PhD THESIS
–ABSTRACT–

PROANGIOTENSINE 12:
BETWEEN THE ROLE OF FORERUNCER
OF THE ANGIOTENSINE II
AND THAT OF DIRECT EFFECTOR.
PARTICULARITIES ON UTERINE SMOOTH MUSCLE

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KEY WORDS

Proangiotensine 12, Angiotensine II, murine myometrium, AT1 receptors, Losartan, captopril, calcium intracellular deposits, IP3, ryanodina, proestrum, estrum, diestrum, gestation, occitoccic effect
GENERAL DATA

Proangiotensin 12 represents the „last income” into the family of angiotensines. It was initially isolated from the rat intestine (Nagata and al., 2006), but its presence was proven in the kidney, liver, CNS, heart, aorta or pancreas. The team of authors that have identified it proved that under laboratory conditions, the compound can induce the increased blood pressure (administered intravenously) and is able to contract the aorta rings isolated by the rat. Both effects are counteracted by captopril (ACE inhibitor- inhibitor of the conversion enzyme) and by CV-11974 (blocker of AT1 receptors). These data argue that, “in vivo”, proangiotensin 12 is first of all converted in angiotensin I, and, further, under the action of the converting enzyme into angiotensin II, the last one being in fact responsible for the hypertensive effect of proangiotensine 12.

Due data demonstrate for the first time that proangiotensin 12 is capable of generating per se effects, completely different at the vascular and uterine level, involving receptors and distinct systems of intracellular signaling, sometimes even completely different from those of angiotensine II, the role of precursor of angiotensine II „in vivo”, being just a part of its importance in the angiotensin system.

These results were possible only because of the exceptional techniques that I had to get in order to obtain the preliminary data: intracellular microinjecting and viewing of the intracellular calcium dynamics.

OBJECTIVES OF THE STUDY

1. Studying the effects of proangiotensin 12 over the smooth uterine muscle.
2. Investigation of functional differences and similarities between proangiotensin 12 and angiotensin II.
3. Study of the uterine smooth muscle sensibility in angiotensin II and in proangiotensin 12, according to hormonal status.
4. Study of different types of intracellular calcium deposits over the ocytocic effects of angiotensin II and proangiotensin 12.
RESULTS AND DISCUSSIONS

I. COMPARATIVE STUDY OF THE OCYTOCIC EFFECT OF ANGIOTENSIN II VERSUS PROANGIOTENSIN 12

Angiotensin II (AG II) had a higher ocytocic effect than proangiotensin 12 (PRO) in extracellular administration, fact proven both by using fragments of the rat uterus and smooth muscle cells in suspension, into which we have used the visualisation technique of the ion of calcium in cytosol (Tab. 4. and Fig. 1).

But, in intracellular administration, the PRO effect was much higher to the one induced by AGII.

The possible explanation of this phenomenon will be discussed immediately below.

Figure 1. Variation of angiotensin II (AG II) and of proangiotensin 12 effect (PRO), depending on the concentration, on a fragment of female rat uterus, found in diestrum, quantified according to the area from under the concentration curve.
II. TYPE AND LOCATION OF INVOLVED RECEPTIVE STRUCTURES

In extracellular administration, both by using fragments of rat uterus, as well as cell suspension, the AGII effect is cancelled by losartan, which is a selective blocker of the receptors for angiotensin of type I – AT1. This demonstrates that AGII acts exclusively through setting this type of receptors. Losartan inhibits only partially (37±2,4%) the PRO action, administered extracellular (through the two modalities just as AGII), fact that proves that PRO partially owes its effect to the stimulation of AT1 receptors and, first of all, to the stimulation by specific receptors, whose characteristics remain to be discovered later.

They are mandatory membranar, the substance having peptide structure is not able to get through the cell membrane.

In extracellular administration, AGII has a superior PRO effect, which proves that the substance fixes more intensely the AT1 receptors, the stimulation of the other (others) type(s) of specific receptors by PRO is resulting in a lower effect.

In intracellular administration, by intracellular micro-injection technique, PRO generates a greater effect than AGII. In the same time, the intracellular injection by losartan cancels the AGII effect, which shows that the AGII effect in intracellular administration is due to the fixation on intracellular receptors, as shown by Brailoiu and al., in 1999[120]. In the same time, losartan inhibits with only 24±2,4% the PRO action.

III. DESENSIBISATION OF THE RECEPTORS FOR ANGIOTENSIN II AND FOR PROANGIOTENSIN 12

The AT1 receptors, both the membranar and the intracellular ones, undergo a rapid desensibilisation process, two administrations of AG II being able to diminish with up to 91±2,4% the contractile response (value determined through the technique of visualisation on intracellular calcium dynamics).

