α -Adrenergic stimulation induces phosphorylation of retinoblastoma protein in neonatal rat ventricular myocytes

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Mammalian cardiac myocytes become postmitotic shortly after birth, and the subsequent myocardial growth in adaptation to increasing workloads becomes primarily dependent on hypertrophy of existing myocytes. Although hypertrophic growth of cardiac myocytes has been extensively studied by using both *in vitro* and *in vivo* models, the molecular mechanism controlling the switch from hyperplastic to hypertrophic growth of cardiac myocytes is largely unknown. Since the majority of terminally differentiated cardiac myocytes are growth-arrested in G₁/G₀ phase, it has been hypothesized that the retinoblastoma protein (Rb) or its related pocket proteins which block G₁/S transition becomes constitutively active during myocardial terminal differentiation. To test this hypothesis, we studied the regulation of Rb activity by α -adrenergic stimulation in neonatal rat ventricular myocytes which are mostly postmitotic in culture. Our results

demonstrate that Rb is predominantly in the active hypophosphorylated state in control neonatal ventricular myocytes. α -Adrenergic stimulation activates G₁/S transition in foetal but not neonatal rat ventricular myocytes. Although α -adrenergic stimulation does not activate G₁/S transition in neonatal myocytes, it induces hyperphosphorylation of Rb to the same extent as in proliferating skeletal-muscle myoblasts or foetal ventricles. Hyper- but not hypo-phosphorylated Rb in stimulated neonatal myocytes or proliferating skeletal-muscle myoblasts fails to bind to the transcription factor, E2F, suggesting that hyperphosphorylated Rb is inactive. Therefore G₁/S transition could also be blocked at steps in addition to Rb inactivation during terminal differentiation and these blockades are refractory to α -adrenergic stimulation.

INTRODUCTION

The retinoblastoma protein (Rb) and its related pocket proteins such as p107 and p130 block G₁/S transition in a variety of cell types by binding and thereby sequestering the transcription factor, E2F, which is essential for entering S phase [1–3]. The activity of Rb is regulated by cyclin-dependent kinases, which inactivate Rb by phosphorylating multiple tyrosine residues and release the transcription factor E2F in late G₁ phase, allowing cells to enter S phase. Since over 95 % of terminally differentiated cardiac myocytes are growth-arrested in G₁/G₀ phase [4], it has been hypothesized that withdrawal of cardiac myocytes from the cell cycle is induced by constitutive activation of Rb or its related proteins during early neonatal development. This hypothesis is supported by a recent observation that re-activation of DNA synthesis in neonatal rat ventricular myocytes can be induced by overexpression of the oncogenic adenovirus E1A protein [5], which is also known to disrupt the function of pocket proteins by binding to the pocket domain and thereby releasing E2F [6]. Thus sequestering of E2F by pocket proteins is at least partially responsible for withdrawal of cardiac myocytes from the cell cycle. Further supportive evidence came from the observation that the effects of E1A overexpression on neonatal ventricular myocytes can be mimicked by overexpression of the E2F-1 gene [7]. However, it is still not clear whether inactivation of Rb is necessary and/or sufficient to re-activate DNA synthesis in neonatal ventricular myocytes. Therefore, we initiated the study to investigate whether G₁/S transition is blocked in neonatal

myocytes by active Rb and whether inactivation of Rb would allow these myocytes to re-enter S phase.

In the current study, we show that α -adrenergic stimulation can activate G₁/S transition in foetal but not neonatal rat ventricular myocytes. Rb is predominantly in an active hypophosphorylated state in the control neonatal rat ventricular myocytes. Although DNA synthesis is not re-activated by α -adrenergic stimulation, Rb becomes hyperphosphorylated to the same extent as in proliferating skeletal-muscle myoblasts or foetal ventricles. The hyperphosphorylated Rb from stimulated ventricular myocytes or proliferating skeletal-muscle myoblasts fails to bind to E2F protein, suggesting that it is inactive. Therefore steps in addition to Rb inactivation before DNA synthesis are possibly blocked in terminally differentiated cardiac myocytes and these blockades are refractory to α -adrenergic stimulation.

