-coagulant MPs shedding. Results are presented as representative immunoblots (upper panels) and corresponding cumulative data (lower panels) and are shown as mean ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control.

Figure 5. Replicative and premature senescence are associated with the induction of a pro-fibrotic phenotype in AECs. Passaging of AECs from P1 to P4 and exposure of AECs at P1 to either L-NAME (1 mM) or H_2O_2 (100 µM) for 48 h increased the protein expression level of (A, D) TGF-β1, (B, E) MMP-2 and (C, F) MMP-9 as assessed by Western blot analysis. Results are presented as representative immunoblots (upper panels) and corresponding cumulative data (lower panels) and are shown as mean ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control.

Figure 6. Role of oxidative stress in the induction of AECs senescence. (A) The level of oxidative stress in AECs at P3 was determined in the absence or presence of either N-acetylcysteine (NAC, an antioxidant, 1 mM, 3 h), VAS-2870 (VAS, a NADPH oxidase inhibitor, 5 µM, 30 min), or indomethacin (INDO, a cyclooxygenase inhibitor, 30 µM, 30 min) before the addition of DHE (5 µM) for 20 min and the subsequent analysis of the fluorescent level by confocal microscopy. The level of oxidative stress in P1 AECs is also shown. Upper panel represents ethidium staining; lower panel corresponding cumulative data. (B, C) AECs at P3 and P1 (treated or not with L-NAME 1 mM for 48 h) were either untreated or exposed to NAC, VAS or INDO before the subsequent determination of the SA-β-gal activity using flow cytometry. Results are shown as mean ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control, $^{\#}P$ < 0.05 versus AECs at P3 or L-NAME-treated AECs at P1.

Figure 7 Role of the local angiotensin system in both replicative and premature senescence of AECs. (A, B) AECs at P3 and P1 (untreated or treated with L-NAME 1 mM) were either untreated or exposed to losartan (an AT1R antagonist, 10 µM) or perindoprilat (ACE inhibitor,

10 µM) for 48 h. (C-F) Passaging of AECs from P1 to P4 and exposure of AECs at P1 to either L-NAME (1 mM) or H_2O_2 (100 µM) for 48 h increased the protein expression level of ACE and AT1R as assessed by Western blot analysis. Results are presented as representative immunoblots (upper panels), and corresponding cumulative data (lower panels) and are shown as mean ± SEM of n = 3-4 different experiments. *$P < 0.05$ versus respective control, $^{\#}P < 0.05$ versus L-NAME-treated AECs at P1.

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Figure 8. Ang II-induced senescence promotes pro-inflammatory, pro-coagulant and pro-fibrotic, oxidative stress and up-regulation of local angiotensin system in AECs at P1. AECs were exposed to Ang II (100 nM) for 24 h before determination of senescence (A), protein expression level of target proteins (B-J, N, O), zymography (K, L), and oxidative stress (M). Results are shown as representative immunoblots or gelatinolytic activity (upper panels) and corresponding cumulative data (lower panels) and shown as mean ± SEM, n = 3-4, *$P < 0.05$ versus respective control.

FIGURE 1

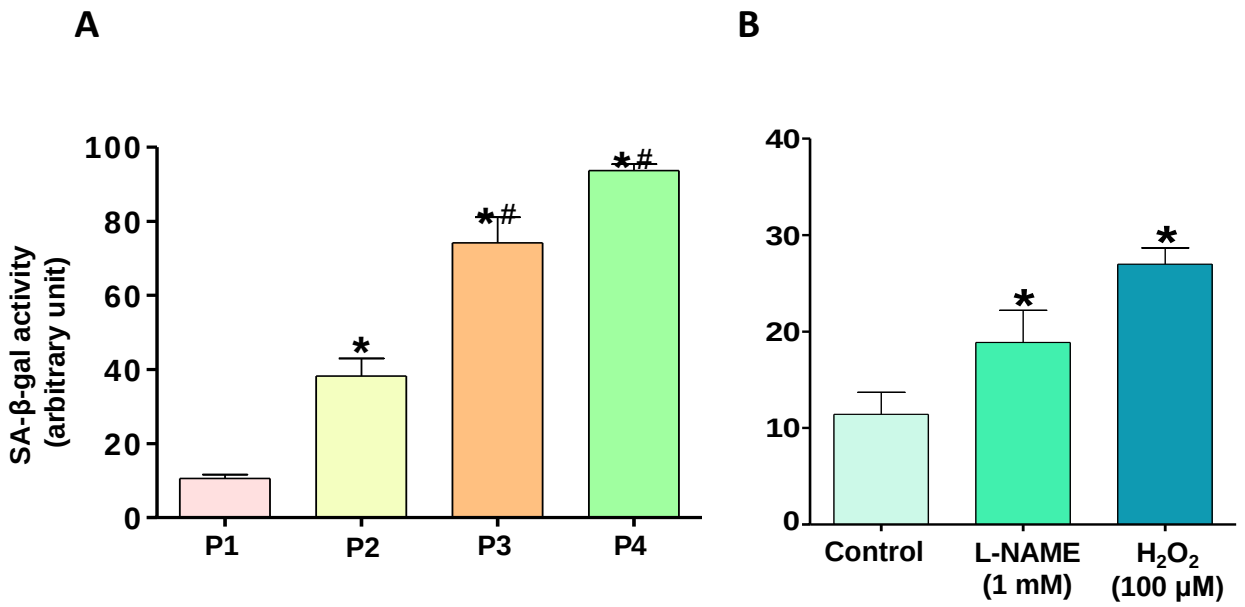

A
B
SA-β-gal activity
(arbitrary unit)
100
80
60
40
20
0
*
*#
*#
P1
P2
P3
P4
40
30
20
10
0
*
*
Control
L-NAME
(1 mM)
H2O2
(100 µM)

FIGURE 2

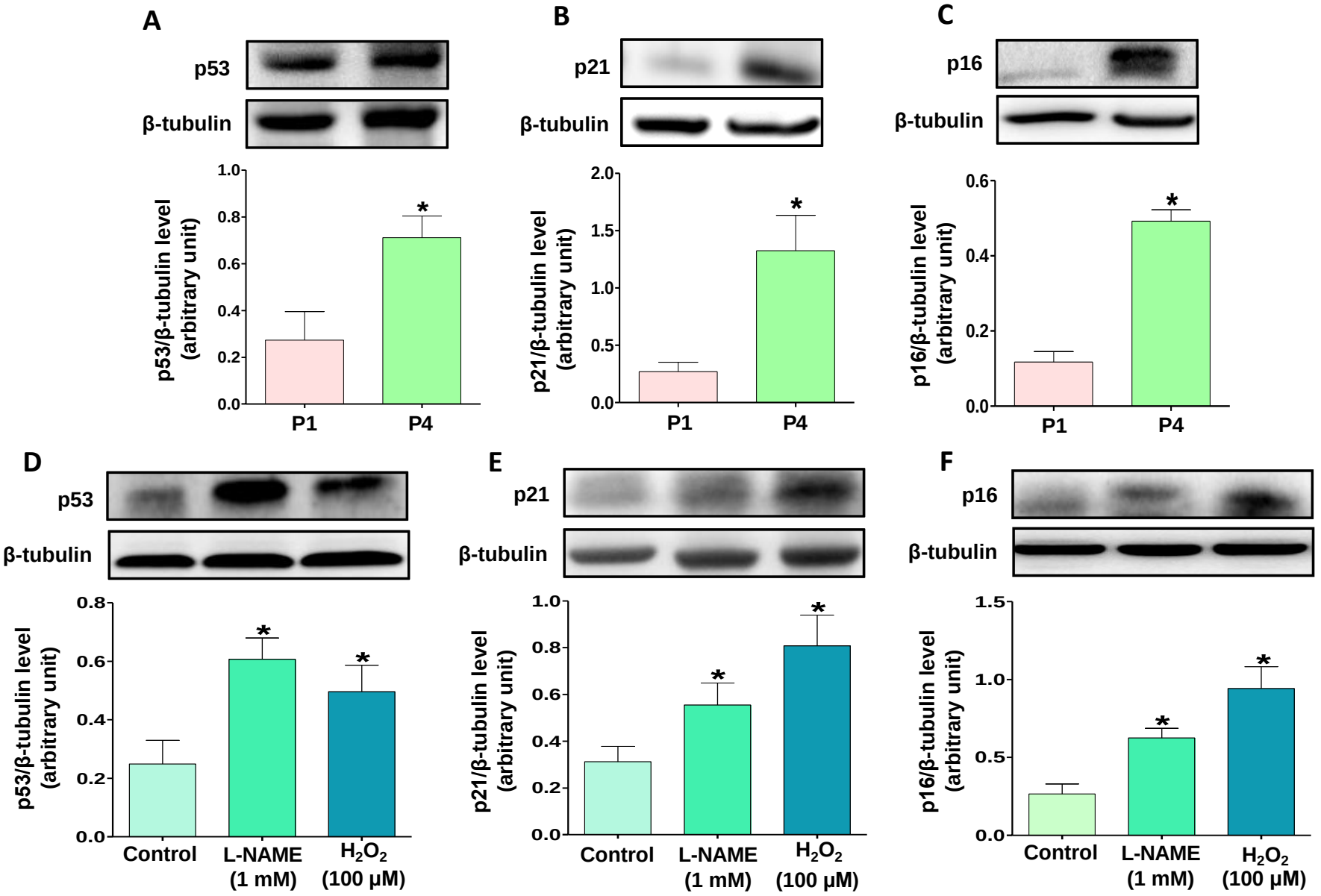

A
p53
β-tubulin
p53/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
P1
P4
B
p21
β-tubulin
p21/β-tubulin level (arbitrary unit)
2.0
1.5
1.0
0.5
0.0
*
P1
P4
C
p16
β-tubulin
p16/β-tubulin level (arbitrary unit)
0.6
0.4
0.2
0.0
*
P1
P4
D
p53
β-tubulin
p53/β-tubulin level (arbitrary unit)
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)
E
p21
β-tubulin
p21/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)
F
p16
β-tubulin
p16/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)

FIGURE 3

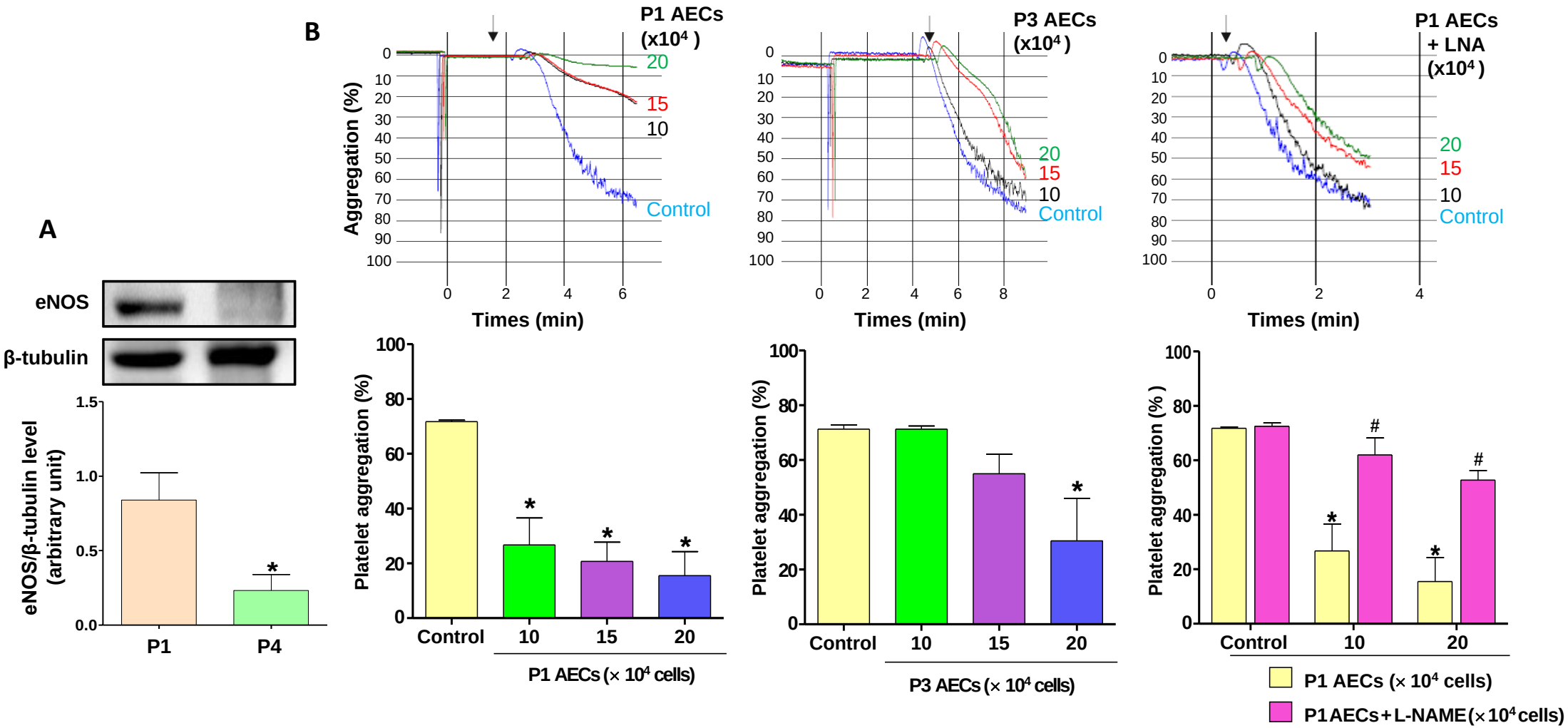

A
eNOS
β-tubulin
eNOS/β-tubulin level (arbitrary unit)
P1
P4
B
P1 AECs (x10⁴)
P3 AECs (x10⁴)
P1 AECs + LNA (x10⁴)
Aggregation (%)
Times (min)
20
15
10
Control
Platelet aggregation (%)
Control
10
15
20
P1 AECs (× 10⁴ cells)
P3 AECs (× 10⁴ cells)
P1 AECs (× 10⁴ cells)
P1 AECs + L-NAME (× 10⁴ cells)

