The Transcriptional Coactivator p300 Plays a Critical Role in the Hypertrophic and Protective Pathways Induced by Phenylephrine in Cardiac Cells but Is Specific to the Hypertrophic Effect of Urocortin

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Anacardic acid is an alkylsalicylic acid obtained from cashew-nut-shell liquid, and is a potent inhibitor of p300 histone acetyltransferase (HAT) activity. We have used anacardic acid to prevent the induction of hypertrophy in isolated neonatal rat cardiomyocytes. Hypertrophy was detected as an increase in cell size, the rearrangement of sarcomeres into a striated pattern, and the induction of embryonic genes β-MHC and ANF. p300 inhibition was equally effective at preventing hypertrophy whether it was induced by treatment with the α1-adrenergic agonist, phenylephrine, or by treatment with urocortin, a member of the corticotrophin-releasing-factor family, which stimulates specific G protein-coupled receptors. Spiruchostatin A is a natural-product inhibitor of histone deacetylases (HDAC) similar to the depsipeptide FK228 molecule. We have recently synthesized spiruchostatin A and now show that, although HDACs act in opposition to HATs, spiruchostatin A has the same effect as anacardic acid, that is, it prevents the induction of hypertrophy in response to phenylephrine or urocortin. Pretreatment with either phenylephrine or urocortin reduced the extent of death observed after the exposure of isolated cardiomyocytes to simulated ischaemia and reoxygenation. Inhibition of p300 or HDAC activity eliminated the protection conferred by phenylephrine; however, it did not affect the protection conferred by urocortin. Therefore, it might eventually be possible to use chemical inhibitors such as these in a therapeutic setting to dissociate the protective effect and hypertrophic effect of urocortin, enhancing the survival of cardiomyocytes exposed to transient ischemia, while inhibiting the hypertrophic pathway that would otherwise be induced concurrently.

Introduction

In response to an increase in hemodynamic pressure or volume, the heart may undergo compensatory hypertrophy. This can also be stimulated by small molecules such as endothelin and phenylephrine and causes the cardiomyocytes to increase their rates of protein synthesis, resulting ultimately in an increase in the size of individual cardiomyocytes and, consequently, an enlargement of the heart. Cardiac hypertrophy is also associated with increased expression of embryonic genes such as atrial natriuretic factor (ANP), B-type natriuretic protein (BNP), the beta isoform of myosin heavy chain (β-MHC), and the alpha skeletal-muscle isoform of actin (αSA). Prolonged hypertrophy is ultimately deleterious and results in dilated cardiomyopathy and heart failure (reviewed in ref. [1]).

Urocortin (UCN) is a forty amino acid peptide that is closely related to corticotrophin-releasing factor and has been shown to potently protect the heart from ischemia/reperfusion injury (reviewed in ref. [2]). However, its therapeutic use could be hampered by the fact that it also induces hypertrophy (reviewed in ref. [2]). However, we have previously shown that UCN's hypertrophic and protective pathways are separable, since the mitogen-activated protein kinase (MAPK) pathway is

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  DOI: 10.1002/cbic.200400246 *ChemBioChem* **2005**, *6*, 162 – 170

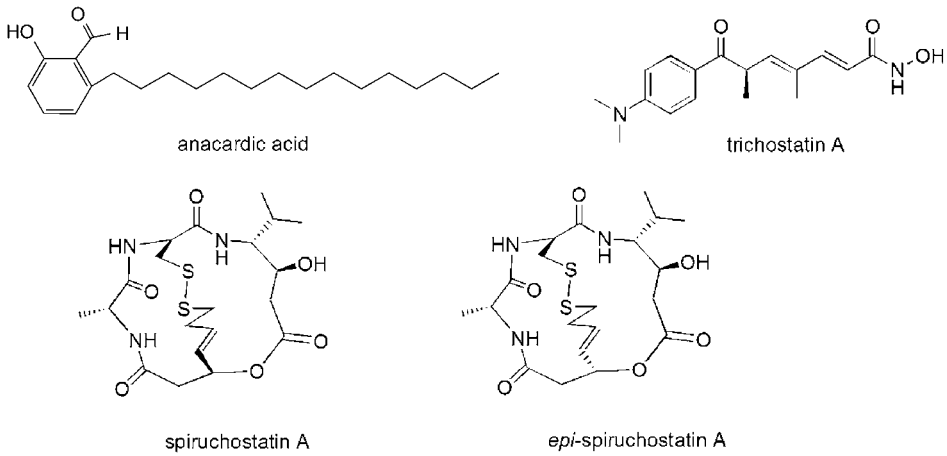
important in protection mediated by UCN,[3] but is not necessary for its induction of hypertrophy.[4]

