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Mesangial Cells are Able to Produce Catecholamines In Vitro

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Abstract Mesangial cells (MC) participate in the control of the glomerular function due to their ability to synthesize hormones and induce cell contraction. Since MC can produce various kinds of hormones, the purpose of the present study was to determine if they are able to synthesize catecholamines. For this evaluation, the levels of norepinephrine, epinephrine, dopamine, and biopterin, the enzymatic cofactor of tyrosine hydroxylase (TH), were analyzed by HPLC in the intracellular compartment and in the medium of primary cultured MC. To identify and locate the enzymes responsible for monoamine synthesis, TH, dopa decarboxylase, and dopamine β-hydroxylase, Western blotting and immunocytochemistry were employed using monoclonal and polyclonal antibodies. Concentrations of NE = 57 ± 8, EPI = 82 ± 10, and DA = 52 ± 9 pg/mg protein (X ± SEM) were found in the cell homogenate. The culture medium showed concentrations of NE = 25 ± 3, EPI = 33 ± 3, and DA = 62 ± 15 pg/mg protein. Western blotting analysis and immunocytochemistry evidenced the presence of all enzymes. Moreover, biopterin was also detected in the intracellular compartment and in the medium (0.28 ± 0.03 and 5.70 ± 2 nmol/mg cell protein, respectively). Overall, the data indicate that MC have the biosynthetic machinery necessary to produce catecholamines, suggesting that they can act as a paracrine/autocrine hormone system, contributing to the regulation of glomerular hemodynamic and renal microcirculation. J. Cell. Biochem. 89: 144–151, 2003.

Key words: mesangial cells; catecholamine production; tyrosine hydroxylase; biopterin

It is currently known that mesangial cells (MC) have a complex biological function integrating responses, such as matrix synthesis, secretion, and endocytosis-phagocytosis functions, as well as cell contraction and proliferation. Moreover, MC contain receptors for a large number of hormones and autacoids, such as angiotensin II, serotonin, thromboxane A2, adenosine triphosphate, norepinephrine (NE), and dopamine (DA), among others. Thus, MC are a target for several vasoactive substances capable of modifying the kidney filtration process. In addition, MC control the glomerular function due to their ability to contract and reduce the glomerular capillary surface area, and also synthesize several hormones. Local generation of these vasoactive substances may profoundly alter renal function, independent of systemic hemodynamic modifications.

The kidney of humans and rats excrete catecholamines, especially dopamine [Goldberg and Weder, 1981; Lee, 1993]. Among extra neural tissues, proximal tubular cells are the primary sites of dopamine production after uptake of filtered L-Dopa [Chan, 1976]. However,

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these studies provide only indirect evidence of DA production by the nephron. The local DA has an effect on sodium and fluid absorption in proximal straight tubules, where these non-neural sources of DA production respond for more than half of this catecholamine, which is excreted in the urine [Stephenson et al., 1982]. DA is the immediate metabolic precursor of NE and EPI. In the kidney, low doses of DA (0.5–2.0 μg/kg/min) increase the glomerular filtration rate, renal blood flow, and Na^+ excretion. High doses (5–20 μg/kg/min) can cause splanchnic and glomerular vasoconstriction [Conger, 1993]. EPI increases renin secretion and renal vascular resistance, and also decreases renal blood flow by as much as 40%. In addition, it maintains the glomerular filtration, decreases Na^+, K^+, and Cl^- excretion, and has practically no effect on urinary volume [Schor et al., 1981]. Thus, it is important to investigate the renal sources of these vasoactive substances.

Catecholamines are synthesized from the amino acid L-tyrosine, where the first step of this reaction involves the hydroxylation of tyrosine to L-dihydroxyphenylalanine (L-dopa). Tyrosine hydroxylase (TH), a rate-limiting enzyme, requires biopterin as cofactor, and its presence in cells indicates the potential for catecholamine biosynthesis. L-dopa is converted to DA by dopa decarboxylase (DDC), DA is converted to NE by dopamine β-hydroxylase (DβH), and finally, NE is converted to EPI by phenylethanolamine *N*-methyltransferase. Therefore, in order to study the catecholamine cascade, it is necessary to evaluate many steps in the metabolic pathway.

