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Cent. Eur. J. Med. • 7(6) • 2012 • 704-712
DOI: 10.2478/s11536-012-0059-z

Central European Journal of **Medicine**

New evidence for vascular interactions between aldosterone, angiotensin II and antioxidants in isolated smooth muscle cells of rats

Research Article

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Received 3 April 2012; Accepted: 31 July 2012

Abstract: Accumulating evidence suggests that the nongenomic cardiovascular actions of aldosterone are produced by varied cellular pathways and mediated by a multitude of messenger systems including the reactive oxygen and nitrogen species. Considering the involvement of the oxidative and nitrosative stress in the pathways leading to the activation of the angiotensin – aldosterone system, in the current study we tried to evaluate the functional interactions between aldosterone, angiotensin II and antioxidants in isolated vascular smooth muscle of aortic rings from rats. Our data provide additional arguments that the nongenomic actions of aldosterone on aortic smooth muscle cells of rats are a question of cross-talk and balance between its rapid vasoconstrictor and vasodilator effects, as result of the activation of reactive oxygen species in the first case and of nitrogen species in the second. In this way, it seems that at low ambient oxidative stress, aldosterone promotes nitric oxide (NO) production and vasodilatation, while in situations with increased oxidative stress the endothelial dysfunction and detrimental effects induced by vasoconstriction will prevail. Thus, aldosterone could be considered both "friend and foe". This could be relevant for the ways in which aldosterone damages cardiovascular functions and could lead to significant therapeutic improvements.

Keywords: *Aldosterone • Angiotensin II • Antioxidants • Smooth muscle • Rat Cardiorenal Metabolic Syndrome*

1. Introduction

Recent data concerning nongenomic effects of aldosterone have identified a specific membrane receptor (nongenomic receptor - NGR), which is distinct from the citosolic mineralocorticoid receptor (MR), accounting for a variety of actions that contribute to the cardiac regulation and blood pressure homeostasis, independent of renal salt and hydro-electrolytic balance [1]. Both types of mineralocorticoid receptors are present in the endothelium and vascular smooth muscle cells [2]. It was established that the presence of 11β hydroxysteroid dehydrogenase ($11\beta HSH_2$) in mineralocorticoid sensitive tissues, protects the MR against the activation by glucocorticoids [3]. At the same time, it has been demonstrated that aldosterone acts as a key hormone which participates in the pathogenesis of some cardiovascular disorders [4]. Aldosterone inhibits contractility in atrial and ventricular trabeculae of the human heart and potentiates the vasoconstrictor response to angiotensin II (Ang II) in the coronary arteries, while upregulating angiotensin receptors [5,6].

Angiotensin II being the main regulator of adrenal aldosterone synthesis, through the membrane AT_1R complex [7], interactions between these two hormones, are also present in cardiac muscle and vascular smooth muscle cells [8,9]. By rapid nongenomic effects, aldo-

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sterone may increase NO (nitric oxide) release from the vascular endothelium [10] or may stimulate ROS production as signaling molecules by activation of NADPH - oxidase, depending on the ambient level of oxidative stress [11,12]. In situations with low levels of oxidative stress, aldosterone promotes vasodilation induced by predominant activation of eNOS and NO release, while in some pre-existing vascular conditions of higher oxidative damage, aldosterone participates in the production of vasoconstriction by increasing the level of oxygen derived free radicals [13]. In this way, by rapid nongenomic vascular actions, aldosterone can cause vasoconstriction, vasodilatation or no effect.

Combined treatment of vascular smooth muscle cells with aldosterone and Ang II exerts a synergistic effect at even their nonefective doses [8]. Inducing oxidative stress and vasoconstriction through nongenomic mechanisms, aldosterone has been proposed to be involved in the pathogenesis of some forms of hypertension in addition to its genomic implications. These new findings highlight the role of aldosterone as a key cardiovascular hormone.

On the other hand, the increased formation of reactive oxygen species due to aldosterone and Ang II [14-16] contributes to cardiac and vascular dysfunction. The free radical scavengers superoxide dismutase, catalase and dimethyl sulfoxide (DMSO) given to rats made acutely hypertensive with Ang II infusion, reduced the vascular damage induced by this type of hypertension, suggesting that reactive oxygen species may play a role in the pathogenesis of some hypertensive vascular disease [17].

