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Retinoid-dependent pathways suppress myocardial cell hypertrophy

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ABSTRACT **Utilizing an *in vitro* model system of cardiac muscle cell hypertrophy, we have identified a retinoic acid (RA)-mediated pathway that suppresses the acquisition of specific features of the hypertrophic phenotype after exposure to the α-adrenergic receptor agonist phenylephrine. RA at physiological concentrations suppresses the increase in cell size and induction of a genetic marker for hypertrophy, the atrial natriuretic factor (ANF) gene. RA also suppresses endothelin 1 pathways for cardiac muscle cell hypertrophy, but it does not affect the increase in cell size and ANF expression induced by serum stimulation. A trans-activation analysis using a transient transfection assay reveals that neonatal rat ventricular myocardial cells express functional RA receptors of both the retinoic acid receptor and retinoid X receptor (RAR and RXR) subtypes. Using synthetic agonists of RA, which selectively bind to RXR or RAR, our data indicate that RAR/RXR heterodimers mediate suppression of α-adrenergic receptor-dependent hypertrophy. These results suggest the possibility that a pathway for suppression of hypertrophy may exist *in vivo*, which may have potential therapeutic value.**

Cardiac muscle hypertrophy is one of the most important adaptive physiological responses of the myocardium. In response to increased demands for cardiac work or after a variety of pathological stimuli that lead to cardiac injury, the heart adapts through activation of a hypertrophic response in individual cardiac muscle cells, which is characterized by an increase in myocyte size, accumulation of contractile proteins within individual cardiac cells, activation of embryonic gene marker expression, and lack of a concomitant effect on muscle cell proliferation (for review, see ref. 1). Although the hypertrophic process can initially be compensatory, there can be a pathological transition in which the myocardium becomes dysfunctional (2).

Studies in an *in vitro* model system of myocardial cell hypertrophy have led to identification of a number of defined stimuli that can activate several independent features of hypertrophy (3–8). Currently, there are at least two signal transduction pathways, involving *ras*- (6) and G_q- (7) dependent pathways, which have been implicated in activation of features of the hypertrophic response in the *in vitro* model system. While a great deal of progress has been made in uncovering the signaling pathways that activate the hypertrophic response, relatively little is known about the mechanisms that might inhibit or suppress the hypertrophic response. The presence of pathways that modify or inhibit hypertrophy has been suspected from experimental studies of the regression of hypertrophy (2) and in patients with familial hypertrophic cardiomyopathy that harbor identical myosin missense mutations and display widely discordant cardiac phenotypes (8). However, no direct evidence for the existence of hypertrophy suppressor pathways has been reported to date.

Recent studies document that *ras* is sufficient to activate hypertrophy in both *in vitro* and *in vivo* model systems of hypertrophy. In other cell types, retinoic acid (RA) and related vitamin A derivatives (retinoids) can inhibit *ras*-dependent pathways for proliferation and differentiation (9, 10). In addition, recent studies in retinoid X receptor α (RXRα) gene-targeted mice have documented a requirement for retinoids for normal maturation of ventricular muscle cells during cardiogenesis (11). RXRα −/− embryos display the persistent, aberrant expression of an atrial marker, myosin light chain 2 (MLC-2a), in the ventricular chamber, a phenotype that is closely associated with an embryonic form of heart failure (12). The clear role of retinoid-dependent pathways in promoting the ventricular phenotype in the embryonic heart suggests the possibility that retinoids might also be important in maintaining the normal ventricular phenotype in the postnatal state as well. Since hypertrophy is associated with the expression of atrial markers in the ventricular chamber [such as atrial natriuretic factor (ANF)], the ability of retinoids to maintain a normal ventricular phenotype in the presence of defined hypertrophic stimuli would offer a direct test of the possible role of retinoids in maintenance of the ventricular muscle phenotype in the postnatal state.

Utilizing the *in vitro* model system of cardiac muscle cell hypertrophy, the present study documents a role for retinoids in suppressing activation of the hypertrophic response by two well-defined hormonal stimuli, α-adrenergic agonists and endothelin 1, and provides direct evidence that hypertrophy suppressor pathways may indeed exist within cardiac muscle cells. In addition, these studies indicate an important role for retinoids in the maintenance of a normal ventricular muscle cell phenotype in the postnatal state.