With the hypothesis that MC are able to produce catecholamines, this study was performed to evaluate: (1) the production of catecholamines by MC in culture devoid of any neural tissue; (2) the presence of enzymes responsible for catecholamine synthesis (TH, DDC, and DβH); and (3) the TH cofactor, biopterin. The concentrations of NE, EPI, and DA were quantified both in the MC homogenate and in the cell culture medium. Western blotting and immunocytochemistry were employed to identify and locate the enzymes.

MATERIALS AND METHODS

The experiments were performed with institutional ethical approval of the protocol and all efforts were made to minimize animal suffering. Moreover, assisted feeding was carried out when necessary.

MC Culture

The culture was done as previously described by our group [Hadad et al., 1995]. Briefly, two normal male Wistar rats were anesthetized with ethyl ether and submitted to bilateral nephrectomy. The kidneys were decapsulated and the cortex was macrodissected. The separated cortex was then sliced and forced through a graded series of stainless steel meshes (60, 100, and 200 mesh), and the glomeruli were collected from the surface of the third sieve and forced through a 25×7 gauge needle for full decapsulation. MC were obtained from collagenase-treated isolated glomeruli in order to remove the epithelial cell component. Washed glomerular remnants were plated onto RPMI 1640 supplemented with 20% fetal bovine serum, 50 U/ml penicillin, 2.6 mM acid HEPES, and 2 mM glutamine. The cultures were allowed to develop in a CO_2 incubator (5% CO_2, 95% air) at 37°C. The medium was replaced every 36 h. At the time of 100% confluence, the cultures were submitted to trypsinization and subcultured in flasks under the same culture conditions. This procedure was repeated up to the third subculture, when the cells were prepared for the experiments, had been washed twice with 5.0 ml of PBS and then incubated with 2.5 ml of RPMI 1640 (Sigma Co., St. Louis, MO) without fetal bovine serum for 24 h. This medium was collected and placed on ice or transferred to a freezer for storage at −80°C. The culture flasks (25 cm^2) were rinsed twice with 5 ml of phosphate-buffered saline (PBS) and scraped in 1 ml PBS. These cells were characterized according to the following criteria: (1) the morphological aspect of stellate cells, (2) the immunofluorescence staining of extracellular matrix for type IV collagen and fibronectin using monospecific anti-serum, (3) the negative immunofluorescence staining for human factor VIII antigens and cytokeratin, and (4) the positive immunofluorescence staining for actin and myosin monospecific anti-serum. Cell viability was monitored with Trypan blue.

Catecholamine Quantification by High Performance Liquid Chromatography (HPLC-ED)

Catecholamines released into the medium and intracellular catecholamines were measured

using ion-pair reverse phase chromatography coupled with electrochemical detection (0.5 V) as described by Cavalheiro et al. [1994]. Fast isocratic separation was obtained using an RP 18 Aquapore cation F micron, Brownlee Column (Applied Biosystems, San Jose, CA) (4.6 × 250 mm) eluted with the following mobile phase: 0.02 M sodium dibasic phosphate, 0.02 M citric acid, pH 2.64, 10% methanol, 0.12 mM Na_2EDTA, and 566 mg/L heptanesulfonic acid. The total time for sample analysis was 30 min. The medium (2.0 ml) and cell lysate (0.8 ml) were previously submitted to the following purification steps: 50 mg Al_2O_5 were weighed out in centrifuge tubes and the samples were added in Tris-buffer, pH 8.8, plus 40 μl (8 ng) DHBA (internal standard, dihydroxybenzylamine). The suspension was vortex-mixed for 10 min. The precipitated alumina was washed three times and vortex-mixed with 1 ml water, and the catecholamines were eluted with 400 μl 0.1 M perchloric acid after 3 min of vortex mixing. After centrifugation for 3 min at 1,500*g*, the supernatant was filtered and 100 μl were injected into the reverse phase column. The monoamine concentration was expressed as ρg/mg cell protein.

