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Angiotensin II-Induced Increase in Inositol 1,4,5-Trisphosphate in Cultured Rat Mesangial Cells : Evidence by Refined High Performance Liquid Chromatography

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Angiotensin II-induced change in inositol phosphates were studied in cultured rat mesangial cells prelabeled with [^{3}H]myo-inositol. By using anion-exchange high performance liquid chromatography, we could analyzed the change in inositol mono-, bis-, and tris-phosphate more rapidly and easily with higher resolution than the previously reported methods. Angiotensin II rapidly increased inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate within 15 sec, followed by an increase in inositol 1-monophosphate at 30 sec. Angiotensin II-induced increases in inositol phosphates were dose-dependent and completely blocked by saralasin. These results indicate that angiotensin II induces the production of inositol phosphates including inositol 1,4,5-trisphosphate, an intracellular Ca^{2+}-releasing factor, in cultured rat mesangial cells.

Angiotensin II(AII) is thought to affect the filtration function of renal glomeruli by causing contraction of glomerular mesangial cells (1,2,3,4.). However, the signal transduction system of AII in these cells has not yet been completely clarified. It is generally accepted that hormones whose action is mediated by increasing cytosolic Ca^{2+} stimulate the metabolism of membrane phosphoinositide, i.e., the phosphoinositide cycle, yielding two second messengers: Diacylglycerol(DG) which activates protein kinase C (5) and inositol 1,4,5-trisphosphate(IP_3) which releases Ca^{2+} from endoplasmic reticulum (6,7). AII has been shown to stimulate phosphoinositide cycle in vascular smooth muscle cells (8,9), adrenal glomerulosa cells (10) and hepatocytes (11,12). We have reported that AII causes a rapid breakdown of phosphatidylinositol 4,5-bisphosphate(PIP_2) and a simultaneous

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increase in DG in cultured rat mesangial cells (13), suggesting the activation of phospholipase C after the binding of AII to mesangial receptor. To confirm this suggestion, it is necessary to analyze the change in inositol phosphates. The conventional method using Dowex column that has been widely used is not suitable for analyzing inositol phosphates, because of complicated procedure and poor reproducibility (14,15). Using this method, Pfeilshifter et al. demonstrated that AII increased IP_3 in cultured rat mesangial cells (19). However, the increase was only minimal (about 100cpm /mg protein) and neither the time course nor the dose dependency was shown. Therefore, we have developed the new high performance liquid chromatography (HPLC) method by which we could analyze inositol phosphates rapidly and easily with high resolution. By using this method, we have clearly demonstrated that AII increases inositol phosphates in cultured rat mesangial cells.

MATERIALS and METHODS

Cell culture. Cultured mesangial cells were prepared from Sprague-Dawley rat kidneys as previously described (13,17,18). In brief, under sterile conditions, renal cortical tissue was removed and consecutively passed through stainless mesh with pore size of 280 μm and 120 μm onto a mesh of 53 μm pore size. The isolated glomeruli were seeded in 60 mm plastic culture dishes, and the cells were grown in RPMI 1640 medium supplemented with 20 % fetal bovine serum and bovine insulin at 1 μg /ml. Culture dishes were kept at 37°C in a humidified atomosphere with 95% air and 5% CO_2. The first subculture was done on day 21 of the primary culture, when almost all of the grown cells were of mesangial origin. The second subculture was seeded on a cover glass (10^5 cells/glass). The cells were used for all experiments 14 days after the seeding.

Labeling. Medium was removed 24 h prior to labeling and the cells were incubated in inositol-free MEM containig 10 % dialyzed fetal bovine serum. Cells were prelabeled for 48 h with [1,2-^{3}H]myo-inositol (20 μCi/ml). The medium was removed and cells were rinsed three times to remove free [^{3}H] inositol. After rinsing, the cover glass was detached, washed in warmed Hanks-Hepes buffer (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 5.6 mM glucose, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 30 mM Hepes, pH 7.4) containing 1 mM myo-inositol and placed onto the glass dish for 20 min at 37°C.

