Original Paper

An Alternative Promoter in Intron1 of the Renin Gene is Regulated by Glucose Starvation via Serum Response Factor

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Key Words

Cytosolic renin • Renin promoter • Serum response factor • Glucose starvation

Abstract

Background/Aims: Renin is known as a secretory glycoprotein that ultimately leads to angiotensin II generation. In this way renin exerts pro-inflammatory effects and promotes cardiac injury. Additional transcripts have been identified encoding for a cytosolic renin isoform that - in contrast to secretory renin - exhibits cardioprotective effects under ischemic conditions. The promoter of these transcripts is unknown. **Methods:** Using qRT-PCR and dual-luciferase reporter assay we examined the expression and promotor activity of cytosolic renin as well as the regulation by glucose starvation in H9c2 cardiomyoblasts. **Results:** We identified a promoter in intron1 of the rat renin gene with two glucose starvation-sensitive regions. One region contains a binding motif for serum response factor (SRF). Under glucose depletion expression of SRF increased prior to cytosolic renin. SRF knock down selectively decreased cytosolic renin expression and attenuated the increase of cytosolic renin expression under glucose depletion. **Conclusions:** Transcripts encoding for secretory and cytosolic renin are differentially expressed. The low basal expression of cytosolic renin as well as its induction under ischemia-related conditions represents an efficient system regulated in accordance with its previously identified unfavorable effects under control situations but protective effects seen after myocardial infarction or glucose depletion.

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Introduction

Renin is a secretory glycoprotein that, after secretion into the extracellular space, generates angiotensin (ANG) I from its only known substrate, angiotensinogen [1]. ANG I is further cleaved to ANG II, the effector peptide of the renin-angiotensin system (RAS). In the heart, ANG II increases oxidative stress, exerts pro-inflammatory effects and promotes hypertrophy [2, 3]. Thus, ANG II leads to apoptosis, necrosis, fibrosis, myocardial remodeling and hence cardiac failure [4, 5].

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In addition to the classical renin transcript, alternative renin transcripts have been identified in several species. In rat these transcripts were denoted as exon(1A-9)renin [E(1A-9)] [6] and in human as renin-b and renin-c [7, 8]. These alternative transcripts lack exon1. Exon1 encodes for the signal sequence required for a co-translational transport to the endoplasmic reticulum (ER) and thus for the sorting of the renin protein to the secretory pathway. Instead, alternative renin transcripts are translated into a truncated prorenin with use of the first in-frame UTG in exon 2. The protein lacks the pre-fragment and the first 15 amino acids of the conventional prorenin [6-8]. Consequently, the translated renin protein cannot be secreted, remains in the cytosol and is taken up by mitochondria [6, 9]. We confirmed the translation into a cytosolic and mitochondrial located renin with enzymatic activity *in vivo* in transgenic rats [10]. In the rat, exon 2 is preceded by exon1A, a short sequence derived from intron1. Up to date the function of exon1A is unknown. It does not contain an in-frame AUG and therefore, cannot code for an alternative 5' extension of the renin protein.

In the rat heart, the expression of cytosolic renin was increased after myocardial infarction [11]. In addition, glucose deprivation caused an increase of the transcript levels of cytosolic renin in cardiac H9c2 cells [12]. Functionally, hearts of transgenic rats overexpressing cytosolic renin were more stable against ischemia in the ex vivo Langendorff preparation and H9c2 cells transfected with cytosolic renin were protected against necrotic cell death under glucose depletion [12]. Nevertheless, basal rates of apoptosis were increased in rat H9c2 cardiomyoblasts overexpressing cytosolic renin [9]. Of considerable advantage in this respect, however, endogenous expression of cytosolic renin in these cells as well as in the rat heart is very low under basal conditions.

A promoter in intron1 of the renin gene has been suggested that drives the expression of cytosolic renin, but such a promoter has not been demonstrated so far. Aim of the present study was therefore to characterize the unknown promoter in intron1 of the rat renin gene and to demonstrate that this promoter is subject to regulation by ischemia related conditions such as glucose deprivation. To unravel the mechanism of regulation on renin mRNA expression, we also studied the influence of the serum response factor (SRF), which has a predicted binding site at the alternative promoter region.