Phenylephrine (PE) binds to α1A-adrenergic receptors,[5] then the Gq- and ras-dependent pathways transduce the hypertrophic signal.[6] Further downstream, a number of protein kinase pathways are activated in cardiomyocytes, including p42/p44 MAPK, p38, SAPK/JNK, and PKB/Akt,[7] and we have shown that p42/p44 MAPK is responsible for the activation of p300 and the highly related CBP (CREB binding protein) in response to PE.[8]

p300 is a transcriptional coactivator that is known to act as a "bridging factor" between basal transcription factors and various transactivators, including cardiac transcription factors such as MyoD, MEF2, and GATA4/5/6 (reviewed in ref. [9]). p300 has a histone acetyltransferase (HAT) activity that modifies associated transcription factors and chromatin by acetylation, thereby relaxing chromatin structure and promoting gene activation. The importance of p300 in normal cardiac transcription is illustrated by p300 knockout mouse embryos that have reduced expression of muscle structural proteins such as myosin heavy chain and α-actinin, and, as a consequence, cardiac structural defects.[10] Further transgenic studies have shown that it is the HAT activity of p300 that is essential for heart formation.[11] In contrast, it should be noted that cardiovascular development is impaired to a much lesser extent in mice harboring a deletion of CBP,[11,12] and these proteins might therefore not be able to compensate for each others' absence during cardiomyocyte differentiation, in vivo. We have previously demonstrated that p300 transcriptional activity is increased during PE-induced hypertrophy,[8] and that inhibition of p300, either by using antisense or dominant negative mutant constructs, inhibits PE-induced hypertrophy.[13] Overexpression of p300 was sufficient to induce hypertrophy, but the HAT domain of p300 was found to be required for this activity.[13] These results suggested the importance of p300 in regulating the hypertrophic program in response to PE and that further investigation of histone-acetylation pathways during hypertrophy was warranted.

Opposing the action of HATs in the cell are histone deacetylases (HDACs), which deacetylate histones, resulting in chromatin condensation and transcriptional repression (reviewed in ref. [14]). Inhibitors of these enzymes are currently under intense investigation as potential anticancer drugs. There are three classes of HDAC enzymes; class I deacetylases include HDACs 1, 2, 3, and 8; class II deacetylases include HDACs 4, 5, 6, 7, 9, and 10; and class III HDACs, which are unique in that they are not inhibited by trichostatin A (TSA), SAHA, or related compounds. There is some confusion regarding the role of HDACs in hypertrophy. Overexpression of class II HDACs in cardiomyocytes prevents their hypertrophic response, and mice lacking HDAC9 are supersensitive to hypertrophic stress. However, we previously found that in vitro inhibition of HDACs resulted in a partial induction of the hypertrophic response (with no increase in ANP levels), while others found that cardiomyocyte hypertrophy was completely blocked by broad specificity HDAC inhibitors such as TSA, NA-butyrate, or HC toxin.[15]

As a first step towards the elucidation of the involvement of HDAC and p300 HAT activities in pathways of cardiomyocytes hypertrophy and protection from ischemia/reperfusion injury, we present the results obtained from using recently isolated, specific chemical inhibitors. Anacardic acid is an alkylsalicylic acid obtained from various natural sources such as cashew-nut-shell liquid (see Scheme 1 for chemical structure), and has been found to be a potent inhibitor of p300 and p300/CBP-associated factor HAT-dependent transcriptional activity, in vitro.[16]

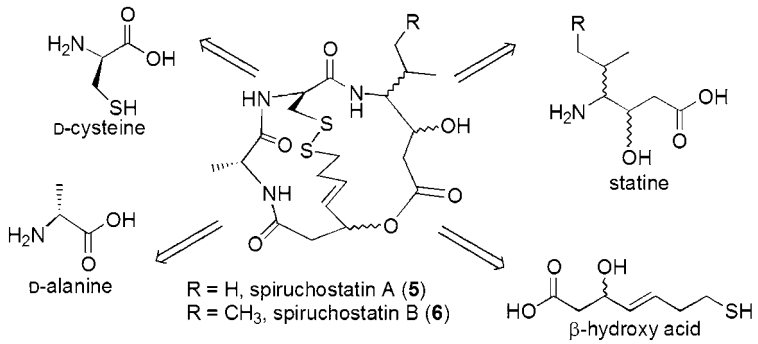


Scheme 1. *Chemical structures of HAT (anacardic acid) and HDAC (trichostatin A, spiruchostatin A) inhibitors.*

Among the HDAC inhibitors, compounds like TSA and SAHA have been widely used in biological assays. Although these hydroxamic acids are highly potent nanomolar inhibitors, their broad spectrum of action makes it difficult to ascertain the relative importance of class I, II, and III HDACs in a specific context. On the other hand, the natural product FK228[14] (also known as FR901228 and depsipeptide), was shown to selectively inhibit class I HDACs, which are precisely the HDACs believed to be of importance in cardiac hypertrophy. Nevertheless, experimental proof of the benefits of a selective class I HDAC inhibitor in hypertrophy models is lacking. FK228 is currently in advanced clinical trials as an anticancer agent, and for these reasons is no longer available as a research tool. Spiruchostatin A is a recently isolated natural product[17] that bears a close structural relationship to FK228. The similarity suggests that spiruchostatin A will also function as a selective class I HDAC inhibitor.

