Cultured neonatal rat cardiac myocytes and fibroblasts do not synthesize renin or angiotensinogen: evidence for stretch-induced cardiomyocyte hypertrophy independent of angiotensin II

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Abstract

Objective: The hypertrophic response of cardiomyocytes exposed to mechanical stretch is assumed to depend on the release of angiotensin (Ang) II from these cells. Here we studied the synthesis of renin–angiotensin system (RAS) components by cardiac cells under basal conditions and after stretch. Methods: Myocytes and fibroblasts were isolated by enzymatic dissociation from hearts of 1–3-day-old Wistar rat strain pups, grown for 1 day in serum-supplemented medium and then cultured in a chemically defined, serum-free medium. Medium and cell lysate were collected 5 days later or after exposure of the cells to cyclic stretch for 24 h. Prorenin, renin and angiotensinogen were measured by enzyme-kinetic assay; Ang I and Ang II were measured by radioimmunoassay after SepPak extraction and HPLC separation. Results: Prorenin, but none of the other RAS components, could be detected in the medium of both cell types. However, its levels were low and the Ang I-generating activity corresponding with these low prorenin levels could not be inhibited by the specific rat renin inhibitor CH-732, suggesting that it was most likely due to bovine and/or horse prorenin sequestered from the serum-containing medium to which the cells had been exposed prior to the serum-free period. When incubated with Ang I, both myocytes and fibroblasts generated Ang II in a captopril-inhibitable manner. Myocyte and fibroblast cell lysates did not contain prorenin, renin, angiotensinogen, Ang I or Ang II in detectable quantities. Stretch increased myocyte protein synthesis by 20%, but was not accompanied by Ang II release into the medium. Conclusion: Cardiac myocytes and fibroblasts do not synthesize renin, prorenin or angiotensinogen in concentrations that are detectable or, if not detectable, high enough to result in Ang II concentrations of physiological relevance. These cells do synthesize ACE, thereby allowing the synthesis of Ang II at cardiac tissue sites when renin and angiotensinogen are provided via the circulation. Ang II is not a prerequisite to observe a hypertrophic response of cardiomyocytes following stretch. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Angiotensin; ACE inhibitor; Myocytes; Renin–angiotensin system; Stretch

1. Introduction

The existence of a local renin–angiotensin system (RAS) in the heart, often invoked to explain the beneficial effects of ACE inhibitors in heart failure [1,2], is still a controversial issue. The presence of RAS components in cardiac tissue [3–5] cannot be taken as direct evidence for local production of these components. One or more components may have been sequestered from the circulation. For instance, circulating renin may bind to cardiac cell receptors and to renin binding proteins in the heart [6–8], and circulating Ang II is known to accumulate in cardiac tissue via \textit{AT} \textsubscript{1} receptor-mediated endocytosis [9]. The levels of renin and angiotensinogen mRNA in the...
heart are low or undetectable [10–13], thereby suggesting that the presence of these components in cardiac tissue may indeed depend on uptake rather than local production. The uncertainties concerning local synthesis arising from tissue measurements can be avoided when measurements are made in cells cultured in the absence of serum. The use of serum-free culture medium is necessary to exclude the uptake of RAS components present in serum.

Most so-called renin-expressing extrarenal cells produce prorenin rather than renin. These cells do not store prorenin and secrete it in a constitutive manner [14–17]. With regard to cardiomyocytes, both Dzau and Re [18] and Dostal et al. [19] reported on the presence of renin in these cells. No distinction between renin and prorenin was made, nor did these authors determine (pro)renin release into the culture medium. Constitutive secretion of angiotensinogen has also been described [20–22]. The single report on the synthesis of angiotensinogen by cardiac cells focuses on its presence in these cells [19]. ACE, a cell membrane-bound enzyme, has been demonstrated in cardiac cells by enzyme-kinetic and immunohistochemical methods [23–26].