Material and Methods

Cell culture

Neonatal cardiomyocyte rat H9c2 cells (ATCC, Manassas, VA, USA) were maintained in DMEM medium (Lonza, Basel, Swiss) supplemented with 10% fetal bovine serum (PAN Biotech, Darmstadt, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO, Life Technologies, Darmstadt, Germany) in a humidified incubator at 5% CO₂ and 37°C. For PCR and functional analyses, cells were seeded in culture plates (Greiner bio-one, Frickenhausen, Germany) for 3 days. They were then exposed to siRNA-mediated SRF knock down followed by glucose depletion for another 24 hours as described previously [12].

Quantitative Real-Time PCR

RNA was extracted using the RNeasy Mini Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions. Quality was checked by spectrophotometry (DS-11+, DeNovix Inc, Wilmington, USA). High Capacity cDNA Kit (Life Technologies, Darmstadt, Germany) was used to reverse transcribed RNA to cDNA, which was stored at -70°C. For qPCR, cDNA was diluted in nuclease-free water. Then duplicates of 20 ng cDNA were mixed with SYBR® FAST Universal 2X Master Mix containing SYBR green dye and optimized primer pairs: for exon(1A-9)renin forward TGAATTTCCCCAGTCAGTGAT and revers GAATTCACCCCAT TCAGCAC and for exon(1-9)renin forward ATGAATTCACCCCATTCAGC and revers CCAGATGGGCGGGAGGAGGATG. For quantification of SRF transcripts we used the QuantiTect Primer Assay (Qiagen, Hilder, Germany). As housekeeping gene Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (YWHAZ) with the primer pairs FOR: CATCTGCAACGACGTACTGTCTCT, REV: GACTGGTCCACAATTCCTTTCTTG

Table 1. Primer sequences for construction of deletion fragments

Position	Direction	Primer Sequences (5'-3')
+53 bp	Antisense	GTTCAAGCTTTGTCCTCCAATGCATC
-12 bp	Sense	TGCAGCCAGTAGCAGCTCAG
-40 bp	Sense	GTATGAGGCTCTTGCCAGGCAG
-88 bp	Sense	GCTGCAATGTCTTGGCTGGAAAA
-95 bp	Sense	CTATGAAGCTGCAATGTCTTGGCTG
-228 bp	Sense	GCTGGCGGCATCTTCCTCCAGAC
-451 bp	Sense	GGAAATAAAAAACACCCATCTGATGGA
-659 bp	Sense	CTATAGACCTGGCTGTCTCAAACCTCG
-769 bp	Sense	GCGCCCTTGCTGAGAGCATCTAG

was applied. The threshold cycle number (CT) in combination with the $2^{-\Delta CT}$ method was normalized against the housekeeping gene *YWHAZ*.

Promoter analysis

For analysing the alternative renin promoter (exon1A) we used the Dual-Luciferase Reporter Assay (Promega, Mannheim, Germany) comprising expression vectors that contain the luciferase reporter gene. Different regions of interest upstream of the exon1A were amplified by PCR using rat cDNA and the primer combination listed in Table 1. After subcloning into the pGEM-TEasy vector, amplified deletion fragments were checked by sequence analyses using Sp6 and T7 primers (GATC, Germany). Correct deletion fragments were then cloned into the pGL4.10 [*luc2*] vector upstream of the firefly luciferase gene. Afterwards, the expression vector was introduced into the H9c2 cells using Fugen HD (Roche, Mannheim, Germany) as transfection reagent. To minimize non-relevant influences an additional vector (pGL4.74 [*hRluc*/TK]) containing the renilla luciferase gene was co-transfected. As negative control H9c2 cells were transfected with both the empty pGL4.10 and the pGL4.74 vectors. After an incubation time of 6 hours or 24 hours, the cells were lysed and incubated with luciferin according to the manufacturer's instructions. The enzymatic activity of luciferases was measured consecutively on a luminometer (LB96V, EG & G Berthold, Germany). The luciferase activity of the promoter-firefly luciferase constructs was then normalized to the renilla luciferase activity derived from the pGL4.74-TK vector and expressed as relative fold increase in comparison to the promoter-less pGL4.10 basis vector.