We have recently communicated a total synthesis of spiruchostatin A.[18] In achieving the target, a series of challenges had to be overcome, which are briefly summarized:

1) Neither the relative nor absolute stereochemistry of the statine and β-hydroxy acid subunits (Scheme 2) in spiruchostatin were reported. Thus, several diastereomeric possibilities need to be considered. During the course of our work, the stereochemistry of the statine was established as 3*S*,4*R*; this simplified the target to either of two enantiomers in the β-hydroxy acid. A superposition of FK228 and spiruchostatin suggests this remaining stereocenter to be *S*, and this was the structure chosen for synthesis.



Scheme 2. *The structures of spiruchostatin A and B, indicating their origin from four building blocks.*

2) When the statine subunit is protected as a carboxylic acid ester, it is prone to intramolecular cyclization by the amine to an undesired lactam. In our initial approach, this was avoided by using a bulky *tert*-butyl ester protecting group. However, neither acidic nor basic deprotection satisfactorily removed the ester later on in the synthesis. Ultimately, the solution relied on using the *t*Boc protecting group for the amine, which upon acidic deprotection produces a *protonated* amine. By neutralizing the amine in situ in the presence of an excess of acylating agent, intermolecular coupling proved to be significantly faster than the intramolecular lactam cyclization, and good yields were achieved.
3) The statine subunit is susceptible to elimination of the β-hydroxy group or migration of protecting groups from the alcohol to the carboxylic acid. For example, as stated above, compounds with the *tert*-butyl ester could not be efficiently deprotected. The trichloroethyl ester proved to be the answer, as it could be removed at essentially neutral pH under buffered reaction conditions.
4) The β-hydroxy acid needs to be synthesized in an enantiopure form. We had difficulty reproducing the enantioselective reagent-controlled Carreira aldol conditions used by Simon et al.[19] in their FK228 total synthesis. As an alternative, we explored Keck[20] conditions using BINOL as the catalyst, but both the chemical yield and enantioselectivity were poor. We then switched to a chiral auxiliary mediated aldol and used the Nagao thaiazolidinethione version[21] of the Evans auxiliary. The Nagao conditions worked well in our case, providing high yields with $>90\%$ diastereoselectivity. The two products were readily separated, and indeed access to the minor diastereomer allowed us to later synthesize an epimer of spiruchostatin A.
5) Fragment coupling and macrocyclisations. The choice of Nagao auxiliary for the aldol reaction enabled a streamlined assembly of the linear precursor to spiruchostatin. The thiazolidinethione is an activated leaving group, and hence the aldol product itself functioned as an acylating agent with amines.[22] In the crucial macrocyclization step of the FK228 synthesis, Simon[19] had observed difficulties with methods involving activation of the carboxylic acid. For these reasons, a Mitsunobu macrocyclization involving alcohol activation was employed instead. In our studies with spiruchostatin, the Yamaguchi method of carboxylic acid activation was successful,[23] after some experimentation and optimization of reaction conditions. It is possible that the differences in structure between spiruchostatin and FK228 favor macrocyclization in our case.

Once spiruchostatin A had been synthesized, biological evaluation confirmed that it was a potent HDAC inhibitor, causing the accumulation of acetylated histone H4 and activating the $p21^{waf1/cip1}$ promoter in breast-cancer cells.[18] We believe that, like FK228, spiruchostatin A acts as a prodrug that is cell permeable in its bicyclic disulfide-bridged form. Intracellular reduction would then release the free thiols, which can act as zinc-binding groups at the HDAC active site. It is likely that the macrocyclic depsipeptide scaffold of FK228 and spiruchostatin results in additional favorable interactions in the HDAC rim outside the active site, and that these help mediate selectivity. We synthesized an epimer of spiruchostatin A containing the *R* enantiomer of the β-hydroxy acid. This compound would be expected to undergo intracellular reduction and release zinc-binding thiols just like spiruchostatin, but the rest of the molecule would be a mirror image in three-dimensional space. *Epi*-spiruchostatin A was completely inactive in our assays; this underscores the importance of such additional rim interactions for HDAC inhibition.

Results

Anacardic acid (AA), an alkylsalicylic acid obtained from various natural sources such as cashew-nut oil (Scheme 1), has recently been described as a potent inhibitor of p300 and p300/CBP-associated factor (PCAF) histone acetyltransferase (HAT) activities.[16] Anacardic acid was shown to inhibit HAT-dependent transcription from a chromatin template by directly inhibiting acetylation of histone H3, while having no effect on transcription from naked DNA.[16] To confirm that anacardic acid inhibits p300 activity in our system, we transfected neonatal rat cardiomyocytes with a construct expressing full-length p300 that was fused to the DNA-binding domain of the Gal4 transcription factor. We have previously shown that the ability of this construct to activate a cotransfected reporter gene containing Gal4 DNA binding sites is stimulated by treatment of the cells with PE.[8] Anacardic acid completely prevented the stimulation of p300 by PE (Figure 1). Similarly, although UCN only weakly stimulated this reporter system in accordance with our previ-