According to several investigations, serum-deprived cardiac cells release angiotensins into the culture medium. The Ang I and II levels in the medium, however, show huge variations, from <10 fmol/ml to >1000 fmol/ml [23,26–29]. Ang II in the medium increased 100-fold after the induction of mechanical stretch [27], possibly by release from intracellular storage sites [23,27], and this cell-derived Ang II may play a role in the stretch-induced hypertrophic response of cardiomyocytes [26–29].

It was the aim of the present study to investigate the synthesis of RAS components by neonatal rat cardiomyocytes and fibroblasts by measuring renin, prorenin, angiotensinogen, Ang I and Ang II in the medium and cell lysate of serum-deprived cells with the help of well-established biochemical techniques. Cellular ACE activity was investigated by quantifying Ang I-to-II conversion by intact cells in the presence and absence of captopril. Stretch-induced release of angiotensin II and its role in cellular hypertrophy were examined in cardiomyocytes exposed to cyclic, circular stretch for 24 h. For comparison, studies were also performed in cells cultured in the presence of serum.

2. Materials and Methods

2.1. Reagents

Fetal calf serum, horse serum, penicillin and streptomycin were purchased from Boehringer Mannheim (Mannheim, Germany). Dulbecco’s modified Eagle’s medium (DMEM) and Medium 199 were from Gibco, Life Technologies (Middlesex, UK). Tryptase (type III) and captopril were from Sigma (St. Louis, MO, USA). Methanol and ortho-phosphoric acid (both analytical grade) were from Merck (Darmstadt, Germany). Ang I was obtained from Bachem (Bubendorf, Switzerland). [3H]-leucine was from Amersham (Buckinghamshire, UK). The rat renin inhibitor CH-732 was a kind gift of Dr. M. Szolka, Ferring Research Institute, (Southampton, UK) [30]. Rat renin was prepared from rat kidneys as described before [31]. Human recombinant prorenin was a gift of Dr. W. Fischli (Hoffmann-La Roche, Basel, Switzerland). Angiotensinogen was prepared from plasma of nephrectomized rats [31].

2.2. Cell Culture

All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus University, Rotterdam, The Netherlands, in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Primary cultures of neonatal ventricular cardiomyocytes and fibroblasts were prepared from 1–3-day-old Wistar strain rats as described before [32]. Briefly, ventricles from newborn 1–3-day-old Wistar rats were minced, and cells were isolated by eight subsequent trypsinization steps at 30°C. Non-cardiomyocytes were separated from the cardiomyocytes by differential preplating. Cardiomyocytes were seeded in 20-cm² culture dishes (Falcon, Becton & Dickinson, Plymouth, UK) at 1.5·10^5 cells/cm², giving a confluent monolayer of spontaneously contracting cells after 24 h. The preplated cells (fibroblast fraction) were passaged after 4 days, using a 0.02% trypsin–0.05% EDTA solution, in 20-cm² culture dishes at 0.75% CO₂, 95% air in 5 ml culture medium consisting of DMEM and Medium 199 (4:1), supplemented with 5% fetal calf serum, 5% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. After incubation for 24 h, cells were either serum-deprived or maintained in serum-supplemented culture medium for 5 days.

2.3. Collection of Medium and Cells for the Measurement of RAS Components

Cardiomyocyte- and fibroblast-conditioned culture medium (5 ml) was collected for the measurement of RAS components after the cells had been maintained with or without serum for 5 days. The RAS component content of unconditioned medium, i.e. medium that had not been in contact with either cardiomyocytes or fibroblasts, was also studied. Medium for the measurement of prorenin, renin and angiotensinogen was frozen at −70°C without the addition of inhibitors. Medium for the measurement of Ang I and II was mixed with 250 μl angiotensinase inhibitor solution (containing 125 mmol/l disodium EDTA and 25 mmol/l 1,10-phenanthroline) and frozen at −70°C.

