Biochemical Effects of Intraliposomal Angiotensins on Isolated Vascular Smooth Muscle Cells

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The intracellular renin-angiotensin effectors (peptides, enzymes, receptors) and their effects are intriguing for a lot of systems. That's why we aimed the effects of intracellularly-administered angiotensins (angiotensin II, angiotensin III, angiotensin IV, angiotensin fragment 1-7), angiotensinogen, CGP-42112A, apelin, and angiotensin receptors blockers (losartan, PD123319), by the means of liposomes, on apoptosis of cultured isolated rat aortic vascular smooth muscle cells. We evidenced that CGP-42112A (a potent AT_2 angiotensin II receptor agonist), administered intracellularly, induced the apoptosis of the cultured isolated vascular smooth muscle cells in a much higher proportion than other agonists and antagonists of angiotensin system: CGP-42112A > angiotensin II> angiotensin III≅ angiotensinogen. Moreover, losartan (an AT_1 angiotensin II receptor antagonist), administered intracellularly, induced an important degree of apoptosis of cultured isolated vascular smooth muscle cells. Losartan, administered as concomitant treatment for other angiotensin peptides and CGP-42112A, did not significantly modified the apoptotic effects of these peptides. On the other hand, PD123319 (an AT_2 angiotensin II receptor antagonist) was able to significantly reduce the losartan effects when administered as co-treatment for 24 h. The same effects were obtained when LY294002, a PI3K/Akt signaling inhibitor, was administered as a co-treatment. We can conclude an involvement of an AT_2 angiotensin II receptor and PI3K/Akt signaling in these apoptotic effects induced by some angiotensin peptides and losartan on cultured isolated rat aortic vascular smooth muscle cells.

Keywords: vascular smooth muscle cells, rat aorta, intracellular angiotensin, losartan, apoptosis

The inflammatory and atherogenic vascular processes are involving in many ways the guanosine-trisphosphate molecules and its metabolites. Such one metabolite of guanosine-trisphosphate is neopterin, released by activated macrophages. When assessed, neopterin concentrations in the plasma and lesions of patients with coronary artery disease were increased. Neopterin reduced proliferative capacities of endothelial cells from human aortic origin. These effects were doubled by the decreasing of upregulated monocyte chemotactic protein 1, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 by tumor necrosis factor. Furthermore, neopterin reduced the adhesion of monocytes to endothelial cells of human aortic origin, promoted by tumor necrosis factor, and blocked the development of macrophages toward the inflammatory phenotype through the downregulated nuclear factor-KB. On the other hand, neopterin blocked the forming of foam cells, induced by oxidized low-density lipoproteins, processes involving the downregulation of CD36 and the upregulation of cassette transporters of A1 and G1 type, driven by ATP, in the human macrophages having the origin in circulating monocytes. In smooth muscle cells of human aortic origin neopterin downregulated the pathways represented by c-Src/Raf-1/ERK1/2 without apoptosis, inducing the same time the suppression of migration and proliferation mediated by angiotensin II. *In vivo*, in mice with apolipoprotein E deficiency, the enhancement of neopterin attenuated the development of aortic atherogenesis. The results of these studies suggested that neopterin, an internal metabolite, could be involved in the attenuation of atherogenesis processes. Meanwhile, it could be used as a novel therapeutics for cardiovascular diseases associated to atherosclerosis [1].

Calcium signaling, fundamental for functioning of all cell types, could be modulated by the so-called stromal interacting molecule-1. The alterations of stromal interacting molecule-1 concentrations in cardiomyocyte will induce cardiac complications. When its corresponding gene is deleted or its concentrations are decreased in endothelium, these results in endothelial dysfunctions. On the other side, the alteration of stromal interacting molecule-1 in vascular smooth muscle cells will not affect the endothelium functioning but will protect vascular functioning toward the infusion with angiotensin II. There are no data on the role of stromal interacting molecule-1 in myocardial infarction of acute or chronic type as a result of acute ischemia-reperfusion injury, as well as of permanent coronary artery occlusion. *In vivo*, the heart infarction was increased when the stromal interacting molecule-1 was augmented. On the other hand, it is of interest to mention that the size of the infarction area was decreased in stromal interacting molecule-1(SMC-/-) mice. The protective effects showed in stromal interacting molecule-1(SMC-/-) mice are associated with the modulatory effects on apoptosis, inflammatory processes, oxidative and endoplasmic reticulum stress, as well as on mitogen-activated protein kinases of ERK1/2 and p38 types. All the above mentioned processes are involved somehow in the protective effects of stromal interacting molecule-1 from smooth muscle cells toward the development and progression of myocardial infarction [2].