Analysis of inositol phosphates. Cells were incubated for various times in 1.5 ml of Hanks-Hepes buffer with or without agents. At indicated times, the buffer was rapidly aspirated and the reaction was terminated by the addition of chloroform / methanol (1:2; v/v). The extraction was done according to Bligh & Dyer (18). Aqueous phase was removed. Chloroform phase was rinsed with 1 ml of water. Combined aqueous phase was dried with the rotary evaporater (Savant), dissolved in water and applied to HPLC. The samples were analysed by HPLC on TSK gel DEAE-2SW anion exchange column (TOYO SODA Co. Ltd., Tokyo) with a flow rate of 1ml/min at 25°C. The solvent

was 0.14 M sodium phosphate buffer, pH 6.8. Volumes of the collected fraction were 0.25 ml for the determination of inositol 1-monophosphate(IP_1) and inositol 1,4-bisphosphate(IP_2), and 0.5 ml for that of IP_3. Each fraction was subjected to liquid scintillating counting in ACS-II scintillation fluid (6 ml) containing water (0.5 ml). For determining the retention time in every analysis, we used the standards of IP_1, IP_2 and IP_3. Detector was a refractive index monitor (Knauer).

Chemicals. (1-Asp, 5-Ile) AII and [1-Sar, 8-Ala] AII (saralasin) were from Peptide Institute, Protein Research Foundation (Osaka). Standards of IP_1, IP_2 and IP_3 were purchased from Amersham Japan (Tokyo). RPMI 1640, inositol-free MEM, fetal bovine serum and dialysed fetal bovine serum were from Gibco Oriental (Tokyo). [1,2-^{3}H] myo-insitol (45-80Ci/mmol) was obtained from New England Nuclear (Boston, MA). All other chemicals and solvent used were reagent grade.

RESULTS

Fig. 1 shows the chromatogram of the standard mixture for IP_1, IP_2, glycerophosphoinositol 4,5-bisphosphate(GroPI(4,5)P_2) contaminated in IP_2 standard, and IP_3. Amounts of each standard were about 64 nmol, 24 nmol, 24 nmol and 40 nmol, respectively. Each inositol phosphate was clearly sepa-

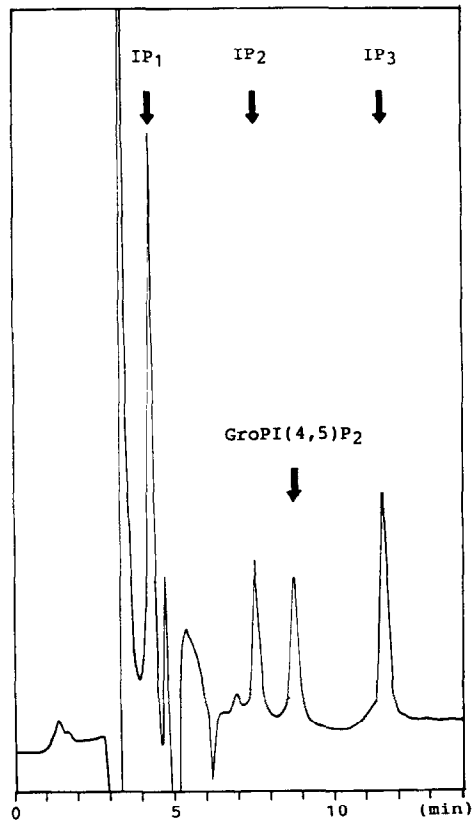

Fig. 1. HPLC of inositol phosphates.
Authentic IP_1, IP_2 and IP_3 were loaded onto a DEAE-2SW column and eluted with 0.14 M sodium phosphate buffer, pH 6.8 with a flow rate of 1 ml/min at 25°C. Detection was done with a refractive index monitor x1.