To measure RAS components in the cells, each well was washed three times with 6 ml ice-cold phosphate buffered
saline (PBS; 140 mmol/l NaCl, 2.6 mmol/l KCl, 1.4 mmol/l KH$_2$PO$_4$, 8.1 mmol/l Na$_2$HPO$_4$, pH 7.4). After washing, cells used for the measurement of prorenin, renin and angiotensinogen were lysed in 0.5 ml ice-cold PBS containing 0.2% Triton X-100, and the cell lysates were quickly frozen on dry ice. Cells used for the measurement of Ang I and II were scraped with a rubber policeman in a volume of 0.5 ml ice-cold PBS. The cell–PBS mixture was centrifuged at 1000 g at 4°C for 1 min, after which the pellet was homogenized in 0.5 ml 0.1 mol/l HCl–80% ethanol using a hand-operated douncer. The ethanol was evaporated under vacuum rotation at 4°C using a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, USA). The concentrated homogenates were dissolved in 0.5 ml 1% ortho-phosphoric acid and applied to SepPak columns (see Section 2.6).

2.4. Angiotensin I-to-II conversion by ACE

To determine whether 5-day-old cardiomyocytes and fibroblasts contain ACE, Ang I-to-II conversion by these cells was studied in the presence or absence of captopril (final concentration in the medium: 0.5 μmol/ml). Ang I was added to the medium (final concentration: 1 pmol/ml), and 150 μl samples were obtained over a period of 40 min (from cells cultured in the presence of serum) or 120 min (from cells cultured in the absence of serum). The samples were rapidly mixed with 10 μl angiotensinase inhibitor solution and frozen at −70°C. No corrections were made for the small volume changes (approximately 3% per sample) occurring as a consequence of fluid sampling. Ang I-to-II conversion was also dissolved in unconditioned medium.

2.5. Angiotensin II generation during stretch of cardiomyocytes

To study the generation of Ang II during prolonged stretch and its contribution to the increased protein synthesis occurring under these conditions, cardiomyocytes were subjected to cyclic, circular stretch for 24 h. Following isolation (see Section 2.2) the cells were seeded in flexible-bottomed 6-well culture plates (type I collagen-coated, 5 cm$^2$/well; Flexcell, Hillsborough, NC, USA). They were maintained at 37°C and 5% CO$_2$–95% air in 1 ml culture medium consisting of DMEM and Medium 199 (4:1), supplemented with 5% fetal calf serum, 5% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. After 24 h the medium was replaced by DMEM and Medium 199 (4:1), supplemented with 4% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. The stretch experiment was performed 48 h later under serum-free conditions. The cells were preincubated for 30 min with 1.5 ml serum-free medium. The 6-well plates were then placed on a Flexcell Strain Unit (FX-2000, Flexcell), and the cells were stretched at 30 cycles per minute (1 s strain, 1 s relaxation) at 20% elongation for 24 h. Control cells, grown on non-flexible-bottomed culture plates, were studied in parallel. For comparison, control cells cultured on non-flexible-bottomed culture plates were also incubated for 24 h with endothelin-1 (ET-1; final concentration in the medium: 10$^{-8}$ mol/l), an agent known to induce protein synthesis in cardiomyocytes [32,33].

The effect of stretch on protein synthesis was evaluated by adding [$^3$H]-leucine to the medium (final concentration: 0.5 μCi/ml) of control-, stretched- and ET-1-treated cells 2 h before the end of the 24 h study period. After 24 h, incorporated [$^3$H]-leucine was determined as described before [33]. Total cellular protein was measured after 24 h in control-, stretched- and ET-1-treated cells that had not been incubated with [$^3$H]-leucine, using the Bradford assay [34]. Cellular hyperplasia was investigated by measuring optical density after incubation of the cells for 2 h at 37°C with (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), an agent which is converted to the colored product formazan via intact mitochondria. A change in optical density correlates directly with a change in cell number [35].

The effect of stretch on Ang I and II synthesis was studied by collecting 0.15 ml samples from the culture medium of each well of two 6-well plates after 1, 2, 6, and 24 h. The twelve 0.15 ml samples obtained at each time point were added together and mixed with 0.1 ml angiotensinase inhibitor solution (final volume 1.9 ml). All samples were stored at −70°C.