MATERIALS AND METHODS

Isolation and culture of foetal and neonatal rat ventricular myocytes

The isolation and culture of foetal and neonatal rat ventricular myocytes has been described previously [8]. In the present report, Sprague–Dawley rats were used for all the studies. Myocytes were dispersed from the ventricles of rat foetuses of 15-day gestation or 4-day old neonates by digestion with collagenase II (Worthington) and pancreatin (Gibco). Myocytes were further

Abbreviations used: Rb, retinoblastoma protein; BrdU, 5-bromo-2'-deoxyuridine; MLC-2v, ventricular isoform of myosin light chain-2; pRb, hypophosphorylated Rb protein; ppRb, hyperphosphorylated Rb protein.

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purified by a discontinuous Percoll (Pharmacia Biotechnology Inc.) gradient to obtain myocardial cell cultures with more than 95 % myocytes, as assessed by immunofluorescence staining with antibody directed against ventricle-specific myosin light chain-2 (MLC-2v). Cardiac myocytes were plated in Dulbecco's modified Eagle's medium + 5 % foetal bovine serum + 10 % horse serum. For immunofluorescence staining, foetal or neonatal myocytes were plated at a density of 10^5 cells per 2×2 cm² well. For Western-blot analysis, neonatal myocytes were plated at the same confluence as above but in 10 cm dishes. The rat skeletal muscle cell line, RMO, was cultured as myoblasts in Dulbecco's modified Eagle's medium + 10 % foetal bovine serum, and cell fusion was induced by switching the cells to serum-free medium when confluent. Myocytes were starved in serum-free medium for 12 h and then stimulated with phenylephrine (Sigma) at a final concentration of 0.1 mM on the fourth day of culture. To monitor DNA synthesis, 5-bromo-2'-deoxyuridine (BrdU) or [³H]thymidine (Amersham) was added with phenylephrine to a final concentration of 20 μ M and 2 μ Ci/ml respectively.

Monitoring DNA synthesis

To monitor DNA synthesis, BrdU was added to the medium at a final concentration of 20 μ M, and the BrdU labelling was continued for 48 h. The incorporated BrdU was then detected by immunofluorescence staining as described below. Alternatively, myocytes were labelled with [³H]thymidine (2 μ Ci/ml) for 48 h. Myocyte lysates were precipitated with an equal volume of 20 % (v/v) trichloroacetic acid and the suspension was filtered on to glass-microfibre disks, washed three times with 5 % trichloroacetic acid and once with 95 % ethanol. After the disks had been dried at room temperature, the radioactivity was measured in a scintillation counter.

Indirect immunofluorescence staining

The indirect immunofluorescence assays were carried out as previously described with some minor modifications [9]. Cells were rinsed with PBS and fixed for 15 min at 25 °C in 2 ml of 3 % paraformaldehyde in buffer A (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM MgCl₂). The cells were changed into 2 ml of 50 mM NH₄Cl in buffer A and incubated for 15 min. After being washed twice with PBS, the cells were permeabilized with 2 ml of 0.2 % Triton X-100 in buffer A for 15 min followed by three additional washes with PBS. The slides were blocked with 1 % BSA for 10 min at 25 °C, incubated with antibody against MLC-2v and BrdU for 60 min at 37 °C, rinsed, and washed four times with PBS. The cells in chamber slides were then incubated with appropriate secondary antibodies at 37 °C for 60 min. After one rinse and four washes with PBS, the slides were mounted on glass coverslips with 90 % glycerol in 0.1 M Tris, pH 9.4, and viewed by fluorescence microscopy.