On muscular preparates, the desensibilisation has been of 63,52±14%, but the interval between the two administration was of 15 minutes and not of 2 minutes as in the case of cellular suspension usage.

Continuing to use suspensions of smooth uterine muscular cells, two successive administrations, at every two minutes of PRO was determined a diminishing of the effect with 64±2,4%. If we take into consideration the
fact that PRO owes its effect on a rate of 24±2,4 % to the stimulation of AT1 receptors and that these are suffering a desensitisation process of about 91±2,4%, it can be easily calculated the degree of desensitisation of the specific receptors for PRO, as being of about 42%, percentage significantly lower than the AT1 receptor targeting.

The administration of PRO, 2 minutes after the AG II, led to a diminished response as compared to AGII, with 66±2,5%, namely with 43±2,4% vs. PRO witness. If we subtract the percentage of AT1 receptors that do not desensitizes after the AT1 administration (9±2,4% – see above) from this last value we reach a value of 34%, similar to the one obtained through the calculation of the impact losartan had over the contraction induced by PRO. So, the share of AT1 receptors, in the contraction induced by PRO, is around the value of 30%.

The administration of AG II every 2 minutes after PRO, determines a reduction with about 88±2,4% from the answer witnessing AG II, that demonstrates that the PRO receptors desensitize the AT1 receptors equipotent with AG II.

IV. ACTIVATED INTRACELLULAR SIGNALING PATHWAYS

It is highly unlikely that specific receptors for PRO to have a higher intracellular density as compared to membranar localisation.

Under these circumstances it seems logical to assume that, in intracellular administration, the more intense effect of PRO towards AG II to be due to the activation of different intracellular signaling pathways, pathways that cannot be initiated by setting PRO on membranar receptors.

V. THE EFFECT OF BLOCKING THE CONVERSION ENZYME OVER THE ACTION OF PROANGIOTENSINE 12

Captopril inhibits the PRO contraction with about 41,26±17%, which shows that some of the PRO is metabolized into angiotensin I (AGI), that being transpormed into AG II, by the conversion enzyme.

If we take into consideration the fact that PRO owed its effect at a rate of 24±2,4% to the stimulation of AT1 receptors, we can say that the PRO effect over the last ones is dwed to AGII, synthetized from PRO.

The difference is probably generated by the non-specificity of captopril action, this being also responsible for other effects, not necessary related to the PRO effect.
So, PRO owes its action, predominantly, to its own effects, his role of precursor of AG II coming second.

VI. THE DEPENDENCE OF UTERINE CONTRACTILE ACTIVITY ON HORMONAL STATUS

From the realised experiments, it has come out a spontaneous maximal activity during the estrum, characterized through values exceeding up to 6 times those measured in proestrum (Tab. 1). This is explained by the fact that during this period, a maximal motility of murine genital channels in order to ensure a rapid ascension of sperm on the one hand, and on the other, a safe intake of eggs produced by the ovaries of female rats.

The characteristic aspect to the waves of spontaneous contraction could be seen, these representing towards their peak, many irregularities, due to the specific amplitude of ionic currents, specific for this stage of maximum motility.

<table>
<thead>
<tr>
<th></th>
<th>ESTRUM</th>
<th>PROESTRUM</th>
<th>DIESTRUM</th>
<th>GESTATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium amplitude (g)</td>
<td>1,86</td>
<td>1,47</td>
<td>1,63</td>
<td>1,13</td>
</tr>
<tr>
<td>Maximum amplitude(g)</td>
<td>2,17</td>
<td>1,52</td>
<td>1,78</td>
<td>1,72</td>
</tr>
<tr>
<td>Medium frequency</td>
<td>15</td>
<td>3</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>(contractions/10 min.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area under the curve (g.s.)</td>
<td>239</td>
<td>40</td>
<td>165</td>
<td>266</td>
</tr>
</tbody>
</table>

Tabel 1. The variation of the main characteristics of spontaneous uterine motility, in the different stages of murine sexual period, respectively during pregnancy
However, as can be seen from Table 2, in this phase of murine sexual period, the myometrium presents a maximal sensitivity to oxytocic agents, these having a powerful contractile effect both on frequency, but especially on the amplitude of contractile waves.