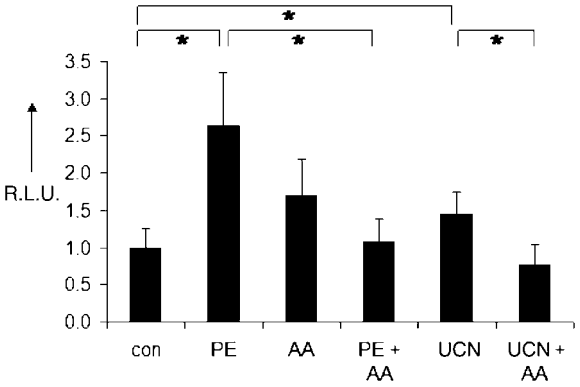


Figure 1. *An inhibitor of p300 HAT activity inhibits the ability of p300–Gal4 to activate transcription from a Gal4-luciferase reporter plasmid transfected into primary neonatal rat cardiomyocytes. The treatments were phenylephrine (PE), anacardic acid (AA), urocortin (UCN) or combinations thereof. Results expressed in relative light units (RLU) were normalized to pCMV-βgal and the control value was set at one. Values are the average of four wells transfected with G4TATAluc (1 μg) and p300full-length (2 μg) and CMV-βgal (0.5 μg) in each of two separate experiments ±S.E.M. Asterisks indicate significant differences between the indicated sample pairs (P < 0.05).*

ous results,[8] anacardic acid returned p300 activity to baseline levels (Figure 1).

Next we wished to determine whether chemical inhibition of p300 HAT activity can suppress the induction of cardiomyocyte hypertrophy. During hypertrophy, cardiomyocytes typically revert to an embryonic pattern of gene expression, with increased expression from several genes including ANF and β-MHC. We measured the level of β-MHC mRNA by reverse transcription followed by polymerase chain reaction (RT-PCR) and normalized to levels of gapdh mRNA. β-MHC mRNA was increased after 24 hours' treatment with PE or UCN, and this was dependent on the HAT activity of p300, since AA completely abolished this increase (Figure 2).

We also used a recently isolated inhibitor of HDACs, the bacterial natural product spiruchostatin A (SpA; Scheme 1). This depsipeptide has recently been prepared by total synthesis, and the synthetic material was used in our assays. In addition, an unnatural analogue of spiruchostatin A, *epi*-spiruchostatin A (*epi*-SpA), was tested. Compared to SpA, *epi*-SpA has altered stereochemistry at the β-hydroxy acid fragment (Scheme 1). Although it should be capable of in vivo disulfide reduction to release a zinc-binding thiol at the HDAC active site, the altered stereochemistry will considerably alter contacts with the HDAC rim residues compared to SpA. The importance of such interactions can be seen by the lack of activity of *epi*-SpA in p21 regulation and growth inhibition in breast-cancer cells.[18] We first verified that SpA induces histone H4 acetylation at 2.5 nM in cardiomyoyctes and that *epi*-SpA is inactive in this assay (M.B., F.H., G.P., data not shown). By RT-PCR, we found that SpA prevents the accumulation of β-MHC mRNA after treatment with PE or UCN, whereas *epi*-SpA had no effect (Figure 2).

Since it might have been anticipated that inhibition of HDACs would have the opposite effect to inhibition of p300 HAT activity, we verified that SpA has the same effect in cardiomyocytes as the classical inhibitor of HDACs, trichostatin A (TSA). TSA behaved in the same way as SpA in inhibiting the increase in β-MHC in response to PE or UCN (Figure 2).

The levels of endogenous transcripts can potentially be regulated by altering their stability, as well as altered transcription. To confirm that the alteration in mRNA level that we observed is due to regulation at the level of transcription, we repeated the above experiment using transiently transfected reporter plasmids that harbored the promoter for ANF or β-MHC upstream of a luciferase reporter gene.

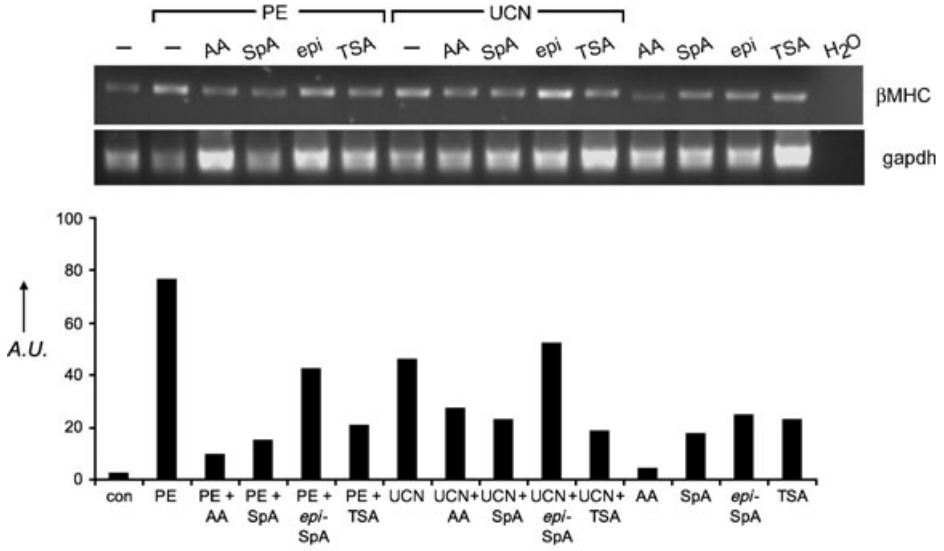


Figure 2. *The levels of endogenous β-MHC RNA are increased by treatment with PE or UCN, but not in the presence of anacardic acid (AA), spiruchostatin A (SpA), or trichostatin A (TSA). The inactive epimer of SpA (*epi*-SpA) had no effect. Cells were treated for 48 h, then RNA was extracted and amplified by RT-PCR by using primers specific to β-MHC. The product was separated on an agarose gel, quantitated with densitometry, and normalized to the level of gapdh in a parallel RT-PCR reaction. This value is presented in arbitrary absorbance units (A.U.).*