SRF downregulation by siRNA

Downregulation of SRF in H9c2 cells was accomplished with the RNA interference method using 20 and 40 nmol/L Stealth RNAi™ siRNA Duplex oligoribonucleotides to SRF according to the manufacturer's instructions (Thermo Fisher Scientific, Dharmacon, Schwerte, Germany). Briefly, transfection was performed for 6 h with the indicated siRNA and scramble control siRNA using OptiMEM (GIBCO, Life Technologies, Darmstadt, Germany) as transfection medium and polyethyleneimine (1 µg/µL) (Sigma-Aldrich, Taufkirchen, Germany) as transfection reagent. Following a varying incubation period of 1 to 3 days, transcript abundance and cell viability were quantified.

Cell-based ELISA to the serum response factor

The calorimetric cell-based SRF ELISA kit was performed according to manufacturer's instructions (LSBio LifeSpan Biosciences, Eching, Germany). Briefly, 20,000 H9c2 cells were plated onto a 96 well plate and cultured for 2 day at 37°C in the CO₂ incubator. After incubation of the cells in complete culture medium or glucose depleted medium for another 3, 6 and 24 hours, respectively, cell culture medium was removed, cells were washed in TBS buffer twice and fixed in 4% formaldehyde for 20 min at room temperature. After washing the plate 3 times with wash buffer, the cells were incubated with quenching buffer for 20 min at room temperature followed by 3 wash steps and addition of blocking buffer for 1 hour. Then, the cells were washed 3 times again and incubated overnight at 4°C with either the primary anti-rabbit SRF or the anti-mouse GAPDH (internal positive control) antibodies. The primary antibodies were removed by 3 times washing and cells were then exposed to the secondary HRP-conjugated anti-rabbit or anti-mouse antibodies

for 1.5 hours at room temperature. Lastly, after 3 times washing the ready-to-use substrate was added to each well for 30 min at room temperature in the dark. Using the microplate reader the OD at 450 nm was measured immediately after adding the stop solution to each well.

Following the calorimetric measurement of HRP activity, the Crystal Violet whole-cell staining was performed to determine cell density in order to normalize SRF data to the cell count. Cristal Violet binds to cell nuclei and gives absorbance readings proportional to the cell number located in the well. Therefore, wells were washed 2 times with wash and TBS buffers followed by incubation with Cristal Violet for 30 min at room temperature. Then the solution was decanted carefully by rinsing the plate with distilled water. After air-drying for 30 min, SDS was added to the wells for 1 hour at room temperature and absorbance was measured at 595 nm.

Statistical analyses

Data are presented as mean $\pm$ SEM. All statistical analyses were performed by t-test or one-way ANOVA followed by Bonferroni comparison test, respectively using Graph Pad Prism software. Differences were considered statistically significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**) and $p < 0.001$ (***).

Results

Glucose depletion increases *Ren(1A-9)* mRNA- but not *Ren(1-9)* mRNA levels

In H9c2 cardiomyoblasts, expression of cytosolic renin (Fig. 1A) was very low but apparently higher than that of secretory renin (Fig. 1B). Under basal conditions, relative cytosolic *Ren(1A-9)* and secretory *Ren(1-9)* expressions, normalized to the housekeeper *YWHAZ*, accounted to $1.33 \pm 0.13 \times 10^{-4}$ and $0.57 \pm 0.04 \times 10^{-4}$, respectively. Glucose deprivation caused a highly significant 4-fold upregulation of *Ren(1A-9)* expression after an exposure time of 6 and 24 hours. Expression of *Ren(1-9)* was not influenced under these conditions.

An alternative renin promoter is located in intron1 of the renin gene

We hypothesized that a promoter region capable of driving transcription is located in intron1 within the 5' vicinity of exon1A. To test this hypothesis, we cloned a number of deletion fragments of the 5'-flanking region of exon1A into the pGL4.10 vector and analyzed their promoter activities in H9c2 cells after 6 and 24 hours of incubation under control condition or glucose deprivation.

As shown in Fig. 2A, the 5'-flanking region from -546bp to -26bp (520bp fragment) caused a significant 2.7-fold increase in the luciferase activity after 6 hours incubation compared to the control cells transfected with the promoter-less pGL4.10 vector or to cells transfected with the shorter 109 or 157bp fragments. This implies that the region between -546 and -183bp could contain a minimal promoter (shaded area).

