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ANGIOTENSIN II RECEPTOR SUBTYPES PLAY OPPOSITE ROLES IN REGULATING PHOSPHATIDYLINOSITOL HYDROLYSIS IN RAT SKIN SLICES

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Summary: Among the many functions of angiotensin II (Ang II) it now appears that Ang II is a growth factor. The concentration of Ang II in rat skin has been shown to increase during wound healing. To investigate the intracellular effect of Ang II in skin we determined the levels of total cytoplasmic inositol phosphates after incubation of skin slices with different doses of Ang II. 10^{-6} M of Ang II increased significantly the phosphatidylinositol (PI) hydrolysis, and the effect was dose dependent up to 10^{-4} M Ang II. The majority of inositol phosphates yielded after 1 hour incubation in the presence of lithium was $InsP_1$, with lesser amount of $InsP_2$. Losartan, the Ang II AT_1 antagonist, at a dose of 10^{-4} M blocked the effect of Ang II, while PD123319, the Ang II AT_2 antagonist, had no antagonistic action; PD123319 at the higher dose of 10^{-3} M, however, potentiated the effect of Ang II on PI hydrolysis. The results suggest that PI hydrolysis is a second messenger system for Ang II in rat skin. Also, the two subtypes of Ang II receptors mediate opposite effects on PI hydrolysis: Ang II binding to AT_1 receptors increases inositol phosphate production, while Ang II binding to AT_2 receptors decreases inositol phosphate production.

Angiotensin-II (Ang II), in addition to its classical role as a circulating hormone, has paracrine effects as a local tissue hormone (1). Ang II has numerous well recognized effector sites such as the adrenal gland (2-4), vascular smooth muscle (5,6), pituitary (7), liver (8,9,10), kidney (11), peripheral nerves (12,13) and brain (1). With the help of the recently developed type-specific Ang II receptor blockers Losartan (formerly DuP753), PD123319 and CGP42112A the Ang II receptors in these organs has been classified as Ang II type-I (AT_1) or type-II (AT_2) receptors (14-17). The receptor has been cloned in vascular smooth muscle and bovine adrenal cortex (18,19). So far, most of the known cellular effects of Ang II were shown to involve AT_1 receptor activation and phosphatidylinositol (PI) hydrolysis, resulting in increased amounts of diacylglycerol in the cell membrane, and increased intracellular inositol phosphate levels. The primary product of PI hydrolysis is inositol(1,4,5)-trisphosphate, which mobilizes intracellular Ca^{2+}. Both diacylglycerol and the

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elevated intracellular Ca^{2+} can take part in eliciting the ultimate cell response. Activation of the AT_2 receptor appear to cause a decrease in basal cGMP levels (20,21).

Recent investigations revealed some unexpected sites of Ang II receptor expression such as fetal skin, connective tissue and skeletal muscle (22,23) as well as wounded skin (24). Ang II was also suggested to act as a growth factor in blood vessels (5,25,26), and to play a role in skin wound healing (27). These findings led us to determine whether binding of Ang II to its receptors in the skin results in changes in PI hydrolysis, and what types of Ang II receptors are involved.