The catecholamines were also quantified by a radioenzymic assay with the method described by Tasseron et al. [1980] at "Central de Radioimunoensaio de São Paulo" (CRIESP) (data not shown).

TH Activity Assay

TH activity was measured based on assay of the L-dopa formed from L-tyrosine by high-performance liquid chromatography with electrochemical detection [Cavalheiro et al., 1994]. TH activity in MC homogenate or in the medium was expressed as percent activity. The cells were lysed with 1.0 ml 0.2 M sodium acetate buffer (pH 6.0) and shaken for 1 min. For the assay, the incubation mixture contained 0.001 M L-tyrosine/400 μl cell lysate or corresponding medium/0.04×10^{-3} M *o*-phenantroline/0.2 M sodium acetate buffer (pH 6.0) to make up a total volume of 1.0 ml. For the control, the incubation mixture contained 0.001 M L-tyrosine/400 μl cell lysate or corresponding medium/0.2 M sodium acetate buffer (pH 6.0) in a volume of 1.0 ml, the TH inhibitor *o*-phenantroline was not used. The reaction was started with addition of tyrosine and carried out at 37°C for 60 min. The reaction was stopped by adding 100 μl of 0.1 M perchloric acid. The L-dopa formed was measured as described above.

To show TH level in the experiment, the incubate (corresponding to 400 μl of cell lysate and 400 μl of culture medium) was dialyzed against water in centricon (Ultrafree-4 centrifugal filter & tube with Biomax 30 K NMWL membrane; Millipore, Bedford, USA), evaporated in a Speed Vac SC 110 (Savant Instruments, Holbrook, NY), reconstituted with 20 μl sample buffer (Laemmli buffer, BioRad, California, USA), and submitted to Western blotting analysis as described below.

Biopterin Assay

The medium (not supplemented with serum) and the lysate were assayed by direct injection of the filtered samples in the HPLC apparatus with UV detection (365 nm), using two Aquapore Applied Biosystems, California, USA OD 300 coupled columns, 5 micron (4.6 × 250 mm), equilibrated with 15 mM potassium phosphate buffer, pH 6.4, at a 0.8 ml/min flow and eluted isocratically with the same buffer for 20 min. The values were collected from a standard curve for biopterin. Data obtained for intracellular biopterin and biopterin released into the culture medium were expressed as μmol/mg cell protein.

Western Blotting Analysis

MC were homogenized in 125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% 2-mercaptoethanol. The homogenates were sonicated for 30 s and placed in a boiling water bath for 5 min. Protein concentration was measured by the Bradford method [Bradford, 1976]. The samples were diluted and 40 μg of protein were submitted to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the proteins were electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Switzerland). The membranes were probed with mouse anti-TH-1 (1:250) (Sigma Co.), mouse anti-DDC-109 (1:250) (Sigma Co.), and mouse anti-DβH (1:250) (Accurate Chemical & Scientific Corporation, New York, USA) monoclonal antibodies. Biotinylated anti-mouse IgG (Sigma Co.) was added to bind to the primary antibodies. The membranes were incubated with streptavidin-biotinylated-AP complex (BioRad) and the color development reagents (NBT/BCIP) were then

added to the blot, as recommended by the manufacturer (BioRad). The *Full Range Rainbow*(Amersham Biosciences) was used as protein molecular weight markers.