Since inhibitors of p300 or HDACs may conceivably have toxic effects, or nonspecific effects on overall transcription levels, we initially normalized the luciferase measurements to *Renilla* luciferase levels expressed from a cotransfected thymidine kinase promoter construct (i.e., tk-*Renilla*). However, we found that HDAC inhibitors dramatically induced tk-*Renilla* expression (about 50-fold). When we used an alternative construct expressing *Renilla* luciferase from an SV40 promoter, this gave much more consistent results and demonstrated that these compounds did not generally affect transcription, and had no toxic effect on cardiomyocytes at the concentrations

used (data not shown). We subsequently normalized the results of luciferase reporter assays to the SV40-*Renilla* luciferase levels.

The expression from ANF and β-MHC reporter plasmids was increased by 24 hours' treatment with PE, and this was dependent on the HAT activity of p300 since AA completely abolished this increase (Figure 3A, B). Similarly, UCN increased the

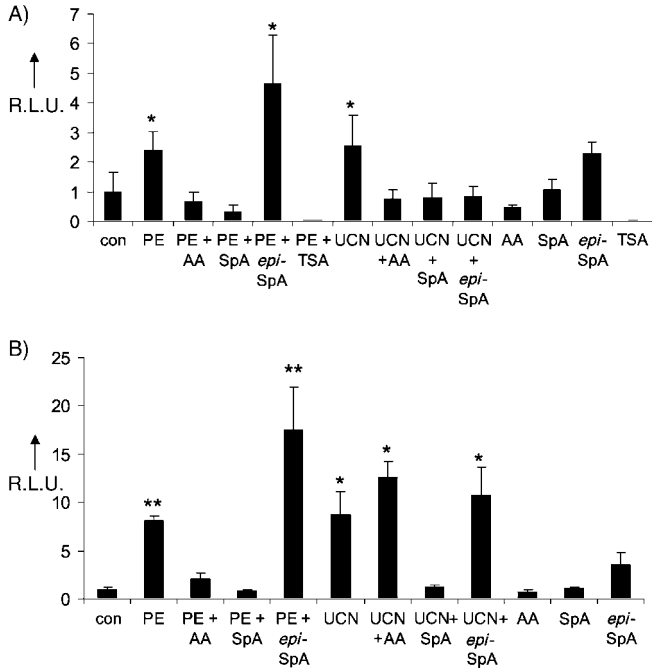


Figure 3. *The expression of ANF and β-MHC reporter plasmids is increased by treatment with hypertrophic agents: PE or UCN. This response is inhibited by anacardic acid (AA), spiruchostatin A (SpA), or trichostatin A (TSA), but not an inactive epimer of SpA (*epi*-SpA). Values are the average normalized luciferase readings (in R.L.U.), from four wells of cardiomyocytes, each transfected with ANF-luc (3 μg) or β-MHC-luc (3 μg) and pSV40-*Renilla *(0.1 μg), averaged over 2–3 separate experiments ± S.E.M. The control value was set at one. Single and double asterisks indicate significant differences from the control (*$P < 0.05$ and $P < 0.01$, respectively).*