MATERIALS AND METHODS

Neonatal rat (1–2 days old) ventricular myocytes were prepared and cultured as described (4, 5). After 24-hr attachment of primary myocytes in 24-well plates, cells were transfected with the plasmid DNA and cultured with serum-free maintenance medium with or without agents and incubated for 48 hr. Then cells were harvested for the luciferase and β-galactosidase assays (6). Luciferase activities were normalized to their corresponding β-galactosidase activities to correct for variation in transfection efficiency. Indirect immunofluorescence assays were performed as described (13). An antiserum,

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Abbreviations: RA, retinoic acid; RAR, RA receptor; hRARα, human RARα; RXRα, retinoid X receptor α; hRXR, human RXR; MLC-2, myosin light chain 2; ANF, atrial natriuretic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CMV, cytomegalovirus; TTNB, synthetic RA agonist; TNI, troponin I.

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TrpE/MLC-2 (13), was used as the first antibody binding to the ventricular MLC-2 (MLC-2v) protein. For Northern blot analysis, total RNA was isolated from primary myocytes cultured in the medium with different agents. Northern blot hybridizations were performed as described (13). The extent of hybridization was quantitated by densitometry of the corresponding autoradiogram and normalized to the signals obtained with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to correct for differences in loading and transfer efficiencies.

The following plasmids were used in transient transfection assays: pANF(-3003)LΔ5′, pSVOALΔ5′, pRSVLΔ5′, pSVOALΔ5′, and pON249 have been described (4–7); CMV-hRARα, composed of a cytomegalovirus (CMV) promoter and human RA receptor α (RARα) cDNA (14); CMV-hRXRα containing CMV promoter and human RXRα cDNA (15). The dominant-negative hRARα cDNA, a C-terminal truncation at amino acid 403 referred to as hRARα403, has been described (16) and is expressed from the CMV promoter. The luciferase reporter constructs, CRBPII–luciferase and βRE1–luciferase, containing RA response elements were made in a truncated thymidine kinase promoter context using sequences from the rat CRBPII gene (17) or the mouse RARβ2 gene (18) promoters.

9-*cis*-Retinoic acid, and synthetic RA agonists (TTNPB, 3-methyl-TTNPB, and LG64) were kindly provided by Richard Heyman (Ligand Pharmaceuticals, San Diego). All compounds were diluted at least 1:1000 in tissue culture media.

RESULTS

Retinoids Inhibit the Increase in Myocardial Cell Size After Treatment with α-Adrenergic Agonists or Endothelin 1. To evaluate the potential effects of retinoids on the hypertrophic response, we utilized an *in vitro* model system in which defined agonists can activate several independent features of myocardial cell hypertrophy (4–8). To monitor changes in cell morphology, indirect immunofluorescence was performed with an antibody directed against MLC-2v. As shown in Fig. 1, treatment with the α-adrenergic agonist phenylephrine results in an increase in myocardial cell size and assembly of MLC-2v protein into organized sarcomeric units (Fig. 1*B*) vs. control (Fig. 1*A*), consistent with previously reported results (4, 8). However, as shown in Fig. 1*C*, the phenylephrine-stimulated increase in cell size is largely prevented by RA, while myofibrillar organization appears to be intact, and the cells continue to exhibit spontaneous contractility (data not shown). Treatment with RA alone had little effect on the myocardial cell phenotype (Fig. 1*D*), indicating that the effect of RA was not secondary to a toxic cellular effect. Features of hypertrophy can also be stimulated by addition of serum (Fig. 1*E*). However, the addition of RA had little effect on serum-treated cells (Fig. 1*F*), suggesting that RA has a selective specificity for inhibition of the α-adrenergic pathway. To more precisely quantitate the effects of RA, we examined multiple fields from independent cultured cell preparations, utilizing a previously described morphometric assay for the hypertrophic phenotype (8). Results are summarized in Table 1. Taken together, these data indicate that pharmacological concentrations of retinoids can selectively suppress the hypertrophic response after α-adrenergic stimulation.