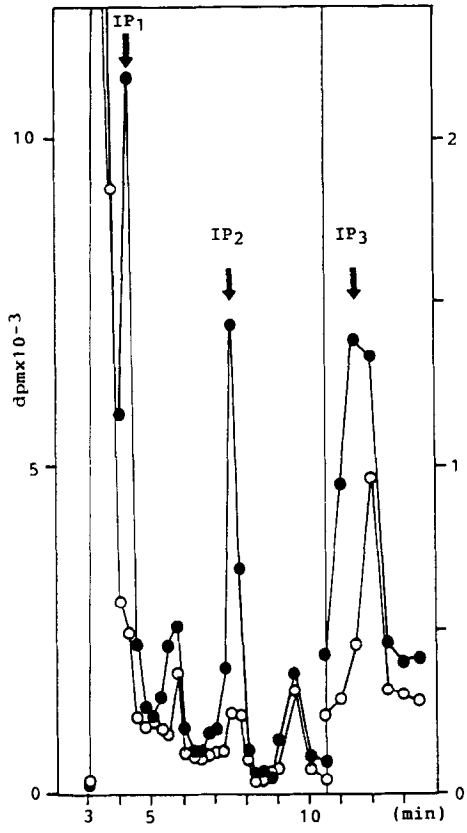

Fig. 2. Separation of [^{3}H]-labeled inositol phosphates from rat cultured mesangial cells with HPLC.
Inositol phosphates were extracted as described under "Materials and Methods". The samples dissolved in water was applied on HPLC. The fractionation was performed: for the determination of IP_1 and IP_2, the fraction volume was 0.25 ml from 3 min to 10 min after the sample injection, for the determination of IP_3, the fraction volume was 0.5 ml from 10 min to 14 min. Data are expressed in dpm. (o-o) unstimulated, (o-o) stimulated by 100 nM AII.

rated with the retention times of 4.3 min, 7.6 min, 8.8 min and 11.5 min, respectively.

In addition to clearly separated three peaks corresponding to IP_1, IP_2 and IP_3, two unknown peaks were detected in the HPLC profiles of aqueous [^{3}H] inositol-labeled extracts prepared from cultured rat mesangial cells stimulated or unstimulated with 100 nM AII for 30 sec in the absence of LiCl (Fig. 2). Of the unkown peaks, one was between IP_1 and IP_2, and another was between IP_2 and IP_3. These peaks didn't coincide with glycerophosphoinositol 4-monophosphate(GroPI4P) and GroPI(4,5)P_2, respectively.

When the cells were stimulated by 100 nM AII, IP_2 and IP_3 rapidly increased at 15 sec and reached maximum at 30 sec (Fig. 3). There was no

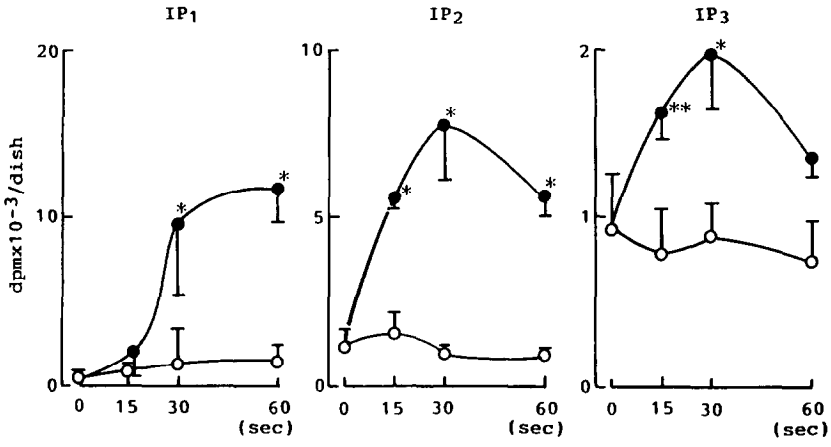

Fig. 3. Time course of AII-stimulated inositol phosphates production. Cultured mesangial cells (10^5/dish) were prelabeled with [^{3}H]myo-inositol (20 μCi/ml) for 48 h and then exposed to 100 nM AII for different intervals. After the rapid aspiration of the medium, the reaction was terminated by the addition of chloroform/methanol (1:2). Inositol phosphates were analyzed by HPLC. (o-o); control, (o-o); AII-stimulated. Each point represents the mean ± S.D. of triplicate determinations. *: $p<0.01$, **: $p<0.05$ vs. control.

significant increase in IP_1 at 15 sec, while it increased at 30 sec (Fig. 3). AII had no effect on [^{3}H] content in the two unknown peaks (data not shown).