A delicate balance also exists between the Ang II vasoconstriction induced by superoxide anion, H_2O_2 and peroxynitrite on one side and vasodilatation caused by NO in vascular smooth muscle cells on the other side [18]. Inhibition of NO synthesis with L-NAME augments Ang II vasoconstriction [19] and causes oxidative stress [20]. Peroxynitrite resulted from NO and superoxide combination contributes to the deleterious cerebrovascular effects of Ang II [21].

Blockade of the aldosterone receptors is frequently used in cardio-myopathies [22], as it reduces the inflammatory/fibrinogenic responses induced by Ang II [23], preventing the apoptosis of endothelial cells [24] and improving the sympathetic nerve activity [25].

While spironolactone inhibits classical mineralocorticoid receptors of aldosterone, eplerenone predominantly blocks its nongenomic effects on the Na^+/H^+ exchange, intracellular Ca^{++} levels and vasoconstriction in mesenteric resistance vessels [26,27].

Accumulating evidence suggest that the nongenomic cardiovascular actions of aldosterone are produced by varied cellular pathways and mediated by a multitude of messenger systems including the reactive oxygen and nitrogen species. Oxidative and nitrosative stress being involved in the pathways leading to the activation of the angiotensin – aldosterone system, we proposed to re-evaluate the functional interactions between aldosterone, Ang II and antioxidants in isolated vascular smooth muscle of aortic rings from rats. It has been demonstrated that in hypertensive patients with heart failure, prolonged treatment with thiazide diuretics, calcium channel blockers, ACE inhibitors and other combination therapies produce increased oxidative stress and endothelial dysfunction even while blood pressure is reduced [28].

The present paper intends to investigate the pharmacologic effects of combined administration of aldosterone, antioxidants and spironolactone on the motility of vascular smooth muscle, in order to identify new pathways for treating eventual inconvenient effects of long-time treatment of high blood pressure and the eventual beneficial effects of antioxidant therapy on the vascular targets of these treatments.

2. Materials and methods

2.1 The subjects

The subjects were experimentally naive, male Wistar rats, weighing approximately 200-250 g at the beginning of the experiment. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. In the day of the experiment, rats were anaesthetized with a lethal dose of pentobarbitone (150 mg/kg, i.v.) and exsanguinated. The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community standards on the care and use of laboratory animals, according to the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The thoracic aorta was dissected, cleaned of any perivascular tissue and cut in rings 3-4 mm long. Each aortic ring was placed in an organ bath (4 mL) intact or with endothelium removed by gentle rubbing with stainless steel wire and perfused with Krebs-Henseleit saline (mmol/L: NaCl 121, KCl 4.7, $NaHCO_3$ 24.7, $MgSO_4$ 12.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, and glucose 5.8) at 38°C, bubbled with 95% O_2 and 5% CO_2.

The experiments were carried out in series of six to ten experiments using coupled preparations of aortic

rings either with or without endothelium for studies of the vascular interactions between aldosterone, Ang II and reactive oxygen or nitrogen species.

The testing of the vascular reactivity and of its endothelial integrity was made with the vasoconstrictor β-adrenergic agonist phenylephrine (10^{-7} M – 10^{-6} M) and with carbachol (10^{-6} M – 10^{-5} M) as releasing factor of myorelaxing endothelial NO [29]. The simultaneous recordings of the contractions were performed using three isometric force transducers coupled with computer acquisition system. In order to avoid the tachyphylaxis of the preparations, the administration of angiotensin II was made at time intervals large enough (90-120 minutes) and after repeated wash-out.

In the first series of experiments, the vascular effects of aldosterone (Merck) in progressive doses (10^{-9} – 10^{-6} M) both proper and on the vasoconstricting actions of Ang II (10^{-6} M) (Sigma Co) or KCl (40 mM) have been investigated in basal relaxation conditions before and 10 min after administration of spironolactone (Boehringer GmbH) as specific inhibitor of mineralocorticoid receptor. The aldosterone and spironolactone were used in various doses (10^{-6} – 10^{-5} M) in intact and in de-endothelized aortic rings.

Taking into account that vascular effects of aldosterone partially depend upon the reactive species of O_2 and NO, in other series of experience we studied its influence on Ang II vasoconstriction in pretreated preparations with amifostine (10^{-8} – 10^{-7} M) (Schering – Plough Ltd), N-acetyl-cysteine (10^{-6} – 10^{-5} M) and L-NAME (10^{-5} M) (Sigma Co).

