Regulation of Renin-Like Enzyme in Cultured Human Vascular Smooth Muscle Cells

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Cultured human arterial smooth muscle cells produced an immunologically specific renin-like enzyme. The renin-like enzyme in the culture medium was mostly an inactive form; the proportion of the active form in the cell was 30 to 75%. Phorbol 12-myristate 13-acetate, N'-O'-dibutyryladenosine 3', 5'-monophosphate and isoproterenol with theophylline increased the renin-like enzyme in the medium and in the cell, dose dependently. Endothelial cell growth supplement also increased the renin-like enzyme produced by cultured vascular smooth muscle cells, and heparin promoted the effects of endothelial cell growth supplement. The existence of the regulation of the renin-like enzyme produced by cultured vascular smooth muscle cells strongly suggests the existence of a local renin angiotensin system in human vascular walls.

AN increasing number of studies suggest the existence of a local renin angiotensin system in peripheral tissues including vascular walls.¹⁻³ The presence of a specific immunoreactive renin in vascular tissues⁴ and vascular smooth muscle cells in culture⁵ and the presence of renin⁶ and angiotensinogen⁷ mRNA in vascular tissues have been demonstrated. And the angiotensin-I converting enzyme (ACE) is known to be localized in the luminal surface of vascular endothelial cells. Thus, vascular tissues can produce every component of the renin angiotensin system.

The antihypertensive effects of ACE inhibitors have been reported to be more closely correlated to the degree of inhibition of tissue ACE than to that of plasma ACE.⁸ A discrepancy between the antihypertensive effects of renin inhibitors and the degree of plasma renin activity inhibition has also been reported.⁹ These findings suggest

the sites of angiotensin production are, at least in part, in peripheral tissues.

We previously demonstrated that cultured human arterial smooth muscle cells of various origin can produce a renin-like enzyme that is inhibited by the antiserum against human renal renin.¹⁰ The purpose of the present study is to investigate the regulation of this renin-like enzyme in human VSMC.

MATERIALS AND METHODS

Culture of vascular smooth muscle cells

Human vascular smooth muscle cells (VSMC) were grown from medial segments of human umbilical arteries, as previously reported.¹⁰ Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (GIBCO). Cells were identified as vascular smooth muscle cells by a typical growth pattern of hills and valleys and a positive staining with anti-vascular smooth muscle cell specific actin monoclonal antibody kindly provided by Dr. Akira Ohshima (Wakayama Medical College). Cells were subcultured with 1 : 4 split ratio when their growth

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TABLE I EFFECTS OF PMA, db-cAMP, AND HEPARIN

	Medium (ng/hr/ml)		Cell (ng/hr/mg protein)	
	Active	Total	Active	Total
Control	0.82 ± 0.12	2.94 ± 0.28	0.43 ± 0.13	0.98 ± 0.21
db-cAMP (1 mM)	0.76 ± 0.20	4.29 ± 0.38*	0.52 ± 0.06	1.99 ± 0.04*
PMA (1 nM)	1.11 ± 0.18	5.82 ± 0.62*	0.64 ± 0.06	3.13 ± 0.14*
Heparin (10 u/ml)	2.02 ± 0.13*	5.05 ± 0.45*	0.47 ± 0.08	1.45 ± 0.14*

Effects of PMA (1 nM), db-cAMP (1 mM), and heparin (10 u/ml) on the renin-like activity in the cell and in the medium. Active = activity before trypsin activation; Total = activity after trypsin activation.

Values are mean ± s.d. (n = 3), *p < 0.05, **p < 0.01

reached confluency.

Experimental Procedures

VSMC between 3rd and 5th passages were used. Cells were cultured on Nunc 6-well multi-culture dishes. VSMC at confluency were washed with phosphate buffer saline (PBS) and maintained for 48h in 1.5 ml/well DMEM supplemented with 10% heat inactivated FBS. At the end of incubation the cells were rinsed with PBS with 0.5 mM ethylene diamine tetraacetic acid disodium salt (EDTA) and scraped with a rubber policeman into PBS-EDTA and then briefly sonicated on ice. The medium and the cell homogenate were stored frozen at -80°C until the assay. To examine the regulation of the renin-like enzyme produced by VSMC, various reagents were introduced to the culture medium during the 48 h incubation.

As the renin-like activity produced by VSMC varied from preparation to preparation, 2 or more separate experiments were performed on each reagent employed in the present study.