MATERIALS AND METHODS

The experiments were carried out on male Harlan Sprague-Dawley rats weighing 100 to 440 g. The rats were anesthetized with sodium pentobarbital (10 mg/100 g body weight). The abdominal skin was depilated and removed. The skin sample was cut by scissors for 3 min and put in Krebs-Ringer Bicarbonate buffer (KRB) (118 mM NaCl, 4.7 mM KCl, 0.75 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.8 mM $NaHCO_3$, 10 mM glucose) which had been gassed with O_2/CO_2 (95:5) and kept in 37° C water bath. The slices were washed five times during 60 min with fresh KRB with continuous gassing with O_2/CO_2. The skin slices were labeled with 30 μCi of myo-[^{3}H]inositol (specific activity 20 Ci/mM, American Radiolabeled Chemicals Inc., St. Louis, MO) for 1 hour at 37° C in 6 ml KRB buffer in a 50 ml tube gassed with O_2/CO_2, and capped tightly. After this incubation the slices were washed and gassed with O_2/CO_2 for 2 x 5 min. The PI hydrolysis assays were carried out in 12 x 75 mm polypropylene tubes. 50 μl of the skin slice suspension was added to each tube containing 170 μl buffer. The stimulation of the skin slices started with the addition of 10 μl LiCl (final concentration: 8 mM), 10 μl Losartan or PD-123319 or buffer (for samples without blockers), and 10 μl angiotensin II (Sigma, St. Louis, MO) or buffer (for control samples). Four parallel tubes were used for each treatment. The tubes were gassed with O_2/CO_2, capped and gently shaken for 60 min in 37°C water bath. The reactions were stopped by the addition of 1 ml of chloroform:methanol:12N HCl (100:200:1), followed by the addition of 0.35 ml chloroform and 0.35 ml water. The tubes were shaken for ten minutes and centrifuged at 800 x g for five minutes to separate the phases. An aliquot (0.75 ml) of the upper aqueous phase containing [^{3}H]inositol phosphates was added to polypropylene columns (Bio-Rad) to which 2 ml of a 1:1 (v:v) slurry H_2O/Dowex anion exchange resin (AG 1X8, 100-200 mesh, Bio-Rad, Richmond, CA) in the formate form had been added. Free [^{3}H]inositol was eluted by washing the columns with 20 ml of water, followed by 6 ml of 5 mM sodium tetraborate/ 60 mM sodium formate to elute [^{3}H]GPI ([^{3}H]glycerophosphoinositol). Total [^{3}H]inositol phosphates were eluted by washing the resin with 5 ml of 1.0 M ammonium formate/0.1 M formic acid directly into a vial. Water (5 ml) was added to each sample. Radioactivity was determined after the addition of 10 ml ScintiVerse I (Fisher) in an LKB (Wallac) 1215 RackBeta II liquid scintillation counter. To determine the radioactivity incorporated into membrane lipids, an aliquot (200 μl) of the lower phase containing the lipid-soluble products was transferred into 5 ml vials. After the evaporation of chloroform 3 ml of CytoScint ES (ICN Biomedicals, Irvine, CA) was added to each vials, and the radioactivity was measured. To determine the composition of total [^{3}H]inositol phosphates formed by the procedure described above, in an experiment the ion exchange column was washed serially with 20 ml of water to elute [^{3}H]inositol, 10 ml of 5 mM sodium tetraborate/ 60 mM sodium formate to elute GPI, 10 ml of 0.15 M ammonium formate/ 0.1 M formic acid to elute inositol monophosphate ($InsP_1$), 10 ml of 0.5 M ammonium formate/ 0.1 M formic acid to elute inositol bisphosphates ($InsP_2$), 20 ml of 0.8 M ammonium formate/ 0.1 M formic acid to elute inositol trisphosphates ($InsP_3$) and 7.5 ml of 1.4 M ammonium formate/ 0.1 M formic acid to elute $InsP_4$. $InsP_3$ containing fractions were shaken and 10 ml aliquot were taken and 2.5

ml of water was added to $InsP_4$ fraction. [^{3}H]inositol phosphates were also separated by high performance liquid chromatography (HPLC), as previously described (28).

Data analysis

For each tube counts were calculated for the total aqueous or lipid phase, and fractional release was determined by dividing the counts of the aqueous phase by the sum of the counts of the aqueous phase and lipid phase. Duncan multiple range test was used for statistical analyses and the results are expressed as means ± S.E.M.

RESULTS

Ang II stimulated PI hydrolysis in skin. Stimulation of PI hydrolysis in rat skin slices by Ang II was dependent on the concentration of Ang II. There was a significant increase in PI hydrolysis at 10^{-6} M concentration of the hormone in the incubation medium, and the effect was dose dependent up to 10^{-4} M Ang II ($p<0.01$, Fig.1).