Immunoprecipitation and Western-blot analysis

Neonatal myocytes, RMO myoblasts or myotubes were washed three times with ice-cold PBS and then scraped off the dishes. Cells from each 10 cm plate were pelleted by centrifugation and lysed with 0.5 ml of lysis buffer (20 mM Tris/HCl, pH 8.0, 1 % Triton X-100, 137 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM PMSF) on ice for 30 min. After centrifugation at 4 °C for 30 min, the supernatant was mixed with 5 μ g of monoclonal antibody against human Rb (Pharmingen) and the mixture was incubated at 4 °C for 12 h. To each tube, 0.05 ml of Protein A-Sepharose (Pharmacia) was added and the mixtures were incubated at 4 °C for 1 h with gentle agitation. The Protein

A-Sepharose-antibody-antigen complexes were pelleted by centrifugation at 25 °C for 20 s. The complexes were washed three times with 0.5 ml of lysis buffer and resuspended with 0.05 ml of Laemmli sample buffer (2 % SDS, 10 % glycerol, 100 mM dithiothreitol, 60 mM Tris/HCl, pH 6.8, 0.001 % Bromophenol Blue). The samples were heated at 90 °C for 10 min and resolved on an SDS/8 % polyacrylamide gel. Western blot was carried out by a standard method and Rb was detected with a polyclonal antibody against mouse Rb (Pharmingen). For co-immunoprecipitation, 5 μ g of polyclonal antibody against all five isoforms of E2F (Santa Cruz Biotechnology) was incubated with each cell extract for 12 h. The following precipitation of antibody-antigen complex with Protein A-Sepharose and Western-blot analysis with the polyclonal anti-Rb antibody were performed as described above.

Statistics

The percentage of BrdU-positive ventricular myocytes are expressed as means $\pm$ S.E.M. For [³H]thymidine-incorporation experiments, the [³H]thymidine incorporation by the control foetal ventricular myocytes was arbitrarily set as 100 %. The [³H]thymidine incorporation by stimulated foetal ventricular myocytes as well as both control and stimulated neonatal ventricular myocytes was expressed as mean $\pm$ S.E.M. Statistical analysis was performed using the Mann-Whitney U test. The differences between control and stimulated myocytes were considered significant if the Mann-Whitney probability value (*P*) was less than 0.05.

RESULTS

Activation of DNA synthesis in foetal ventricular myocytes and hypertrophy in neonatal myocytes by phenylephrine

Previous work by others has shown that the α -adrenergic agonist, phenylephrine, can induce the expression of a panel of immediate early-growth-response genes such as *c-jun*, *c-fos*, *c-myc*, *Nur77* and *erg-1* in neonatal ventricular myocytes before cellular hypertrophy [9,10]. Activation of these genes by various mitogens is necessary for a number of quiescent cell types to re-enter the cell cycle [11]. Therefore we first tested the effects of phenylephrine on DNA synthesis in cultured 15-day foetal rat ventricular myocytes which are mitotic in culture. Isolated foetal rat ventricular myocytes were stimulated with 0.1 mM phenylephrine for 48 h, and DNA synthesis was monitored by either BrdU labelling and immunofluorescence staining or [³H]thymidine labelling and scintillation counting. To detect the BrdU-positive ventricular myocytes, myocyte cultures were stained with a polyclonal antibody against MLC-2v and monoclonal antibody against BrdU. As shown in Figure 1(A), approximately one-third of control foetal ventricular myocytes were labelled with BrdU during the fourth and fifth days of culture. Phenylephrine stimulation was initiated with BrdU labelling on the fourth day of culture. After being stimulated with phenylephrine for 48 h, however, approximately one-half of foetal myocytes became BrdU-positive (Figure 1B), suggesting that DNA synthesis in these myocytes was induced by α -adrenergic stimulation. These results are consistent with the previous observation that increases in [³H]thymidine incorporation, cell number and the expression of proliferating cell nuclear antigen are stimulated by catecholamine in the culture of foetal rat cardiac myocytes [12]. As shown in Figure 1(C), however, under the same experimental conditions, very few control neonatal myocytes were labelled with BrdU and no obvious increase in BrdU labelling was observed after α -adrenergic stimulation (Figure 1D).