The restenosis process following the injury of carotid artery is based on the hyperplasia of intima, involving on a large scale the migratory and proliferative capacities of vascular smooth muscle cells. ATRAP (aka angiotensin II type 1 receptor-associated protein) was suggested to counteract these hyperplasic phenomena, but it was not demonstrated if apoptosis plays any role. When angiotensin

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II type 1 receptor-associated protein was overexpressed by adenoviral means, it induced vascular smooth muscle cells apoptosis, having as result the alleviation of neointima forming in rats' carotid artery, exposed to balloon injury. Moreover, under the stimulation of angiotensin II, the overexpressed angiotensin II type 1 receptor-associated protein also induced the apoptosis of rat vascular smooth muscle cells through the PI3K-Akt pathway. The vascular smooth muscle cells apoptosis was abolished by the upregulated Akt, when PTEN inhibitor was used. The clear conclusion of these studies is that angiotensin II type 1 receptor-associated protein has a regulatory role in the hyperplasic processes affecting the carotid intima. These regulatory roles are involving apoptosis of vascular smooth muscle cells which is mediated by PI3K-Akt signaling [3].

The actual studies were performed to research the effects of intracellularly-administered angiotensins (angiotensin II, angiotensin III, angiotensin IV, angiotensin fragment 1-7), angiotensinogen, CGP-42112A, apelin, and angiotensin receptors blockers (losartan, PD123319) by the means of liposomes, as well as LY294002, a PI3K/Akt signaling inhibitor, on apoptosis of cultured isolated rat aortic vascular smooth muscle cells.

Experimental part

To obtain cultured isolated vascular smooth muscle cells 5 rats were used, applying an anterior developed and adapted technique [4, 5]. Apoptosis of cultured isolated vascular smooth muscle cells from rat aorta was measured in accordance with the protocols applied by our laboratory for several lines of cells and implied the flow cytometry as instrument [6, 7]. To administer intracellularly the active molecules, we used liposomes, prepared and characterized as previously described [8-11].

The apoptosis of cultured isolated vascular smooth muscle cells from rat aorta was assessed after the administration of angiotensin II (amino acid sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), angiotensin III (amino acid sequence Arg-Val-Tyr-Ile-His-Pro-Phe), angiotensin IV (amino acid sequence Val-Tyr-Ile-His-Pro-Phe), angiotensin fragment 1-7 (amino acid sequence Asp-Arg-Val-Tyr-Ile-His-Pro), angiotensinogen, CGP-42112A (amino acid sequence Tyr-Z-Arg-Lys-His-Pro-Ile, a potent AT_2 angiotensin II receptor agonist), losartan (an AT_1 angiotensin II receptor antagonist), PD123,319 (an AT_2 angiotensin II receptor antagonist), apelin-13 (amino acid sequence Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, a synthetic endogenous peptide agonist for the human APJ receptor, a putative receptor protein related to the angiotensin receptor AT_1), all in concentrations of 10^{-3} M in liposomes-entrapped solution. The proportion of administered liposomes was of 1% as related to vascular smooth muscle cells culture medium for 24 h. The apoptotic cells index was referred to that of valinomycin (1 μM), considered to be 100%.

Some experiments were doubled by the concomitant administration of the LY294002, a PI3K/Akt signaling inhibitor, 10 μM for 24 h.

All the protocols involving Wistar rats from Baneasa source were entirely approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy from Iasi.

Results and discussions

When we started the analysis of results, we evidenced that CGP-42112A (amino acid sequence Tyr-Z-Arg-Lys-His-Pro-Ile, a potent AT_2 angiotensin II receptor agonist), administered intracellularly by the means of liposomes, induced the apoptosis of the cultured isolated vascular smooth muscle cells in a much higher proportion than other agonists and antagonists of angiotensin system: CGP-42112A (fig. 1) > angiotensin II (fig. 2) > angiotensin III (fig. 3) ≅ angiotensinogen (fig. 4). Moreover, losartan (an AT_1 angiotensin II receptor antagonist), administered intracellularly by the means of liposomes, induced an important degree of apoptosis of cultured isolated vascular smooth muscle cells (fig. 5). Losartan, administered as concomitant treatment for other angiotensin peptides and CGP-42112A, did not significantly modified the apoptotic effects of these peptides. On the other hand, PD123319 (an AT_2 angiotensin II receptor antagonist) was able to significantly reduce the losartan effects when administered as co-treatment for 24 hours (fig. 6). The same effects were obtained when LY294002, a PI3K/Akt signaling inhibitor, was administered as a co-treatment.