Retinoid Suppression of Induction of a Marker of the Embryonic Gene Program, ANF. Induction of an embryonic gene program is a hallmark of the hypertrophic phenotype in both *in vitro* and *in vivo* model systems. In this regard, the reactivation of expression of the ANF gene is one of the most well-studied and conserved genetic markers of this response, seen in every form of hypertrophy and in all species thus far examined (3). To examine the effects of RA on induction of the ANF gene, total RNA was isolated from ventricular cells cultured in maintenance medium alone or in maintenance medium supplemented with phenylephrine, RA, or both agents. As previously reported, phenylephrine stimulation induces ANF mRNA expression significantly, by ≈6-fold vs. control (Fig. 2*A*) (quantitative estimation was done by densitometric analysis of the intensity of bands on the autoradiographic film of the Northern blot and normalized by the intensity of the bands probed by GAPDH). In contrast, combined treatment with both RA and phenylephrine results in levels of ANF mRNA comparable to unstimulated cells, consistent with an effect of RA on inhibiting the hypertrophic phenotype. RA suppression of phenylephrine induction of the ANF mRNA was dose dependent (Fig. 2*B*), with a 50%

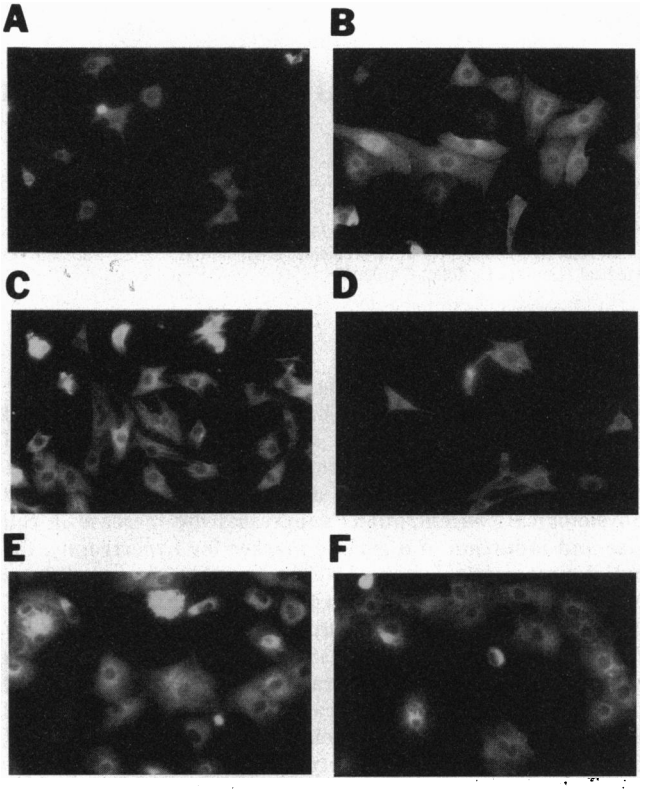

FIG. 1. Effect of RA on cell size of ventricular myocardial cells. After ventricular myocardial cells attached to the chamber slides, cells were cultured in different media. Cells cultured in maintenance medium (serum-free medium) alone (*A*), supplemented with 5×10^{-6} M phenylephrine (*B*), 5×10^{-6} M phenylephrine and 1×10^{-6} M all-*trans*-RA (*C*), RA alone at 1×10^{-6} M (*D*), 10% fetal bovine serum (*E*), both 10% fetal bovine serum and RA at 1×10^{-6} M (*F*). After 64 hr of culture in conditioned medium, slides were stained by indirect immunofluorescence with TrpE/MLC-2v antisera. (×40.)

Table 1. Summary of the RA effect on cell size

Medium	No. of cells	Relative cell size
Maintenance	23	100 ± 6
RA (1×10^{-7} M)	31	140 ± 14
RA (1×10^{-6} M)	40	120 ± 15
Phe (5×10^{-6} M)	35	320 ± 21
Phe (5×10^{-6} M) + RA (1×10^{-7} M)	39	210 ± 20
Phe (5×10^{-6} M) + RA (1×10^{-6} M)	31	160 ± 17
FBS (10%)	26	300 ± 25
FBS (10%) + RA (1×10^{-6} M)	25	290 ± 34

Cell size was estimated by measuring the area of individual cells attached on the tissue culture chamber slides. Data are means ± SEM of values from two experiments. Phe, phenylephrine; FBS, fetal bovine serum.