FIGURE 4

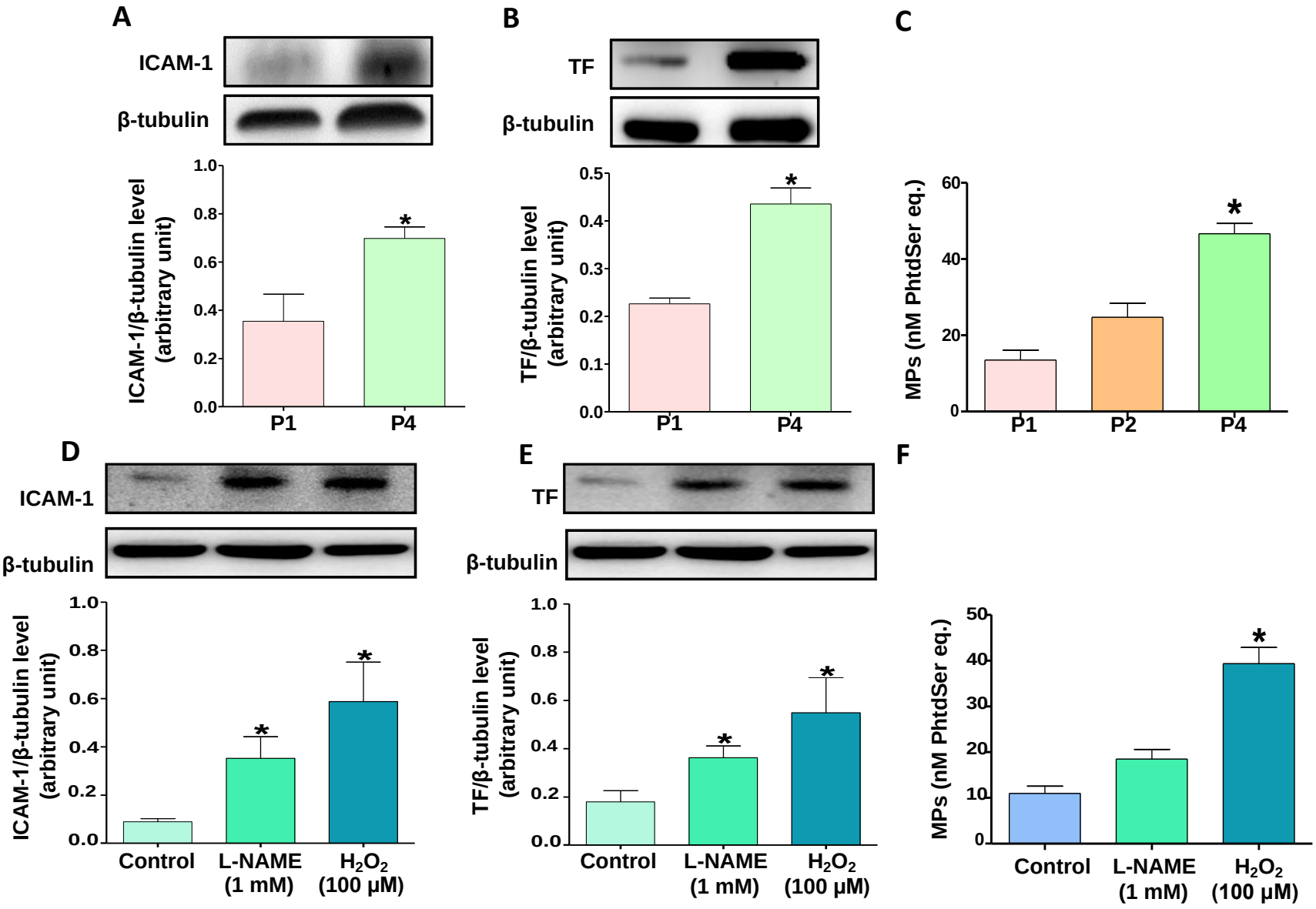

A
ICAM-1
β-tubulin
ICAM-1/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
P1
P4
B
TF
β-tubulin
TF/β-tubulin level (arbitrary unit)
0.5
0.4
0.3
0.2
0.1
0.0
*
P1
P4
C
MPs (nM PhtdSer eq.)
60
40
20
0
*
P1
P2
P4
D
ICAM-1
β-tubulin
ICAM-1/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)
E
TF
β-tubulin
TF/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)
F
MPs (nM PhtdSer eq.)
50
40
30
20
10
0
*
Control
L-NAME (1 mM)
H2O2 (100 μM)

FIGURE 5

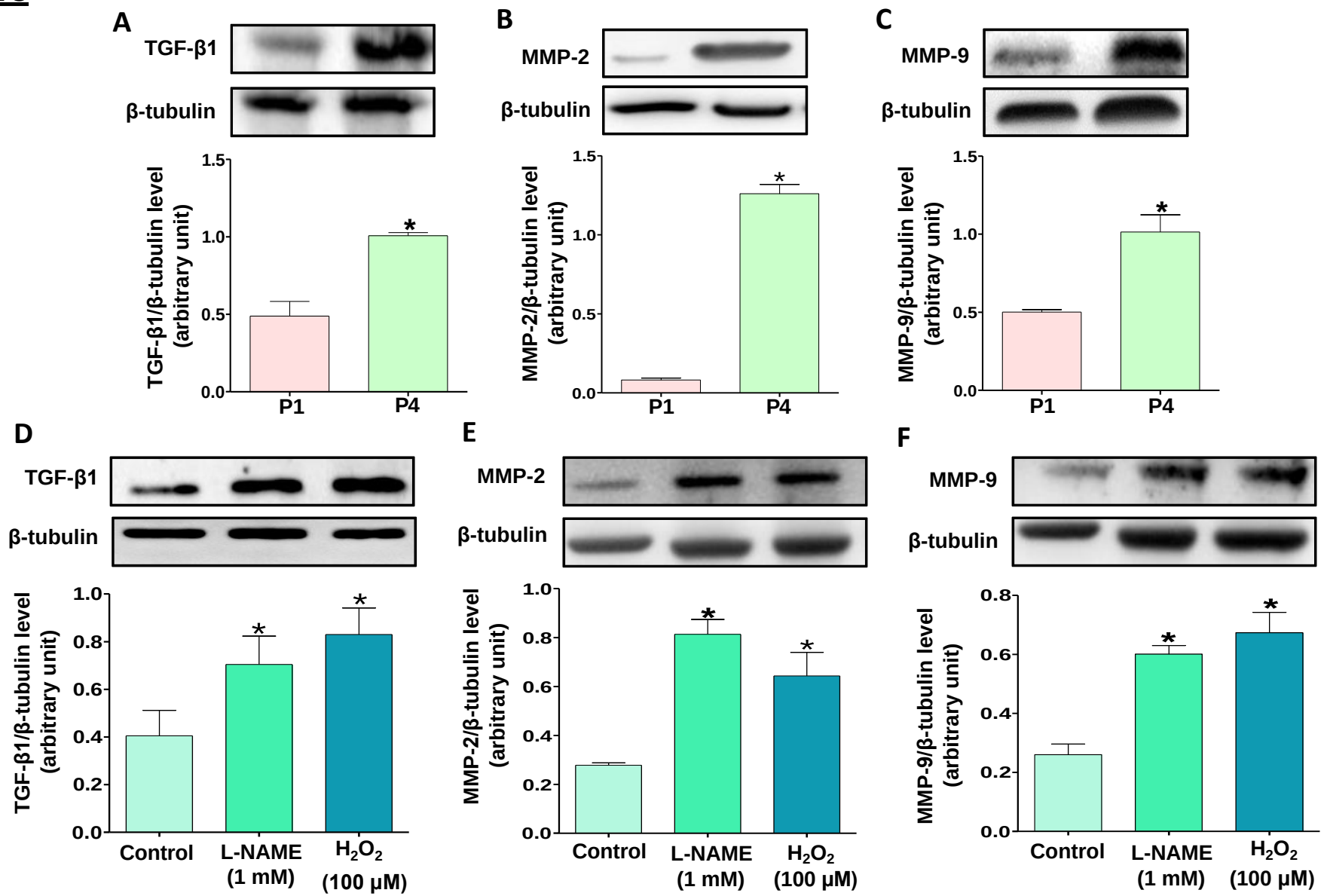

A
TGF-β1
β-tubulin
TGF-β1/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
P1
P4
B
MMP-2
β-tubulin
MMP-2/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
P1
P4
C
MMP-9
β-tubulin
MMP-9/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
P1
P4
D
TGF-β1
β-tubulin
TGF-β1/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)
E
MMP-2
β-tubulin
MMP-2/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)
F
MMP-9
β-tubulin
MMP-9/β-tubulin level (arbitrary unit)
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)

FIGURE 6

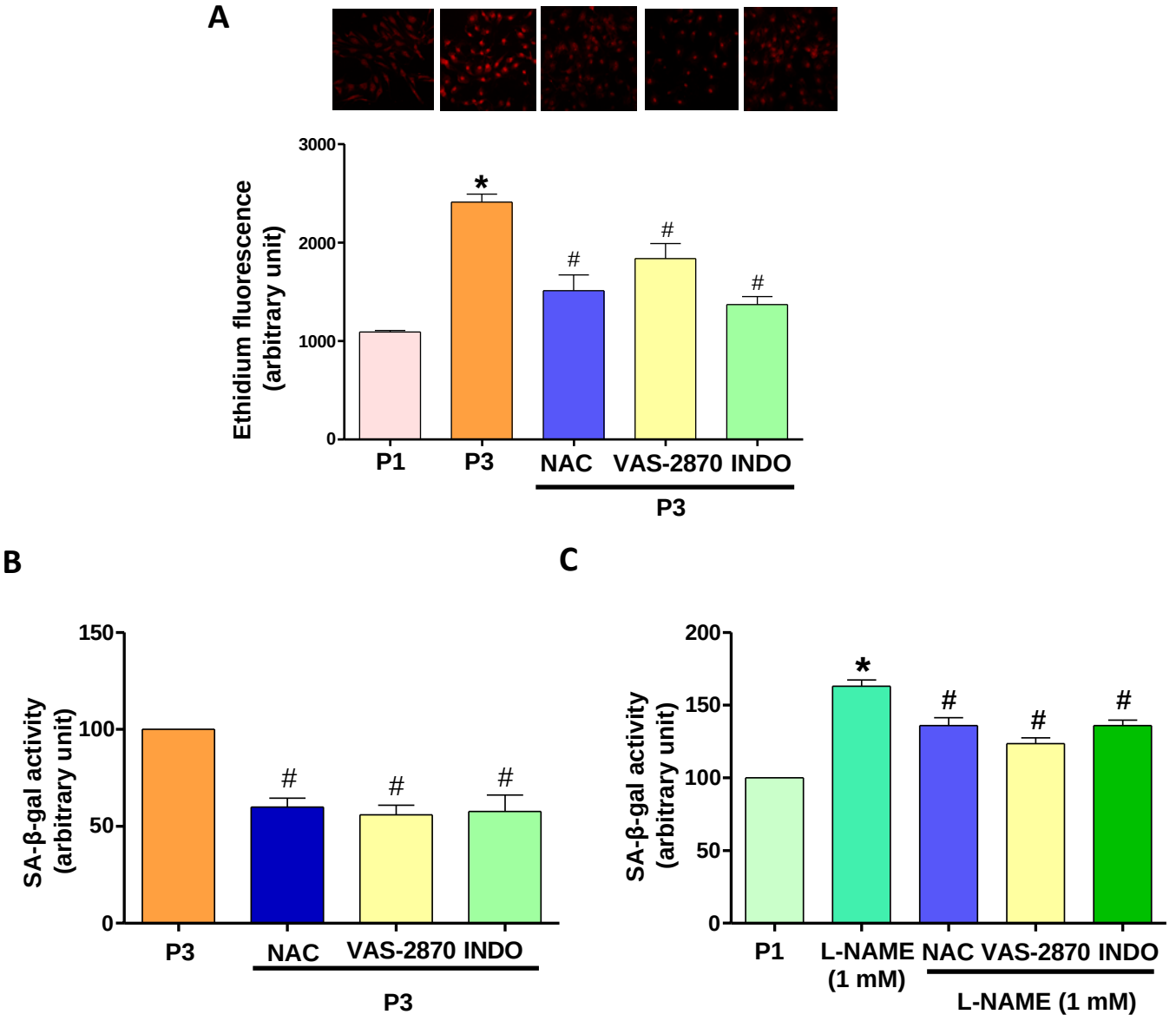

A
Ethidium fluorescence (arbitrary unit)
3000
2000
1000
0
*
#
#
#
P1
P3
NAC
VAS-2870
INDO
P3
B
SA-β-gal activity (arbitrary unit)
150
100
50
0
#
#
#
P3
NAC
VAS-2870
INDO
P3
C
SA-β-gal activity (arbitrary unit)
200
150
100
50
0
*
#
#
#
P1
L-NAME (1 mM)
NAC
VAS-2870
INDO
L-NAME (1 mM)