Very important from the point of view of analysis were the inhibitory effects of 10^{-3} M PD123319 (an AT_2 angiotensin II receptor antagonist), entrapped in liposomes, on the apoptosis induced by 10^{-3} M CGP-42114A (an AT_2 angiotensin II receptor agonist), encapsulated in

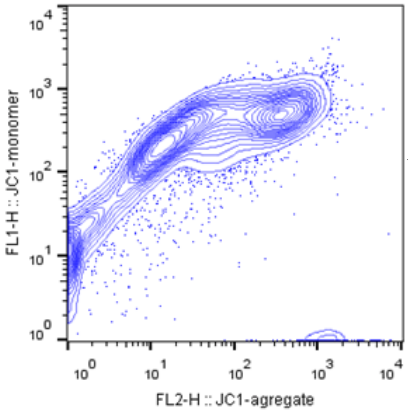

Fig. 1. When were treated with 10^{-3} M CGP-42114A, encapsulated in liposomes, for 24 h, the cultured isolated vascular smooth muscle cells were associating apoptosis in a proportion of 45% on average (representative experiment presented)

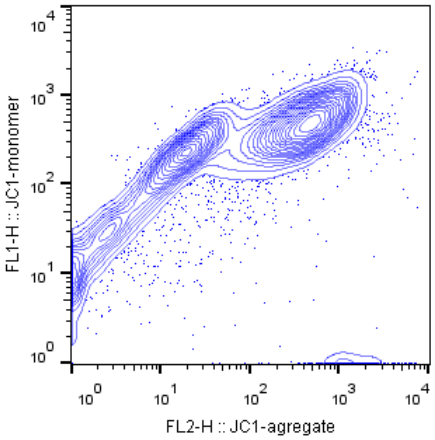

Fig. 2. Angiotensin II, 10^{-3} M, administered intracellularly by the means of liposomes, induced an apoptosis proportion of 25% on average of cultured isolated vascular smooth muscle cells (representative flow cytometry)

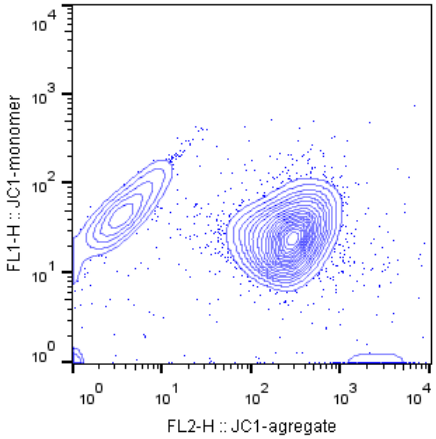

Fig. 3. When were treated with 10^{-3} M angiotensin III, encapsulated in liposomes, for 24 h, the cultured isolated rat aortic vascular smooth muscle cells were demonstrating apoptosis in a proportion of 15% on average (representative experiment presented)

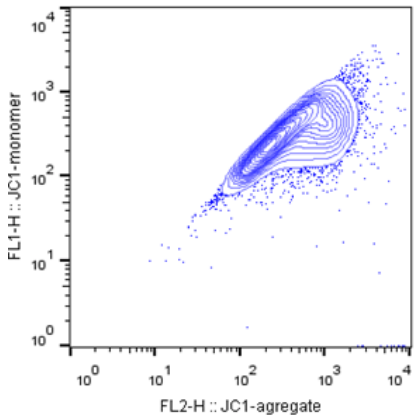

Fig. 4. Angiotensinogen, 10^{-3} M, renin substrate, administered intracellularly by the means of liposomes, induced an apoptosis index of 8% on average of cultured isolated vascular smooth muscle cells (representative flow cytometry)