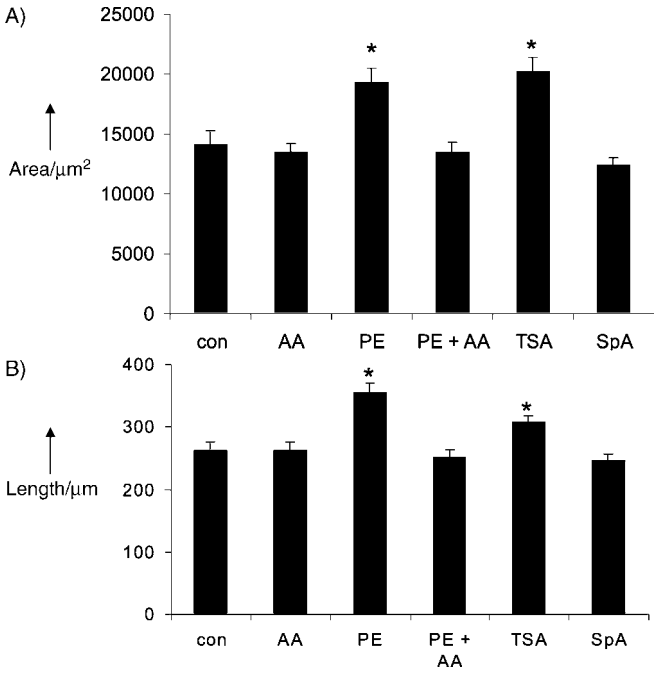


Figure 4. *The increase in cell surface area after treatment with phenylephrine (PE) is inhibited by anacardic acid (PE + AA). TSA alone causes an increase in cell size, but SpA does not. Cardiomyocytes were transfected with EGFP. The following day, the medium was replaced with the indicated agents and left for 24 h, then cells were fixed and the surface area and maximum cell length were measured as described in the Experimental Section. Results represent the average and S.E.M. of at least fifty cells, and similar results were obtained in a separate experiment. A) Planimetric area of GFP positive cardiomyocytes. B) Maximum length of GFP posistive cardiomyocytes. Asterisks indicate significant difference from the control ($P < 0.05$).*

expression from ANF and β-MHC reporter plasmids. AA inhibited the effect of UCN on ANF, although the induction of β-MHC was unaffected (Figure 3A, B). We found that SpA prevents the effect of PE on the ANF and β-MHC promoters, but *epi*-SpA does not. The effect appeared to be similar when UCN was used instead of PE, although *epi*-SpA may have had nonspecific inhibitory effects on the ANF reporter (Figure 3A).

A defining feature of hypertrophy is an increase in both cell surface area and cell length. We transfected cardiomyocytes with an EGFP-expression plasmid in order to highlight their outline, and measured the surface area and length of cells imaged using a fluorescent microscope. When cultures were stimulated with PE for 24 hours, the average size of cells increased significantly ($P < 0.05$), and inhibition of p300 activity by treatment with AA prevented this increase (Figure 4A). No change in cell size was observed when cells were treated with AA alone (Figure 4A). Similar results were obtained with respect to cell length (Figure 4B). This indicates that chemical inhibition of p300 HAT activity is sufficient to inhibit hypertrophy induced by PE. Interestingly, although TSA caused an increase in cell area and width, SpA had no effect (Figure 4A, B).

Another commonly used method for detecting hypertrophy in isolated cardiomyocytes is to use immunofluorescence microscopy to visualize sarcomeric reorganization. We stained cardiomyocytes for desmin intermediate filaments, which become striated after treatment with PE or UCN (Figure 5). Treatment of cultures with AA, TSA, or SpA, prevented the reorganization of sarcomeres by either PE or UCN, although the inactive epimer of SpA did not (Figure 5).

In combination, these results indicate that cardiomyocyte hypertrophy induced by PE or UCN can be prevented by a chemical inhibitor of HAT activity of p300 or by an inhibitor of HDAC activity. Both urocortin and phenylephrine are known to have cardioprotective properties.[3,24] Therefore, we investigated whether the same pathway is involved in protection against the damage induced by treatment with transient simulated ischemia and reoxygenation (IR). Cells were pretreated for 24 hours with the indicated chemicals, then exposed to simulated ischemia for 4 hours and returned to maintenance medium to recover overnight. They were then fixed and stained with Hoechst 33258 to enable visualization of nuclear morphology, and labeled by using the TUNEL reaction, which stains the nuclei of cells that are undergoing apoptosis. There

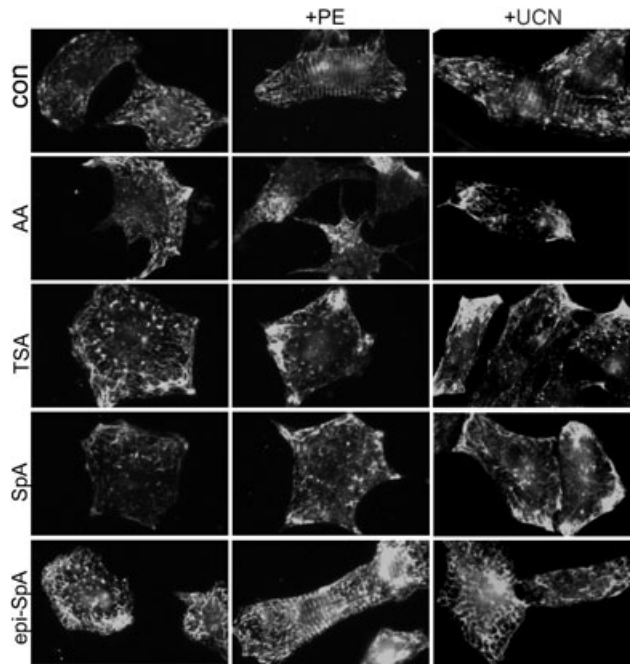


Figure 5. *Cardiomyocyte sarcomeres are reorganized after 24 h treatment with PE or UCN, and this response is blocked by anacardic acid (AA), spiruchostatin A (SpA), or trichostatin A (TSA), but not an inactive epimer of SpA (*epi-*SpA). Treatment of unstimulated cells with the HAT and HDAC inhibitors had no effect. Sarcomeric organization was visualized by immunofluorescent staining of fixed and permeabilized cells with an antibody against desmin, as described in the Experimental Section.*

was typically a background level of 5–10% dead cells in untreated cultures, and most of these appeared to be apoptotic (Figure 6); this is typical of primary cardiomyocyte cultures.[25]