Serial elution of inositol phosphates from the Dowex anion exchange resin showed that the majority of inositol phosphates yielded after 60 min incubation with 10^{-4} M Ang II in the presence of lithium was $InsP_1$, while some $InsP_2$ was also detectable. To determine the inositol phosphate isomers formed during Ang II stimulation we have separated [^{3}H]inositol phosphates by SAX-HPLC. The $InsP_3$ fraction consists of 3 isomers: Ins(1,4,5)P_3, Ins(1,3,4)P_3 and an unidentified peak, possibly Ins(2,4,5)P_3 (Fig.2). No Ins(1,3,4,5)P_4 was detected in our assay. The $InsP_2$ fraction consists of Ins(1,4)P_2 and Ins(1,3)P_2. Ins(1)P and Ins(4)P are the two $InsP_1$ isomers formed during Ang II stimulation (Fig.3).

To determine which Ang II receptor subtype is coupled to PI hydrolysis, the AT_1 receptor blocker Losartan or the AT_2 receptor blocker PD123319 were administered

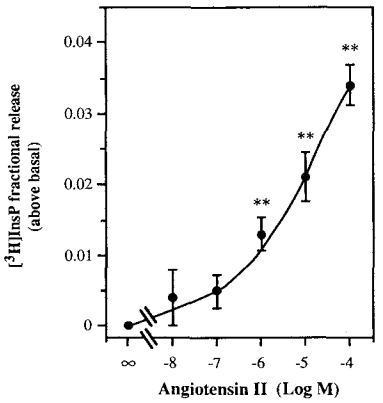


Figure 1. Stimulation of inositol phosphate formation by increasing concentrations of angiotensin II (Ang II) in rat skin slices. Each data point represents the mean ± S.E.M. of 22 experiments (except 10^{-8} M Ang II, where n=12). ** $p<0.01$.

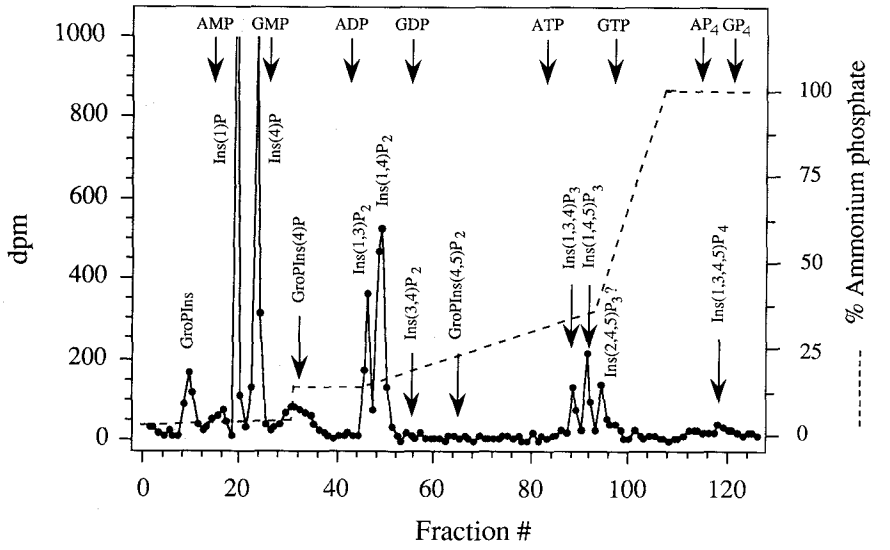


Figure 2. HPLC separation of [^{3}H]inositol phosphates in rat skin slices. [^{3}H]myo-inositol and [^{3}H]inositol phosphates were extracted and spiked with the indicated nucleotides and separated by SAX HPLC. Identification of sample [^{3}H]GroPIns, [^{3}H]Ins(1)P $_1$, [^{3}H]Ins(4)P $_1$, [^{3}H]GroPIns(4)P, [^{3}H]Ins(1,4)P$_2$, [^{3}H]GroPIns(4,5)P $_2$, [^{3}H]Ins(1,3,4)P$_3$, [^{3}H]Ins(1,4,5)P$_3$, [^{3}H]Ins(1,3,4,5)P$_4$, were based on co-elution with authentic [^{3}H] labeled standards in separate runs. Ins(1,3)P$_2$, Ins(3,4)P$_2$ and Ins(2,4,5)P$_3$ were identified with their relative position to the authentic standards (AP$_4$ and GP$_4$; adenosine and guanosine tetraphosphate. ---- % ammonium phosphate, pH 3.7).

