# INTERACTIONS BETWEEN ANGIOTENSIN II AND AGMATINE IN EXPERIMENTAL INFLAMMATION

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**Keywords:** agmatine, angiotensin II, rat, air pouch, inflammation.
**Abstract:** Agmatine (decarboxylated arginine) significantly reduced the experimentally carageenan inflammatory process enhanced by Ang II in rats. Noteworthy was the strong action of agmatine, very close to that of spermine, although surpassed by that of indomethacin. In contrast, some other polyamines (spermidine, cadaverine and putrescine) had no significant reducing effects. Inhibition of polyamines synthesis by DL-α-Difluoromethylornithine (DFMO) has further significantly enhanced the pro-inflammatory effects of Ang II at 6 hours. The above findings might demonstrate the involvement of agmatine synthesis (beyond other polyamines) as a reactive response to pro-inflammatory action of angiotensins.

## INTRODUCTION

The critical role played by the renin-angiotensin-aldosterone system (RAAS) in the regulation of blood pressure and body fluid homeostasis has been well recognized. Angiotensin (Ang) II and aldosterone are the most powerful biologically active products of the RAAS, although there are also other bioactive Ang peptides involved in this system, including Ang III, Ang IV, and Ang1-7. In addition to their physiological roles, Ang II and aldosterone induce inflammation, cell growth, mitogenesis, apoptosis, migration, and differentiation; regulate gene expression of bioactive substances; and activate multiple intracellular signaling pathways, all of which contribute to cardiovascular tissue injury (Nishiyama and Kim-Mitsuyama, 2010).

Ang II displays inflammatory activity and is implicated in several cardiovascular disorders. Primary (essential) hypertension has been shown to be mediated by a relative impairment in sodium excretion by the kidney, but the mechanisms responsible for this defect are still being clarified. Increasing evidence suggests a role for subtle acquired renal injury in mediating this process. Microvascular injury is present in the majority of subjects with hypertension. The development of arteriolosclerosis, primarily of the afferent arteriole, may interfere with glomerular autoregulation, whereas the loss of peritubular capillaries may facilitate local ischemia. These changes favor the localization of T cells and macrophages into the interstitium, which, coupled with local oxidative stress and Ang II generation, may contribute to the impaired pressure natriuresis observed with salt-sensitive hypertension. Consistent with this hypothesis, therapies that are aimed at blocking the immune response, including thymectomy, genetic alterations in mice resulting in impaired immune responses, or the use of immunosuppressive agents, can protect against the development of hypertension in experimental models. Preliminary data in humans also suggest that the inhibition of the renal inflammatory response may reduce blood pressure. The actual investigations are directed to gain insight in the role of the intrarenal T-cell reactivity and autoimmunity in driving the tubulointerstitial inflammation and its participation in the pathogenesis of salt-sensitive hypertension (Rodriguez-Iturbe and Johnson, 2010).

Some studies determined the effect of a short-term Ang II signaling blockade on vascular endothelial growth factor (VEGF), soluble intercellular adhesion molecule-1 (sICAM-1), nitric oxide (NO), and malondialdehyde (MDA) (index of lipid peroxidation) levels in the systemic circulation and on peroxynitrite generation and insulitis development in the streptozotocin (STZ) diabetic rats' pancreas. Diabetes was induced in Wistar rats by intraperitoneal STZ injection. Diabetic rats were treated for 1 week with losartan (20 mg/kg/body weight/day in the drinking water), and pancreas and blood were collected for histochemical, immunohistochemical, and biochemical studies. Diabetic rats showed greater VEGF, sICAM-1, NO, and MDA levels, a high score of insulitis, increased nitrotyrosine staining, and markedly reduced pancreatic insulin content when compared with controls. Losartan treatment suppressed the excessive NO and lipid peroxidation production systemically without restoring them to that of healthy subjects and reduced VEGF levels while leaving sICAM-1 levels unchanged. The insulitis score and nitrotyrosine staining were reduced, whereas the pancreatic islets and the beta-cell area were increased significantly in the treated group, indicating the reduction of inflammation and nitrosative stress and an early regeneration of beta-cell mass in the pancreas. Conclusively, in the STZ diabetic rat model, even a short-term losartan treatment improves oxidative and nitrosative stress systemically and locally, improving the islets' environment and accelerating beta-cell regeneration (Kamper *et al*., 2010).

Production of reactive oxygen species is regulated by several cytokines and growth factors, including Ang II, which increase $O_2^{\cdot-}$ and $H_2O_2$ in cardiac cells, vascular smooth muscle, endothelial, adventicial and mesangial cells.

Generation of oxygen free radicals has been implicated in the pathogenesis of hypertension induced by Ang II (Androulakis *et al*., 2009).