2.6. Biochemical measurements

2.6.1. Renin and prorenin

Renin was quantified in duplicate by measuring Ang I generation at pH 7.4 during incubation at 37°C for 2–4 h with a saturating concentration of rat angiotensinogen in the presence angiotensinase-, ACE- and serine protease-inhibitors [4,7]. Two different inhibitor solutions were used, one with and one without the rat renin inhibitor CH-732 (final concentration in the incubation mixture: 5 μmol/l). Inhibition of rat kidney renin is >95% at this concentration (Fig. 1). Ang I was measured with a sensitive radioimmunoassay [36]. The lowest renin level that could be detected was 1.0 fmol Ang I/min per ml medium and 0.5 fmol Ang I/min per 10$^6$ cells.

Prorenin was first converted into renin by proteolytic activation and then also measured with the above assay. Based upon our experience with the activation of prorenin in tissues [4,7], two different activation procedures were tested, i.e. acidification only or acidification followed by treatment with plasmin at neutral pH. Medium or cell lysate were acidified by dialysis at 4°C for 48 h against 0.05 mol/l glycine buffer, pH 3.3, containing 0.001 mol/l disodium EDTA and 0.095 mol/l NaCl. This was followed by either 1) dialysis at 4°C for 24 h against 0.1 mol/l phosphate buffer, pH 7.4, containing 0.001 mol/l disodium
limit of detection for Ang I and Ang II in the culture medium were 0.2 and 0.1 fmol/ml, respectively. In cell homogenates, it was 0.3 and 0.2 fmol/10⁶ cells.

2.7. Calculations

Ang I is eliminated by conversion to Ang II by ACE, and by breakdown into small biologically inactive peptides by various other enzymes. The latter process is referred to as degradation of Ang I. The first order rate constants for Ang I degradation (k₁) and conversion (k₂) were calculated as described before [36]. The percent contribution of conversion to the total metabolism of Ang I is defined as follows:

\[
\text{Contribution of conversion to metabolism (\%) = } \left( \frac{k_2}{k_1 + k_2} \right) \times 100\%.
\]

2.8. Statistical analysis

Results are expressed as mean±S.D. One-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (Student’s t-test for paired observations, with Bonferroni correction) was used for comparison between groups. Values of P<0.05 were considered significant.

3. Results

3.1. Renin, prorenin and angiotensinogen

Renin and prorenin were detectable in unconditioned fetal calf serum- and horse serum-supplemented medium (Fig. 2). The Ang I-generating activity corresponding with these renin and prorenin levels was not inhibited by the rat renin inhibitor CH-732 (5 µmol/l), which suggests that CH-732, at this concentration, does not inhibit bovine or horse renin. Following 5 days of incubation with either cardiomyocytes or fibroblasts, the levels of renin and prorenin in serum-supplemented medium were unchanged.

Renin was undetectable in cardiomyocyte- and fibroblast-conditioned, serum-deprived medium. Low levels of prorenin were present in the cardiac cell-conditioned, serum-free media, but they were not inhibited by CH-732, indicating that the Ang I generation we measured after in vitro prorenin activation was not caused by rat renin. Both renin and prorenin were undetectable in cell lysates of serum-deprived cardiomyocytes and fibroblasts.

Low levels of angiotensinogen were detected in unconditioned, serum-supplemented medium, which did not change after 5 days of incubation with cardiac cells (Fig. 2). These levels therefore most likely represent bovine and horse angiotensinogen. Angiotensinogen was undetectable in cardiomyocyte- and fibroblast-conditioned serum-free medium and in the lysates of these cells.
3.2. Angiotensin I-to-II conversion by ACE

Ang I added to unconditioned serum-supplemented medium was rapidly metabolized, Ang II being a major metabolite (Fig. 3). Captopril prevented the formation of Ang II completely. On the basis of the difference in Ang I metabolism with and without captopril it could be calculated that more than 90% of the Ang I metabolism in unconditioned serum-supplemented medium was due to ACE-dependent Ang I-to-II conversion (Table 1). During incubation with unconditioned serum-deprived medium, no significant Ang I metabolism could be demonstrated over a period of 2 h.