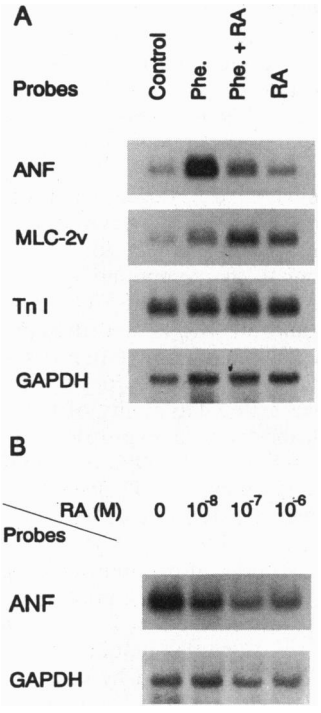

FIG. 2. Northern blot analysis of the effect of RA on cardiac gene expression. (*A*) Total RNAs isolated from cells cultured in maintenance medium alone (control) or in maintenance medium containing 5×10^{-6} M phenylephrine (Phe.), 5×10^{-6} M phenylephrine and 1×10^{-6} M all-*trans*-RA (Phe. + RA), or containing 1×10^{-6} M RA were analyzed by Northern blot analysis. The blotting filter was probed with radiolabeled cDNAs of ANF, MLC-2v, TnI, and GAPDH. (*B*) Total RNAs were isolated from cells cultured in phenylephrine-containing maintenance medium (5×10^{-6} M) and with different concentrations of RA as shown. RNAs on the filter were hybridized with ANF and GAPDH cDNA probes.

suppression seen with a RA concentration of $<10^{-8}$ M, which is within physiological levels and comparable to the dose–response profile for transcriptional activation mediated by retinoid receptors (19). Treatment with RA alone had little effect on the basal expression of ANF mRNA in the control cells (Fig. 2*A*). We also examined the effects of phenylephrine and RA treatment on expression of troponin I (TnI) and MLC-2v, both constitutively expressed cardiac muscle genes. As shown in Fig. 2*A*, RA had no detectable effect on inhibiting expression of the TnI or MLC-2v gene during phenylephrine stimulation. Similar results were also obtained at the protein level in studies using dual immunofluorescence with MLC-2v and ANF antibodies (data not shown). Taken together, these results indicate that RA treatment can selectively inhibit expression of a genetic marker of the hypertrophic response, ANF, at both the protein and RNA levels.

A series of studies have identified *cis* sequences within the ANF promoter region that can confer α-adrenergic inducibility to a luciferase reporter gene in transient transfection assays (4, 20, 21). When introduced into ventricular cardiomyocytes cultured in maintenance media, phenylephrine or endothelin 1 induces a 15- to 20-fold induction of an ANF promoter–luciferase fusion gene (Fig. 3). This induction by phenylephrine or endothelin 1 is suppressed by addition of RA back toward basal levels. In contrast, RA had little effect on suppressing induction of the ANF luciferase reporter gene after serum stimulation, similar to previous studies examining the effects on cell size (Fig. 1*F*). The inability of RA to suppress serum inducibility of the ANF–luciferase reporter gene suggests that retinoid signaling pathways do not directly inhibit ANF promoter activity but rather may induce a functional blockade at