Fig. 1. Glucose starvation time dependently increased *Ren(1A-9)* expression in H9c2 cells. Real-time PCR was performed from H9c2 cells exposed to control conditions (white columns) or glucose starvation (grey columns) for the indicated time periods. Expression pattern of (A) cytosolic *Ren(1A-9)* and (B) secretory *Ren(1-9)*. Data represent mean $\pm$ SEM of 6 experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni comparison test with *** $p < 0.001$.

Fig. 2. Intron 1 of the renin gene contains an alternative promoter activated by glucose starvation. Promoter analyses were performed with deletion fragments of the 5' flanking region of exon1A cloned into two different pGL vectors upstream of the firefly luciferase gene. After transfection, H9c2 cells were exposed to (A) control conditions and (B) to glucose starvation for 6 hours. Luciferase activity was determined using the dual luciferase assay. Data represent mean $\pm$ SEM of 6 experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni comparison test with *** p <0.001 and ** p <0.01 compared to pGL4.10 control vector and the shorter 109 and 157bp fragments. Shaded areas represent the presence of the minimal promoter within the 520bp fragment as well as the location of glucose depletion-sensitive elements within the 520 and 838bp fragments.

Fig. 3. A minimal promoter is located 157bp upstream of exon1A. The localization of a minimal promoter was determined by promoter analyses using different deletion fragments of the 5' flanking region of exon1A cloned into two different pGL vectors upstream of the firefly luciferase gene. After transfection, H9c2 cells were exposed to control conditions for 24 hours. Luciferase activity was measured using the dual luciferase assay. Data represent mean $\pm$ SEM of 6 experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni comparison test with *** p <0.001 and ** p <0.01 compared to pGL4.10 control vector and ### p <0.001 compared to the fragments with a length of 838 and 520bp.

Additionally, we identified glucose deprivation-sensitive domains within the 5'-flanking promoter region of exon1A between positions -564 and -183bp as well as -864 to -754bp upstream of exon1A (Fig. 2B). The enhanced luciferase activity was seen after a starvation period of 6 hours, while after prolongation of glucose depletion to 24 hours this effect disappeared (data not shown).

To narrow the minimal promoter region, we extended the incubation time to 24 hours and investigated fragments closer to the transcription starting point of exon1A. The results shown in Fig. 3 push the minimal promoter closer towards exon1A (157bp fragment). Interestingly, within the range of -754 to -546bp a negative regulatory element(s) may exist because the promoter activity decreased highly significant. Compared to this fragment the longer 838bp fragment displayed a significant higher promoter activity indicating the existence of a positive regulatory element(s).

SRF upregulates the expression of Ren(1A-9)

By computational analysis using the AliBaba2.1 program, we identified numerous potential transcription factors that can bind in the alternative promoter region such as SRF. The structure of rat renin gene as presented in Fig. 4 comprises 9 exons whereby in intron1 an alternative exon1 (exon1A) is located at position 3833 to 3911 [6]. Because this exon1A

Fig. 4. Structure of the 5'-region of the renin gene. The rat renin gene comprises 9 exons (striated boxes) and 8 introns (black arrows). An alternative "exon1" (exon1A) is located within intron1. Numbers denote the positions of exon1, exon1A and exon2 in the genomic sequence [6]. Upstream of exon1A closed to the black box one SRF binding site (CARG-like sequence) and two specific protein1 (SP1) binding sites were identified by computational analysis using the AliBaba2.1 program.

Fig. 5. Relative SRF expression increased time dependently in response to glucose starvation. Real-time PCR was performed from H9c2 cells exposed to control conditions or glucose starvation for the indicated time periods. (A) Expression pattern of SRF mRNA and (B) SRF protein. Data represent mean $\pm$ SEM of 6 and 9 experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni comparison test with *** $p < 0.001$ and ** $p < 0.01$.

lacks the AUG sequence within the reading frame the translation starts with exon 2 resulting in a truncated prorenin. Our described promoter analyses were performed with different fragments covering the range between 26bp and 864bp upstream of exon1A. Close to the black box we identified one serum response element (CARG-like sequence) and two specific protein1 binding sites.