On the other hand, polyamines are multifunctional molecules with anti-inflammatory potential, acting both by modulating respiratory combustion and by adjusting the lymphocyte multiplication. Polyamines inhibit NADPH-oxidase complex formation by 2 different mechanisms: binding of spermine to fosfatidilinositol-4-phosphate (PIP), making it inaccessible to phospholipase C (PLC), or inhibition of protein kinase C (PKC) coupling process to the membrane (Zhu *et al*., 2009).

Starting from this point, the goal of the actual study was represented by the interactions between Ang II and agmatine in inflammation, as compared to spermine, spermidine, cadaverine, putrescine, DL-α-Difluoromethylornithine (DFMO) and indomethacin.

## MATERIALS AND METHODS

For the experiments we used 48 Wistar adult male rats (Băneasa source), weighing 150-200 g, kept under normal and the same laboratory conditions.

Experiments were carried out by the model of inflammation named "*air pouch*". To achieve the "air bag", rats were injected with 10 ml of sterile air into their back shaved skin, subcutaneously (the default and relatively the same region for all rats), consecutively for 3 days. Rats were then divided into equal series and relatively homogeneous in weight, 6 each series. Inflammation was further induced by injecting the air pockets with 2% carrageenan in saline.

Series I witnessed and received only saline, 1 ml i.p. To series II 25 mg/kg b.w. spermine was administered i.p. in 1 ml saline. Series III received i.p. 25 mg/kg b.w. spermidine in 1 ml saline. Series IV received 25 mg/kg b.w. putrescine i.p. in 1 ml saline. Series V received cadaverine in 1 ml saline i.p. 25 mg/kg b.w., series VI agmatine i.p. in 1 ml saline 25 mg/kg b.w., and series VII DL-α-Difluoromethylornithine hydrochloride hydrate (DFMO) i.p. in 1 ml saline 25 mg/kg b.w. For comparison, series VIII was given i.p. in 1 ml saline indomethacin 10 mg/kg b.w.

Meanwhile, Ang II was administered to all above groups intravenously (into a vein outside of the rear feet), 100 nM in 10 μl sterile saline for 2-3 minutes, 30 minutes after the specific treatments already mentioned. Moreover, all rats received Evans blue, 20 mg/kg b.w., i.p., in 200 μl saline. After 6 hours the rats were killed and the granulomatous tissue or the existing liquid in the air bag were harvested, and then introduced, after weighing, in formamide for 48 hours to extract the Evans blue.

Evans blue was spectrophotometrically quantified at 620 nm using a UV-VIS spectrophotometer HP 8453. Evans blue values were expressed in μg/g tissue. Quantification was done using a standard curve made with 2 concentrations of Evans blue (0.1 μM and 10 μM), while knowing that Evans blue absorbance is linear with concentration in these areas. Concentration calibration equation was as follows: concentration = 12.79100 μM/ml x absorbance (A).

The statistical significance of test results was highlighted using the Variance One-Way ANOVA (possibly complemented by Bonferroni test) and Student t-test and the results were expressed as mean ± S.E.M (n = 6). Value of $p<0.05$ was considered statistically significant always.

Present studies were carried out in accordance with the "Guide for Care and Use of Animal Experiments" of U.S. National Institutes of Health (NIH), published by the U.S. National Academy in 1996 and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr T. Popa" Iași.

Chemicals, compounds and reagents used: spermine, spermidine, agmatine sulfate (argamine), cadaverine (1,5-diaminopentan), putrescine (1,4-diaminobutan), angiotensin II (Ang II), DL-α-Difluoromethylornithine (DFMO), Evans blue, formamide, indomethacin and carrageenan were purchased from *Sigma-Aldrich Co*., St. Louis, U.S.A. The remaining reagents used were of analytical grade.

## RESULTS AND DISCUSSIONS

Administrations of agmatine and spermine significantly reduced the extrusion of Evans blue into the granulomatous tissue, enhanced by Ang II in rats (in fact, the amplifying of the experimentally inflammatory process). Noteworthy is the strong action of agmatine. On the other hand, spermidine, cadaverine and putrescine had no significant reducing effects on the amplification of the inflammatory process by Ang II (figure no. 1).

Inhibition of polyamines synthesis by DL-α-Difluoromethylornithine (DFMO) has further amplified the pro-inflammatory effects of Ang II at 6 hours. This demonstrates the

involvement of polyamine synthesis as a reaction response to pro-inflammatory action of angiotensins (figure no. 1).