Figure 1 Effect of phenylephrine on DNA synthesis in foetal and neonatal ventricular myocytes

Foetal and neonatal rat ventricular myocytes were stained with a rabbit polyclonal anti-MLC-2v antibody and a monoclonal anti-BrdU antibody. The secondary antibodies used for staining were fluorescein-labelled goat anti-rabbit IgG and rhodamine-labelled donkey anti-mouse IgG. (A) Control foetal ventricular myocytes; (B) phenylephrine-stimulated foetal ventricular myocytes; (C) control neonatal ventricular myocytes; (D) phenylephrine-stimulated neonatal ventricular myocytes. Myocytes were cultured in serum-free medium for 12 h before a 48 h stimulation with phenylephrine at a final concentration of 0.1 mM. BrdU was added to the medium with phenylephrine to a final concentration of 0.01 mM. Scale bar: 40 μ m.

To quantify the effects of phenylephrine on DNA synthesis in both foetal and neonatal ventricular myocytes, the percentage of BrdU-positive myocytes was determined by counting 500 myocytes in each experiment. Phenylephrine increases the percentage of BrdU-positive foetal myocytes from $28 \pm 1.9\%$ to $71 \pm 8.4\%$, but has no effect on the percentage of BrdU-positive neonatal myocytes ($3.3 \pm 0.6\%$ compared with $2.7 \pm 0.5\%$). Similar results were obtained by [3 H]thymidine labelling, i.e. [3 H]thymidine incorporation was increased by approx. 2.5 ± 0.6 -fold in stimulated foetal myocytes, whereas no significant change was observed in stimulated neonatal myocytes. These results indicate that G_1/S transition is stimulated by α -adrenergic stimulation in foetal but not in neonatal ventricular myocytes.

Induction of Rb hyperphosphorylation in neonatal ventricular myocytes by phenylephrine

On the basis of the above results, we speculated that Rb becomes constitutively active in neonatal ventricular myocytes and can no longer be inactivated by α -adrenergic stimulation. To test this, the phosphorylation status of Rb was compared in control and phenylephrine-stimulated neonatal ventricular myocytes. Rb was

first isolated by immunoprecipitation with a monoclonal antibody against Rb from lysates of either control or phenylephrine-stimulated neonatal ventricular myocytes. The isolated Rb was then subjected to Western-blot analysis with a polyclonal antibody against Rb. Hyperphosphorylated Rb (inactive form) migrates more slowly than hypophosphorylated Rb (active form) in an SDS/polyacrylamide gel. As shown in Figure 2(A), a single band of Rb was detected in the control neonatal ventricular myocytes. The molecular mass of this Rb was estimated to be 110 kDa, which is consistent with that predicted from the cDNA sequence. Thus Rb is predominantly in an active hypophosphorylated state (pRb) in control neonatal ventricular myocytes. This result is consistent with the above observation that cultured neonatal ventricular myocytes are mostly postmitotic. However, after a 12 h stimulation with 0.1 mM phenylephrine, almost 50% of the Rb became approx. 140 kDa, suggesting that these Rb molecules were hyperphosphorylated (ppRb). This phosphorylation pattern was sustained for at least 24 h. Rb became predominantly hypophosphorylated again at the 36 h time point of stimulation. Therefore the machinery responsible for Rb phosphorylation in response to external stimuli may not be permanently turned off in these neonatal ventricular myocytes.

Figure 2 Induction of hyperphosphorylation of Rb in neonatal ventricular myocytes by phenylephrine