together with Ang II. 10^{-4} M Losartan shifted the dose-response curve of inositol phosphates to increasing concentrations (10^{-7}-10^{-4} M) of Ang II to the right, significantly decreasing the effect of 10^{-7} and 10^{-5} M Ang II ($p<0.05$, Fig. 4/A). PD123319 at the same

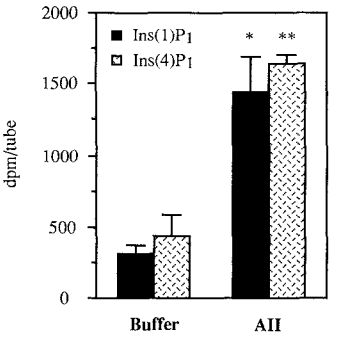
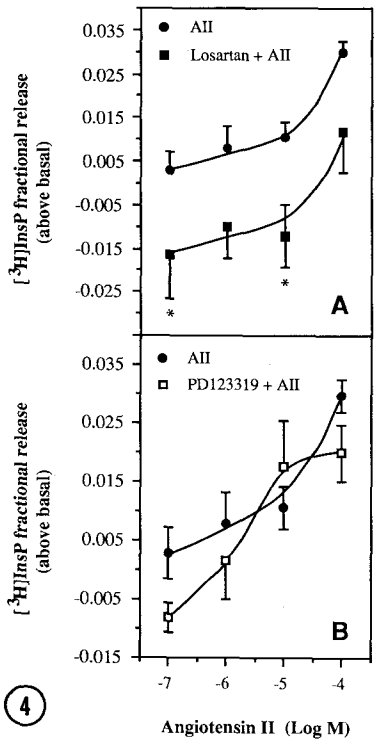


Figure 3. Angiotensin II stimulates [^{3}H]Ins(1)P $_1$ and [^{3}H]Ins(4)P $_1$ formation. Rat skin slices were labeled with [^{3}H]inositol for 60 min and stimulated with 10^{-4}M Ang II for 60 min. [^{3}H]inositol phosphates were extracted and separated as described in methods. The results shown are mean + S.E.M. of 3 determinations in a single experiment.



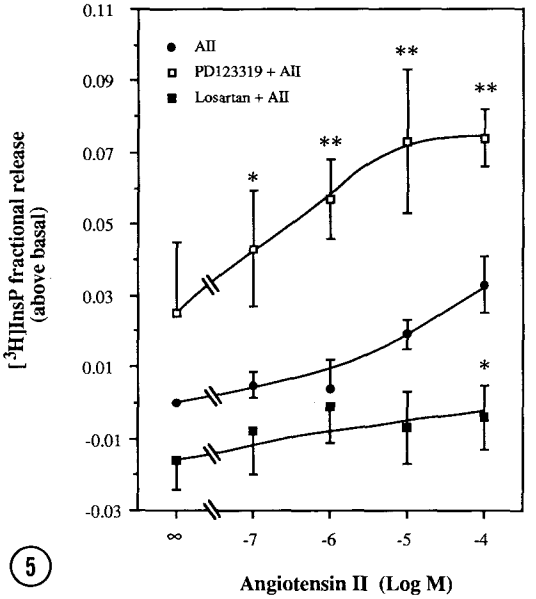


Figure 4. Inhibition of the effect of Ang II on PI hydrolysis with 10^{-4}M Losartan. Each data point represents the mean ± S.E.M. of 3 experiments. *P<0.05(A). 10^{-4} M PD123319 could not produce a significant change on the dose-response curve of PI hydrolysis to increasing concentrations of Ang II. Each data point represents the mean + S.E.M. of 4 experiments (B).