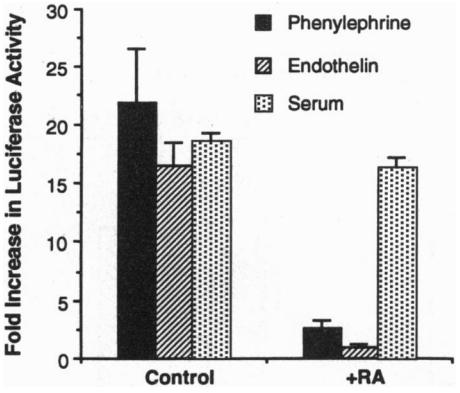

FIG. 3. Transient transfection analysis of the ANF–luciferase fusion gene in ventricular myocardial cells cultured in different conditioned media. Plasmids containing the ANF promoter fused with the luciferase reporter were separately transfected into ventricular cells. Cells were then cultured in medium containing different agents: 2×10^{-6} M phenylephrine, 1×10^{-8} M endothelin, or 10% fetal bovine serum, with or without 1×10^{-6} M all-*trans*-RA. Bars represent effects of these agents relative to unstimulated cells. Luciferase activities were normalized by β-galactosidase activities through co-transfection with a CMV–β-galactosidase plasmid.

a step in α-adrenergic and endothelin 1 signal transduction pathways of myocardial cell hypertrophy.

Retinoid-Dependent Suppression Is Blocked by Cotransfection of a Dominant-Negative Retinoid Receptor Expression Construct. The effects of RA are mediated through specific nuclear receptors of the nuclear receptor family (22). There are two subfamilies of RA receptors, the RARs and the RXRs, each of which contains three members, designated α, β, and γ. Two types of active receptor complexes form on appropriate response elements: a homodimer of RXR, activated by 9cRA, and a heterodimer of RXR and RAR, activated by 9cRA or atRA; RAR alone is unable to bind DNA with high affinity. DNA sequences that confer unique responsiveness to either the homodimer or the heterodimer have been identified, indicating two distinct physiological pathways through which retinoids affect biological processes (23, 24).

To assay for the activity of RARs in cultured ventricular muscle cells, response elements specific for activation by the RXR homodimer (CRBPII) (17) or by the RAR–RXR heterodimer (βRE) (18) were cloned into an enhancer-dependent heterologous reporter context and introduced by transfection. As shown in Fig. 4*A*, the CRBPII reporter construct was not activated by endogenous receptors in the presence of 9cRA, yet it was upregulated when cotransfected with a RXR expression construct. In contrast, the βRE heterodimer reporter gene is functional in the presence of ligand using the endogenous complement of receptors, and this activity can be further increased by cotransfection of receptor expression constructs. These results demonstrate that cultured neonatal ventricular cardiomyocytes express a functional RAR complex of both RARs and RXRs, which can transactivate through at least one of the known RA response pathways.

Recently, a dominant-negative form of the RARα (hRXRα403) has been described (16), which is able to block both the RXR–RAR heterodimer and RXR homodimer pathways. As shown in Fig. 4*B*, RA is ineffective in suppression of the reporter gene in the presence of the dominant-negative mutant receptor. Cotransfection of hRXRα403 with the ANF–luciferase reporter gene in the presence of RA retains 83% of the phenylephrine activity in induction of the ANF promoter, in comparison with only 23% of the phenylephrine-inducible activity observed in the absence of hRXRα403. In contrast, forced expression of either RAR or RXR results in enhancement of the suppressing activity. These results demonstrate