3. Result

The results of the preliminary experiments have shown that aldosterone alone did not significantly modify the basal tone of the aortic smooth muscle rings in the first 10-15 minutes from administration. However, an evident modification of the vascular reactivity tested with Ang II and potassium has been obtained in the pretreated preparation with different doses of aldosterone.

3.1 Influence of aldosterone on vascular effects of Ang. II

Ang II vasoconstriction was inhibited by acute aldosterone exposure in the majority of our experiments (75%), while the vascular contractions induced by potassium were invariably potentiated. An increased angiotensin vasoconstriction induced by aldosterone was inconstantly obtained only in 25 % of experiences. Different effects of aldosterone on angiotensin II and potassium vasoconstriction are presented in Figure 1.

To determine whether the endothelium is involved in these vascular actions of aldosterone, its influence on angiotensin vasoconstriction was comparatively studied in normal and de-endothelised rings. The integrity of the endothelium was tested in the beginning with carbachol on precontracted rings by phenylephrine. The prevalent inhibitory actions of aldosterone on Ang II vasoconstriction occurred both in normal and in desendothelised rings. The increased angiotensin vasoconstriction induced by aldosterone in some normal preparation was also inhibited in denudated rings (Figure 2).

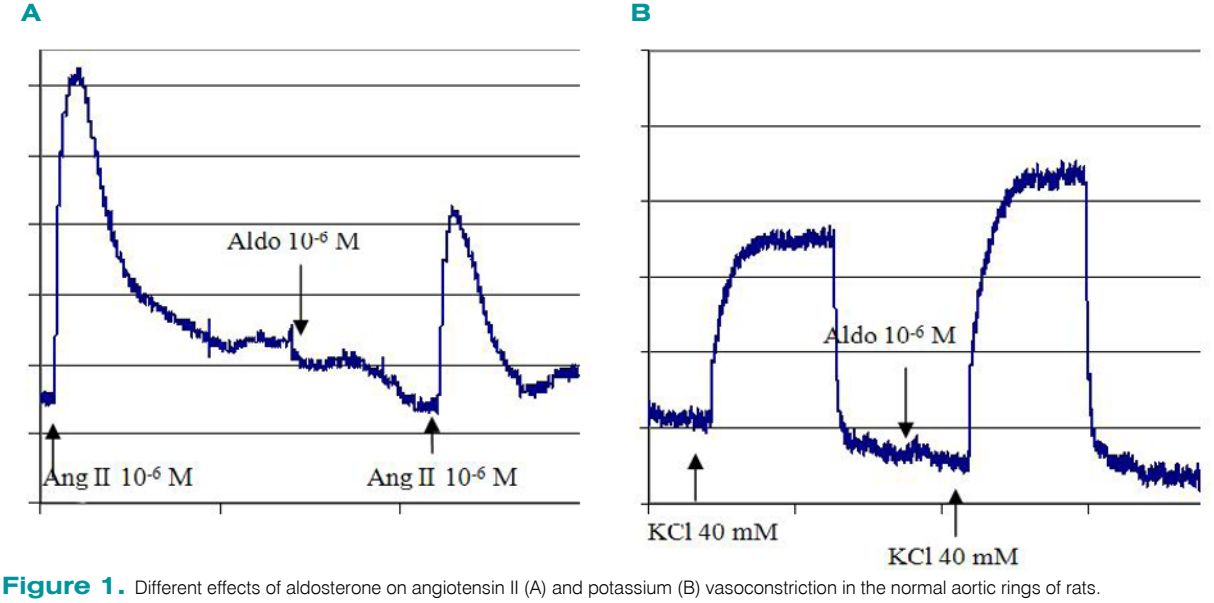

Figure 1. Different effects of aldosterone on angiotensin II (A) and potassium (B) vasoconstriction in the normal aortic rings of rats.