Figure 5. Effect of 10^{-3} M PD123319 and 10^{-3} M Losartan on the dose-response curve of PI hydrolysis to increasing concentrations of Ang II. Data points represents the mean + S.E.M. of 3-4 experiments. * p<0.05,** p<0.01.

dose did not have significant effect (Fig. 4/B.). 10^{-3} M of PD123319 however, potentiated the effect of Ang II on PI hydrolysis, significantly increasing the release of inositol phosphates at Ang II concentrations of 10^{-7} M ($p<0.05$) and 10^{-6}-10^{-4} M ($p<0.01$, Fig. 5).

Because of the differences in wound healing with aging, we investigated the effects of age on the Ang II response. Skin samples of young (body weight: 102 - 117 g, 5-6 weeks of age) and adult (body weight: 390 - 430 g, 12-15 weeks of age) rats were taken and PI hydrolysis were compared for the two groups. The skin slices of both groups produced a dose-dependent increase in PI hydrolysis to 10^{-8}-10^{-4} M Ang II, and there was no significant difference between the two groups at any Ang II concentrations.

DISCUSSION

This report demonstrates that Ang II stimulates PI hydrolysis in skin and that the two Ang II receptor subtypes have opposite effects. Ang II binding to AT_1 receptors increases

inositol phosphate production, whereas AT_2 receptors inhibit inositol phosphate production. Ang II and its receptors in the skin show dynamic changes during certain physiological and pathological events. The major receptor type in rat embryo skin is AT_2 (23), but these receptors seem to disappear within a few days after birth (22). In adult rat skin membrane preparations the predominant Ang II receptor type is AT_1 (24). Recently it has been discovered that Ang II receptors are involved in wound healing of skin (27) and AT_2 receptors reappear during wound healing after skin injury (29). We can conclude from our experiments that PI hydrolysis is a possible second messenger system for actions of Ang II in the rat skin. Although we were measuring total inositol phosphates instead of Ins(1,4,5)P_3-trisphosphate itself, substantial experimental data suggest that most of the inositol phosphates in the Ang II stimulated cell are products of the rapid metabolism of Ins(1,4,5)P_3, which is viewed as the second messenger molecule (2,30,31). Also, the majority of inositol phosphates detected by HPLC was Ins(4)P, which is suggested to be the preferred metabolic product of Ins(1,4,5)P_3 in the presence of lithium (2). Since PI hydrolysis is thought to be implicated in the processes that control cell growth (25,27), this finding can provide further support for the hypothesized role of Ang II in skin wound healing (27).

To discuss the role of the Ang II receptor subtypes, we have to consider that the skin slices used in these experiments should be viewed a model for wounded skin rather than for intact skin. Our results show that the AT_1 receptor blocker Losartan in a dose of 10^{-4} M was able to block the effect of Ang II on PI hydrolysis, while the AT_2 blocker PD123319 in the same dose was ineffective. 10^{-3} M of PD123319 however, potentiated the effect of Ang II. These results suggest that both the AT_1 and AT_2 receptors are involved, being the AT_1 dominant at the time of the incubation which happened 2.5-3.5 hours after skin slicing. These results also suggest that the two types of Ang II receptors have an opposite role in rat skin slices: Ang II binding to AT_1 increases inositol phosphate production, while Ang II binding to AT_2 inhibits it. However, the role of inositol phosphates is not yet clear. Considering that the number of AT_1 receptors decrease during the first day of wound healing (24), which would lead to decreasing PI hydrolysis, while AT_2 reappear at the third day of wound healing, possibly further decreasing PI hydrolysis, we can conclude that the cell tends to attenuate the Ang II's effect on PI hydrolysis by modifying the number and composition of its Ang II receptors. It has been proposed that in endothelial cell injury Ang II stimulates a growth inhibitory factor, TGFß and a growth promoting factor, PDGF (32). For hyperplasia to occur, the PDGF must predominate over the TGFß. Further studies will show if the PI hydrolysis is a selective second messenger in TGFß production and if reducing PI hydrolysis by AT_1 downregulation and by AT_2 upregulation during wound healing would decrease TGFß activity in favor of PDGF activity. Whether these changes happen on the same cell type or on different ones, is not known yet.

The outcome of the interplay between the Ang II receptor changes and the elevated skin Ang II level during wound healing (27) also need to be elucidated.

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