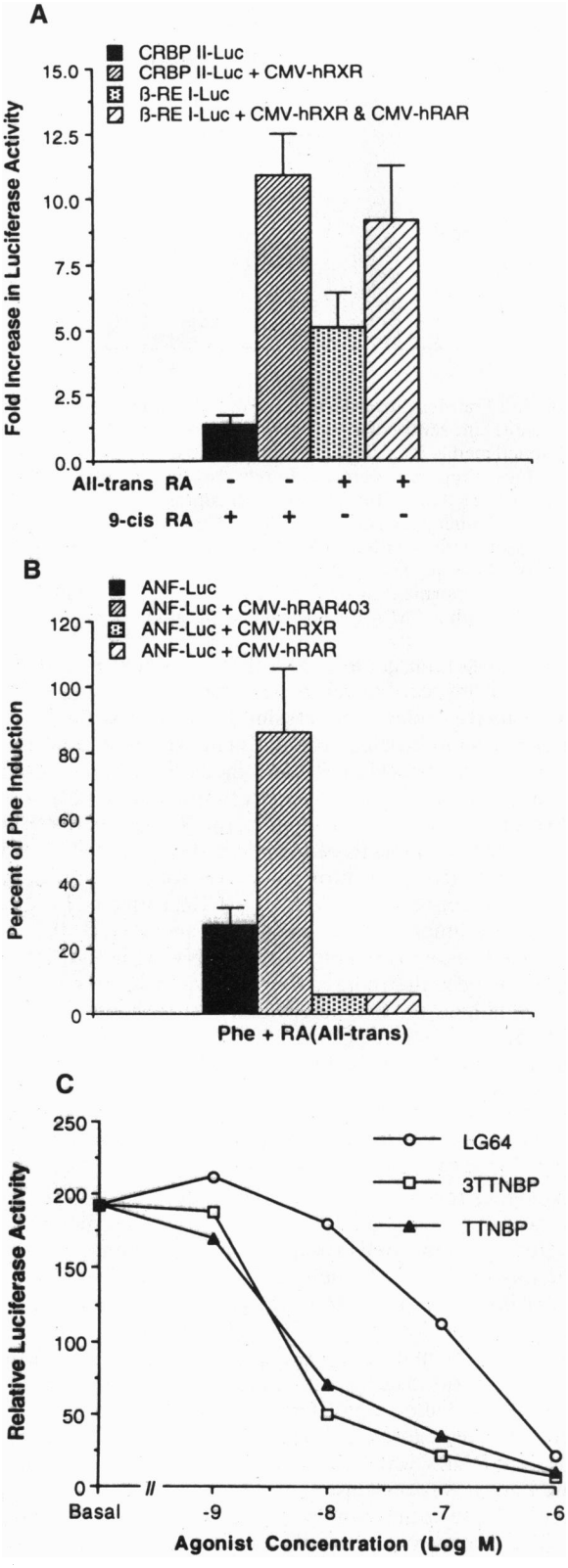

FIG. 4. Trans-activation analysis of endogenous RARs. (*A*) Reporter plasmids containing CRBPII–luciferase (Luc) or βRE1–luciferase were separately transfected into ventricular myocardial cells without or with cotransfected expression plasmids that contain human RXRα or RARα cDNA driven by a CMV promoter. Ligands 9-*cis*- and all-*trans*-RA were separately added to the cultures to activate CRBPII or βRE1 promoters (see ref. 23). Bars represent effects of ligands on promoter activities relative to reporter gene activity in the absence of ligand. (*B*) Reporter construct containing the ANF promoter was transfected alone or with a dominant-negative reporter construct (CMV-hRAR403), the wild-type receptor construct (CMV-hRXR), or CMV-hRAR into ventricular cells cultured in phenylephrine (Phe) (2×10^{-6} M)-containing medium with or without all-*trans*-RA (1×10^{-7} M). The percentage of phenylephrine induction is expressed as the ratio of -fold increase in the reporter gene by phenylephrine in the presence of RA to the -fold increase in the reporter gene observed after phenylephrine stimulation in the absence of RA. (*C*) Ventricular cells were transfected with the ANF–luciferase reporter gene and activated by 2×10^{-6} M phenylephrine with a series of concentrations of three agonists: LG64, TTNBP, and 3-methyl-TTNBP. Curves represent relative luciferase activities normalized by β-galactosidase activities.

that the suppression of phenylephrine-induced ANF expression by RA is mediated through the endogenous RARs.