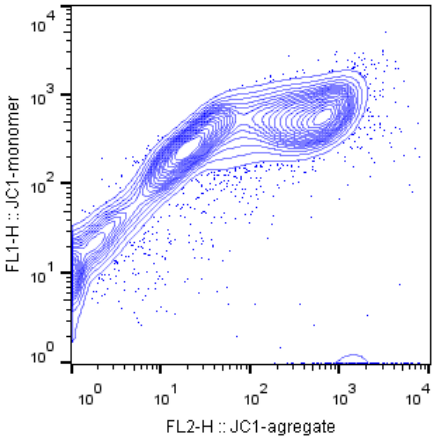

Fig. 5. Interestingly, **w**hen were treated with 10^{-3} M losartan (an AT_1 angiotensin II receptor antagonist), entrapped in liposomes, for 24 h, the cultured isolated rat aortic vascular smooth muscle cells were evidencing apoptosis in a proportion of 32% on average (representative experiment presented)

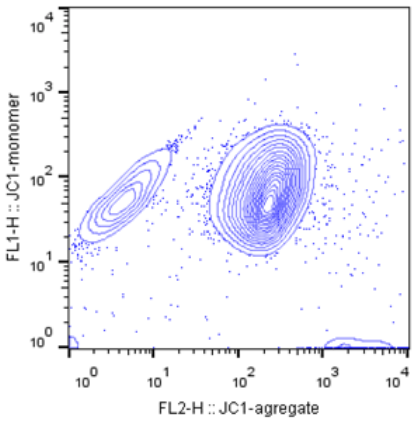

Fig. 6. LY294002, a PI3K/Akt signaling inhibitor, reduced significantly the apoptosis index of cultured isolated vascular smooth muscle cells toward 12% on average, when was administered as 10 μM co-treatment for 24 h for losartan entrapped liposomes (representative flow cytometry)

liposomes (almost 50% blocking, data not shown). Moreover, apelin-13 (amino acid sequence Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe, a synthetic endogenous peptide agonist for the human APJ receptor, a putative receptor protein related to the angiotensin receptor AT_1), administered intracellularly by the means of liposomes, induced no apoptotic effects on cultured isolated vascular smooth muscle cells. The same was for angiotensin IV, as well as for angiotensin fragment 1-7.

The deep molecular mechanisms ascribed to the above particular effects remain to be explored by our future studies.

There are data demonstrating that the expressed angiotensin II, fundamental peptide of the renin-angiotensin system, is really closely related to the appearance and development of cancer diseases. Telmisartan, a widely specific angiotensin II type 1 receptor blocker, has been suggested to possess anticancer potential in renal cancer. When tested also on non-small cell lung cancer cells, telmisartan induced their apoptosis through PI3K/Akt signaling pathways. Thus, telmisartan, might be considered as a novel therapy for non-small cell lung cancer [12].

Very interesting from the point of view of the functioning of intracellular renin-angiotensin system would be the application of the actual studies and methods to other mature and precursor cellular systems [13-18].

It is evident that plastics represent a revolutionary trend in medicine, especially for medical devices, hematological care, cardiology procedures, etc. Beside these clear advantages, studies demonstrated that phthalate chemicals migrate out of them and affects cardiovascular system functioning [19]. Thus, we must be able to recycle the vast majority of wastes we produce, improving the quality of our life [20-27].

The diseases from the cardiovascular and metabolic groups have as basal pathophysiological mechanism the oxidative stress and its altered control. One very important source for H_2O_2 generation is the angiotensin peptides system, acting both intracellularly or extracellularly [28].

The analysis of mixtures realized simultaneously and quantitatively, could involve the bivariate and multivariate spectral calibrations. Such calibrations are suitable for example for atorvastatin-amlodipine and telmisartan-hydrochlorothiazide mixtures from tablets. The conditioning of various pharmaceutical forms is important from the point of view of pharmacological effects [29].

The angiotensin-aldosterone system is closely functionally related with oxidative stress and especially with free radicals of oxygen [30]. From this angle, the components of the intracellular angiotensin peptides system must be extensively researched to find if there is any relationship with the oxidative stress-antioxidant systems.

Conclusions

The actual studies were performed to research the effects of intracellularly-administered angiotensins (angiotensin II, angiotensin III, angiotensin IV, angiotensin fragment 1-7), angiotensinogen, CGP-42112A, apelin, and angiotensin receptors blockers (losartan, PD123319) by the means of liposomes, as well as LY294002, a PI3K/Akt signaling inhibitor, on apoptosis of cultured isolated rat aortic vascular smooth muscle cells.

The deep molecular mechanisms ascribed to the above particular effects remain to be explored by our future studies.

Acknowledgements: Diana Tatarciuc is a Ph.D. Student at the Grigore T. Popa University of Medicine and Pharmacy of Iasi, 16, University Str., 700115, Romania

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Manuscript received: 15.11.2017