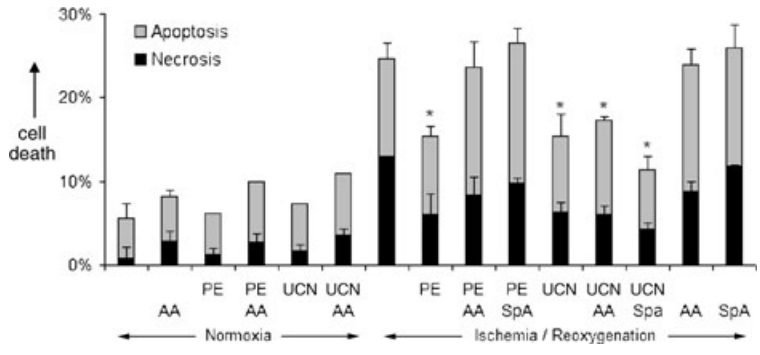


Figure 6. *Inhibition of p300 HAT activity with AA eliminates the protection conferred by PE to cardiomyocytes exposed to IR, but does not eliminate the protection conferred by UCN. Inhibition of HDACs with SpA has the same effect. Black bars represent the percentage of all cells that were necrotic. Grey bars represent the percentage of cells that were apoptotic. Asterisks indicate significant difference from IR alone ($P < 0.05$).*

After IR, 23.9 ± 1.9% of cells were dead, due to an increase in both apoptosis and necrosis (Figure 6). When cells were pretreated for 24 h with either PE or UCN, the extent of death was significantly reduced to 15.4 ± 1.2% and 15.4 ± 2.7%, respectively ($P < 0.05$; Figure 6). PE and UCN appeared to reduce both apoptosis and necrosis to a similar extent (Figure 6). When AA was included during the pretreatment, the protection mediated by PE was completely eliminated; however, protection by UCN was unaffected (Figure 6). This indicates that p300 HAT activity is common to both the hypertrophic and protective pathways induced by PE, but that the protection conferred by UCN does not depend on p300 HAT activity or UCN-induced hypertrophy. As had been observed with hypertrophy, SpA had a similar effect to that of AA, in this case eliminating protection from PE, but not UCN (Figure 6).

Discussion

We have previously shown that that p300 and the related protein CBP are necessary for the induction of cardiomyocyte hypertrophy by PE,[8,13] and that PE elevates CBP HAT activity.[8] p300 or CBP overexpression results in hypertrophy only when the HAT domain is intact.[13] Here, we show that inhibition of endogenous p300 HAT activity by anacardic acid can prevent the induction of hypertrophy in primary neonatal cardiomyocytes in response to two different stimulatory agents. Pretreatment with either PE or UCN conferred resistance to a subsequent transient exposure to simulated ischemia followed by reoxygenation. However, p300 activity was only involved in the protection mediated by PE and not by UCN. We also tested a recently isolated inhibitor of HDACs, spiruchostatin A, and found that it inhibited the induction of hypertrophy. This is despite the fact that a HDAC inhibitor might be expected to have the opposite effect to that of a HAT inhibitor. We verified that SpA had the same effect on the levels of transcripts of hypertrophic marker genes as a classical HDAC inhibitor, the hydroxamic acid trichostatin A (TSA). Interestingly, however, TSA and SpA differed fundamentally in that only TSA induced a hypertrophic increase in cell size.

We and others have previously shown that a dominant negative construct of p300 inhibits the induction of hypertrophy by PE in terms of ANP protein production, protein-to-DNA ratio, mean cell area and mean cell length.[13,26] p300 levels are increased by PE treatment of cardiomyocytes, and its nuclear hyperacetylation also increases.[26] We found that PE-treated cells increased in both cell area and cell length, as well as inducing sarcomeric reorganization, visualized by immunofluorescent staining of desmin. UCN also causes an increase in hypertrophy markers, such as ANP and BNP secretion, and [3H]-leucine uptake.[27] We show here that it also results in the reorganization of the sarcomere. All of these aspects of PE- and UCN-stimulated hypertrophy are dependent on the activity of p300. The prohypertrophic activity of p300 might occur through different pathways. For example, it has been shown to act as a coactivator for various cardiac-specific transcription factors, such as MEF2[28] and GATA4,[29] and also for hypoxia-inducible factor (HIF1).[30]

TSA has recently been shown to block hypertrophy induced by PE, endothelin 1 or serum stimulation, in vitro, while having no effect on its own.[15] This contrasts with our previous findings—in our system, treatment of cardiomyocytes with TSA

alone resulted in a partial induction of the hypertrophic response, that is, an increase in protein-to-DNA ratio and cell size and length, without an increase in ANP levels.[13] Here we show that there was also no sarcomeric rearrangement (Figure 5). An understanding of the role of HDACs in hypertrophy is made all the more confusing by observations that overexpression of class II (but not class I) HDACs in cardiomyocytes prevents the activation of MEF2 (part of the hypertrophic response),[31] and mice lacking HDAC9 (class II) are sensitized to hypertrophic signals.[32] These contradictory results are potentially resolved by a model in which the tendency towards hypertrophy is governed by the opposing actions of class I and class II HDACs. In this model, class I HDAC activity would be permissive for p300-mediated activation of transcription from the loci of prohypertrophic genes (Figure 7 A). Our results,