Effects of Selective Synthetic Retinoids on Myocardial Cell Hypertrophy. In cultured cells, atRA and 9cRA are reversibly isomerized; consequently, in transactivation assays, treatment with either compound potentially activates both the RXR–RAR heterodimer and RXR–RXR homodimer pathways. Recent advances have led to development of stable synthetic retinoids that are selective agonists for either the RARs or the RXRs and that are not subject to metabolic conversion. Among such agonists, the compound TTNPB is selective for the RARs, the compound 3-methyl-TTNPB is a panagonist for both the RARs and the RXRs, and the compound LG64 is selective for the RXRs, although there is some degree of crossover at higher concentrations for both TTNPB and LG64 (30, 31). We have tested the ability of these compounds to block phenylephrine-induced expression of the ANF promoter. As shown in Fig. 4*C*, TTNPB and 3-methyl-TTNPB are equivalent in their potency to block induction of the ANF promoter, whereas the RXR-selective compound LG64 is ≈40-fold less potent in this assay. Similarly, the hypertrophic increase in cell size induced by phenylephrine was prevented by TTNPB and not by LG64 (data not shown). This indicates that ligand activation of the RAR is the essential component in blocking the α-adrenergic induction of hypertrophy, as monitored both by cell size and by expression of the ANF promoter. To explore the possibility that other hormonal signaling pathways might also impact on phenylephrine-induced hypertrophy, we tested the effect of dexamethasone, estrogen, vitamin D_3, and linoleic acid on phenylephrine-induced ANF promoter activity (data not shown). None of these agents displayed suppressor activity, indicating that the α-adrenergic induction of hypertrophy is specifically blocked by the RA pathway.

DISCUSSION

The specific signal transduction pathways that ultimately result in induction of cardiac muscle cell hypertrophy are currently unknown. Since RA is selective in blocking both the α-adrenergic and endothelin 1 pathways of *in vitro* hypertrophy but is ineffective on the serum-induced hypertrophic response, it is clear that the cardiomyocyte is able to modulate its growth response through multiple parallel pathways. Furthermore, while RA blocks the increase in cell size and the induction of ANF expression after α-adrenergic stimulation, RA does not prevent myofibrillar reorganization or induction of contractility. This suggests that multiple effector pathways emerge from the α-adrenergic receptor, as has in fact been previously documented (6, 7), and that the inhibitory effects of RA are manifest at an intermediate point in a portion of the downstream α-adrenergic pathways, but RA does not block all of the α-adrenergic receptor-dependent signals.

In addition to directly regulating gene expression, an additional pathway in which retinoids have biological consequences has been described in which ligand-activated receptors block the activity of the transcription factor AP-1 (25, 26). This

process, termed cross-coupling, does not require DNA binding of the receptor but rather appears to be a protein–protein interaction between a retinoid receptor and c-jun and likely additional proteins as well. AP-1 activity is induced in many signal transduction pathways, and the ANF promoter has sequences that are similar to AP-1 binding sites (4). However, cross-coupling is probably not sufficient to explain at a mechanistic level the suppression of hypertrophy caused by RA. While multiple hormone receptors can engage in cross-coupling, including the glucocorticoid receptor, our results indicate that suppression of hypertrophy is not seen after addition of glucocorticoids (data not shown). Consequently, the most likely explanation for the observed results is that RA induces the expression of a select subset of cardiac genes through the RXR–RAR heterodimer and that these induced gene products are able to block specific aspects of downstream signal transduction stimulated by the α-adrenergic and endothelin receptors, leading to suppression of the hypertrophic phenotype.

RA has additional consequences on cardiomyocytes in addition to simply suppressing α-adrenergic receptor-mediated hypertrophy. In neonatal cells, RA induces expression of MLC-2v, as shown in this report, and has been previously reported to induce the adult form of α-myosin heavy chain (27). During the hypertrophic response, ventricular muscle cells express embryonic markers, such as the ANF, skeletal α-actin, and β-myosin heavy-chain genes, and thereby exit from the mature, adult ventricular phenotype (for review, see ref. 3). The ability of retinoids to suppress the expression of atrial and embryonic markers during hypertrophy in the postnatal state, coupled with their ability to promote the ventricular phenotype in the embryonic state, raises the question of whether retinoid-dependent pathways might function in the adult myocardium to actively maintain the ventricular phenotype. In this manner, the activation of a hypertrophic response might be related, in part, to the relief of retinoid suppression.

This report represents a clear demonstration of the existence of defined signaling pathways that can suppress myocardial cell hypertrophy. Since recent studies have suggested that this *in vitro* model system of hypertrophy can have predictive value for generation of *in vivo* hypertrophy in transgenic animals harboring either MLC-Ras (28) or receptor transgenes (29), additional studies should examine the effects of retinoids in the *in vivo* context.

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