(A) Neonatal ventricular myocytes were stimulated with 0.1 mM phenylephrine for 12, 24 and 36 h. Rb was isolated by immunoprecipitation with a monoclonal anti-(human Rb) antibody and subjected to Western-blot analysis with a polyclonal anti-(mouse Rb) antibody. +PE, phenylephrine-stimulated myocytes; -PE, control myocytes. (B) Top panel, Western-blot analysis was carried out with extracts of RMO blasts and tubes as described in (A). Extracts of control myocytes and myocytes stimulated with phenylephrine for 12 h were included for comparison. Bottom panel, Western-blot analysis was carried out with tissue extracts of foetal (F.V.) and neonatal (N.V.) rat ventricular muscle as described in (A). (C) Co-immunoprecipitation was performed with a polyclonal anti-(all five E2F isoforms) antibody and extracts of RMO blasts (lane 1) and neonatal ventricular myocytes stimulated with phenylephrine for 12 h (lane 2). The immunoprecipitates were then subjected to Western-blot analysis with the polyclonal anti-Rb antibody. Lane 3, the immunoprecipitate of the monoclonal anti-Rb antibody from neonatal ventricular myocytes stimulated with phenylephrine for 12 h was included in the Western blot as a control.

To evaluate the degree of Rb phosphorylation in these stimulated neonatal ventricular myocytes, Rb phosphorylation pattern was further compared in myocytes and rat skeletal-muscle myoblasts or myotubes (RMO blasts and tubes). RMO blasts are mitotic when cultured in serum-containing medium at a low density (< 70 % confluence), whereas RMO tubes induced by culturing RMO blasts in serum-depleted medium at a high density (100 % confluence) are postmitotic. As shown in the top panel of Figure 2(B), Rb is predominantly in the hypophosphorylated (pRb) state in RMO tubes as in control neonatal ventricular myocytes. The faint band of hyperphosphorylated Rb (ppRb) in the sample of RMO tubes could possibly be due to the remaining RMO blasts. These results indicate that Rb becomes predominantly hypophosphorylated in both terminally differentiated skeletal- and cardiac-muscle cells. In RMO blasts, however, approx. 50 % of Rb was hyperphosphorylated, which is highly comparable with the Rb phosphorylation state in neonatal ventricular myocytes stimulated with phenylephrine for 12 h (Figure 2B). Therefore Rb phosphorylation in these stimulated neonatal myocytes has probably reached the threshold for entering S phase. The attempts to determine the Rb phosphorylation state in isolated foetal ventricular myocytes by the same method were unsuccessful because of limited amounts of tissue. As a further support, approx. 50 % of Rb is hyperphosphorylated in foetal rat ventricular muscle where cardiac myocytes are mitotic, whereas the Rb is predominantly hypophosphorylated in neonatal rat ventricular muscle (Figure 2B, bottom panel).

Failure of ppRb in either phenylephrine-stimulated neonatal ventricular myocytes or skeletal-muscle myoblasts to bind to the transcription factor E2F

Since Rb inactivated by hyperphosphorylation is unable to bind to E2F [1–3], we determined whether the ppRb from phenylephrine-stimulated neonatal ventricular myocytes can bind to E2F by co-immunoprecipitation. A polyclonal antibody against all five isoforms of E2F was incubated with cell lysates prepared from either RMO blasts or neonatal ventricular myocytes stimulated with phenylephrine for 12 h to immunoprecipitate E2F proteins. The immunoprecipitates were then subjected to Western-blot analysis using the polyclonal antibody against Rb. For a control, the immunoprecipitate from stimulated neonatal myocyte lysate using the monoclonal antibody against Rb was included in the Western-blot analysis (Figure 2C, lane 3). As shown in Figure 2(C), only p-Rb was co-immunoprecipitated with the anti-E2F antibody in RMO blasts (lane 1) and stimulated neonatal myocytes (lane 2), indicating that ppRb protein fails to bind to E2F protein. Hence these results confirm that ppRb in either RMO blasts or phenylephrine-stimulated neonatal ventricular myocytes is inactive.