Ang I metabolism in cardiomyocyte and fibroblast cell cultures that had been maintained for 5 days in the presence of serum tended to be more rapid than Ang I metabolism in unconditioned serum-supplemented medium (Fig. 3). Ang II was again a major metabolite, and captopril prevented its formation completely. In both cell cultures, approximately 80% of Ang I metabolism was due to ACE-dependent Ang I-to-II conversion (Table 1).

Ang I added to serum-deprived cardiomyocyte and fibroblast cell cultures was also converted to Ang II, and captopril inhibited the formation of Ang II completely (Fig. 3). This indicates that, in the absence of serum, cardiomyocytes and fibroblasts contain detectable ACE activity. Approximately 60–70% of the Ang I metabolism was due to ACE (Table 1).

3.3. Angiotensin I and II

Low levels of Ang I and II were present in unconditioned serum-supplemented medium (Table 2). These levels remained low or decreased to levels below the detection limit after 5 days of incubation with cardiomyocytes or fibroblasts. The cellular levels of Ang I and II at that time were also close to or below the detection limit of our assays (Table 2). Ang I and II were undetectable in cardiomyocyte- and fibroblast-conditioned serum-deprived medium and could also not be demonstrated in cell homogenates of cardiomyocytes and fibroblasts that had been serum-deprived for 5 days (Table 2).

Ang I and II were also undetectable in medium collected from cells that had been serum-deprived for 1 h (n=3, data not shown). This excludes the possibility that Ang I and II are released by the cells at the start of the 5-day period and subsequently metabolized during further incubation.
Ang I, Without serum Ang II 0.4
Ang I 1.3
6
With serum 5-day-old fibroblasts cultured in the presence or absence of serum Angiotensin I and II levels in unconditioned medium (n = 4) (Table 2). Angiotensin levels in the medium were below the detection limit.

<table>
<thead>
<tr>
<th></th>
<th>Unconditioned medium</th>
<th>Cardiomyocytes (n = 6)</th>
<th>Fibroblasts (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t_{1/2} without captopril (h)</td>
<td>0.63±0.35</td>
<td>0.27±0.03</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>t_{1/2} with captopril (h)</td>
<td>4.35±0.90*</td>
<td>1.53±0.37*</td>
<td>2.22±1.08*</td>
</tr>
<tr>
<td>k_1 (h^{-1})</td>
<td>0.17±0.03</td>
<td>0.62±0.16</td>
<td>0.46±0.15</td>
</tr>
<tr>
<td>k_2 (h^{-1})</td>
<td>1.69±0.77</td>
<td>2.13±0.22</td>
<td>1.92±0.09</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td>93±10</td>
<td>79±4</td>
<td>81±6</td>
</tr>
<tr>
<td><strong>Without serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t_{1/2} without captopril (h)</td>
<td>No metabolism</td>
<td>1.42±0.33</td>
<td>3.22±0.10</td>
</tr>
<tr>
<td>t_{1/2} with captopril (h)</td>
<td>No metabolism</td>
<td>4.15±1.52*</td>
<td>14.0±1.97*</td>
</tr>
<tr>
<td>k_1 (h^{-1})</td>
<td>–</td>
<td>0.31±0.09</td>
<td>0.05±0.01§</td>
</tr>
<tr>
<td>k_2 (h^{-1})</td>
<td>–</td>
<td>0.42±0.17§</td>
<td>0.17±0.01§</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td>–</td>
<td>56±9</td>
<td>76±4</td>
</tr>
</tbody>
</table>

*The culture medium was either serum-deprived or contained 5% fetal calf serum and 5% horse serum. The percentage of metabolism due to angiotensin I-to-II conversion by ACE is given for each condition. Values are mean±S.D. *P<0.01 without captopril vs. with captopril. §P<0.05 without serum vs. with serum.