Assay of Renin Activity

The activity was determined according to a method already reported!¹⁰ Generated angiotensin I from sheep substrate at pH 7.4 at 37°C for 1–3 h, depending on the expected activity, was measured by radioimmunoassay with a commercial kit (Sorin Biomedica, France). The inactive renin was activated by trypsin (1 mg/ml, 25°C for 10 min) and the reaction was stopped by soy bean trypsin inhibitor (1 mg/ml). The renin activity was expressed in terms of nanograms of angiotensin-I generated in 1h per ml of the medium and per mg protein of the cell homogenate. The protein content of cell homo-

genate was determined with BIO-RAD protein assay kit (BIO-RAD, CA), with bovine serum albumin as a standard.

As FBS had some renin like activity, which varied with the lot of FBS as is seen in Table II, all values of the medium renin activity were corrected by subtracting the mean value of triplicate assays of the renin activity in the medium alone which was simultaneously incubated for 48h at 37°C at each experiment.

To rule out the possibility that the active renin-like enzyme in the cell was an artifact due to the activation during experimental process, a cell homogenate (stimulated by 10 nM PMA) was divided into 2 portions and the one was incubated at 37°C and the other was left on ice for 30 min and then the active renin-like activity was compared. The activity incubated at 37°C was 1.55 ± 0.01 ng/h/mg, and that on ice was 1.54 ± 0.11 ng/h/mg (triplicate assay). Gradual loss of active and total renin-like activity was brought about by repeated freeze-thawing (data not shown).

Effectiveness of an antiserum against human renin

The ability of an antiserum against human renin¹¹ to inhibit the enzymatic activity was tested by incubating the medium and cell homogenates for 1h at 37°C in the presence and the absence of the antiserum (1 : 300) prior to the renin assay.

Materials

Phorbol 12-myristate 13-acetate (PMA), N'-O'-dibutyryl adenosine 3', 5'-monophosphate (db-cAMP), endothelial cell growth supplement (ECGS), trypsin (type III), and soy bean trypsin

TABLE II EFFECTIVENESS OF AN ANTISERUM

a) Total renin like activity in the medium

	Antiserum (—)	Antiserum (+)
10% FBS	1.49	1.66
Control	5.67	1.89
ISO (0.1 mM) + THEO (0.1 mM)	8.79	1.70
Heparin (10 u/ml)	6.23	1.76

Effectiveness of an antiserum against human renin to suppress the trypsin activated total renin-like activity in the medium. 10% FBS denotes the total renin-like activity of the culture medium employed in this experiment. Values are the mean of duplicate samples of duplicate assays (ng/h/ml), and are not corrected by the intrinsic renin-like activity in the medium.

b) Renin-like enzyme in the cell and in the medium

Cellular renin-like activity

	Control		PMA (10 nM)	
	Total	Active	Total	Active
Antiserum (—)	1.42	0.51	3.07	1.03
Antiserum (+)	0.82	0.47	1.09	0.53

Medium renin-like activity

	10% FBS*		Control		PMA (10 nM)	
	Total	Active	Total	Active	Total	Active
Antiserum (—)	4.08	0.51	6.14	0.61	18.20	2.72
Antiserum (+)	4.66	0.54	5.64	0.58	4.89	0.65

Effectiveness of the antiserum to suppress the renin-like activity induced by PMA. Cellular renin-like activity: PMA (10 nM) and Control denote the renin-like activity of the cell homogenates stimulated or not stimulated by 10 nM PMA, respectively. Values are the mean of duplicate samples of duplicate assays (ng/h/mg). Medium renin-like activity: *The culture medium employed in this experiment was supplemented with a different lot of FBS from that in the experiment a). Values are the mean of duplicate samples of duplicate assays (ng/h/ml), and are not corrected by the intrinsic renin-like activity in the medium.

Active = activity before trypsin activation

inhibitor (type I-S) were from Sigma. Isoproterenol (ISO), theophylline (THEO), and heparin were from Nakarai Chemicals, Ltd. (Kyoto, Japan).

Statistical Analysis

Results were expressed as the mean $\pm$ SEM of *n* experiments. Student-Newman-Keul's multiple comparison were used for the analysis of variance, taking *p* = 0.05 as the limit of significance.¹²

RESULTS

Effectiveness of An Antiserum Against Human Renin

Renin-like enzyme produced by VSMC was increased in the culture medium and in the cell by db-cAMP (1 mM), PMA (1 nM), and heparin (10 u/ml) (Table I). However, the extent of the increases by these 3 reagents varied from preparation to preparation. The media and cell homogenates of relatively high renin-like activity were selected for the following experiments to

Fig.1. Dose responsiveness by PMA.

(a): medium renin-like activity. * $p < 0.01$ (b): cellular renin-like activity. * $p < 0.05$, ** $p < 0.01$

'T' and 'A' represent the total and the active renin-like activity, respectively.

Vertical bars represent s.d. ($n = 3$).