Immunocytochemistry

The culture flasks (25 cm^2) were rinsed twice with 5 ml PBS and gently scraped in the presence of 1 ml PBS. The MC pellet was fixed in Bouin solution, embedded in paraplast, and sectioned at 5 μm with a rotary microtome. Prior to use, the thin sections were dewaxed, rehydrated, and rinsed in 0.05 mol/L Tris-phosphate-saline buffer (TPS), pH 7.6. Immunostaining was performed by the peroxidase–anti-peroxidase (PAP) (Sigma Co.) method, as previously done for the localization of other enzymes in the kidney [Velarde et al., 1995; Vio et al., 1997] with mouse anti-TH-1 and anti-DDC-9 monoclonal antibodies, and rabbit anti-DβH polyclonal antibody (see below). The cells were separately incubated with each primary anti-serum (1:100) overnight at 25°C, followed by the secondary antibody (1:10) and the PAP complex (1:100) for 30 min each at 25°C, all diluted in TPS containing 0.25% (vol/vol) Triton X-100 and 0.7% (wt/vol) lambda-carrageenan. Between incubations, the sections were rinsed with TPS buffer. When a monoclonal antibody was used, the procedure was followed by incubation with a rabbit anti-mouse IgG antibody as a link between the primary and secondary antibody. Peroxidase activity was visualized by incubating the sections in 0.1% (wt/vol) 3,3′-diaminobenzidine and 0.01% (vol/vol) hydrogen peroxide. The cells were counter-stained with hematoxylin, then dehydrated, cleared with xylene and coverslipped. The cell sections were observed and photographed with a Nikon Optiphot microscope equipped with a Nikon Microflex UFX IIA photographic system.

Anti-serum against DβH was obtained by standard procedures immunizing rabbits with an intradermal injection of purified dopamine β-hydroxylase (200 μg/animal) emulsified in complete Freund's adjuvant, followed by booster injections of dopamine β-hydroxylase (100 μg/animal) emulsified in incomplete Freund's adjuvant every 3–4 weeks. Blood was obtained prior to immunization (preimmune serum) and during the immunization protocol by periodical bleeding to test the antibody titer. Purified DβH of bovine origin was purchased from Sigma Co.

Immunocytochemical Controls

Omission of the primary antibodies and their replacement with pre-immune serum or non-immune serum were used as immunocytochemical controls. Substantia nigra and adrenal glands from normal rats were used as positive control. They were fixed in Bouin's, embedded in paraplast, and processed as described for the MC pellets (data not shown).

Statistical Analysis

Results are presented as means ± SEM. Data were analyzed statistically by the Student's t test with the level of significance set at $P < 0.05$.

RESULTS

The presence of NE, EPI, and DA was demonstrated in the intracellular MC compartment as well as in the extracellular compartment. As shown in Figure 1, NE and EPI levels (X ± SEM), measured by HPLC-ED, were lower in the medium (24.50 ± 3.32, $n = 12$, and 32.72 ± 2.79 pg mg^{-1} cell protein, $n = 12$, respectively, $P < 0.001$) than in the intracellular compartment (56.52 ± 7.75, $n = 12$, and 82.46 ± 10.34 pg mg^{-1} cell protein, $n = 12$, respectively, $P < 0.001$). No statistically significant difference in DA levels was found between the medium and the intracellular compartment (61.63 ± 14.68, $n = 8$, and 51.73 ± 8.92 pg mg^{-1} cell protein, $n = 8$, respectively, $P > 0.05$). Our results were obtained based on the number of culture dishes from two rats. When we repeated our experiments with other MC cultures, we found the

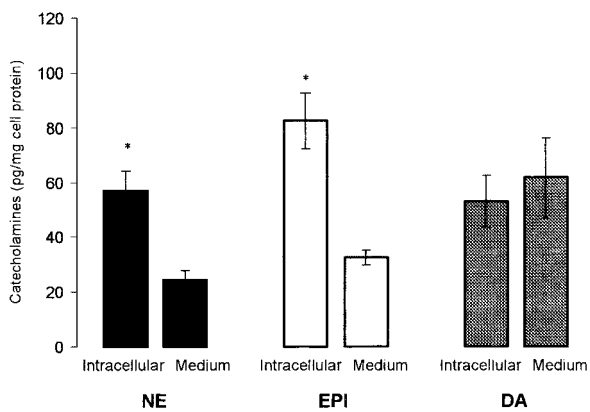

Fig. 1. Distribution of catecholamines in the intracellular compartment and in the culture medium of MC. All values are means ± SEM, $n = 8$, based on the number of culture dishes from two rats. NE = norepinephrine; EPI = epinephrine; DA = dopamine. *$P < 0.001$.