AII increased IP_3 formation in a dose-dependent manner at the range from 10 nM to 1 μM (Fig. 4). The increasing effect of AII upon the formation of IP_1 and IP_2 was also dose-dependent (Fig.4).

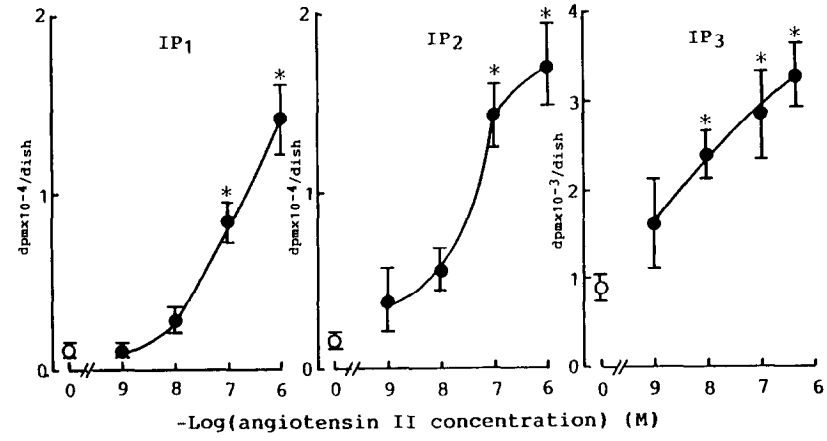

Fig. 4. Effect of various concentrations of AII on inositol phosphates accumulation. Cultured mesangial cells (10^5/dish) were prelabeled with [^{3}H]inositol (20 μCi/ml) for 48 h. Experiments were initiated by the addition of various concentrations of AII. After 30 sec of incubation, medium was rapidly aspirated and chloroform/methanol (1:2) was added to terminate the reaction. Inositol phosphates were analyzed by HPLC. Each point represents mean ± S.D. of triplicate determinations. *: $p<0.01$ vs. control.

Table 1. Effect of saralasin on AII-induced inositol phosphates accumulation

	10^{-3} x Radioactivity (dpm/dish)		
	IP_1	IP_2	IP_3
control	1.10±0.21	1.56±0.33	0.89±0.15
100nM angiotensin II	8.38±1.20*	14.30±2.28*	2.84±0.53*
100nM angiotensin II + 10μM saralasin	1.32±0.49	2.27±0.38	0.87±0.32

Cultured mesangial cells (10^5/dish) were labeled with [^{3}H] inositol (20 μCi/ml) for 48 h. Experiments were initiated by the addition of 100nM AII with or without 10 μM saralasin. After 30 sec of incubation, the reaction was terminated by the rapid aspiration of the medium and the addition of chloroform/methanol (1:2). Inositol phosphates were analyzed by HPLC. Each point represents the mean ± S.D. of triplicate determinations. *: $p<0.01$, vs. control.

The increase in inositol phosphates by 100 nM AII for 30 sec was completely blocked by 10 μM saralasin (Table 1).

DISCUSSION

By using HPLC on anion exchange resins, we could clearly separate inositol phosphates within 15 min. The use of isocratic elution buffer has made it possible to ensure the retention time of each inositol phosphate just before the analysis by a refractive index monitor.

Applying this refined HPLC method, we studied the metabolism of inositol phosphates in cultured rat mesangial cells stimulated by AII. We detected two unknown peaks different from IP_1, IP_2 and IP_3. Several possible substances produce these peaks. First, they may be GroPI4P and GroPI(4,5)P_2 which are deacylated products of PIP and PIP_2, respectively. However, neither of the peaks coincided with authentic standards for them. Second, they may be isomers of IP_2 and IP_3, such as inositol 1,3-bisphosphate and inositol 1,3,4-trisphosphate (14,15). Third, they may be cyclic IP_2 and cyclic IP_3 because we used the neutral condition for the assay. However, we couldn't identify these two peaks because of unavailability of the authentic standards for the isomers or cyclic inositol phosphates. Anyway, these two peaks showed no significant change by the stimulation with AII. Therefore,

they don't seem to be so important components in the signalling mechanism of AII in the cells.