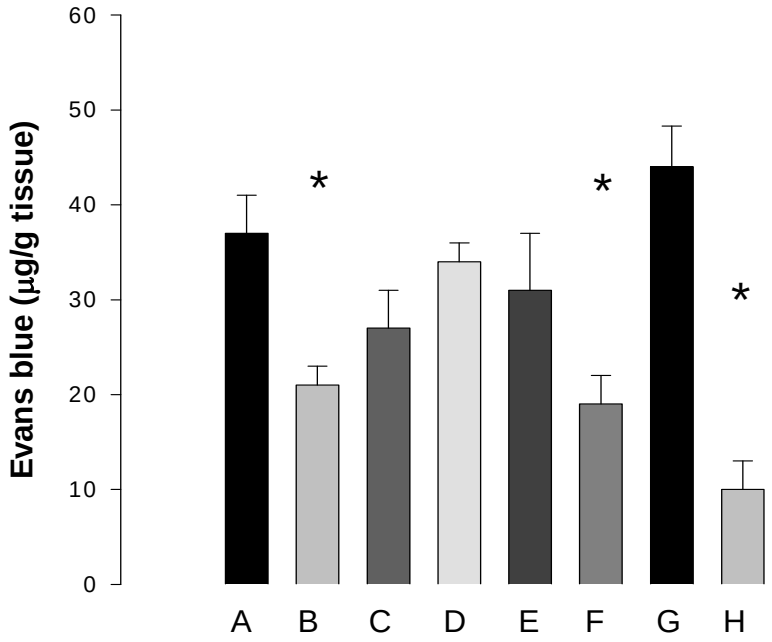


**Figure no. 1**: The effects of spermine (B), spermidine (C), putrescine (D), cadaverine (E), agmatine (F), DL-α-Difluoromethylornithine (DFMO) (G) and indomethacin (H) on the amplification of the carageenan inflammatory process in rats, induced by 100 nM Ang II, as compared to Ang II in vehicle-saline (A). * $p < 0.05$ as compared to Ang II in saline (mean ± S.E.M, n=6).

However, none of these answers is as strong as that of indomethacin, an extremely potent anti-inflammatory pharmacodynamic (figure no. 1).

As already mentioned Ang II is a critical mediator of vascular inflammation and remodeling in a number of diseases including hypertension and atherosclerosis. ESE-1 knockout mice were used to evaluate the role of the epithelium-specific ETS transcription factor-1 (ESE-1), a member of E26 transformation-specific sequence (ETS) transcription factors, in regulating Ang II-mediated vascular inflammation and remodeling. ESE-1 levels are low to undetectable under basal conditions but rapidly increase in response to Ang II. Intimal medial thickness and perivascular fibrosis of the aorta were significantly greater in ESE-1 knockout mice compared with the wild-type littermate controls. Proliferating cell nuclear antigen (PCNA) staining was also greater in the aorta of the Ang II-infused ESE-1 knockout mice compared with the controls. The infiltration of T cells and macrophage into the vessel wall of the aorta was dramatically

enhanced in the ESE-1 knockout mice compared with the controls. Finally, Ang II-induced expression of a known downstream target of ESE-1, nitric oxide synthase 2 (NOS2), was significantly blunted in ESE-1 knockout mice compared to littermate controls. The alterations in vascular inflammation and remodeling were associated with an exaggerated systolic blood pressure response to Ang II in ESE-1 knockout mice. It was thus concluded that ESE-1 is an Ang II-inducible transcription factor that plays an important counter-regulatory role in the setting of vascular inflammation and remodeling (Zhan *et al*., 2010).

Furthermore, a recent study evaluated the effect of cis- and trans (t)-resveratrol (RESV) in two *in vivo* models of vascular inflammation and identified the cardioprotective mechanisms that underlie them. *In vivo*, Ang-II-induced arteriolar leukocyte adhesion was inhibited by 71% by t-RESV (2.1 mg/kg, i.v.), but was not affected by cis-RESV. These effects were accompanied by reductions in monocyte and endothelial CAM expression, chemokine release, phosphorylation of p38 MAPK, and phosphorylation of the p65 subunit of NF-κB. Upregulation of PPAR-γ also appears to be involved in the cardioprotective effects of t-RESV (Rius *et al*., 2010).