FIGURE 7

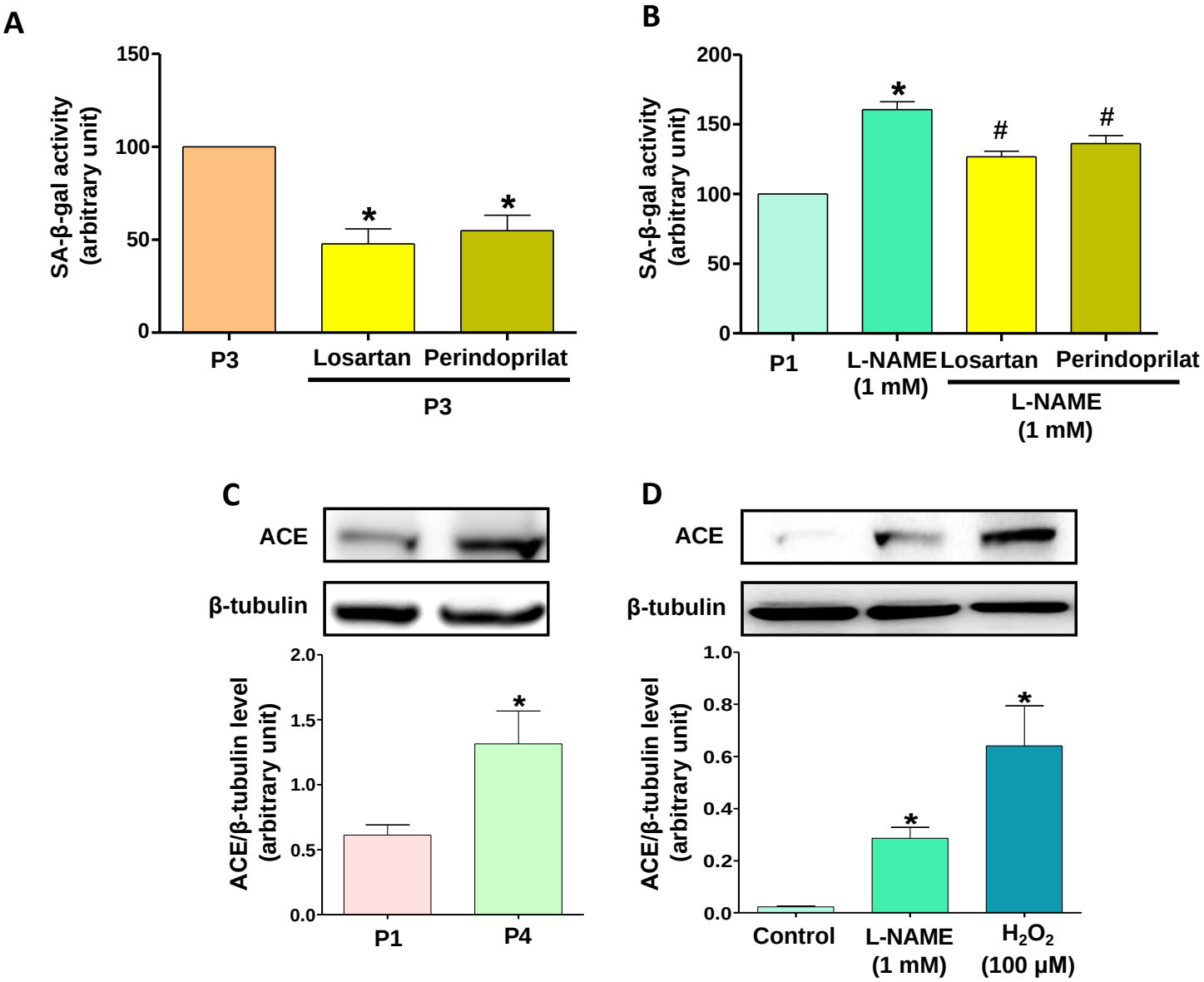

A
SA-β-gal activity (arbitrary unit)
150
100
50
0
*
*
P3
Losartan
Perindoprilat
P3
B
SA-β-gal activity (arbitrary unit)
200
150
100
50
0
*
#
#
P1
L-NAME (1 mM)
Losartan
Perindoprilat
L-NAME (1 mM)
C
ACE
β-tubulin
ACE/β-tubulin level (arbitrary unit)
2.0
1.5
1.0
0.5
0.0
*
P1
P4
D
ACE
β-tubulin
ACE/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)

FIGURE 7

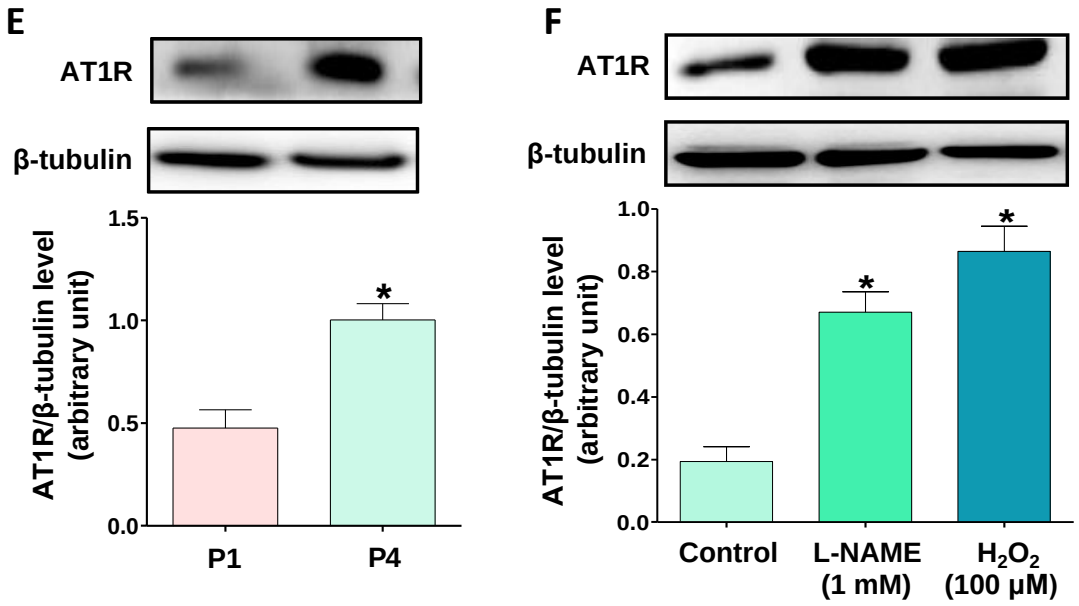

E
AT1R
β-tubulin
AT1R/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
P1
P4
F
AT1R
β-tubulin
AT1R/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
L-NAME (1 mM)
H2O2 (100 μM)

FIGURE 8

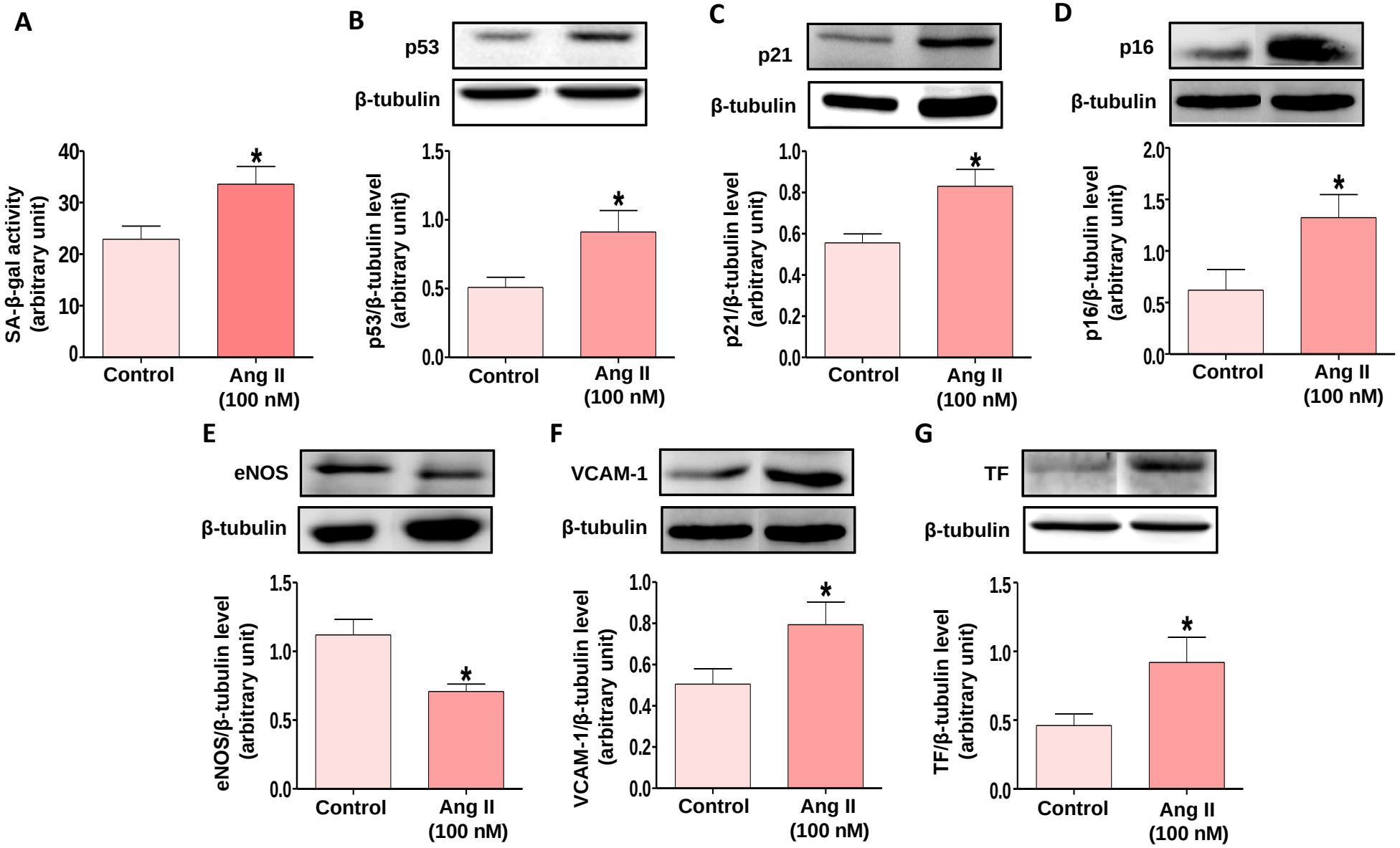

A
SA-β-gal activity (arbitrary unit)
40
30
20
10
0
*
Control
Ang II (100 nM)
B
p53
β-tubulin
p53/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
Control
Ang II (100 nM)
C
p21
β-tubulin
p21/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Ang II (100 nM)
D
p16
β-tubulin
p16/β-tubulin level (arbitrary unit)
2.0
1.5
1.0
0.5
0.0
*
Control
Ang II (100 nM)
E
eNOS
β-tubulin
eNOS/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
Control
Ang II (100 nM)
F
VCAM-1
β-tubulin
VCAM-1/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Ang II (100 nM)
G
TF
β-tubulin
TF/β-tubulin level (arbitrary unit)
1.5
1.0
0.5
0.0
*
Control
Ang II (100 nM)

FIGURE 8

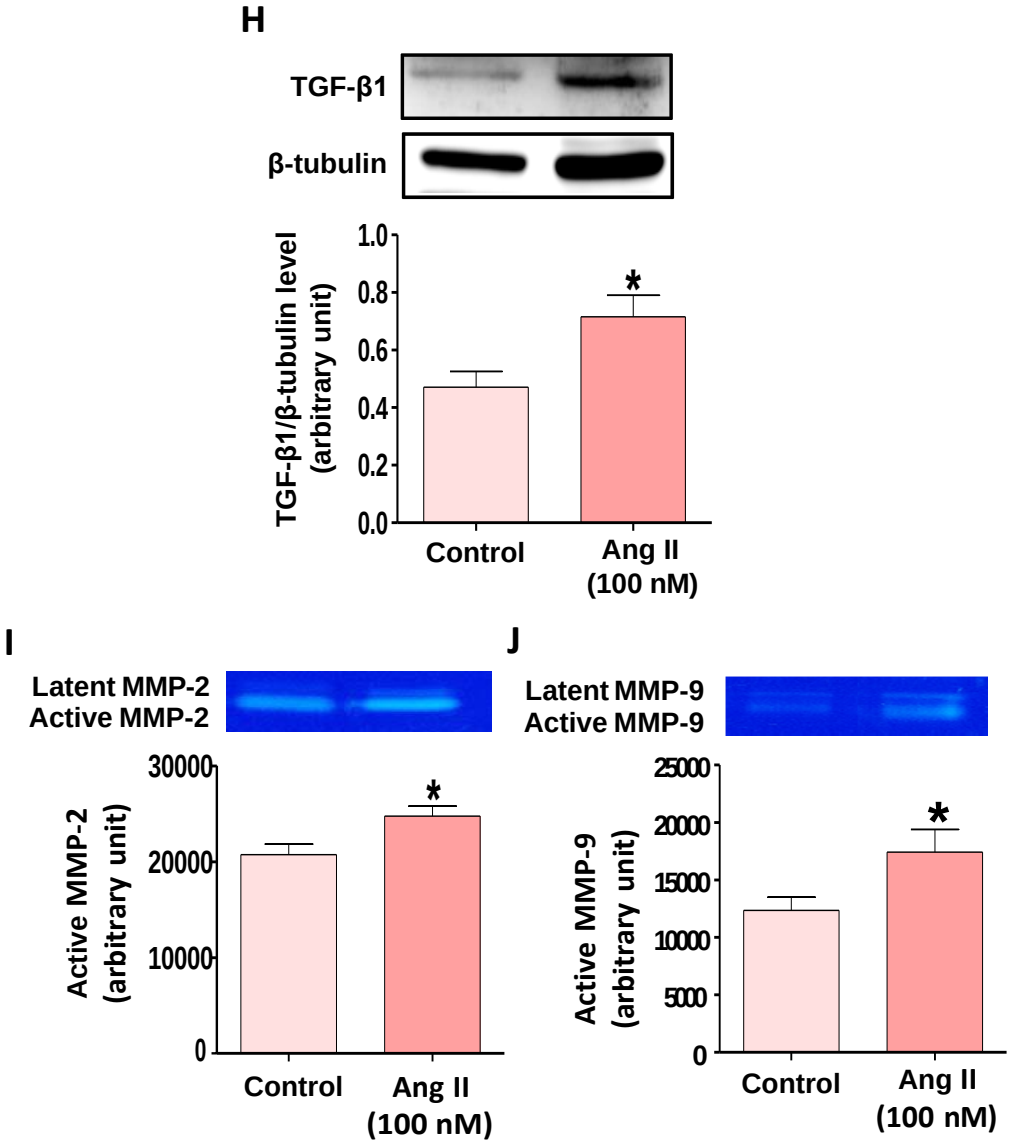
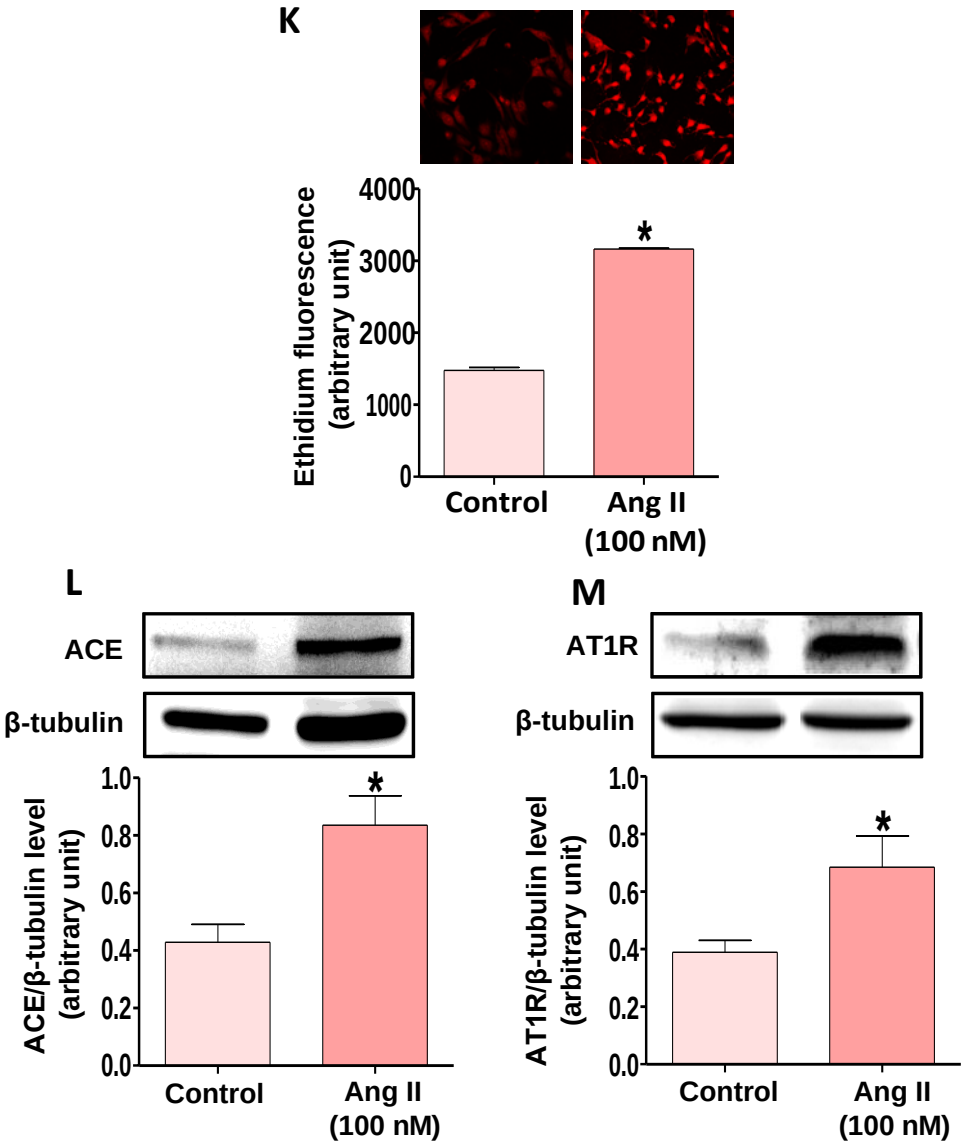