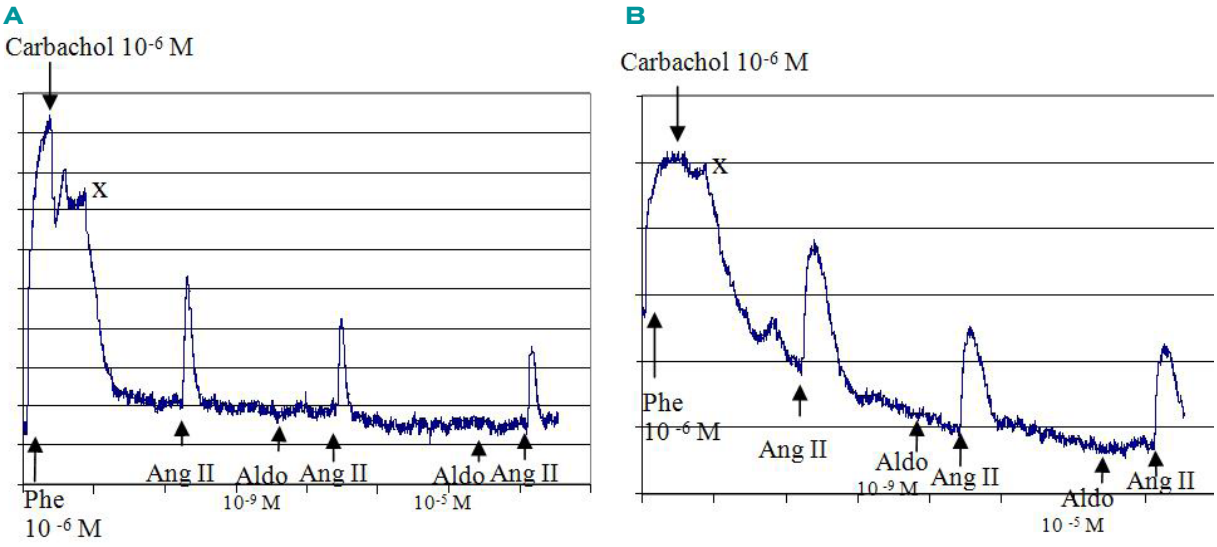

Figure 2. Influence of aldosterone on angiotesnin II (10-6 M) vasoconstriction in normal (A) and desendothelised (B) aortic rings.

The next step was to test the effects of aldosterone on Ang II vasoconstriction in the pretreated aortic rings with spironolactone. The blockade of aldosterone receptors by spironolactone did not significantly modify the vascular reactivity of the normal or of the denudated rings to Ang II vasoconstriction induced by aldosterone. Comparative effects of aldosterone and spironolactone on angiotensin II vasoconstriction in normal and denudated aortic rings are presented in Figure 3. In this way, while the administration of aldosterone 10^{-6} ($F(1,10)=695$, $p<0.01$) and aldosterone 10^{-8} ($F(1,10)=530$, $p<0.01$) in normal rings resulted in a significant decrease of the percentage contraction to Ang II 10^{-6}, the aldosterone 10^{-9} ($F(1,10)=5$, $p=0.99$) did not result in any significant changes (Figure 3A). More importantly, this was a dose-effect response, since post-hoc analysis showed significant differences between aldosterone 10^{-6} vs. aldosterone 10^{-8} groups ($p<0.01$), as well as between aldosterone 10^{-8} vs. aldosterone 10^{-9} groups ($p<0.01$) and aldosterone 10^{-6} vs. aldosterone 10^{-9} groups ($p<0.01$).

Regarding the results of the aldosterone administration in the denudated rings, we also noticed a significant decrease of the percentage contraction to Ang II 10^{-6} in the aldosterone 10^{-6} ($F(1,10)=632$, $p<0.01$) and aldosterone 10^{-8} ($F(1,10)=125$, $p<0.01$) groups, while the aldosterone 10^{-9} ($F(1,10)=1$, $p=0.81$) did not result in any significant changes (Figure 3A). Again, this was also a dose-effect response in the denudated rings, since post-hoc analysis showed significant differences between aldosterone 10^{-6} vs. aldosterone 10^{-8} groups ($p<0.01$), as well as between aldosterone 10^{-8} vs. aldosterone 10^{-9} groups ($p<0.01$) and aldosterone 10^{-6} vs. aldosterone 10^{-9} groups ($p<0.01$).