same profile. For method standardization, catecholamines were first quantified in the PBS washing before incubation of MC in 5.0 ml of serum free medium. A 1.0 ml sample was collected at: 0, 2, 8, 12, and 24 h, and a linear release of catecholamines into the medium was observed, but no catecholamine concentration was detected in the PBS (data not shown). Our results showed that MC were able to produce catecholamines in vitro, thus, in order to confirm the results obtained by HPLC-ED, the catecholamines were also detected and measured by a radioenzymic assay, which revealed similar results to those obtained by HPLC-ED (data not shown).

Figure 2 shows, in percentage, the TH enzymatic activity from MC lysate and culture medium with and without the TH inhibitor, *o*-phenantroline. For both samples, a 39% inhibition (39% ± 1.47, n = 4) of L-dopa formation was observed.

Using HPLC determination, the biopterin values obtained for intracellular and extracellular spaces are summarized in Table I. A high value was detected in the medium (5.70 ± 1.98 nmol mg^{-1} cell protein, n = 4, $P < 0.05$), whereas a lower concentration was detected in the intracellular compartment (0.28 ± 0.03 nmol mg^{-1} cell protein, n = 4, $P < 0.05$).

Western blotting analysis of MC homogenates revealed the presence of (a) TH as one band of 75 kDa (Fig. 3A); (b) DDC antibody with three bands located at 75, 30, and 23 kDa (Fig. 3B); (c) dopamine β-hydroxylase antibody with two bands corresponding to 32 kDa (Fig. 3C).

Enzymes TH (Fig. 4A), DDC (Fig. 4B), and DβH (Fig. 4C) in rat MC pellets could also be shown by immunocytochemistry. High magnification showed heavy staining over the cytoplasm and in the perinuclear area of the cells. No staining was observed when the primary antibodies were omitted or when they were replaced with preimmune or nonimmune serum (data not shown).

DISCUSSION

MC in vitro are a target and a source of hormones, cytokines, and autacoids. They are capable of synthesizing and releasing a variety of substances that may exert autocrine, paracrine, or endocrine effects. But in vivo evidence for this production in MC has not yet been provided [Ardaillou et al., 1996].

We report here, for the first time, detection of significant catecholamine (NE, EPI, DA) levels in MC in culture. Surprisingly, catecholamines were detected both in the intracellular and extracellular compartments by HPLC-ED and/or radioimmunoassay. Our results demonstrated that the concentrations of NE and EPI secreted into the medium were significantly