H
TGF-β1
β-tubulin
TGF-β1/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Ang II (100 nM)
I
Latent MMP-2
Active MMP-2
Active MMP-2 (arbitrary unit)
30000
20000
10000
0
*
Control
Ang II (100 nM)
J
Latent MMP-9
Active MMP-9
Active MMP-9 (arbitrary unit)
25000
20000
15000
10000
5000
0
*
Control
Ang II (100 nM)

FIGURE 8


K
Ethidium fluorescence (arbitrary unit)
4000
3000
2000
1000
0
*
Control
Ang II (100 nM)
L
ACE
β-tubulin
ACE/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Ang II (100 nM)
M
AT1R
β-tubulin
AT1R/β-tubulin level (arbitrary unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Ang II (100 nM)

Thrombin induces oxidative stress and atrial endothelial cells senescence: Impact on pro-thrombotic, pro-inflammatory, pro-fibrotic and pro-remodeling patterns

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Short title: Thrombin induces premature atrial endothelial cells ageing and thrombogenicity

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Abstract

Background: Within the atria, besides its role in thrombus formation, recent findings have highlighted that thrombin conveys 'non-hematological" functions through PAR-1 activation. This study examined the potential of thrombin to promote premature atrial endothelial cells (ECs) ageing and thrombogenicity and the shift towards pro-inflammatory, pro-fibrosis and pro-remodeling patterns.

Methods: Primary porcine atria ECs were isolated from the left atrial tissue. The level of endothelial senescence was assessed as senescence-associated beta-galactosidase (SA-β-gal) activity using flow cytometry, oxidative stress using the redox-sensitive probe dihydroethidium, protein level by Western blot analysis, and matrix metalloproteinases (MMPs) activity using zymography.

Results: Atrial endothelial senescence, as assessed by SA-β-gal activity, was induced by thrombin at clinically relevant concentrations. Likewise, thrombin induced the up-regulation of p53, a key regulator in cellular senescence, and of p21 and p16, key cyclin-dependent kinase inhibitors, in atrial ECs. NADPH oxidase, cyclooxygenases and the mitochondrial respiration complex contributed to thrombin-induced oxidative stress and senescence. Senescence-associated secretory phenotype of atrial ECs activated by thrombin was characterized by enhanced expression level of VCAM-1, tissue factor, TGF-ß and MMP-2 and 9. In addition, the pro-senescence endothelial response to thrombin was associated with an overexpression of both ACE and AT1 receptors and was inhibited by perindoprilat and losartan.

Conclusions: The present findings indicate that thrombin promotes premature ageing and senescence of atrial ECs and may pave the way to structural changes of the underlying atrial tissue by a local up-regulation of the angiotensin system and by promoting pro-inflammatory,

pro-fibrotic and pro-remodeling responses. They further suggest that targeting the angiotensin system may be of interest to delay thrombin-induced atrial endothelial senescence.

Keywords: Ageing, thrombosis, atrial fibrillation endothelium, metalloproteinase, TGF

Clinical Perspective

1) **What is known?** Arial fibrillation (AF) activates the coagulation cascade with possible formation of intra-atrial thrombi and carry an intrinsic risk of cardioembolism and ischemic stroke.
2) **What is new?** Thrombin induces atrial endothelial cells senescence and may pave the way to structural changes of the underlying atrial tissue by a local up-regulation of the angiotensin system and by promoting pro-thrombotic, pro-inflammatory, pro-fibrotic and pro-remodeling responses.
3) **What are the clinical implications?** These findings suggest that targeting thrombin and/or the angiotensin system may be of interest to delay thrombin-induced atrial endothelial senescence.

Introduction

Atrial fibrillation (AF) is the most common sustained arrhythmia especially during ageing and portends an increased risk of thromboembolism, ischemic stroke and mortality [1]. In atrial fibrillation, the classic paradigm involves thrombogenesis associated blood stasis in poorly contractile atria together with a hypercoagulable state, as witnessed by high circulating levels of fibrinolytic degradation products, plasminogen activator inhibitor (PAI)-1, thrombin-antithrombin complex and procoagulant microparticles levels (MPs; [1, 2]. Detected at high concentrations in AF patients [3] and in patients with ischemic stroke [4], circulating MPs provide within the vasculature an additional phospholipidic surface enabling the assembly of tenase and prothrombinase complexes, ultimately leading to thrombin generation [5]. Thrombin, a serine protease central to blood coagulation, converts soluble plasma fibrinogen into insoluble clot-forming fibrin polymers. Besides its role in thrombus formation, recent findings have highlighted that thrombin conveys 'non-hematological" functions through PAR-1 activation and modulates key processes involved in AF initiation and maintenance such as atrial remodeling and tissue inflammation but also altered electrical and mechanical properties of atria [6-9]. Because thrombin is short-lived in blood flow, most of its effects are exerted locally, near its site of generation within the atrium. As a consequence, neighboring endothelial cells (ECs) closed to a site of tissue injury and coagulation represent an important target of thrombin action [10]. At the interface between blood flow and atria tissue, ECs play a determinant role in the tuning of hemostatic functions but the underlying mechanisms remain poorly investigated. Recent advances in the understanding of the purported mechanisms for thrombogenesis in cardiovascular diseases have suggested a key role for endothelial senescence in the modulation of important biological responses including thrombogenicity. In the setting of AF, two recent reports have highlighted the link between senescence, AF occurrence or extent of atrial fibrosis as a marker of tissue remodeling associated to AF

maintenance [11, 12]. In addition to fibrotic remodeling of the atrial tissue, previous studies have emphasized that the senescence process is characterized by the acquisition of senescence-associated secretory phenotype (SASP) witnessed by cytokines release. In addition, besides this shift towards a pro-inflammatory pattern, we have recently demonstrated that endothelial senescence favors thrombogenicity through several mechanisms including tissue factor up-regulation, pro-coagulant MPs release, endothelial nitric oxide synthase down-regulation and reduced ECs-mediated nitric oxide-dependent inhibition of platelet aggregation [13].

In the present study, taking advantage of an original model of culture of primary atrial ECs, we sought to examine whether thrombin, could promote atrial endothelial senescence. In addition, the possibility that atrial endothelial senescence shifts the cell phenotype towards pro-thrombotic, pro-fibrotic, pro-inflammatory and pro-remodeling patterns was examined. Moreover, since angiotensin II (Ang II) via NADPH oxidase-derived oxidative stress is a potent inducer of premature endothelial senescence and ECs express both angiotensin-converting enzyme (ACE) and angiotensin type 1 receptors (AT1R, [13], the potential role of the local Ang II/AT1R in the noxious impact of thrombin on premature endothelial senescence and dysfunction was determined.

Materials and methods

Chemicals

Unless indicated, all chemicals and solvents were from Sigma-Aldrich (Sigma-Aldrich SARL, St Quentin Fallavier, France). Human thrombin was obtained from Etablissement Français du Sang-Alsace (EFS), Strasbourg, France. Losartan was obtained from Merck Research Laboratories (NJ, U.S.A) and perindoprilat from Servier, Paris, France.

Isolation and culture of atrial endothelial cells

Atrial ECs were isolated from left atria after collection of porcine hearts from the local slaughterhouse (SOCOPA, Holtzheim, France). Heart-lung blocks were dissected to obtain complete left atrium. At the level of mitral valve, left ventricle was removed and ligation of pulmonary veins was done. The luminal surface of the left atrium was cleaned and flushed with phosphate-buffered saline (PBS) without calcium to discard remaining blood. To prepare primary cultures, ECs were isolated by collagenase treatment (type 1, Worthington, 1 mg/ml for 40 min at 37 °C) and cultured in culture dishes containing MCDB131 medium (Invitrogen) supplemented with penicillin (100 UI/ml), streptomycin (100 UI/ml), fungizone (250 µg/ml), L-glutamine (2 mM, all from Lonza, St Quentin en Yvelines, France) and 15% fetal calf serum and grown for 3-4 days (passage 0). The medium was changed every 48 h. Premature atrial ECs senescence was induced at passage 1 by incubating cells with either thrombin (1 or 3 U/ml) or Ang II (100 nM) for 24 h in serum-free medium 15 h after seeding. In some experiments, pharmacological modulators were added to atrial ECs, 30 min before adding thrombin (1 U/ml).

Determination of SA-β-gal activity by flow cytometry

SA-β-galactosidase activity was determined by flow cytometry using the fluorogenic substrate C_{12}FDG (5-dodecanoylaminofluorescein Di-β-D-galactopyranoside, Invitrogen, Life Technology, SAS) as described previously [14]. Atrial ECs were pretreated with 300 µM chloroquine for 1 h to induce lysosomal alkalinization. C_{12}FDG (33 µM) was then added to the incubation medium (without phenol red) for 1 h. At the end of the incubation period, ECs were washed with ice-cold PBS, re-suspended following trypsinization and analyzed using a flow cytometer (FACScan, BD Bioscience, CA, USA). Data were acquired and analyzed using the Cellquest software (Becton Dickinson). Light scatter parameters were used to eliminate dead cells and subcellular debris. The C_{12}-fluorescein signal was measured on the

FL1 detector, and the proportion of ECs with SA-β-gal activity was estimated using the median fluorescence intensity of the population. Autofluorescence assessed in parallel in atrial ECs not exposed to C_{12}FDG was negligible.

Western blot analysis

Atrial ECs were washed with PBS and then lysed in extraction buffer (composition in mM: NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), Tris/HCl 20 (pH 7.5; QBiogene), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen)). Equal amounts of proteins were separated on denaturing SDS (10-12%) polyacrylamide gel. Separated proteins were transferred electrophoretically onto nitrocellulose membranes (GE Healthcare Life Sciences). Blots were blocked at room temperature with 5% bovine serum albumin in PBS plus 0.1% Tween 20 (Sigma). For detection of proteins, membranes were incubated with the respective primary antibody: mouse monoclonal anti-eNOS (diluted 1:2500; BD Transduction Laboratories; cat. nº #610297), rabbit polyclonal anti-p53 (diluted 1:1.000; Santa Cruz; cat. nº SC-6243), mouse monoclonal anti-p21 (diluted 1:1000; Santa Cruz; cat. nº SC-817), rabbit polyclonal anti-p16 (diluted 1:1000; Delta Biolabs; cat. nº DB018), mouse monoclonal anti-tissue factor (TF, diluted 1:5000; Sekisui Diagnostics, LLC; cat. nº 4509), rabbit polyclonal anti-VCAM-1 (diluted 1:5000; Abcam; cat. nº ab134047), mouse monoclonal anti-tranforming growth factor β (TGF-β1, diluted 1:2500; Abcam; cat. nº ab27969), rabbit polyclonal anti-cyclooxygenase-1 (COX-1, diluted 1:1.000; Abcam; cat. nº ab109025), rabbit polyclonal anti-COX-2 (diluted 1:1000; Abcam; cat. nº ab15191), rabbit polyclonal anti-angiotensin-converting enzyme (ACE, diluted 1:1000; Abbiotec; cat. nº 250450), rabbit polyclonal anti-angiotensin type 1 receptor (AT1R, diluted 1:1000; Abcam cat. nº ab124505), or mouse monoclonal anti-β-tubulin (diluted 1:20000; Sigma-Aldrich; cat. nº T7816) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or

anti-mouse immunoglobulin G, dilution of 1:5000; Cell Signaling Technology; cat. nº #7074, #7076, respectively) at room temperature for 60 min. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunocomplexes were detected by chemiluminescence reaction (ECL; Amersham, Les Ulis, France) followed by densitometric analysis using the software Image J.

Determination of oxidative stress

Atrial ECs were seeded in Millicell EZ SLIDE 8-well glass slide for 15 h, before exposure to serum-free MCDB 131 (Invitrogen) for 2 h. The redox-sensitive fluorescent dye dihydroethidium (DHE) was used to evaluate the formation of reactive oxygen species (ROS; [15]. ECs were incubated with DHE (5 μM) for 20 min at 37 °C in a light protected manner. To determine the sources of ROS, cells were untreated or exposed to either N-acetyl cysteine (NAC, an antioxidant, 1 mM, 3 h), VAS-2870 (VAS, a NADPH oxidase inhibitor, 1 μM, 30 min), indomethacin (INDO, a cyclooxygenase inhibitor, 10 μM, 30 min), SC-560 (a cyclooxygenase-1 inhibitor, 0.3 μM, 30 min), NS-398 (a cyclooxygenase-2 inhibitor, 3 μM, 30 min), mitochondrial inhibitory complex (Rotenone, 1 μM, 30 min; KCN, 1 μM, 30 min; Myxothiazol, 0.5 μM, 30 min) before the addition of thrombin (1 U/ml) for 1 h in serum-free medium. In some experiments, ECs were incubated with either losartan (10 μM) or perindoprilat (10 μM) 30 min before the addition of thrombin (1 U/ml) for 24 h. ECs were then washed and mounted with fluorescent mounting medium (DAKO, S3023) and examined under confocal microscope (Leica SP2 UV DM Irbe). Images were analyzed using Image J software.