<table>
<thead>
<tr>
<th></th>
<th>ESTRUM</th>
<th>PROESTRUM</th>
<th>DIESTRUM</th>
<th>GESTATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGII</td>
<td>3.96</td>
<td>2.38</td>
<td>2.13</td>
<td>2.07</td>
</tr>
<tr>
<td>PRO</td>
<td>3.39</td>
<td>2.19</td>
<td>2.04</td>
<td>1.61</td>
</tr>
<tr>
<td>Maximal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amplitude (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>1343</td>
<td>924</td>
<td>755</td>
<td>843</td>
</tr>
<tr>
<td>Area under the</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>curve (g.s.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth (with %)</td>
<td>462</td>
<td>1.202</td>
<td>358</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>287</td>
<td>885</td>
<td>276</td>
<td>147</td>
</tr>
</tbody>
</table>

Table 2. The effect of angiotensin II (AGII) and proangiotensin 12 (PRO), over spontaneous uterine contraction, according to the different stages of murine sexual period.

The proestrus phase is being characterized through a minimal of the uterine spontaneous activity. This aspect according to the physiological development of the sexual murine cycle, the genital apparatus having a period of „calm”, „preparation”, before the estrum and, eventually, before a pregnancy.

Notable is that in proestrus, although there are long periods without spontaneous activity, the contractile waves are not much lower than in the other phases of murine sexual period, which suggests the fact that, this period is characterised through the extremely marked decrease of excitability of the myometrum and, to a lesser extent of the contraction force. Furthermore, the amplitude of the contractile waves is superior to those in the diestrum phase.

The reduction of excitability is also proven by the decreased sensitivity to contractile agents (with about 50% as compared to the diestrum and with about 300% facing the estrum) (Tab.2). This aspect is correlated to the fact mentioned above, that this stage is a „breathing space”, before the phase of hyperactivity that characterises the estrum.
The diestrus phase is characterized by a great consistency of spontaneous activity, reason that determined its election as phase for the collection of biological material for study. Spontaneous activity returns “to normal”, if fecundation has not occurred, the gynaecological apparatus preparing for a new sexual cycle. Excitability in ocytic agents is medium and, most important, is being characterized by a marked consistency.

Gestation fundamentally modifies spontaneous uterine motility. Murine uterus has an increased spontaneous activity, but with a reduced amplitude. But what mostly characterizes the myometrial automatism in this stage is the complete irregularity of the waves. All these are explained by the fact that murine uterus, unlike the human one is bicornual and can contain up to 10 foetuses (even more eventually). Or, under this circumstance a perpetuous movement of uterine structures is required in order to adapt all foetuses in murine genital ducts and their correct positioning in view of birth. Also, this chart of spontaneous activity, is the mirror of membrane ionic currents (and not only), extremely complex and broad in pregnancy. Now new type of ionic ducts appear and the characteristics of the existing ones are fundamentally modified.

VII. THE DEPENDENCE OF CONTRACTILE UTERINE ACTIVITY ON THE DYNAMICS OF THE ION OF CALCIUM

The automatism is partly dependent of the mobilized calcium deposits from endoplasmic deposits, sensitive to inozitol 1,4,5 triphosphate (IP₃), especially in what the contraction form is concerned and, to a lesser extent, the frequency of spontaneous oscillations. The role of calcium deposits sensitive to ryanodine, lysosomal calcium deposits respectively in the spontaneous contractility remains to be established.

<table>
<thead>
<tr>
<th>Percentage (%)</th>
<th>Endoplasmic deposits sensitive to IP₃</th>
<th>Endoplasmic deposits sensitive to RYANODINE</th>
<th>Lysosomal calcium deposits</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.25</td>
<td>0-10</td>
<td>0-15</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. The percentage of different intracellular calcium deposits during spontaneous contractile activity
Both angiotensin II (AG II), as well as proangiotensin 12 (PRO) induce a contractile effect depending on the calcium concentration in the cytosol.

<table>
<thead>
<tr>
<th>Endoplasmic deposits sensitive to IP3</th>
<th>Endoplasmic deposits sensitive to RYANODINE</th>
<th>Lysosomal calcium deposits</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGII</td>
<td>PRO</td>
<td>AGII</td>
</tr>
<tr>
<td>Percentage (%)</td>
<td>34,12</td>
<td>38,79</td>
</tr>
</tbody>
</table>

Table 4. The percentage of different intracellular calcium deposits during the contractile activity induced by angiotensin II (AGII) $10^{-7}$M, respectively by proangiotensin 12 (PRO) $10^{-7}$M, on a fragment of female rat uterus, found in the diestrum.

Endoplasmic calcium from the deposits sensitive to IP$_3$ interferes in diastolic depolarisations, thus in the frequency of contractile waves, but its main impact is on the contraction force.

Endoplasmic calcium from the deposits sensitive to ryanodine has a lower percentage in the contractions induced by AGII, namely PRO. The effect is more intense on the amplitude of contractile waves, having an insignificant impact on their frequency.