3.4. Angiotsensin II generation during stretch of cardiomyocytes

Cyclic, circular stretch of cardiomyocytes for 24 h led to the expected increase in protein synthesis rate and total cellular protein, although the effects were modest as compared to those observed after 24 h of exposure to ET-1 (Table 3). Using the MTT assay, no change in cell number was observed following stretch (n = 3, data not shown). This indicates that stretch induced cellular hypertrophy rather than cellular hyperplasia. Ang I in the medium of control cells and cells exposed to stretch was close to the detection limit at 1 h after the start of the experiment (<0.2–0.7 fmol/ml and <0.2–0.8 fmol/ml, respectively; range of 3 experiments) and decreased to undetectable levels during prolongation of the experiment. Ang II was undetectable at all time points, both in the medium of control cells and in the medium of stretched cells. Exposure to ET-1 also did not result in Ang I or Ang II release (Table 3).

4. Discussion

This study indicates that cultured neonatal rat cardiomyocytes and fibroblasts do not synthesize renin.

Table 3

Table 3

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Control</th>
<th>Stretch</th>
<th>ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1H]-Leucine incorporation, total cellular protein, and the angiotensin I and II levels in the medium of untreated cardiomyocytes (control), cardiomyocytes exposed to cyclic stretch for 24 h (stretch) and cardiomyocytes incubated with 10^{-8} mol/l endothelin-1 (ET-1) for 24 h*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incorporated ([1H]-leucine (dpm/well))</td>
<td>19</td>
<td>5992±215</td>
<td>7300±288*</td>
<td>9479±397*</td>
</tr>
<tr>
<td>Total cellular protein (mg/well)</td>
<td>19</td>
<td>0.15±0.01</td>
<td>0.17±0.01*</td>
<td>0.18±0.01*</td>
</tr>
<tr>
<td>Ang I (fmol/ml medium)</td>
<td>3</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Ang II (fmol/ml medium)</td>
<td>3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* Ang, angiotensin. Values are mean±S.D. *P<0.05 vs. control. Angiotensin levels in the medium were below the detection limit.

Table 2

Angiotensin I and II levels in unconditioned medium (n = 3), and in conditioned medium (n = 5) and cell lysates (n = 5) of 5-day-old cardiomyocytes and 5-day-old fibroblasts cultured in the presence or absence of serum*<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unconditioned (fmol/ml)</td>
<td>Cardiomyocyte-conditioned (fmol/ml)</td>
</tr>
<tr>
<td>With serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang I</td>
<td>1.3±0.3</td>
<td>2.1±1.2</td>
</tr>
<tr>
<td>Ang II</td>
<td>0.4±0.3</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>Without serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang I</td>
<td>&lt;0.2</td>
<td>&lt;0.7±0.3</td>
</tr>
<tr>
<td>Ang II</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* Ang, angiotensin. Values are mean±S.D. If one or more values was below the detection limit, this is denoted as <mean±S.D. If all values were below the detection limit this is denoted as less than detection limit.
prorenin, or angiotensinogen in concentrations that are detectable or, if not detectable, high enough to result in Ang I or Ang II concentrations of physiological relevance. Both types of cardiac cells do appear to synthesize ACE, and thus are capable of converting Ang I into Ang II. Evidence for the presence of other enzymes capable of converting Ang I into Ang II (e.g., chymase [37]) was not obtained.

All RAS components are present in serum-containing medium, and will therefore be detected when measurements are made in the medium of cells cultured in the presence of serum. We used the specific rat renin inhibitor CH-732 [30,38] to distinguish Ang I generation by rat renin from Ang I generation by other enzymes, such as bovine renin and/or horse renin. The latter two are present in the fetal calf serum and horse serum applied in the present study to obtain cell adherence and confluency prior to serum-deprivation, and both renins are known to react with rat angiotensinogen [17,39].

No CH-732 inhibitable Ang I-generating activity could be detected in the medium of cells maintained in the presence of serum, whereas Ang I generation in medium of serum-deprived cells was below the detection limit. A possible explanation for this lack of renin release from rat cardiac cells might be that extrarenal cells release prorenin rather than renin [14–17]. However, although the Ang I-generating activity of medium obtained from cells cultured with serum increased nearly 10-fold following prorenin activation, it could again not be inhibited by CH-732. Moreover, the levels of prorenin measured in serum-supplemented conditioned medium did not differ from those in serum-supplemented unconditioned medium. Thus, the increase in Ang I-generating activity following activation is most likely due to the activation of bovine and/or horse prorenin.