Determination of MMP activity by zymography

MMP-2 and MMP-9 activities in conditioned medium of cultured atrial ECs were analyzed by substrate-gel electrophoresis (zymography) with the use of SDS-PAGE (8%) containing 0.1%

gelatin, as described previously [16]. Then, gels were washed in denaturing buffer for 30 min and incubated in developing buffer for 24 h at 37 ˚ C. Thereafter, gels were stained in 30 % Coomassie blue for 30 min and then destained with destaining buffer. Gelatinolytic activity appeared as bands with blue background. Images were taken and bands were analyzed using Image J software.

Statistical analyses

Data are presented as mean ± SEM of n different experiments. Mean values were compared using Student's paired *t* test or an analysis of variance followed by the post-hoc Bonferroni test to identify significant differences between treatments using GraphPad Prism (v5.0). The difference was considered to be significant when the *P* value was less than 0.05.

Results

Thrombin induces atrial endothelial cells senescence

Thrombin induced atrial ECs senescence as depicted by the increased level of SA-beta-gal activity, in a concentration-dependent manner (Figure 1A). Likewise, thrombin induced the up-regulation of the protein expression level of p53, a key regulator in cellular senescence, and of p21 and p16, key cyclin-dependent kinase inhibitors, in atrial ECs (Figures 1B-D). Similar responses were also observed in atrial ECs in response to Ang II, a strong inducer of premature endothelial senescence (Figure 1).

Thrombin increases oxidative stress within atrial endothelial cells

Since reactive oxygen species (ROS) are strong inducers of senescence [13], experiments were performed to determine whether oxidative stress is involved in thrombin-induced premature senescence using DHE. Indeed, thrombin (1 U/mL) increased the level of ethidium

fluorescence in ECs (Figure 2A). The source of ROS was characterized using inhibitors of major vascular sources of ROS including NADPH oxidase (VAS-2870), cyclooxygenases (COXs, indomethacin, INDO), COX-1 (SC-560), COX-2 (NS-398), and the mitochondrial respiration complex (MIT INH), and the antioxidant N-acetylcysteine (NAC). All pharmacological tools blunted the thrombin-induced formation of ROS (Figure 2A). Similarly, NAC, VAS-2870, INDO and MIT also prevented the thrombin-induced SA-ß-gal activity (Figure 2B). These findings suggest that NADPH oxidase, COXs and the mitochondrial respiration complex contribute to thrombin-induced oxidative stress and senescence in atrial ECs. In addition, Western blot analysis indicated that thrombin up-regulated COX-2, but not COX-1, in atrial ECs (Figure 3).

Critical role of the local angiotensin system in thrombin-induced oxidative stress in atrial ECs

Since many reports have underscored that endothelial senescence is critically dependent on the redox activation by the local angiotensin system [13], experiments were performed to examine the interplay between thrombin and the local activation of the angiotensin system. We first established that thrombin increased the expression level of ACE and AT1R in atrial ECs (Figures 4A, B). To determine whether Ang II mediates the thrombin-induced ECs oxidative stress, we examined the effect of losartan (AT1R antagonist) and perindoprilat (ACE inhibitor). The induction of oxidative stress-mediated senescence by thrombin was significantly blunted by pretreatment with the ACE inhibitor and also the AT1R antagonist (Figure 4C-D).

Thrombin induces endothelial dysfunction, and pro-thrombotic and pro-inflammatory pattern

Because endothelial senescence is characterized by endothelial dysfunction and a reduced formation of NO [13], the effect of thrombin on eNOS protein expression was examined. A decreased in eNOS expression level was evidenced when ECs were exposed to thrombin (3 U/mL) to the same extent that induced by Ang II (100 nM, Figure 5A). To underscore pro-thrombotic and pro-inflammatory pattern associated with ECs senescence, VCAM-1 and TF expression was examined. A concentration-dependent increased in VCAM-1 and TF expression level was observed in atrial ECs in response to thrombin and Ang II (Figures 5B,C).

Thrombin increases pro-fibrotic and pro-remodeling responses

Since atrial remodeling is a key feature of AF [7], we investigated the link between thrombin-induced ECs senescence, TGF-ß, and the metalloproteinases MMP-2 and MMP-9. Previous studies have emphasized that TGF-ß is involved in the remodeling of extracellular matrix and is up-regulated in AF [17]. As shown in Figure 6A, thrombin induced a concentration-dependent increase in the expression level of TGF-ß. In addition [18], experiments were performed to determine the impact of thrombin on the expression level of active MMP-2 and 9. As depicted in Figures 6B and C, thrombin-increased the active MMP-2 and 9 expression levels to a greater extent as Ang II in atrial ECs. Altogether, these findings indicate that thrombin-induced atrial endothelial senescence contributes to the acquisition of a pro-fibrotic and pro-remodeling pattern.

Discussion

The salient findings of the present study are as follows (i) thrombin induces oxidative stress and premature atrial endothelial senescence, (ii) phenotypical changes associated with thrombin-induced atrial endothelial senescence comprise the acquisition of pro-thrombotic,

pro-adhesive, pro-fibrotic and pro-remodeling patterns, (iii) pro-senescence endothelial response to thrombin is associated with an overexpression of both ACE and ATR1 and inhibited by perindoprilat and losartan, and (iv) the local angiotensin system acts as an important amplifying system to accelerate the acquisition of senescence-associated secretory phenotype. Altogether, these findings substantiate the view that thrombin, beyond its role in the coagulation process, shifts the atrial endothelium phenotype towards pro-thrombotic, pro-inflammatory, pro-fibrotic and pro-remodeling patterns and thereby, could contribute to further worsen the function of the atrial tissues paving the way to AF perpetuation.

Recent data have challenged the classical causality of AF causing hypercoagulability by demonstrating that hypercoagulability *per se* induces pro-fibrotic and pro-inflammatory responses in adult atrial fibroblasts contributing to structural remodeling in the atria [6, 7, 9, 19]. At the interface between blood flow and atrial tissue, the precise role of endothelium in response to thrombin activation remains conspicuously unexplored. Up to now, the understanding of endothelial dysfunction during AF was mainly based on studies performed on human umbilical vein ECs (HUVECs). To circumvent the possibility that endothelial function may vary according to the vascular bed, we have developed an original model of primary cultures of porcine atrial ECs, and the possibility that thrombin acts as a key effector of premature atrial senescence was examined. The present findings indicate that thrombin, at relevant concentrations achieved during vascular injury associated to thrombus formation [20], induced oxidative stress, premature ageing of atrial ECs and enhanced thrombogenicity. Using several pharmacological tools, the thrombin-induced oxidative stress in atrial ECs has been shown to involve several major sources of ROS including NADPH oxidase, cyclooxygenases, and the mitochondrial respiratory chain, and to promote ECs senescence. These pro-oxidant sources have also been involved in replicative senescence in coronary artery ECs [13], and both NADPH oxidase and the mitochondria are involved in endothelial

MPs-mediated oxidative stress in mouse aortic ECs [21]. In other cell lineages (HUVECs), another group group has recently established that activated Factor Xa, another mediator of hypercoagulability, induces endothelial senescence through the up-regulation of insulin-like growth factor binding protein 5 and early growth response protein1 [22].

The impact of thrombin on the expression of eNOS remains highly controversial. Previous reports have suggested that thrombin activates PAR-1 and stimulates eNOS with subsequent NO formation and vascular relaxation [23, 24]. By contrast, in the setting of paroxysmal AF, Akar et al., have demonstrated that the acute onset of AF in humans is associated within minutes with thrombin generation and concomitant platelet aggregation together with a swift decreased in NO formation as a marker of endothelial dysfunction [25]. Likewise, in rabbits, short term paroxysmal AF was evidenced to induce oxidative stress, thrombin generation and endothelial dysfunction [26]. Consistent with these findings, we could establish that thrombin induced in a concentration-dependent manner a significant decreased of eNOS expression in atrial ECs. Another characteristic feature of the acquisition of a pro-thrombotic pattern by the senescent atrial ECs is represented by an enhanced expression of tissue factor. Altogether, it is therefore likely that at the senescent atrial endothelial surface, both blunted NO-mediated inhibition of platelet aggregation and enhanced tissue factor expression contribute to the acquisition of a pro-thombotic pattern ultimately leading not solely to thrombin generation but also to amplification loops favoring further endothelial senescence.

Besides its role in thrombus formation, recent data have highlighted that “non hemostatic effects” mediated by thrombin through PAR-1 activation could perpetuate AF by inducing inflammatory burden, atrial remodeling and modulation of the electrophysiological characteristics of pulmonary vein and/or left atrium tissue [7-9]. The relevance of these findings was for instance emphasized by the demonstration that hypercoagulability promotes the development of a substrate for AF in transgenic mice and in goats with persistent AF [9]. The

major importance of this pathway is demonstrated by the fact that pro-remodeling effects of thrombin could be blunted by pharmacological blockade of thrombin using dabigatran. In isolated rat atrial fibroblasts, recent findings by Spronk et al., has depicted that, thrombin enhanced the phosphorylation of the pro-fibrotic signaling AKT and ERK pathways, and increased the expression of TGF-ß and the pro-inflammatory factor monocyte chemo-attractant protein-1 [27]. The present findings extent these data by demonstrating that atrial ECs, when subjected to pro-senescent stimuli, constitute another importance source of bio-effectors involved in inflammatory cell infiltration, fibrosis and extracellular matrix proteolysis. Because central to the characterization of endothelial dysfunction is the expression of various cytoadhesins or selectins together with the production of proinflammatory chemokines, we investigated the impact of thrombin-induced endothelial senescence on VCAM-1 expression. Thrombin was evidenced as a potent inducer of VCAM-1 expression by senescent atrial ECs that may promote inflammatory cells infiltration within atrial tissues. Such an observation extends previous findings demonstrating that thrombin induces endothelial NF-κB-dependent expression of ICAM-1 and VCAM-1 in SVEC4 mouse ECs [10]. VCAM-1 is an 81 kDa sialoglycoprotein expressed by cytokine-activated vascular endothelium, that mediates cell adhesion and transendothelial diapedesis, and was recently evidenced as a potent marker of post operative AF [28]. Other data have underlined that both inflammation and oxidative stress are responsible of electrical and structural changes that promoted increased automaticity and autonomic dysfunction leading to an increased risk of AF [29, 30].

Another important determinant of structural changes occurring during AF is represented by TGF-ß. Recent data obtained by transgenic goat model overexpressing TGF-ß1 have emphasized the view that cardiac overexpression of TGF-ß leads to increased fibrosis within the atria tissue and favors not only P wave prolongation but also AF vulnerability [31]. Other reports have previously established that mice that overexpress TGF-ß1 have pronounced atrial

fibrosis and increased susceptibility to AF induction via rapid atrial pacing [32]. In human, senescence markers such as SA-ß-gal activity and p16 were positively correlated with the extent of atrial fibrosis [12]. The impact of thrombin on endothelial TGF-ß expression remains controversial. The present demonstration of significant up-regulation of TGF-ß expression on thrombin-induced senescent atrial ECs challenges previous reports by Tang and coworkers describing a down-regulation of TGF-ß signaling on aortic or venous human ECs when submitted to thrombin stimulation [33]. Recent data by Altieri et al., have underlined that sustained stimulation by thrombin induced the synthesis of TGF-ß by human atrial fibroblasts, which was counteracted by dabigatran, a direct thrombin inhibitor [6]. Collectively, these novels data reinforce the view that atrial tissues including atrial ECs constitute a potent source of pro-fibrotic mediator TGF-ß when submitted to thrombin stimulation.

Another important regulator of structural changes during AF is represented by MMPs. For instance, it was recently established that fibrosis remodeling as assessed by total collagen in the left atrium is positively correlated to pro-fibrotic cytokines but also MMP-2 and 9 [34]. The striking increase of atrium content in MMP-2 and 9 described in AF patients contrasting with normal content of MMP-1 and 3 emphasizes the paradigm that MMP-2 and 9 are key effectors of matrix remodeling during AF [35]. Our observation of a thrombin concentration-dependent up-regulation of active MMP-2 and 9 by senescent atrial ECs is in line with former studies performed on rat cardiac fibroblasts [36]. By contrast, in human atrial fibroblasts, thrombin was found to mediate opposite effects by decreasing MMP-2 activity [6] (Although species dissimilarities in the nature of atrial tissue response to thrombin appear likely and hamper general extrapolation, our findings substantiate the view that the senescent atrial ECs may participate to extracellular remodeling when subjected to thrombin stimulation. Previous data have highlighted that enhanced fibrosis, as a consequence of TGF-ß signaling stimulation, paralleled the increase in collagen deposition and alterations in extracellular

matrix remodeling by MMPs. These structural alterations of the atrium tissue pave the way to perpetuation of arrhythmia because fibrosis is a determinant mechanism in the disruption of connectivity between myocytes and impair normal electrical conduction, thereby decreasing the wavelength of reentry [37].

Another important determinant of structural changes associated to AF is represented by the angiotensin system [37]. Epidemiological studies have extensively demonstrated the association between AF and hypertension or elevated Ang II [38]. The fact that Ang II is a potent inducer of endothelial senescence in several cell lineages including coronary artery ECs and also atrial ECs, and because ECs express high levels of ACE promoting Ang II formation, prompted us to test the hypothesis that the local angiotensin system may be involved in the pro-senescent effect of thrombin. We could establish that thrombin induced both ACE but also AT1R expression in atrial ECs. The importance of the local activation of the angiotensin system by thrombin was emphasized by the demonstration that losartan, an AT1R antagonist, and also perindoprilat, an ACE inhibitor, blunted thrombin-induced oxidative stress and induction of ECs senescence. Altogether, these findings highlight a pivotal role of the local angiotensin system in the thrombin-mediated induction of premature endothelial senescence via activation of AT1R. Collectively, our data substantiate a new paradigm linking thrombin and AF to each other, in a vicious amplification loop where AF favors thrombin generation and thrombin *per se*, by inducing premature ageing of the atrial ECs, shifts the phenotype of senescent endothelial cells towards pro-thrombotic pro-thrombotic, pro-inflammatory, pro-fibrotic and pro-remodeling patterns, promoting structural changes of the atrial tissue and AF maintenance.