In this study, we could clearly show the rapid increase in IP_3 within 15 sec. Taken this result together with our previous finding that AII induced a rapid breakdown of PIP_2 with a simultaneous increase in DG in the cells, we conclude that AII activates phospholipase C to induce the hydrolysis of PIP_2. Concurrently with the increase in IP_3, IP_2 increased within 15 sec, suggesting a rapid hydrolysis of PIP or a rapid dephosphorylation of IP_3, or both. IP_1 increased after 30 sec, but not within 15 sec, suggesting that PI is not hydrolyzed by phospholipase C as early as PIP_2. PI has been also shown to be hydrolyzed only following the breakdown of PIP and PIP_2 in the membrane fraction of vascular smooth muscle (9). Again, it is unknown whether the increase in IP_1 results from hydrolysis of PI or from dephosphorylation of IP_2, or from both.

AII-induced change in inositol phosphates showed dose dependency and was completely blocked by saralasin, suggesting that this action of AII is mediated by its specific receptor. In conclusion, in glomerular mesangial cells, AII induces the production of inositol phosphates including IP_3 which may be an important messenger in the hormone action.

REFERENCES

1. Mahieu, P. R., Foidart, J. B., Dubois, C. H., Dechenne, C. A. and Deheneffe, J. (1980) Invest. Cell Pathol. 3, 121-128.
2. Ausiello, D. A., Kreisberg, J. I., Roy, C. and Karnovsky, M. J. (1980) J. Clin. Invest. 65, 754-760.
3. Scharshmidt, L. A. and Dunn, M. J. (1983) J. Clin. Invest. 71, 1756-1764.
4. Fujiwara, Y., Kikkawa, R., Kitamura, E., Takama, T. and Shigeta, Y. (1984) Renal Physiol. 7, 344-348.
5. Nishizuka, Y. (1984) Nature 308, 693-698.
6. Streb, H., Irvine, R. F., Berridge, M. J. and Schultz, I. (1983) Nature 306, 67-69.
7. Berridge, M. J. and Irvine, R. F. (1984) Nature 312, 315-321.
8. Smith, J. B., Smith, L., Brown, E. R., Barnes, D., Sabir, M. A., Davis, J. S. and Farese, R. V. (1984) Proc. Natl. Acad. Sci. USA 81, 7812-7816.
9. Griending, K. K., Rittenhause, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A., Jr. and Alexander, R. W. (1986) J. Biol. Chem. 261, 5901-5906.
10. Farese, R. V., Larson, R. E. and Davis, J. S. (1984) Endocrinology 114, 302-304.

11. Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. and Kirk, C. J. (1983) Biochem. J. 212, 733-747.
12. Charest, R., Prpic, V., Exton, J. H. and Blockmore, P. F. (1985) Biochem. J. 227, 79-90.
13. Orita, Y., Fujiwara, Y., Ochi, S., Tanaka, Y. and Kamada, T. (1985) FEBS Lett. 192, 155-158.
14. Pfeilschifter, J. Kurtz, A. and Bauer, C. (1986) Biochem. J. 234, 125-130.
15. Michell, B. (1986) Nature 319, 176-177.
16. Downes, C. P. (1986) Biochem. Soc. Trans. 13, 1107-1110.
17. Foidart, J. B., Dechenne, C. A., Maheu, P., Creutz, C. and De Mey, J. (1979) Invest. Cell Pathol. 2, 15-26.
18. Tanaka, T., Fujiwara, Y., Orita, Y., Sasaki, E., Kitamura, H. and Abe, H. (1984) Jpn. Circ. J. 48, 1017-1029.
19. Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917.