The calcium in lysosomes has a more intense role during the generation of the contraction form and, secondary in the diastolic depolarisations, thus in the frequency of uterine activity.

PRO is more sensitive to the variations of the calcium concentration from cytosol, as compared to AG II, having the same concentrations.
CONCLUSIONS

1. In extracellular administration, angiotensin II (AGII) has a more intense oxytocic effect than proangiotensin 12 (PRO).

2. In extracellular administration, AGII owes completely its effect of fixation from the AT1 receptors, unlike the PRO, whose effect is owed only in a proportion of 37±2.4% to AT1 receptors activation, the rest of the contractile effect being the result of fixation on specific receptors, whose characteristics remain to be clarified.

3. In intracellular administration, through intracellular injection, PRO generates a more intense oxytocic effect than AGII.

4. The AGII effect, in intracellular administration is completely dependent on the activation of the AT1 intracellular receptors (Brailoiu and al. 1999), unlike PRO, whose effect is only slightly due to the fixation on AT1 receptors (24±2.4%) and, in a greater measure, to the activation of specific intracellular receptors. (identical to the membranar ones?).

5. Being unlikely for the receptor specific intracellular structures for PRO to be more numerous than the extracellular, we concluded that PRO activates specific signaling pathways distinct from those initiated by AGII (in intra or extracellular administration) or by the very PRO, in extracellular administration.

6. AT1 receptors, both membranar and intracellular, undergo an intense process of desensitization, whether they are repetitively activated by AGII or PRO.

7. PRO-specific receptor structures also suffer a process of desensitization, but much lower than for AT1.

8. AT1 receptor desensitization and that of those specific for PRO is not a cross-type one.

9. The inhibition of angiotensine conversion (AGI) in AGII, diminishes with PRO activity, thing that proves the fact that, PRO is turned into AGI and, subsequently, in AGII, at the level of smooth uterine muscular fiber.

10. The fact that the impact of blocking the conversion enzyme over the contraction induced by PRO approximately corresponds to the impact of losartan, we can conclude that PRO acts on specific receptors, and the stimulation of AT1 receptors, by PRO, is due only to AGII, the results from PRO metabolisation.
11. It can be concluded that PRO owes its action, predominantly to its effects, its role of precursor of AG II being secondary.

12. Uterine contractility, both spontaneous and induced by AGII, namely PRO, is strongly dependent on the hormonal status. A maximum in the estrum phase is being registred, when a maximal motion of genital murine ducts is necessary in order to ensure, on one hand, a rapid ascent of spermatozoids, and on the other, a safe intake of the eggs, produced by the ovaries of the female rat. The minimum is being registred in the proestrum stage, a period of “quitness”, „respiro” being necessary before the phase of hyperactivity, that characterises the estrum and eventually a pregnancy. The diestrum phase is characterized through a special consultance of spontaneous activity, reason for which it has been chosen as a phase for the gathering of biological matherial for the present study. Gestation fundamentally modifies the spontaneous uterine motility. Contrary to the human uterus, characterised in this situation by a practically absent spontaneous activity and a basal tosus in a continuous decrease, murien uterus presents a intense uterine activity in frequency but with a reduced amplitude and, completely irregular. This specific feature is needed to adapt and position the multiple fetuses in utero ducts, in view of developing and then , finally, their birth.

13. Uterine contractility, both spontaneous and the one induced by AGII, respectively PRO, are dependent on the dynamic of calcium ion in the cytosol. From the two peptides, PRO is more sensitive at the variations of \([Ca^{2+}]_i\). The calcium in the endoplasmatic deposits, sensitive to IP3 interfere, especially in generating the force of contraction, but also in the spontaneous dyastolic depolarisations, thus in the frequency of spontaneous oscillations. The calcium in the endoplasmic deposits , sensible to ryanodine has a reduced percentage in myometrial contratility, both spontaneous and induced through AGII or PRO, the effect being almost exclusive over the tonic component. Lysosomal calcium interferes, especially in the generation of the force of contraction.
SELECTIVE BIBLIOGRAPHY


CURRICULUM VITAE

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Papers presented at Congresses / Conferences / Symposiums - 53
of which at events:
- National -26;
- International / national with international participation -27.

Articles published "in extenso" in peer-reviewed journals - 5,
of which:
- ISI journals - 2;
- ISI indexed journals - 2;
- MEDLINE indexed journals / BDI - 1;

Summaries / abstracts published - 18, of which:
- ISI journals - 3;
- MEDLINE indexed journals / BDI - 6;
- journals / indexed NURC - 9;

Books published - five of which:
- First author - 2;
- co-author - 3;

Grants / research contracts:
- international - 1;
- National - 4;