DISCUSSION

The current results demonstrate that α -adrenergic stimulation induces DNA synthesis in foetal but not in neonatal rat ventricular myocytes. In cultured neonatal rat ventricular myocytes, which are mostly postmitotic, Rb is predominantly in the active hypophosphorylated state, suggesting that it plays a critical role in arresting growth of these myocytes in G_1 phase. Although DNA synthesis is not re-activated in neonatal ventricular myocytes by α -adrenergic stimulation, hyperphosphorylation of Rb is stimulated in these myocytes to the same extent as in proliferating skeletal-muscle myoblasts. Hyperphosphorylated Rb from either the stimulated neonatal ventricular myocytes or skeletal-muscle myoblasts fails to bind to E2F, suggesting that it is inactive. Our data showing that phenylephrine stimulates G_1/S transition in foetal ventricular myocytes appear to be consistent with the previous observation by Marino et al. [12] that shows that catecholamine stimulates [3 H]thymidine labelling, expression of proliferating cell nuclear antigen and an increase in cell number in cultured foetal rat cardiac myocytes [12]. Thus adrenergic innervation in heart during prenatal development could play a critical role in regulating myocardial growth and morphogenesis.

Rb belongs to a family of pocket proteins which all contain the pocket domains involving protein–protein interaction. For instance, the A/B pocket of Rb is involved in binding to simian virus 40 large T-antigen, adenovirus E1A and the transcription factor, E2F [13,14]. It has been shown by a number of *in vivo* studies that Rb plays a critical role in regulating proliferation and differentiation of multiple cell types during prenatal development. For instance, null mutations at both alleles of the Rb gene result in death at mid-gestation from abnormal growth and differentiation in many tissues [15,16]. On the other hand, transgenic mice that overexpress the Rb gene are significantly smaller than wild-type mice, and the level of Rb is inversely proportional to the size of the transgenic mice [17]. In particular, Rb is involved in the regulation of the commitment, myogenesis and terminal differentiation of skeletal-muscle cells, which are also derived from mesoderm as are cardiac-muscle cells [18,19]. However, the function of Rb in cardiac-muscle commitment, myogenesis and terminal differentiation is much less clear. Recent evidence has strongly suggested that Rb is required for regulation of cardiac myocyte proliferation during development. Transgenic

mice that specifically overexpress simian virus 40 large T-antigen in heart develop hyperplasia in both atria and ventricles, suggesting that large T-antigens disturbs cardiac myocyte withdrawal from the cell cycle. Like E1A, large T-antigen can disrupt the pocket proteins by binding to the pocket domains, thus releasing E2F [20]. Furthermore overexpression of large T-antigen in cultured neonatal rat ventricular myocytes induces re-entry into the cell cycle [21]. As described above, overexpression of either E1A or E2F-1 in cultured neonatal rat ventricular myocytes can re-activate DNA synthesis in 80 % of cells [5,7]. Therefore it is possible that Rb, or the related pocket proteins, induces cardiac myocytes to withdraw from the cell cycle by sequestering E2F and thereby blocking G₁/S transition during terminal differentiation. This hypothesis is supported by our observation that Rb is predominantly in the active hypophosphorylated state. Although Rb is inactivated by α -adrenergic stimulation in neonatal ventricular myocytes to the same extent as in mitotic RMO blasts, DNA synthesis is not re-activated. These results are consistent with the observation by Sadoshima et al. [22] that Rb hyperphosphorylation is induced in neonatal rat ventricular myocytes by angiotensin II without concomitant re-activation of DNA synthesis. Therefore steps in addition to Rb inactivation are possibly blocked in these myocytes, and these blockades are refractory to α -adrenergic and angiotensin II stimulation. One possibility is that steps downstream of Rb inactivation are blocked in neonatal ventricular myocytes. The argument against this is that overexpression of E2F-1 in neonatal rat ventricular myocytes can re-activate DNA synthesis. The other possibility is that redundancy for Rb activity exists in neonatal ventricular myocytes which is not inactivated by α -adrenergic or angiotensin II stimulation. The redundant factors should also contain similar pocket domains to Rb in order to function similar to Rb and which could be disrupted by either large T-antigen or E1A protein. The known Rb-related pocket proteins such as p107 and p130 all contain these similar pocket domains [23] and are expressed in cardiac myocytes. Therefore the regulation of these proteins in postmitotic cardiac myocytes in response to α -adrenergic or angiotensin II stimulation needs to be investigated.

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