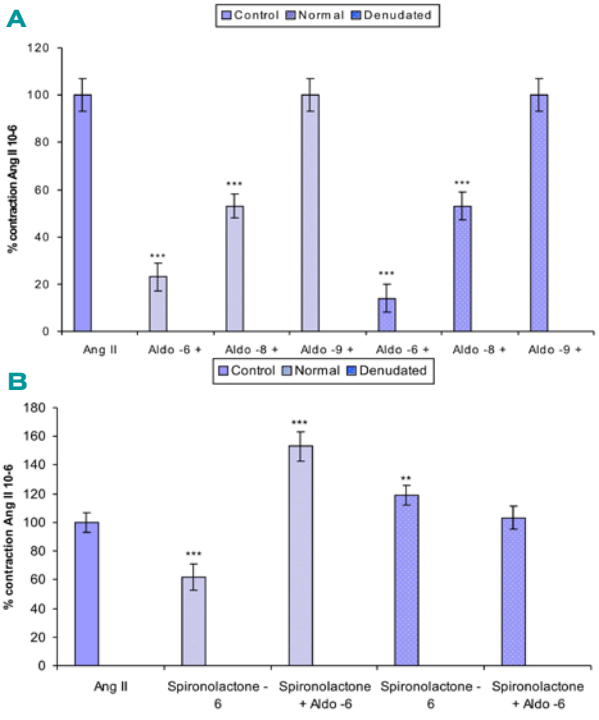

Figure 3. Comparative effects of aldosterone (A) and spironolactone (B) on angiotesnin II (10-6 M) vasoconstriction in normal and denudated aortic rings. The values are mean ± S.E.M. (n=6 animals per group). **p = 0.001 vs. Ang II, ***p< 0.01 vs. Ang II.

Concerning the percentage contraction to Ang II 10^{-6} after the administration of spironolactone, we observed a significant decrease in the contraction after spironolactone 10^{-6} ($F(1,10)=265$, $p<0.01$) administration and a significant increase in the contraction after spironolactone 10^{-6} + aldosterone ($F(1,10)=130$, $p<0.01$) administration, in the normal rings (Figure 3B). Addition-

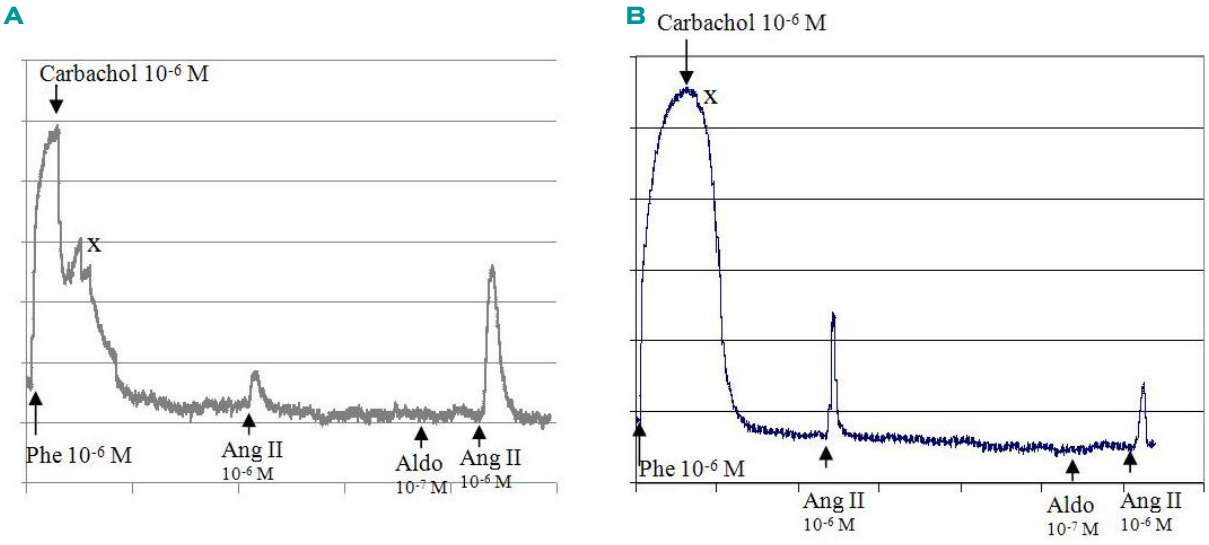

Figure 4. Effects of aldosterone on angiotensin II vasoconstriction in normal (A) and denudated (B) aortic rings.

ally, *post-hoc* analysis revealed significant differences between spironolactone 10^{-6} vs. spironolactone 10^{-6} + aldosterone groups ($p<0.01$)

However, in the case of the denudated rings we found a decrease in the contraction to Ang II 10^{-6} in both spironolactone 10^{-6} ($F(1,10)=18$, $p=0.001$) and spironolactone 10^{-6} + aldosterone ($F(1,10)=1$, $p=0.4$) groups, only the first being, of course, statistically significant (Figure 3B). Also, post-hoc analysis revealed significant differences between spironolactone 10^{-6} vs. spironolactone 10^{-6} + aldosterone groups ($p=0.023$) in the denudated rings.