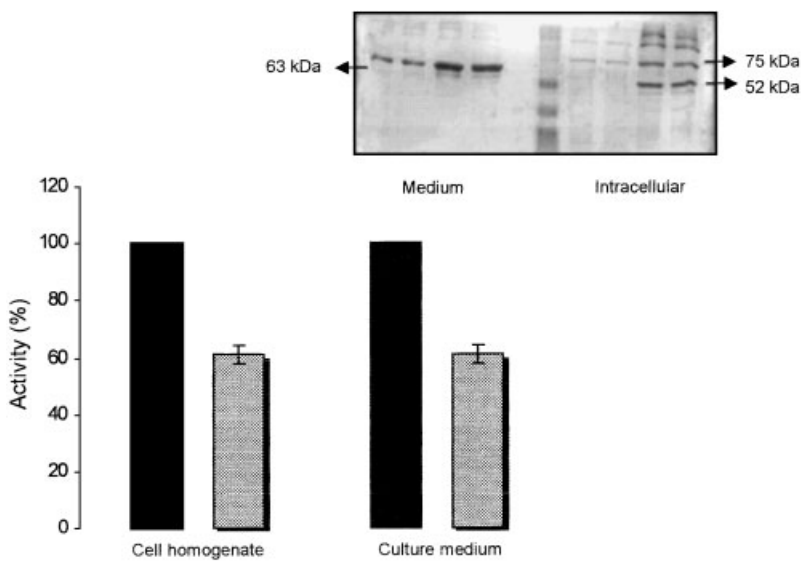

Fig. 2. Percentage of enzymatic activity based on the formation of L-dopa from L-tyrosine. The values are means, n = 4, based on the number of culture dishes from two rats. ■ Control (total activity); ▨ actvity measured in the presence of the inhibitor, *o*-phenantroline. In the box, TH level in the incubate was shown using Western blotting analysis.

TABLE I. Biopterin Values in the Intracellular and Extracellular Compartments of Rat Mesangial Cells (MC) in Culture

	Biopterin (nmol mg^{-1} cell protein)	
	Intracellular	Medium
N	4	4
Mean	0.28	5.70*
SEM	0.03	1.98

Values are reported as means ± SEM, based on the number of culture dishes obtained from two rats.
*$P < 0.05$.

lower ($P < 0.001$) than the values detected in the intracellular compartment, but no differences in DA levels were observed. Our data, expressed as pg/ml (EPI = 2.0 and NE = 6.6), showed that the concentration of these monoamines in MC

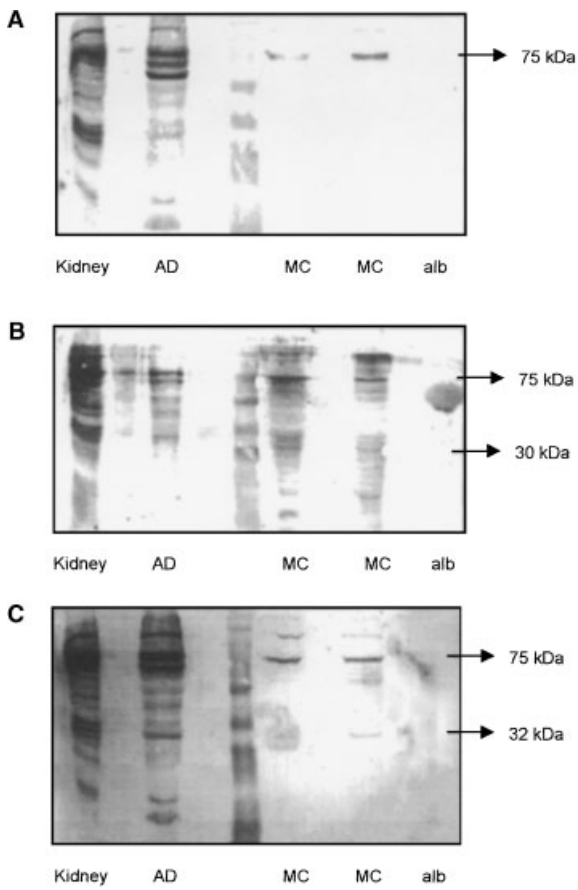

Fig. 3. Western blotting of rat MC. A nitrocellulose membrane containing homogenates from rat kidney (kidney), rat adrenal gland (AD), and from MC was reacted with (**A**) monoclonal TH-2 antibody; (**B**) monoclonal DDC-109 antibody; and (**C**) monoclonal DβH antibody. Arrows indicate the molecular mass in kDa. Bovine serum albumine (alb; 40 μg) was used as negative control.