To test the hypothesis that SRF regulates the expression of the alternative renin transcript, we investigated time dependently the expression pattern of SRF itself. Here, we observed a significant upregulation of SRF already 3 hours after glucose deprivation and reaching a maximum after 6 hours, which was followed by a significant reduction 18 hours later (Fig. 5A). Compared to the expression pattern of *Ren(1A-9)* as seen in Fig. 1, upregulation of SRF mRNA by glucose starvation occurred before that of *Ren(1A-9)*. Simultaneously to the mRNA expression, we also found a glucose starvation-induced increase of SRF protein

Fig. 6. *Ren(1A-9)* expression decreased in response to SRF knock down. Real-time PCR was performed from H9c2 cells exposed to siRNA-mediated SRF knock down for the indicated time periods. (A) Time dependent efficiency of SRF knock down normalized to the housekeeping gene YWHAZ and the scramble control. (B) Expression pattern of cytosolic *Ren(1A-9)* and (C) secretory *Ren(1-9)* each normalized to the housekeeping gene YWHAZ and the scramble control. Data represent mean $\pm$ SEM of 6 experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni comparison test with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

expression (Fig. 5B). Starting 3 hours after glucose deprivation SRF protein expression remained upregulated up to 24 hours.

To demonstrate that SRF indeed regulates *Ren(1A-9)* expression, we next performed a SRF knock down by siRNA followed by the analyses of SRF and renin expressions to determine knock down efficiency and to confirm an interrelationship (Fig. 6). Efficiency of knock down was normalized to both the housekeeping gene YWHAZ and the scramble control. Using 20 nM siRNA the efficiency of SRF knock down accounted to $68 \pm 10\%$, $55 \pm 4\%$ and $55 \pm 3\%$ 24 hours, 48 hours and 72 hours post transfection, respectively. 40 nM siRNA decreased the SRF expression to $40 \pm 4\%$, $46 \pm 7\%$ and $47 \pm 4\%$ in the indicated time period (Fig. 6A). In comparison, *Ren(1A-9)* expression decreased as well (Fig. 6B), whereas the expression of *Ren(1-9)* mostly remained unchanged or even increased (Fig. 6C), indicating a possible selective interrelationship between the transcription factor SRF and the expression of cytosolic *Ren(1A-9)*.

In a next step, we analysed whether SRF mediates the glucose starvation-induced increase in *Ren(1A-9)* expression in addition to its effect on basal expression. Therefore, we investigated the effect of glucose starvation on the expression of renin isoforms in control and SRF-depleted H9c2 cells. Fig. 7A shows that SRF knock down was successful under basal conditions but also under glucose starvation. SRF expression decreased significantly from $6.17 \pm 0.70 \times 10^{-3}$ and $8.00 \pm 0.54 \times 10^{-3}$ in the corresponding controls to $3.86 \pm 0.44 \times 10^{-3}$ in siRNA-treated cells. After glucose deprivation, SRF expression decreased from $10.03 \pm 0.99 \times 10^{-3}$ and $11.21 \pm 1.03 \times 10^{-3}$ in controls to $3.62 \pm 0.35 \times 10^{-3}$ in siRNA-treated cells, indicating an efficient SRF knock down under starvation conditions. Concerning renin expression, SRF knock down caused a significant reduction of *Ren(1A-9)* mRNA levels from

Fig. 7. SRF knock down attenuated the increase of *Ren(1A-9)* expression in response to glucose starvation. Real-time PCR was performed from H9c2 cells exposed to siRNA-mediated SRF knock down for 24 hours followed by culture under control conditions or glucose starvation for another 24 hours. (A) Relative SRF expression in response to glucose starvation and siRNA-mediated SRF knock down. (B) Expression pattern of cytosolic *Ren(1A-9)* and (C) secretory *Ren(1-9)* in dependence on the indicated conditions. Data represent mean $\pm$ SEM of 7 experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni comparison test with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

$0.95 \pm 0.12 \times 10^{-4}$ and $0.94 \pm 0.12 \times 10^{-4}$ in controls to $0.54 \pm 0.06 \times 10^{-4}$ in siRNA-treated cells (Fig. 7B). The glucose starvation-mediated increase of *Ren(1A-9)* expression ($2.81 \pm 0.22 \times 10^{-4}$ and $2.41 \pm 0.19 \times 10^{-4}$ in controls) was retarded to $1.00 \pm 0.13 \times 10^{-4}$ in siRNA-treated cells. Secretory *Ren(1-9)* mRNA levels were not influenced neither under basal conditions nor under glucose starvation confirming the selective interference with the alternative promoter of Exon1A (Fig. 7C).