In its turn, the potentiation of the vasoconstricting effects of Ang II by aldosterone was obvious only in the aortic rings with normal endothelium (Figure 4).

3.2 Modulation of vascular interplay between aldosterone and angiotensin II by amifostine, N-acetylcisteine and L-NAME

Starting from the well-known fact that both aldosterone and Ang II activate at the cardiovascular level the synthesis of SRO [11,17] and nitrogen reactive species [30] activation which is inhibited by the scavengers of free radicals and other endogenous or exogenous antioxidants [31] we investigated the modulatory properties of some antiradical substances on the vascular effects of aldosterone and Ang II in the isolated aortic rings.

In the case of amifostine, a reactive oxygen species scavenger provided with chemo- and radioprotective effects [32] the vasoconstricting action of Ang II was significantly influenced by the radical species of oxygen (O-, H_2O_2), being mostly diminished for the de-endothelized preparations (Figure 5A). In this way, while for the

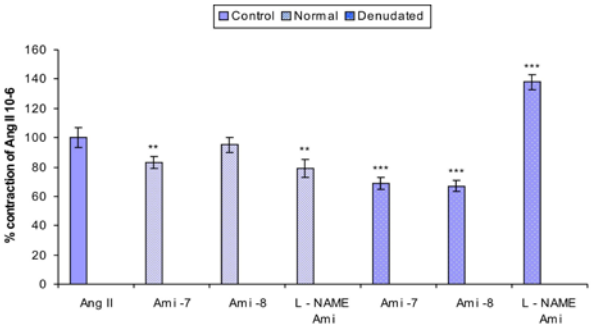

Figure 5A. Influence of amifostine on angiotesnin II (10-6 M) vasoconstriction in pretreated normal and denudated aortic rings with aldosterone. *p = 0.01 vs. Ang II, **p< 0.001 vs. Ang II, ***p< 0.01 vs. Ang II.

normal rings we could only see a significant decrease of the percentage contraction to Ang II 10^{-6} in the amifostine 10^{-7} group ($F(1,10)=27$, $p=0.0004$) and L-NAME + amifostine group ($F(1,10)=25$, $p=0.0005$), in the case of the denudated rings this significant decrease was seen in both amifostine 10^{-7} ($F(1,10)=112$, $p<0.01$) and amifostine 10^{-8} ($F(1,10)=133$, $p<0.01$) groups. However, we noticed also a significant increase in the percentage of contraction to Ang II 10^{-6} in the case of the L-NAME + amifostine group ($F(1,10)=117$, $p<0.01$) (Figure 5A). Post-hoc analysis also showed significant differences between amifostine 10^{-7} vs. amifostine 10^{-8} groups ($p=0.006$) and amifostine 10^{-8} vs. L-NAME + amifostine group ($p= 0.004$), in the case of the normal rings, and also between amifostine 10^{-7} vs. L-NAME + amifostine group ($p<0.01$) and amifostine 10^{-8} vs. L-NAME + amifostine group ($p<0.01$), in the case of the denudated rings.

Similar reactions appeared in aortic ring pretreated with N-acetylcystein, which has antioxidant properties recently demonstrate in oxidative stress [33].

As mentioned, pretreatment with L-NAME induced an increase of the vascular reactivity to Ang II, which was more intense for the normal preparation than for the ones with the endothelium removed.

NOS inhibition with L-NAME produced a significant increase in vascular reactivity, potentiated by aldosterone only in aortic rings with intact endothelium. By removing the vasodilator component of the eNOS-NO couple with L-NAME, the actions of the constrictor oxygen radical species was augmented. This hypothesis is favored by the inhibition of the vasoconstricting effects of angiotensin II in normal aortic rings pretreated with amifostine and L-NAME (Figure 5B). In this way, while in the normal rings the percentage contraction to Ang II 10^{-6} was significantly increased in both L-NAME + Ang II ($F(1,10)=161$, $p<0.01$) and L-NAME + aldosterone+ Ang II ($F(1,10)=1364$, $p<0.01$) groups, in the denudated rings we could observe a significantly decrease in both L-NAME + Ang II ($F(1,10)=10$, $p=0.01$) and L-NAME + aldosterone+ Ang II ($F(1,10)=1364$, $p=0.007$) groups (Figure 5B). Additionally, *post-hoc* analysis showed significant differences in the case of normal rings between L-NAME + Ang II vs. L-NAME + aldosterone+ Ang II groups ($p<0.01$).