The CARMA1, Bcl10, and MALT1 proteins together constitute a signaling complex (CBM signalosome) that mediates antigen-dependent activation of NF-κB in lymphocytes, thereby representing a cornerstone of the adaptive immune response. Although CARMA1 is restricted to cells of the immune system, the analogous CARMA3 protein has a much wider expression pattern. Emerging evidence suggests that CARMA3 can substitute for CARMA1 in non-immune cells to assemble a CARMA3-Bcl10-MALT1 signalosome and mediate G protein-coupled receptor activation of NF-κB. The study showed that one G protein-coupled receptor, the type 1 receptor for Ang II, utilizes this mechanism for activation of NF-κB in endothelial and vascular smooth muscle cells, thereby inducing pro-inflammatory signals within the vasculature, a key factor in atherogenesis. Further, it was demonstrated that Bcl10-deficient mice are protected from developing angiotensin-dependent atherosclerosis and aortic aneurysms. By uncovering a novel vascular role for the CBM signalosome, these findings illustrate that CBM-dependent signaling has functions outside the realm of adaptive immunity and impacts pathobiology more broadly than previously known (McAllister-Lucas *et al*., 2010).

If angiotensins are known as pro-inflammatory molecules, polyamines have antioxidant and anti-inflammatory capacities (Rhee et al., 2007; Eisenberg et al., 2009). Moreover, there are already described some interactions between Ang II and polyamine agmatine (decarboxylated arginine). Thus, our previous study showed that the administration in pre-treatment of some polyamines (especially spermine and spermidine and almost null agmatine, putrescine and cadaverine) reduced the contractile effects of Ang II in the isolated rat aorta (Costuleanu *et al*., 2003).

On the other hand, vascular smooth muscle cells of rat aorta express imidazoline receptors whose stimulation, by drugs or an endogenous ligand, agmatine, inhibits serum-stimulated proliferation. The study investigated whether imidazoline receptors are expressed in human vascular smooth muscle cells and if their stimulation is antiproliferative. Cultured human coronary artery vascular smooth muscle cells express a nonadrenergic binding site for $^{3}$H-idazoxan and an imidazoline receptor-binding protein as revealed by immunocytochemical and immunoblot analyses with a specific antibody. Idazoxan and agmatine dose-dependently inhibited serum-stimulated proliferation of these cells as measured by the incorporation of $^{3}$H-thymidine and serum-stimulated expression of proliferating cell nuclear antigen and cell numbers. These agents inhibited proliferation of human and rat (aorta) smooth muscle cells stimulated by either norepinephrine, Ang II, or platelet-derived growth factor, indicating

inhibition of mitosis mediated by G-protein or receptor tyrosine kinase pathways. It was concluded that human vascular smooth muscle cells express imidazoline-receptors whose activation inhibits proliferation by interacting at a distal step in an intracellular signal cascade common to G-protein and receptor tyrosine kinase mitogenic pathways. Agmatine, synthesized in endothelium, may act as a paracrine regulator of vascular smooth muscle cell proliferation through imidazoline receptors, and agents acting at this site may be useful in treating vascular hyperplasia (Regunathan and Reis, 1997).

In acute inflammatory responses, such as wound healing and glomerulonephritis, arginine is the precursor for production of the cytostatic molecule nitric oxide (NO) and the pro-proliferative polyamines. NO is an early phase response whereas increased generation of polyamines is requisite for the later, repair phase response. The temporal switch of arginine as a substrate for the inducible nitric oxide synthase (iNOS)/NO axis to arginase/ornithine decarboxylase (ODC)/polyamine axis is subject to regulation by inflammatory cytokines as well as interregulation by the arginine metabolites themselves. It was described the capacity of another arginine pathway, the metabolism of arginine to agmatine by arginine decarboxylase (ADC), to aid in this interregulation. Agmatine is an antiproliferative molecule due to its suppressive effects on intracellular polyamine levels, whereas the aldehyde metabolite of agmatine is a potent inhibitor of iNOS. It was proposed that the catabolism of agmatine to its aldehyde metabolite may act as a gating mechanism at the transition from the iNOS/NO axis to the arginase/ODC/polyamine axis. Thus, agmatine has the potential to serve in the coordination of the early and repair phase pathways of arginine in inflammation (Satriano, 2003).

## CONCLUSIONS

Agmatine (decarboxylated arginine) significantly reduced the experimentally carageenan inflammatory process enhanced by Ang II in rats. Noteworthy is the strong action of agmatine, very close to that of spermine, although surpassed by that of indomethacin. In contrast, some other polyamines (spermidine, cadaverine and putrescine) had no significant reducing effects. Inhibition of polyamines synthesis by DL-α-Difluoromethylornithine (DFMO) has further significantly enhanced the pro-inflammatory effects of Ang II at 6 hours. The above findings might demonstrate the involvement of agmatine synthesis (beyond other polyamines) as a reactive response to pro-inflammatory action of angiotensins.

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**ACKNOWLEDGEMENTS**
This study was partially supported by C.N.C.S.I.S. grants IDEI-PCE-1273/2007 (Project director Lect. Dr. Bogdan Gurzu) and IDEI-PCE-2670/2008 (Project director Prof. Dr. Gheorghe Petrescu).

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