Discussion

In the last years, important advances were made in understanding the expression and regulation of the transcripts encoding for secretory renin [13, 14]. Considerably less is known about the regulation of the transcripts encoding for cytosolic renin. We hypothesized that an alternative promoter exists in intron 1 of the renin gene that drives expression of the alternative renin transcript [E(1A-9) in rat]. In the present study, we firstly identified the alternative promoter in intron 1 of the rat renin gene. This promoter drives the expression of cytosolic *Ren(1A-9)* and can be activated by glucose depletion. Secondly, we identified at least two glucose dependent regulatory regions 5' of the promoter. Thirdly, we provide evidence that serum response factor (SRF) is induced by glucose depletion and takes part in the regulation of cytosolic *Ren(1A-9)*, but not of secretory *Ren(1-9)*.

In more detail, using the luciferase reporter gene expression assay we now identified a minimal promoter which is located 135 to 183bp (157bp fragment) upstream of exon1A. The promoter exhibited only weak responses in the luciferase reporter assay. However, the same holds true for the known promoter of secretory renin [15, 16]. For the classical promoter of *Ren(1-9)* five inhibitory- and two stimulatory elements have been detected [17]. However, those elements are far 5' of the alternative promoter for *Ren(1A-9)*. They unlikely play a role in *Ren(1A-9)* regulation, since the compositions of the promoters are completely different (see below). The weak promoter activity for *Ren(1A-9)* is well reflected by the poor basal

expression of *Ren(1A-9)* transcripts in H9c2 cells. Furthermore, it may explain the lacking response in the luciferase assay under basal conditions after an incubation time of 6 hours. Thus, only the prolongation of incubation time to 24 hours was sufficient to accumulate enough luciferases and to record completely the activity of the minimal promoter.

By computational analysis, we could neither find a classic TATA (5' TATAAAA 3') box nor an INR (TCA(G/T)T(T/C)) core promoter element in the region immediately flanking the exon1A, indicating a TATA- and INR-less promoter. Such promoters occurring in nearly 46 % of human genes are enriched in specific protein 1 (SP1) binding sites [18]. Indeed, in the 864bp upstream of the exon1A we identified 10 possible binding sites for SP1 with help of the transcription factor binding site program AliBaba2.1. SP1, however, appeared not to be regulated by glucose depletion in our hands (data not shown). TATA-less genes are frequently involved in basic housekeeping processes, while TATA-containing genes tend to be highly regulated by extracellular stress-related responses [18, 19]. Despite TATA-less, the exon1A promoter was regulated by extracellular stress signals especially in response to glucose deprivation as well documented by the luciferase assay and the upregulation of the *Ren(1A-9)* expression. Here, we found two glucose deprivation-sensitive domains in the 5' upstream promoter region that could explain the observed upregulation of *Ren(1A-9)* expression. Although the promoter activation by glucose deprivation was limited in time to 6 hours and disappeared at 24 hours, we observed a selective upregulation of *Ren(1A-9)* at both time points. This discrepancy could be attributed to a stabilization of the renin mRNA over the indicated time period.