In conclusion, the present findings indicate that thrombin promotes premature ageing and senescence of atrial ECs and may pave the way to structural changes of the underlying atrial tissue by a up-regulation of the local angiotensin system and by promoting pro-inflammatory,

pro-fibrotic and pro-remodeling responses. They further suggest that targeting the angiotensin system may be of interest to delay thrombin-induced endothelial atrial senescence.

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Figure legends

Figure 1. Thrombin and Ang II induce senescence in atrial ECs at passage 1 and are associated with an up-regulation of major cell cycle regulatory proteins: p53, p21 and p16. Atrial ECs were either untreated or exposed to thrombin (1 or 3 U/ml) or Ang II (100 nM) for 24 h before determination of senescence by SA-β gal activity (A), protein expression level of (B) p53, (C) p21 and (D) p16 as assessed by Western blot analysis. Results are presented as representative immunoblots (upper panels), and corresponding cumulative data (lower panels) and are shown as ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control.

Figure 2. Thrombin induces oxidative stress promoting senescence in atrial ECs. (A) Atrial ECs were either untreated or exposed to N-acetylcysteine (NAC, an antioxidant, 1 mM, 3 h), VAS-2870 (VAS, NADPH oxidase inhibitor, 5 μM, 30 min), indomethacin (INDO, COX inhibitor, 30 μM, 30 min), SC-560 (COX-1 inhibitor, 0.3 μM, 30 min), NS-398 (COX-2 inhibitor, 3 μM, 30 min) or a mitochondrial inhibitory complex (MIH; rotenone, 1 μM, 30 min; KCN, 1 μM, 30 min; myxothiazole, 0.5 μM, 30 min) before the additon of thrombin (1 U/ml, 1 h) and DHE (5 μM) during the last 20 min to determine the level of oxidative stress by confocal microscopy. Upper panels represent ethidium staining; lower panel corresponding cumulative data. (B) Atrial ECs were either untreated or exposed to NAC, VAS, INDO or MIH before the addition of thrombin (1 U/ml, 24 h) and subsequent determination of SA-β-gal activity using flow cytometry. Results are shown as mean ± SEM of n = 3-4 different experiments. *P < 0.05 versus respective control, $^{\#}$P < 0.05 versus thrombin-treated atrial ECs.

Figure 3. Thrombin and Ang II induce the expression of cyclooxygenase-2 in atrial ECs. Atrial ECs were either untreated or exposed to thrombin (1 or 3 U/ml) or Ang II (100 nM) before determination of the expression level of (A) COX-1 and (B) COX-2 as assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels) and shown as mean ± SEM, n = 3-4, *P < 0. 05 versus respective control.

Figure 4. Thrombin-induced senescence promotes up-regulation of the local angiotensin system in atrial ECs. Atrial ECs were either untreated or exposed to losartan (an AT1R antagonist, 10 μM) or perindoprilat (ACE inhibitor, 10 μM) for 30 min before the addition of thrombin (1 U/ml) or Ang II (100 M) for 24 h, and the subsequent determination of the expression level of target proteins (A, B) as assessed by Western blot analysis, (C) oxidative stress by confocal microscopy, and (D) SA-β-gal activity using flow cytometry. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels) and shown as mean ± SEM, n = 3-4, *P < 0. 05 versus respective control, $^{\#}$P < 0.05 versus thrombin-treated atrial ECs.

Figure 5. Thrombin- and Ang II-induced senescence promotes pro-inflammatory and pro-coagulant phenotype in atrial ECs. Atrial ECs were untreated or exposed to either thrombin (1 or 3 U/ml) or Ang II (100 nM) before determination of the expression level of target proteins as assessed by Western blot analysis. Results are shown as representative immunoblots (upper panels) and corresponding cumulative data (lower panels) and shown as mean ± SEM, n = 3-4, *P < 0. 05 versus respective control.

Figure 6. Thrombin- and Ang II-induced senescence promotes pro-fibrotic phenotype in atrial ECs. Atrial ECs were untreated or exposed to either thrombin (1 or 3 U/ml) or Ang II (100 nM) before determination of the expression level of TGF-ß (A) as assessed by Western blot analysis, and MMP-2 and 9 activities by zymography (B, C). Results are shown as representative immunoblots or gelatinolytic activity (upper panels) and corresponding cumulative data (lower panels) and shown as mean ± SEM, n = 3-4, *P < 0. 05 versus respective control.

Figure 1

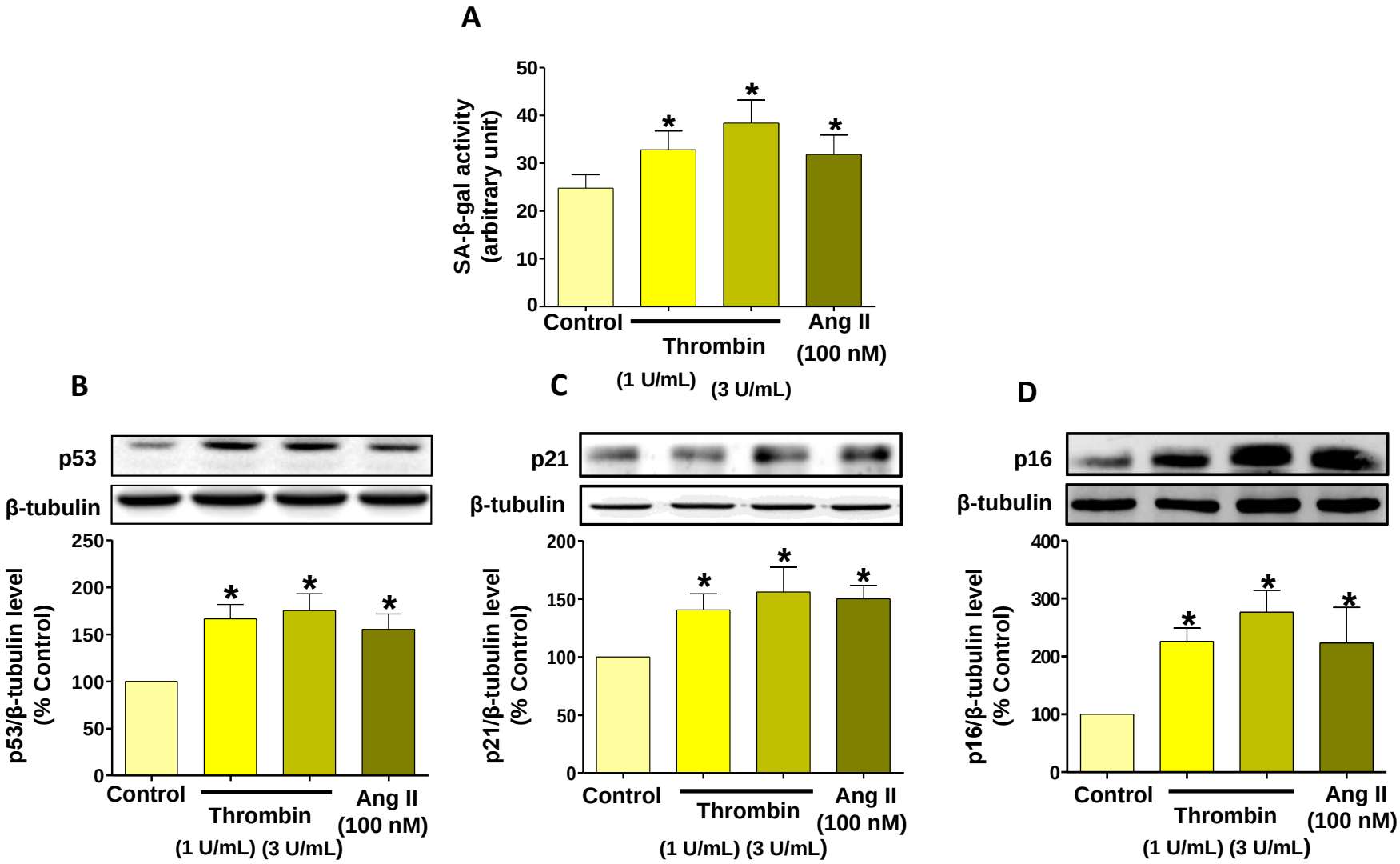

A
SA-β-gal activity (arbitrary unit)
50
40
30
20
10
0
Control
Thrombin
(1 U/mL)
(3 U/mL)
Ang II (100 nM)
B
p53
β-tubulin
p53/β-tubulin level (% Control)
250
200
150
100
50
0
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II (100 nM)
C
p21
β-tubulin
p21/β-tubulin level (% Control)
200
150
100
50
0
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II (100 nM)
D
p16
β-tubulin
p16/β-tubulin level (% Control)
400
300
200
100
0
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II (100 nM)

Figure 2

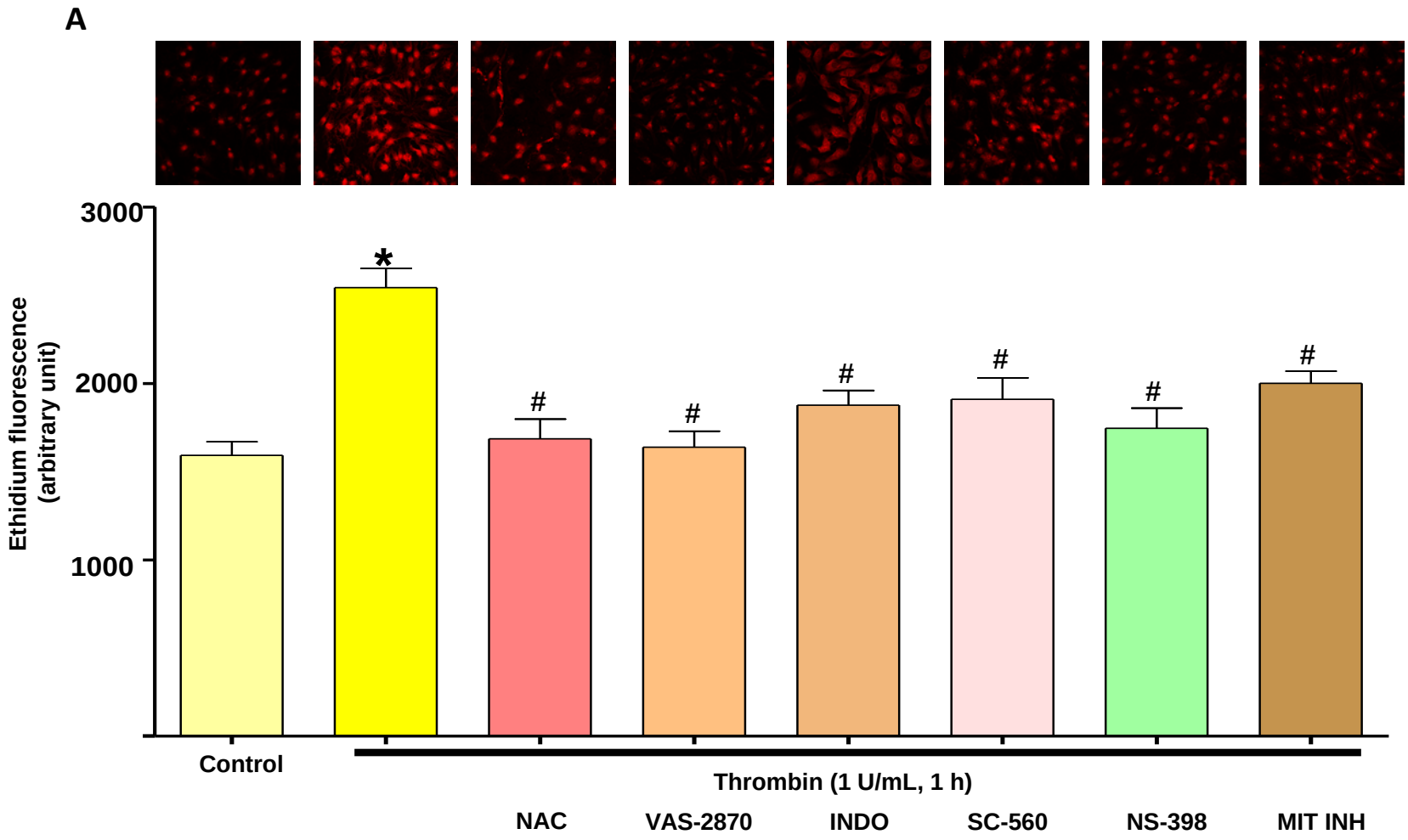

A
3000
2000
1000
Ethidium fluorescence
(arbitrary unit)
*
#
#
#
#
#
#
Control
Thrombin (1 U/mL, 1 h)
NAC
VAS-2870
INDO
SC-560
NS-398
MIT INH

Figure 2

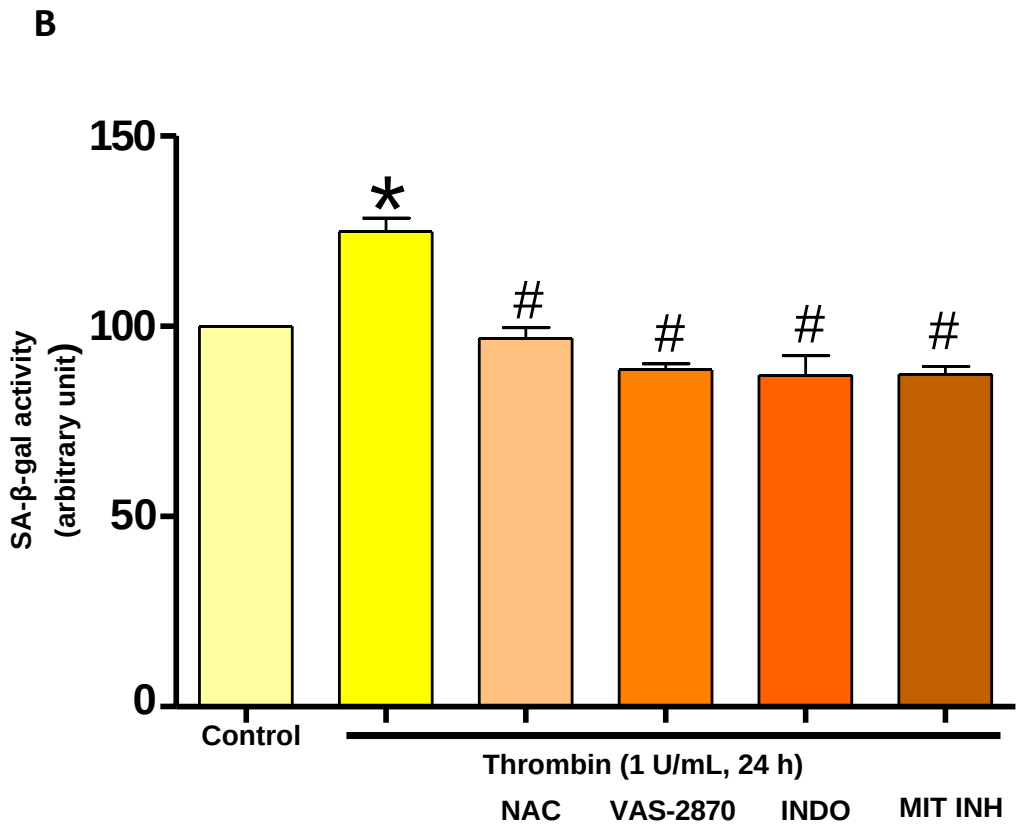

B
150
100
50
0
SA-β-gal activity
(arbitrary unit)
*
#
#
#
#
Control
Thrombin (1 U/mL, 24 h)
NAC
VAS-2870
INDO
MIT INH