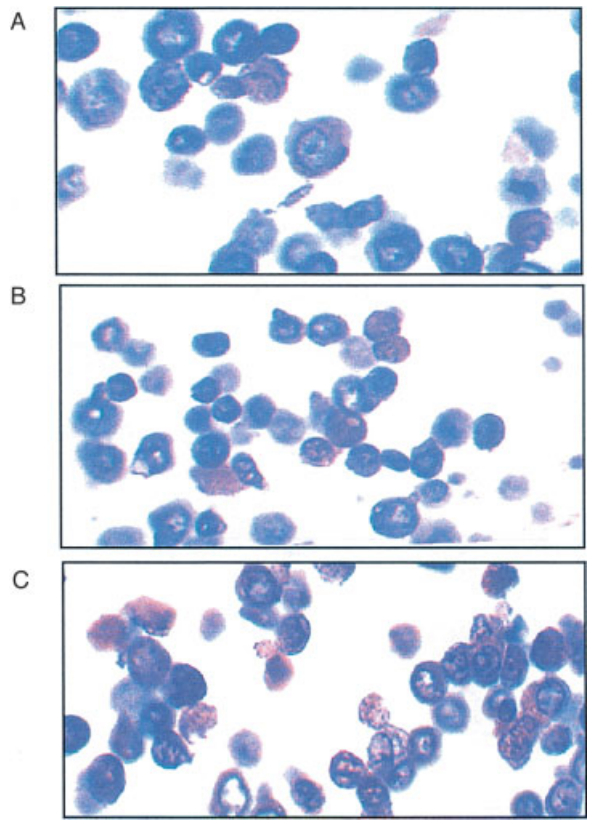

Fig. 4. Photomicrographs showing TH, DDC, and DβH immunostaining in rat MC. Thin MC sections (5 μm) were stained for monoclonal TH-2 antibody (1:100) (5A), monoclonal DDC-109 antibody (1:100) (5B), and polyclonal DβH antibody (1:100) (5C).

was similar to that found in rat red blood cells and substantially lower (40-fold) than values obtained for rat plasma [Gupta et al., 1997].

The linear release of catecholamines in the serum-free medium after two washes with PBS suggests that MC are able to produce and release these monoamines independently of the medium conditions, and that this process is not a consequence of serum accumulation.

Using the present protocol, we detected components of the catecholamine cascade production (Fig. 5). The presence of these enzymes was assessed by two specific and complementary methods: Western blotting analysis of cell homogenates and immunocytochemistry of pelleted cells. The meaning of the different TH molecular masses detected can be elucidated by two enzymes fraction (I and II) with different molecular masses and different kinetic properties from bovine adrenal medulla and caudate nucleus purified on a DEAE-Sephacel column. The molecular mass of the subunit of Fraction I