TABLE III DOSE RESPONSIVENESS BY db-cAMP

	Medium (ng/h/ml)		Cell (ng/h/mg)	
	Active	Total	Active	Total
Control	0.96 ± 0.25	2.83 ± 0.65	0.42 ± 0.11	1.21 ± 0.25
db-cAMP (1 mM)	0.79 ± 0.18	$4.87 \pm 0.64^*$	0.57 ± 0.06	$1.69 \pm 0.06^*$
db-cAMP (0.2 mM)	0.70 ± 0.11	$3.92 \pm 0.54^*$	0.49 ± 0.08	$1.52 \pm 0.06^*$

Values are the mean $\pm$ s.d. ($n = 3$).Active = activity before trypsin activation; Total = activity after trypsin activation. * $p < 0.05$

examine the effectiveness of an antiserum against human renin. Most of the total renin-like activity in the medium induced by heparin (10 u/ml), and isoproterenol (0.1 mM) combined with theophylline (0.1 mM) was inhibited by an antiserum against human renin. The total and active renin-like activity in the medium alone (10% FBS) was not inhibited by the antiserum (Table IIa, b). The active and total renin-like activities in the control cells were not as effectively inhibited by the antiserum as those in the cells stimulated by PMA (Table IIb). The activities in the cells not inhibited by the antiserum may be ascribed to some nonspecific proteolytic enzymes other than human renin.

Effects of PMA

PMA increased the inactive renin-like enzyme in the medium dose dependently. The active renin-like enzyme in the medium, although the level of the active form was much lower than that of total activity, was also increased by PMA (Fig. 1a). While the proportion of the active renin-like enzyme to the total in the medium was 10% (1 nM PMA) or less in this experiment, it was 45% (10 nM PMA) to 75% (1 μ M PMA) in the cell. The cellular active and total renin-like enzymes were increased dose dependently by PMA. With the higher dose of PMA (0.1 μ M–1.0 μ M), the increase of the active form was more prominent than the inactive form (Fig. 1b).

Effects of Adenosine 3', 5'-Cyclic Monophos-

Fig.2. Dose responsiveness by isoproterenol.

Dose responsiveness by isoproterenol combined with theophylline (0.1 mM).

(a): medium renin-like activity. * $p < 0.05$

(b): cellular renin-like activity. * $p < 0.05$, 'T' and 'A' represent total and active renin-like activity, respectively. Vertical bars represent s.d. (n = 3).

TABLE IV PROMOTING EFFECT OF HEPARIN ON ECGS

	Medium (ng/h/ml)		Cell (ng/h/mg)	
	Active	Total	Active	Total
Control	0.41 ± 0.17	1.74 ± 0.12	0.87 ± 0.07	1.01 ± 0.03
Heparin (10 u/ml)	0.25 ± 0.02	1.81 ± 0.13	0.81 ± 0.22	$1.35 \pm 0.15^*$
ECGS (20 μ g/ml)	0.27 ± 0.11	$2.45 \pm 0.20^{**}$	$1.17 \pm 0.13^{**}$	$1.49 \pm 0.07^*$
ECGS + Heparin	0.48 ± 0.16	$3.13 \pm 0.34^{**}$	$1.58 \pm 0.07^{**}$	$4.61 \pm 0.29^{**}$

The effects of heparin (10 u/ml), ECGS (20 μ g/ml), and ECGS (20 μ g/ml) with heparin (10 u/ml) on the renin-like activity.

Values are the mean $\pm$ s.d. (n = 3). Active = activity before trypsin activation; Total = activity after trypsin activation. * $p < 0.05$, ** $p < 0.01$

phate (cAMP)-related agents

Db-cAMP increased the inactive renin-like enzyme dose dependently both in the cell and in the medium (Table III). The renin-like enzyme in the medium was mostly an inactive form, while the proportion of the active form in the cell was 30% or more. As cAMP is known to be

a second messenger of beta adrenergic agonist, the effects of isoproterenol was studied. Isoproterenol (0.1 mM) alone had no effects on the renin-like enzyme produced by VSMC (data not shown). However, isoproterenol (ISO) with 0.1 mM theophylline (THEO) mimicked db-cAMP (Fig. 2a, b). An inactive renin-like enzyme

in the medium and in the cell was increased with 0.1 mM ISO with 0.1 mM THEO ($p < 0.05$). The increase of the active renin-like enzyme in the cell was statistically not significant.

Effects of Heparin and ECGS

Heparin was reported to inhibit renin activity by the competition with renin substrate.¹³ However, 10 u/ml heparin had no inhibitory effects on the renin activity in our assay conditions (data not shown). In the experiment shown in Table I, heparin induced the renin-like enzyme in the cell and in the medium. However, this effect was not always reproducible. On the other hand, heparin always promoted the effects of endothelial cell growth supplement (ECGS), which can by itself increase the renin-like enzyme in the medium and in the cell (Table IV).