Figure 3

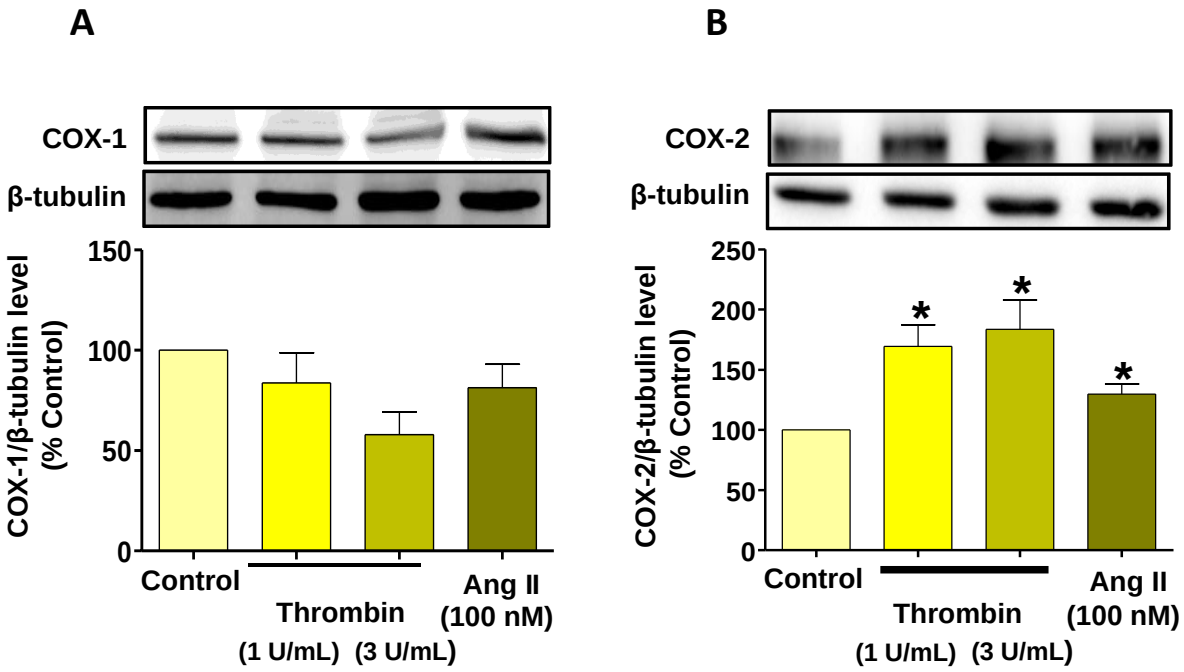

A
COX-1
β-tubulin
COX-1/β-tubulin level
(% Control)
150
100
50
0
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II
(100 nM)
B
COX-2
β-tubulin
COX-2/β-tubulin level
(% Control)
250
200
150
100
50
0
*
*
*
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II
(100 nM)

Figure 4

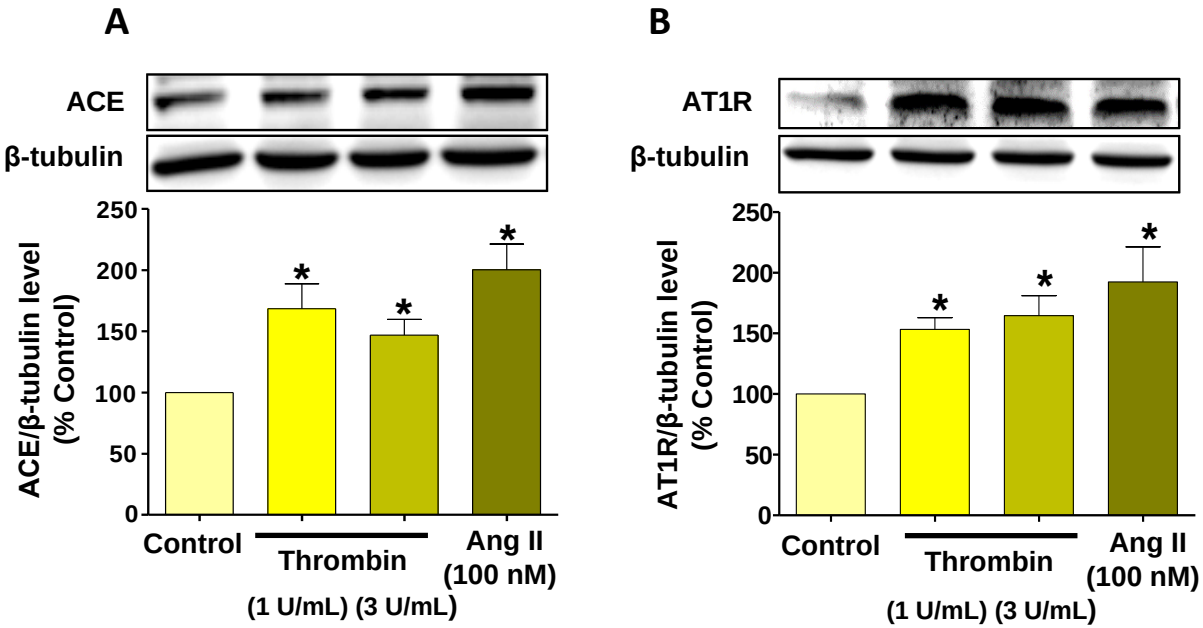

A
ACE
β-tubulin
ACE/β-tubulin level
(% Control)
250
200
150
100
50
0
*
*
*
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II
(100 nM)
B
AT1R
β-tubulin
AT1R/β-tubulin level
(% Control)
250
200
150
100
50
0
*
*
*
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II
(100 nM)

Figure 4

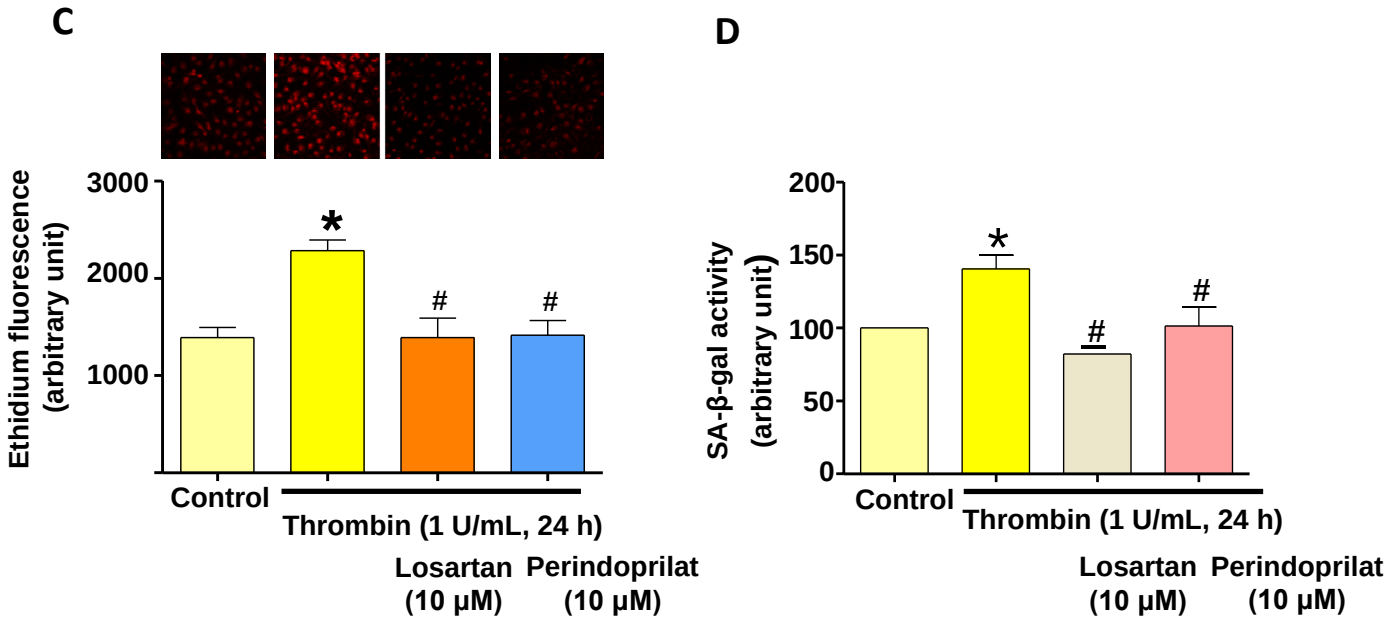

C
Ethidium fluorescence (arbitrary unit)
3000
2000
1000
*
#
#
Control
Thrombin (1 U/mL, 24 h)
Losartan (10 μM)
Perindoprilat (10 μM)
D
SA-β-gal activity (arbitrary unit)
200
150
100
50
0
*
#
#
Control
Thrombin (1 U/mL, 24 h)
Losartan (10 μM)
Perindoprilat (10 μM)

Figure 5

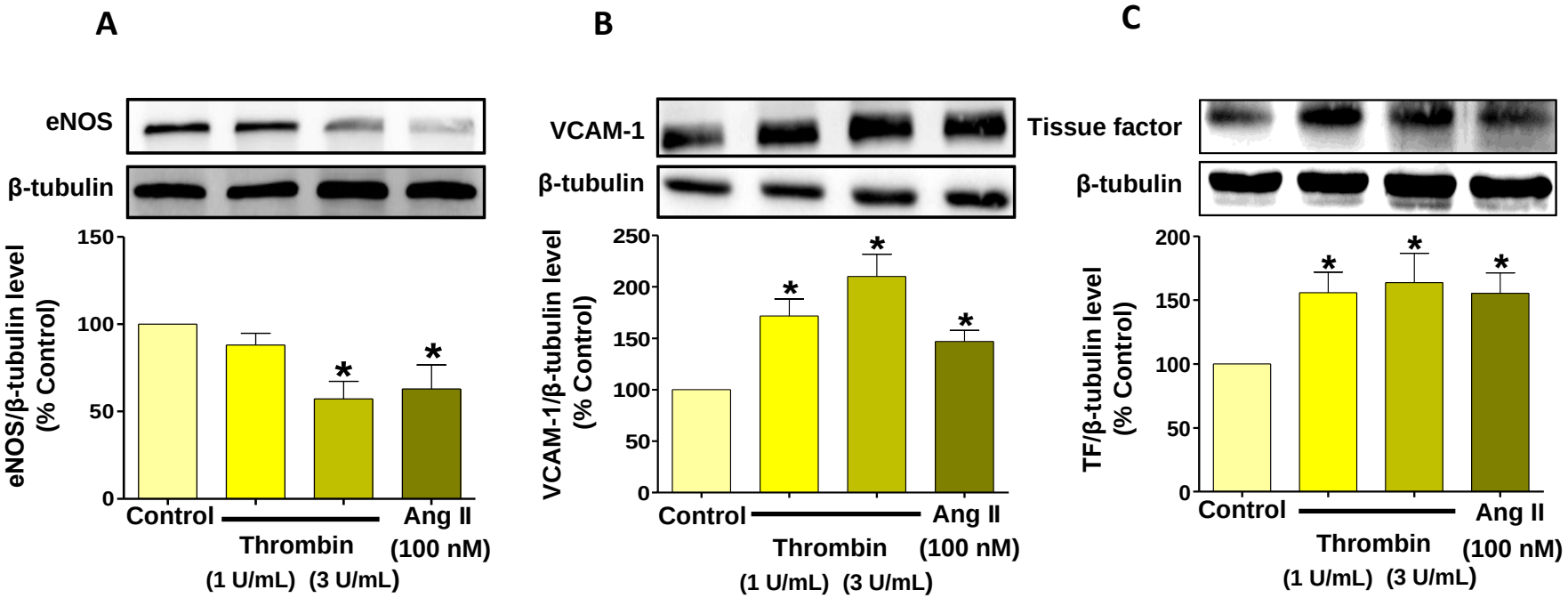
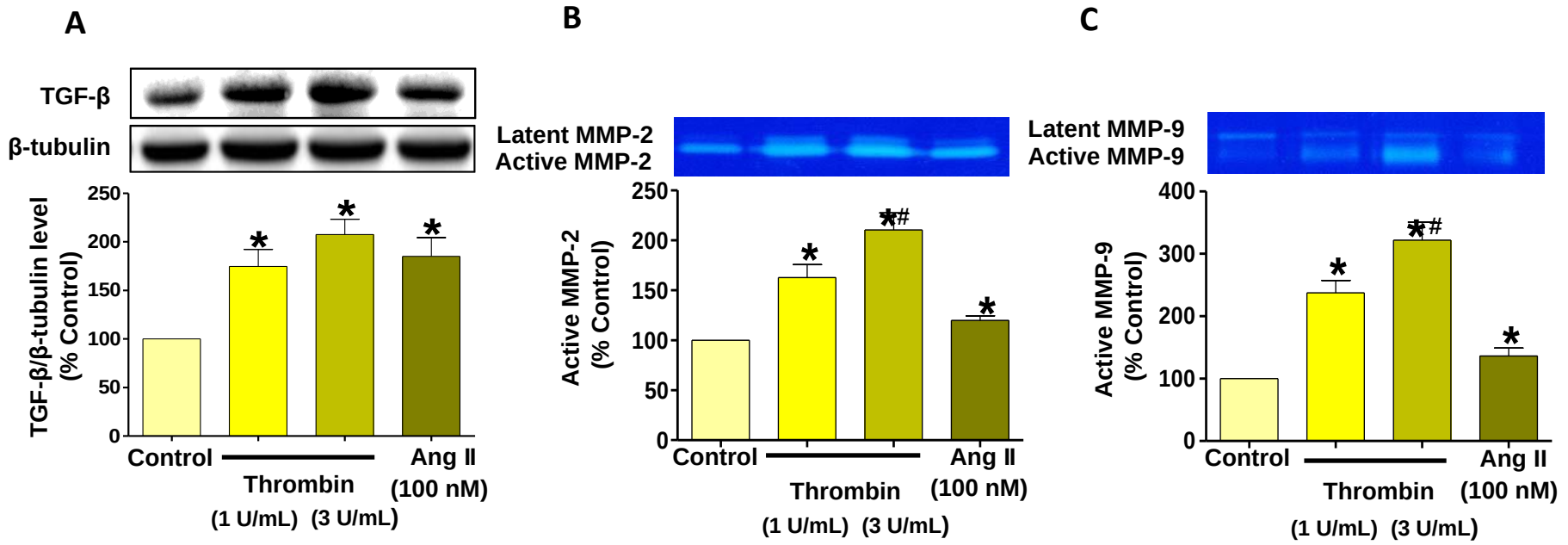