Interestingly, medium of cells cultured in the absence of serum also contained low levels of prorenin. None of the Ang I-generating activity corresponding with these prorenin levels could be inhibited by CH-732, nor did the prorenin levels differ between cardiomyocytes and fibroblasts. Most likely therefore, this prorenin represents bovine and/or horse prorenin trapped or bound by the cells during their incubation in the presence of serum and released back into the medium during incubation under serum-deprived conditions. In support of this assumption, we have recently shown that neonatal rat cardiac cells, during incubation with prorenin, are capable of binding and internalizing prorenin, and that membrane-bound, non-internalized prorenin is released back into the medium when the cells are subsequently incubated with fresh medium without prorenin [8]. Uptake of renin and/or prorenin might also explain the presence of renin in rat cardiac cells described by others [18,19]. The lack of Ang I-generating activity in lysates of serum-deprived cells in the present study may be due to metabolism of (pro)renin following internalization.

The low levels of angiotensinogen present in unconditioned serum-supplemented medium (corresponding to <5% of the normal plasma angiotensinogen levels in the rat) did not change during incubation with cardiac cells, nor did the cells release angiotensinogen into the medium when incubated under serum-free conditions. All other cells described to synthesize angiotensinogen [20–22] release this substrate into the medium, without storing it intracellularly. In vivo, angiotensinogen also appears to be limited to the extracellular fluid compartment [4,5,31]. Therefore, our data do not support synthesis and/or release of angiotensinogen by neonatal rat cardiomyocytes or fibroblasts.

In view of the absence of (pro)renin and angiotensinogen synthesis by cardiac cells, it is not surprising that Ang I and II were below the detection limit in medium samples obtained from cardiomyocytes and fibroblasts incubated in the absence of serum. This finding contrasts with data obtained by others [23,26–29], who found angiotensin levels ranging from <10 to >1000 fmol/ml in medium of serum-deprived cardiomyocytes and fibroblasts. Part of this discrepancy may be due to the fact that angiotensins in other studies were measured directly by radioimmunoassay, an approach which may result in an overestimation of the true angiotensin levels [40]. Furthermore, it must be kept in mind that, in view of the levels measured in cardiac tissue in vivo (Ang I ≈ 5 fmol/g; Ang II ≈ 20 fmol/g) [3,4,41,42] even levels of 5–10 fmol per ml medium are very high, since in most studies only 1 to 4 million cells are incubated with a few milliliters of medium.

Sadoshima et al. [27] found the Ang II concentration in the medium of serum-deprived neonatal rat cardiomyocytes to increase nearly 100-fold upon stretch. This Ang II, which is assumed to be responsible for the hypertrophic response of cardiomyocytes after the induction of stretch [26–28], appeared to originate from intracellular storage sites. Since neonatal rat cardiomyocytes do not synthesize renin and angiotensinogen in detectable quantities, the Ang II in these storage sites may be derived, via AT₁-receptor mediated endocytosis [9], from the serum-containing medium used to culture the cells prior to stretch. We studied intracellular storage and stretch-induced release of Ang II by measuring angiotensin levels in the cells after 5 days of incubation with serum and in the serum-free medium at various time points after the initiation of stretch. Ang II was below the detection limit under all conditions. Yamazaki et al. reported that the concentration of exogenous Ang II needed to exert a similar hypertrophic response as stretch is ≈ 10⁻⁸ mol/l [28]. In our cells, such concentrations of Ang II were found to induce near-maximal hypertrophic effects [33]. Although in the present study Ang II was undetectable (i.e., < 10⁻¹⁵ mol/l), the well-known increase in cellular protein synthesis did occur in response to stretch. It appears therefore that the stretch-induced hypertrophic response of car-
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References