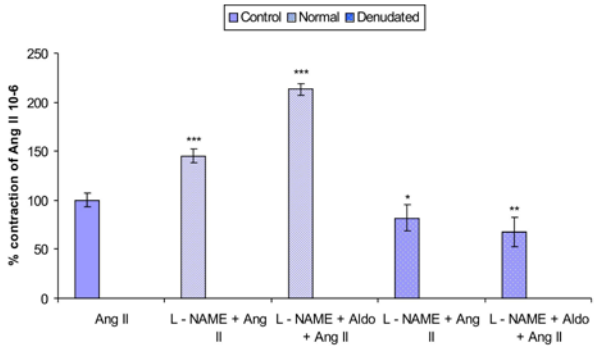

Figure 5B. Influence of L-NAME (B) on angiotesnin II (10-6 M) vasoconstriction in pretreated normal and denudated aortic rings with aldosterone. *p = 0.01 vs. Ang II, **p< 0.001 vs. Ang II, ***p< 0.01 vs. Ang II.

4. Discussion

Many investigations in recent years have demonstrated that beside the activation of the unidirectional transepithelial sodium transport, aldosterone participates in the regulation and modulation of the cardiovascular activity, completing its renal and volemic effects. The two main actions of aldosterone, being produced through cytosol/nuclear mineralocorticoid receptors (genomic) and membrane-linked receptors (nongenomic), they are based on different mechanisms. Unlike the genomic mineralocorticoid receptors, which slowly activate the nuclear genic transcription, generating effector proteins, the nongenomic membrane receptors trigger electrochemical reactions that determine rapid activating or inhibiting cellular responses with participation of the endothelial reactive oxygen and nitrogen species.

Thus, aldosterone inhibits the vasoconstrictor effects of angiotensin in a dose-dependent manner, without the involvement of the endothelium. This effect does not appear to be mediated by the genomic receptors for aldosterone, as the blockade of these receptors with spironolactone does not affect the angiotensin vasoconstriction.

In agreement with others [13] these results provide new evidence that aldosterone modulates by its rapid nongenomic effects the reactivity of some vascular territories with participation of varied signaling molecules, including the reactive oxygen and nitrogen species.

These vasoconstrictor effects can be attributed to the generation of free radicals by the angiotensin, as the pre-treatment with free-radical scavenging agents like amifostine or n-acetylcysteine significantly reduced the constricting effects of angiotensin. Aldosterone in this case proved to be a protector against the effects of endothelium removal or NO-synthase blockade with L-NAME.

Our data provide additional arguments that the nongenomic actions of aldosterone on aortic smooth muscle cells of rats are a question of cross-talk and balance between its rapid vasoconstrictor and vasodilator effects, as result of the activation of reactive oxygen species in the first case and of nitrogen species in the second one.

The chemical cascade of the vascular interactions between aldosterone, Ang II and antioxidants in the aortic smooth muscle cells is schematically presented in Figure 6.

Several other authors have emphasized the role of the aldosterone in the angiotensin-induced free radical damage in the Cardiorenal Metabolic Syndrome [34,35] and of aldosterone excess in the development and progression of cardiovascular disease states including hypertension, metabolic syndrome, cardiac hypertrophy, heart failure, and cardiorenal fibrosis [36].

The biological effects of aldosterone should be interpreted in this context. At low ambient oxidative stress, aldosterone promotes NO production and vasodilatation, while in situations with increased oxidative stress the endothelial dysfunction and detrimental effects induced by vasoconstriction will prevail. Thus, aldosterone could be considered both “friend and foe”.

The results of the present study indicate that a more determined use of aldosterone blockade, both of the genomic and of non-genomic receptors, combined with a more extensive use of antioxidants as adjuvant therapy in the above-mentioned pathological entities would improve the therapeutic outcome.