DISCUSSION

The present study confirmed the production of a specific immunoreactive renin-like enzyme by human vascular smooth muscle cells in culture. This renin-like enzyme was positively regulated by PMA, cAMP-related agents and ECGS. And heparin promoted the effects of ECGS.

The renin-like enzyme in the culture medium had enzymatic¹⁰ and immunological properties almost identical to human renin. The trypsin-activated renin-like activities in the medium were almost completely inhibited by the antiserum against human renin (Table II). The cellular renin-like activity in the basal state was inhibited to a lesser degree, which may be ascribed to some nonspecific proteolytic enzymes other than true human renin. However, the cellular renin-like activity stimulated by PMA was more effectively inhibited (Table IIb).

PMA has been reported to inhibit the renin release from rat renal juxtaglomerular cells in culture,¹⁴ while our results indicated that PMA increased the production of the renin-like enzyme in VSMC. These are not necessarily contradictory, since the regulation of the secretion is a short term phenomenon which possibly has nothing to do with the regulation of the renin gene, while the regulation of the production is a more long term phenomenon.

Our conclusion that db-cAMP and isoproterenol with theophylline increased the production of renin-like enzyme in VSMC was in good agreement with the results obtained in cultured human mesangial cells,¹⁵ and in human

transfected juxtaglomerular cells.¹⁶

The effects of heparin on the production of renin-like enzyme by VSMC varied from preparation to preparation and with the lot of fetal bovine serum (compare Table I, II, IV). However, heparin always promoted ECGS, which can by itself increase the production of the renin-like enzyme in VSMC. This synergistic effect was also observed in the growth rate of cultured human endothelial cells.¹⁷ Since heparan sulphate is a product of vascular endothelial cells,¹⁸ it is probable that vascular endothelial cells may influence the effects of some serum factors on vascular smooth muscle cells through such a mechanism.

The existence of the regulation of the renin-like enzyme in VSMC is a strong evidence for a local renin angiotensin system in vascular walls. What is more, we have recently reported that the angiotensin I converting enzyme produced by cultured human umbilical vein endothelial cells is positively regulated by PMA and cAMP-related reagents,¹⁹ as is the renin-like enzyme in VSMC. We also detected the angiotensinogen mRNA in the cultured human umbilical artery smooth muscle cells (unpublished observations). These findings increase our confidence. The problem is that the renin-like enzyme released into the medium was almost all the inactive form, while the proportion of the active form to the total in the cell was 30 to 75% in our present study. These findings are in general agreement with those reported in cultured human juxtaglomerular tumor cells,²⁰ immortalized human juxtaglomerular cells,²¹ and cultured human mesangial cells.¹⁵ The existence of the active renin-like enzyme in the cell suggests the existence of a processing enzyme in the cell. However, as described in Materials and Method, the freeze-thawing and the incubation of the cell homogenates did not lead to the activation of the inactive renin-like enzyme. Although it is conceivable that a renin processing enzyme is active in some sequestered circumstance in the cell, the inactive renin seems not to be activated during our experimental processes. The renin-like activity in the cell may be overestimated in our assay, for the antiserum could not completely inhibit it. So, the proportion of the active form to the total in the cell may also be slightly overestimated in the present study.

The amount of the renin-like enzyme produced by VSMC varied from preparation to preparation and with the lot of FBS (data not shown). In

the present study we employed VSMC in the 3rd to 5th passages, because these cells produced much renin-like enzyme than those in primary and secondary passages, as we have previously reported²². Vascular smooth muscle cells were reported to take various phenotypes according to the cell density, the generations of the cells and so on^{23,24}. The variability of the amount of the renin-like enzyme from preparation to preparation in the present study may be ascribed to the phenotypic differences of VSMC.

The pathophysiological meaning of this possible vascular renin angiotensin system is not clear at present. Taking into consideration that this system seems to be activated by cAMP-related agents or, in other words, beta adrenergic agonists, it might serve to control vascular wall tonus, in line with classical concepts of the pathophysiological meaning of the renin angiotensin system. Alternatively, it might affect local angiogenesis or cell proliferation in such conditions as arteriosclerotic plaque, where the phenotypes of the vascular smooth muscle cells closely resemble those of the cultured vascular smooth muscle cells in the 'synthetic' state²⁴.

The vascular wall tissues are comprised of various cells, i.e. smooth muscle cells, endothelial cells, fibroblasts and so on. The phenotypes of these cells would be defined under the complex interactions among these cells. To clarify the pathophysiological meanings of this possible local vascular renin angiotensin system, more sophisticated methods such as co-culture of vascular smooth muscle cells with vascular endothelial cells or the detection of mRNA in vascular walls *in situ* will be required.

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