A
eNOS
β-tubulin
eNOS/β-tubulin level (% Control)
150
100
50
0
Control
Thrombin (1 U/mL) (3 U/mL)
Ang II (100 nM)
B
VCAM-1
β-tubulin
VCAM-1/β-tubulin level (% Control)
250
200
150
100
50
0
Control
Thrombin (1 U/mL) (3 U/mL)
Ang II (100 nM)
C
Tissue factor
β-tubulin
TF/β-tubulin level (% Control)
200
150
100
50
0
Control
Thrombin (1 U/mL) (3 U/mL)
Ang II (100 nM)

Figure 6


A
TGF-β
β-tubulin
TGF-β/β-tubulin level (% Control)
250
200
150
100
50
0
Control
Thrombin
(1 U/mL) (3 U/mL)
Ang II (100 nM)
B
Latent MMP-2
Active MMP-2
Active MMP-2 (% Control)
C
Latent MMP-9
Active MMP-9
Active MMP-9 (% Control)
400
300

DISSCUSSION AND PERSPECTIVES

DISCUSSION

The present study provides a new paradigm in which atrial endothelial cells (AECs) senescence enhances thrombogenicity, inflammation, matrix remodeling and the up-regulation of the local angiotensin system. The findings further indicate that thrombin, in addition to its role in the coagulation process, play an important role in the induction of AECs premature senescence resulting in endothelial dysfunction associated with the down-regulation of the protective NO pathway and the induction of pro-infiltrative and pro-fibrotic responses. Altogether, these data provides novel mechanistic insights by which, thrombin as a result of hypercoagulability, may promote tissue remodeling and favour AF susceptibility. Our study also demonstrates a significant role of the local angiotensin system in the perpetuation of senescence-associated secretory phenotype. This can be documented by the fact that both perindoprilat (ACE) and losartan (AT1R) prevented AECs senescence.

Atrial fibrillation AF has now become a serious epidemic across the world as its incidence is expected to double during the next 20 years besides the considerable progression in the diagnosis and treatment of AF (Andrade et al., 2014; Chugh et al., 2014). AF has a pronounced societal burden in terms of diminished quality of life, cost of care, risk for stroke, heart failure, dementia, and mortality. The prevalence of AF is strongly age dependent (Boriani, 2016) and a hallmark of aging is short telomere length. Several studies also suggest the possible role of short telomere length in AF (Carlquist et al., 2016; Denil et al., 2014). Replicative senescence which is associated with DNA damage and telomere erosion plays a fundamental role in aging and accompanied with cardiomyocyte hypertrophy, increased apoptosis and myocardial fibrosis (Cannata et al., 2016). On the other hand, premature senescence primarily with the focus to the elimation of damaged cells, is characterized by an irreversible form of cell cycle arrest (Campisi and d'Adda di Fagagna, 2007; Cannata et al., 2016; Munoz-Espin and Serrano, 2014). *In vivo*, endothelial cell senescence has been documented in several types of pathological arteries including overlying atherosclerotic plaques of human coronary arteries and thoracic aortas of patients with ischemic heart diseases (Minamino et al., 2002). One study conducted on spontaneously hypertensive rats has shown high levels of vascular senescence in the aortic arch (Han et al., 2012) and in another study in the aorta of diabetic rats (Chen et al., 2002). Previous studies conducted with cultured cells have shown that endothelial cell senescence is associated with the down-regulation of endothelial eNOS, the induction of a proinflammatory state, and DNA damages (Botden et al., 2012). In another study it

was found that the overexpression of endothelial p53 induces endothelial dysfunction and decrease nitric oxide (NO) bioavailability in rat aortic sections, and the down-regulation of eNOS in cultured endothelial cells (Kumar et al., 2011).

The identification of senescent cells, both in culture and in pathological arteries, relies predominantly on biomarkers and/or effectors of senescence including increased SA-β-gal activity (Abbas et al., 2017; Dimri et al., 1995) and the up-regulation of p53/p21 and/or p16-retinoblastoma signaling pathways (Khemais-Benkhiat et al., 2016b; Silva et al., 2017). Also, several lines of evidence suggest that the decreased NO bioavailability is a crucial early event triggering the signal transduction cascade leading ultimately to endothelial senescence. In good agreement with these previous observations, the present models of replicative endothelial senescence (induced by serial passaging of AECs) and premature endothelial senescence (induced by L-NAME, H_2O_2, Ang II and thrombin) showed an increased proportion of senescent AECs as assessed by SA-β-gal activity and the down-regulation of the expression of eNOS that was associated with the up-regulation of the cyclin-dependent kinase inhibitors p53, p21, p16, resulting in cell cycle arrest in the G0/G1 phase (Burger et al., 2012; Sherr and McCormick, 2002).

Although the major characteristic of senescent cells is irreversible cell-cycle arrest, recent studies conducted revealed several important functions including secretion of various secretory proteins such as inflammatory cytokines, chemokines, growth factors, and MMPs, into the surrounding extracellular fluid, termed senescence-associated secretory phenotypes (SASPs) (Watanabe et al., 2017). In addition, there are convincing evidences indicating a strong relationship between left atrial (LA) inflammation and interstitial fibrosis to the pathogenesis of AF (Issac et al., 2007; Scott et al., 2018). Endothelial dysfunction is associated with increased endothelial expression of adhesion molecules, such as E-selectin, ICAM-1, and VCAM-1 thus exhibiting a proinflammatory, pro-oxidant and proadhesion features, which facilitate AF substrate formation (Casaclang-Verzosa et al., 2008; Pathak et al., 2013). Several studies have suggested that inflammation exerts its remodeling effects through ROS (Dandana et al., 2011; Kotur-Stevuljevic et al., 2007). ROS can result in increased expression of matrix metalloproteinases, resulting in an imbalance between accumulation and breakdown of extracellular matrix, enhancing LA fibrosis (Ait-Benali et al., 2018; Rajagopalan et al., 1996). In fact, inflammatory cells have been demonstrated to infiltrate atrial tissue (Kume et al., 2017). Others authors have emphasized the view that cellular senescence is a direct signalling event which leads to endothelial dysfunction (Bhayadia et al., 2016). In line

with previous studies conducted on different cell cultures, the present findings underline that both replicative and premature AECs senescence is associated with pro-thrombotic, pro-inflammatory, pro-adhesive and pro-fibrotic cell phenotype. These phenoypical changes depicts fundamental structural alterations of the atrial tissue linked to senescence which ultimately ends up in overall structural remodeling thus impairing normal electrical conductivity and paving the way to atrial fibrillation (Johnston and Gillis, 2017; Kume et al., 2017; Nattel et al., 2008).

Previous studies conducted on coronary artery endothelial cells showed that that oxidative stress is a major inducer of endothelial cell senescence and that it mediates both replicative and premature endothelial senescence (Khemais-Benkhiat et al., 2016b; Silva et al., 2017). These observations are in good agreement with the present study conducted on AECs. ROS can be generated by several sources such as mitochondrial respiratory complex I and III, xanthine oxidase, NADPH oxidase, COXs, and uncoupled eNOS. The pharmacological characterization of the enzymatic sources of ROS in the senescent AECs has indicated a significant contribution of NADPH oxidase, COXs, and the mitochondrial respiratory chain. This is in consistent with previous studies conducted showing involvement of NADPH oxidase subunits Nox1 and Nox4 in the excessive formation of ROS in senescent human endothelial cells (Schilder et al., 2009). In addition, one study also demonstrated an upregulation of NADPH oxidase subunits p47 phox, Nox2, and Nox4 in $JunD^{-/-}$ mice hence featuring premature vascular senescence linked with an impairment of the O2 •-/NO balance (Paneni et al., 2013). In another study, replicative senescence in human umbilical vein endothelial cells was shown to be delayed following in Nox4 gene interference using small-hairpin RNA (Lener et al., 2009). Previous studies also documented the important role of COX-2 in the establishment and maintenance of senescence of human fibroblasts (Martien et al., 2013). Indeed, a low dose of aspirin was found to delay the onset of senescence in circulating endothelial progenitor cells (Hu et al., 2008). Altogether, the previous studies and the present findings indicate that the expression of pro-oxidant enzymes promoting a sustained level of oxidative stress, which leads to the down-regulation of the eNOS-derived NO pathway and the subsequent induction of senescence.

Many studies conducted previously depict a strong associated between the angiotensin system and structural remodeling link to AF perpetuation and maintenance. In line with this concept, activation of the local angiotensin system has been shown to contribute to the increased level of oxidative stress observed in pathological arteries such as in old arteries and in arteries from hypertensive

animals and humans mostly by an AT1R-mediated activation of NADPH oxidase (Doughan et al., 2008; Harrison et al., 2003). Moreover, Ang II has been reported to be a potent inducer of premature senescence in endothelial cells (Abbas et al., 2017; Shan et al., 2014). In the present study, endothelial senescence was associated with an upregulation of the local angiotensin system as indicated by increased expression levels of ACE and AT1 receptors in both replicative and premature senescent AECs. The local angiotensin system has a determinant role in the induction of both replicative and premature endothelial senescent as indicated by the fact that both the ACE inhibitor and the AT1 receptor blocker reduced SA-β-gal activity.

The present findings also point out an important role of the coagulation cascade components in the induction of senescence and in the acquisition of senescence-associated secretory phenotype. Thrombin has been shown to be a strong procoagulant and proinflammatory serine protease that contributes to the various cardiovascular pathologies by increasing the expression of cell adhesion molecules, stimulating the secretion of pro-inflammatory cytokines, activating inflammatory responses in atherosclerotic plaques, stimulating proliferation of smooth muscle cells, and exacerbating vascular lesions at sites of injury (Jaberi et al., 2018). The present study revealed the pro-inflammatory, pro-thrombotic and pro-fibrotic signaling functions of thrombin associated with the induction of senescence in AECs. This is consistent with a previous study with FXa (another component of the coagulation cascade) showing non-hematologic functions beyond blood coagulation including an inflammatory response and tissue remodeling and, hence, indicating that hyper-coagulation, cell senescence, and inflammation are linked. Altogether, our data substantiate the view that atrial endothelial senescence promotes thrombogenicity, inflammation, matrix remodeling and the up-regulation of the local Ang II system. The present findings further indicate that thrombin is a potent inducer of premature senescence in AECs leading to an endothelial dysfunction with the down-regulation of the protective NO pathway and the induction of pro-infiltrative and pro-fibrotic responses. They further suggest the involvement of the local angiotensin system and that targeting the Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial ageing.

Numerous studies have emphasized the view that thrombin has pleiotropic cellular effects through the cleavage of protease-activated receptor (PAR)-1, including hemostasis, inflammation, cellular growth, and proliferation. The importance of this pathway was highlighted by the demonstration that direct thrombin inhibitors and PAR-1 antagonists prevent atrial remodeling and reduce AF

susceptibility (Jumeau et al., 2016b). In the light of these observations, the possible role of PAR-1 antagonists in the prevention of senescence and senescence-associated-secretory phenotype remains to be explored in AECs. Moreover, the impact of other coagulation cascade components on the induction of AECs should be investigated. Another area of intense research relies on the role of microparticles (MPs), generated at high levels in AF, on the induction of AECs senescence. Furthermore, all experiments were conducted on atrial endothelial cells taken from porcine which is considered not a pertinent pathological model regarding atrial fibrillation. Thus more experimental studies need to be performed using other models and also to mimic the original in vivo shear stress conditions. Studies can also be in pathological models of hypertension, diabetes in rabbits, dogs and goats to assess the score of atrial fibrillation. In addition ex vivo studies can be conducted on atrial appendages and localization of senescent cells can be done.

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Induction de la sénescence endothéliale auriculaire par l'angiotensine II et la thrombine : Rôle du stress oxydant et caractérisation du phénotype pro-thrombotique, pro-adhésif, protéolytique et pro-fibrotique

RESUME

De nombreuses études soulignent une relation directe entre la prévalence de la fibrillation auriculaire (FA) et le vieillissement. La senescence cellulaire et le phénotype sécrétoire associé semblent jouer un rôle central dans le développement de l'inflammation auriculaire. Cette inflammation est à l'origine d'un remodelage auriculaire délétère (stress oxydant, fibrose) favorable à la perpétuation et au maintien de la FA. Par ailleurs, il est connu que la FA favorise la coagulation locale et systémique. Cependant, l'impact des facteurs de la coagulation, notamment la thrombine, sur la FA est peu connu. L'objectif de cette étude était de déterminer le lien entre la sénescence des cellules endothéliales atriales et le phénotype pro-inflammatoire et pro-adhésif, la fibrose et le remodelage auriculaire tout en évaluant l'impact de la coagulation, et en particulier le rôle de la thrombine.

RESUME EN ANGLAIS

Many studies documented strong relationship between ageing and development of atrial fibrillation (AF). Moreover, it has been found that senescence and senescence-associated- secretory-phenotype play an important role in development of overall atrial inflammation which can ultimately ends up in atrial structural remodeling paving the way to AF perpetuation and maintenance. Moreover, it has been known for decades that AF has been associated with the activation of local and circulating coagulation factors. However, little is known about the impact of coagulation-derived factors, in particular thrombin, on the onset of AF. The aim of the present study was to determine the link between atrial endothelial cells (AECs) senescence and the induction of pro-inflammatory, pro-adhesive, pro-fibrotic and pro-remodelling AECs patterns and also to evaluate the contribution of coagulation derived-factors such as thrombin.