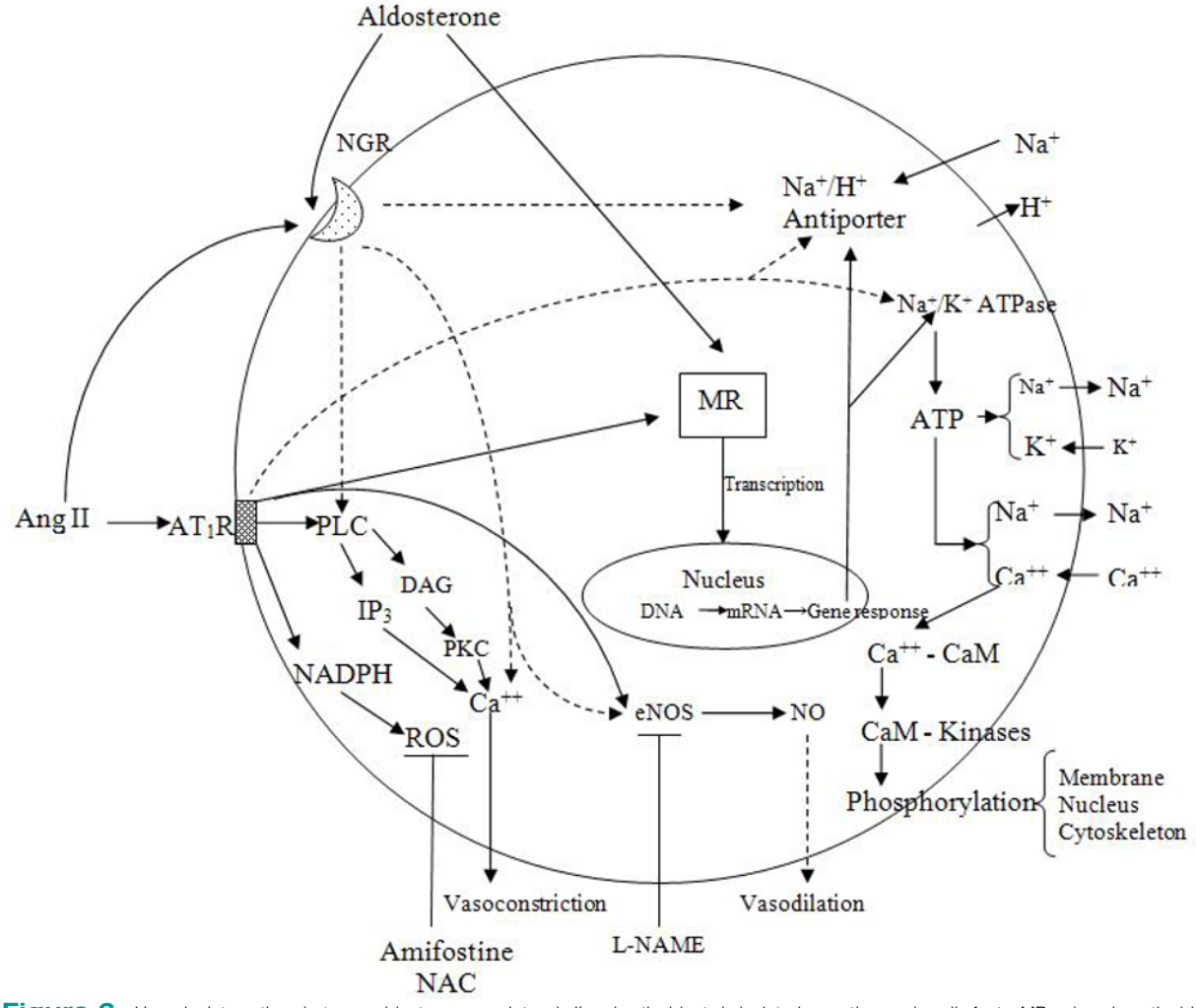

Figure 6. Vascular interactions between aldosterone, angiotensin II and antioxidants in isolated smooth muscle cell of rats. MR- mineralocorticoid receptor, NGR- non-genomic receptor, AT1R- AT1 angiotensin II receptor, CaM- calmodulin.

Acknowledgments

Ciobica Alin is supported by a POSDRU grant /89/1.5/S/49944, "Developing the innovation capacity and improving the impact of research through post-doctoral programs" Alexandru Ioan Cuza University, Iasi.

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