SRF is an important cardiac transcription factor necessary for the regulation of genes involved in cardiac structure and function [20] but also in metabolism, ion transport, and stress responses [21]. In fibroblasts the SRF gene is rapidly but only transiently induced in response to serum stimulation [22]. Thus, SRF levels reach their peak at approximately 2 hours and return to basal levels 6 hours after serum stimulation. In our system, we found a delayed upregulation of SRF transcript and protein expression starting at 3 hours and reaching a maximum at 6 hours. However for the first time, this upregulation was observed after glucose starvation which could explain the temporal discrepancy. SRF activates gene expression by binding to the serum response element which contains a consensus sequence of CC(A/T)₆GG (CArG box or a CArG-like sequence). Such a CArG-like sequence was predicted by the AliBaba2.1 program in the promoter region 761 to 771bp upstream of exon 1A, suggesting a SRF-mediated regulation of *Ren(1A-9)* expression. Using siRNA interference method, we demonstrate that SRF is required for the selective upregulation of *Ren(1A-9)* expression. However, SRF is also expressed at high level in H9c2 cells cultured under basal conditions, where *Ren(1A-9)* expression is very low. This implicates that SRF-dependent *Ren(1A-9)* expression is modulated in a complex manner by (patho)physiological mechanisms during cells adapt to glucose starvation. SRF has, indeed, different co-activators such as the ets domain-containing ternary complex factors [23] and the myocardin-related transcription factors (MRTF's) [24] that modulate SRF activity. Although we did not analyse such co-activators, it is known that SRF-induced target gene expression increases for example by upregulation of MRTF-A expression during hypoxia and ischemia-reperfusion in cardiac cells [25] as well as in rat cortical and hippocampal neurons [26]. Of special interest is the fact that in immediate proximity of the SRF binding site there are also two SP1 binding sites. In this respect Spencer and Misra [27] demonstrated that SP1 can potentiate SRF-mediated gene expression. SP1 activity in turn depends on its phosphorylation following numerous extracellular stimuli [28]. However, we did not further investigate the role of these two SP1 sites yet.

To further confirm a SRF-mediated upregulation of cytosolic renin, SRF knock down should prevent or limit the upregulation of *Ren(1A-9)* observed after glucose starvation. Despite efficient SRF knock down, exposure of cells to glucose starvation still increased *Ren(1A-9)* expression considerably, albeit less strongly than in the presence of regular SRF levels. This implicates that beside SRF there are further transcription factor(s) required for upregulation of *Ren(1A-9)* expression during starvation. Data of the luciferase assay

confirm this hypothesis. Thus, the 520bp construct lacking the SRF binding site exhibited also a strong response to glucose starvation. In this context, we still need to identify other transcription factor(s) that bind in this region and glucose starvation-dependently regulate *Ren(1A-9)* expression.

In an attempt to identify the functions of renin in the brain Lavoie et al. [29] investigated double transgenic mice expressing human cytosolic or secretory renin, each together with human angiotensinogen driven by the astrocyte-specific glial fibrillary acidic protein promoter. A significant amount of human renin could be detected in the cerebrospinal fluid of mice expressing secretory renin but not in mice expressing cytosolic renin. However, mean arterial blood pressure was significantly increased in both transgenic mice strains. Furthermore, plasma renin activity (PRA) was elevated in mice overexpressing the cytosolic renin construct. Since cytosolic renin cannot be secreted, this increase of PRA remained unexplained, but may have contributed to the increased blood pressure. In contrast, in our transgenic rats overexpressing cytosolic renin, renin levels were increased in the cytosol and mitochondria but not in the circulation. Thus, there was no effect on blood pressure in our four independently generated transgenic rat lines [10]. Later, Shinohara et al. [30] demonstrated that the specific ablation of cytosolic renin in the brain resulted in hypertension. In this study, it turned out that there was an inverse regulation of the renin transcripts. The decrease of renin-b [*Ren(1A-9)*] expression resulted in an increase in renin-a [*Ren(1-9)*] expression in the brain. The authors, therefore, suggest that the function of renin-b [*Ren(1A-9)*] consists in the inhibition of renin-a [*Ren(1-9)*] expression. Our own observations partially support this view. Thus, glucose depletion increased *Ren(1A-9)* and decreased *Ren(1-9)* mRNA levels [12]. However, in the present study the expression of *Ren(1-9)* was already very low under basal conditions (near background levels) and we could not detect any further decrease under glucose depletion. In this context, downregulation of SRF decreased expression of *Ren(1A-9)* and at the same time increased expression of *Ren(1-9)*. Thus, it remains an attractive hypothesis that *Ren(A1-9)* negatively controls *Ren(1-9)* expression.

Taken together, cytosolic renin seems to be part of an endogenous regulatory system whose alternative promoter in intron1 of the renin gene is suppressed under basal conditions and partly activated by SRF under glucose starvation. This type of regulation is in accordance both with the unfavorable effect under healthy conditions as well as with the protective effects of cytosolic renin seen after myocardial infarction.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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