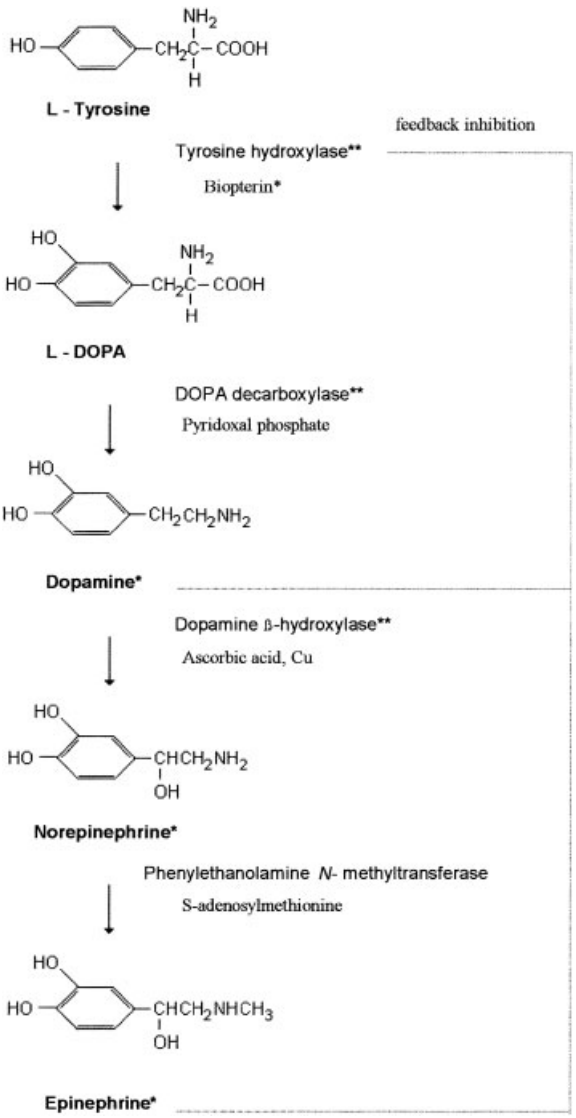

Fig. 5. The main biosynthetic pathway of catecholamines and its regulation. Data obtained by direct measurement (*) or detected by distinct methods (**).

was estimated to be 60 kDa by SDS–PAGE. Fraction II was judged to be composed of several enzymes forms with different molecular masses. In summary, Oka et al. [1982] demonstrated that TH from adrenal medulla is composed of at least two forms, and each enzyme has a different apparent K_m value for biopterin cofactor. Dopamine β-hydroxylase is a tetrameric glycoprotein, and each monomer has an expected molecular mass of 29 kDa [Pendleton et al., 1998]. In the literature, DDC can present three different molecular masses: 54, 40, and 21 kDa [Christenson et al., 1970]. In addition, a special feature of the primary structure of rat DDC was a repeating structure consisting of 29 amino acid residues. A sequence of 58 amino acid residues, including this repeating structure of rat DDC, was found to show homologies with those of rat TH, human DβH, and bovin phenylethanolamine *N*-methyltransferase. These results indicate that catecholamine biosynthetic enzymes are structurally related and suggest that their homologous domains are important for catechol–protein interactions [Tanaka et al., 1989].

In addition to showing TH immunoreactivity, MC were able to convert L-tyrosine to L-dopa. This fact is an evidence that MC have the enzymatic activity associated with TH, the rate-limiting step in the biosynthesis of catecholamines. It is also a strong evidence of the presence of biopterin, which is a cofactor of TH in the synthesis of L-dopa in MC. Biopterin was also detected by HPLC, as shown in the Table I.

In addition, in our laboratory, we isolated and purified to homogeneity a TH isoform from MC in culture; the enzyme presented a molecular mass of 60 kDa and immunoreaction against mouse anti-TH-1 monoclonal antibody [Arita et al., 2002a].

Based on our results, we postulate that MC are able to produce, release, and store catecholamines in vitro, suggesting a potential functional participation of these vasoactive substances in the renal dopaminergic/catecolaminergic sytem, and in the regulation of glomerular hemodynamics in the renal microcirculation and in tubular function.

Although in the present study we did not evaluate the factors involved in the control of production or release of these hormones, the importance is known of modulates basal CA secretion for maintaining a physiologic state, for example, the normotensive state. The fact that TH and Nitric Oxide Sintase, enzymes from a vasoconstrictor and vasodilator system, respectively, use the same cofactor, biopterin, suggests that they can act as counter-regulatory mechanism, which is stimulating issue to be clarified in futher studies. In addition, our laboratory has widely studied the participation of local catecholamines (autocrine and paracrine functions) in some pathophisiologies, such as (1) diabetes, that presents increased CA levels and TH expression, and decreased MAO and COMTE activities (umplublished data), and (2) exposure to radiographic contrast media (ioxaglate sodium meglumine), which increased

L-DOPA and dopamine levels [Arita et al., 2002b].

In summary, we show here a new pathway for catecholamine formation in MC in culture and the identification of their biosynthetic cascade (Fig. 5) by determining the enzymes and a cofactor that mediate their production. The regulation of this hormonal cascade and its potential contribution to the intrarenal control of glomerular function and microcirculation are still unknown, however they are under investigation.

Thus, cultured MC provide a convenient model for the study of renal synthesis and release of catecholamines lacking neuronal contribution. Finally, MC can be a useful tool to study possible alterations in the synthesis and levels of catecholamines in experimental physiological and pathophysiological models of renal alterations.

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