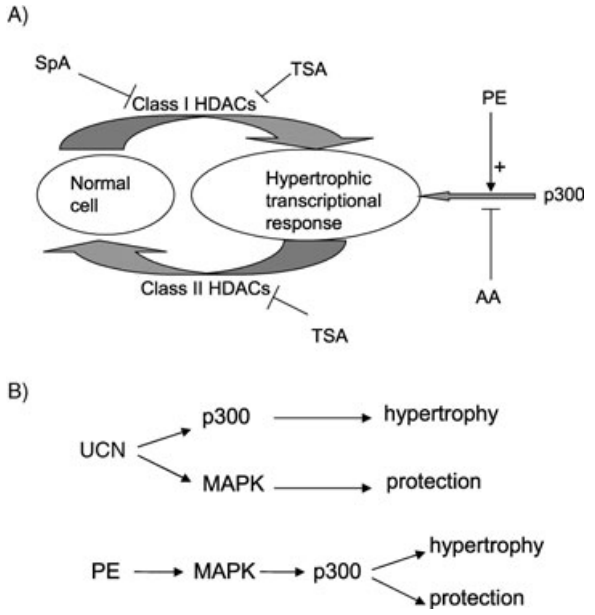


Figure 7. *A) Hypothetical model in which HDAC class I and class II oppose each other in inducing hypertrophic transcriptional response (i.e., activation of transcription factors such as MEF2). Class I HDAC activity would permit access by HATs such as p300 to activate transcription, and HDAC inhibitors such as TSA or SpA would prevent this. See text for further detail. B) There appear to be at least two alternative pathways between agonist stimulation and p300 activation of hypertrophy and/or protection. See text for details.*

which use different HDAC inhibitors, support this model. TSA inhibits both class I and class II HDACs to a similar degree,[33] thus preventing the cell from ever entering a state in which it can respond to hypertrophic signals. SpA inhibits HDAC1 and HDAC8 (both class I HDACs), in vitro, (F.H., G.P., data not shown) and does not appear to inhibit HDAC6 (class II) since it does not promote accumulation of acetylated tubulin in cells (F.H., G.P., data not shown). Thus, the relative activity of SpA against different HDAC families is similar to that of the highly related natural product, FK228, which is relatively specific for class I members (i.e., HDACs 1 and 2) and not representatives of class II HDACs (i.e., HDACs 4 and 6).[33] Therefore, from our results, inhibition of class I HDACs alone appears to be at least as effective as TSA at preventing hypertrophy.

Note that, in this model (Figure 7 A), class I HDACs are unlikely to directly activate gene transcription, but could inhibit transcription from loci encoding antihypertrophic genes or modify the activity of a transcription factor(s) by deacetylation, for example. Indeed, a similar model has recently been proposed[15] in which "antihypertrophic genes", which would normally be repressed by inhibitors of class I HDAC, are also proposed to exist, although these genes remain to be elucidated.

There is recent evidence that p300 activity is also regulated at other levels. For example, treatment of HeLa cells for 20 hours with 160 mM TSA causes loss of p300, apparently by proteasomal degradation.[34] However, it is not known whether this is a general phenomenon or is related to the viral oncoproteins expressed in HeLa cells, which are known to interact with p300. We found that the level of p300 was not dramatically affected by TSA or SpA (S.D., data not shown). Furthermore, we have previously shown that the hypertrophic increase in cell size and length caused by treatment of neonatal cardiomyocytes with TSA is eliminated by expression of a dominant negative p300 construct.[8] This indicates that there must be sufficient levels of p300 for the action of TSA, at least in terms of cell-size increase. It would be interesting to test whether different aspects of cardiomyocyte hypertrophy have differing degrees of sensitivity to p300 levels.

Interestingly, the binding of cardiac transcription factor MEF2 to p300 and class II HDACs, HDAC4 and HDAC5, is mutually exclusive.[35,36] This suggests that class II HDACs inhibit hypertrophy by binding MEF2 in the place of p300, hence, preventing the transactivation of MEF2 target genes (reviewed in ref. [37]). This might explain why inhibition of class II (and class I) HDACs with TSA induces some aspects of hypertrophy, while inhibition solely of class I HDACs with SpA does not.

It seems likely that a subtle balance of competitive binding lies at the heart of hypertrophic regulation—one in which the balance of p300 and different HDAC family members compete for binding to the same transcriptional activators. Indeed, we have previously shown that overexpression of p300 in cardiomyocytes is sufficient to activate a hypertrophic response.[13]

The rational redesign of SpA to preferentially inhibit different HDAC isoforms will permit further dissection of the pathways involved in cardiomyocytes hypertrophy as well as perhaps leading to the development of a clinically useful compound that inhibits the development of hypertrophy. It will also be important to investigate whether inhibition of certain HDACs (e.g., with TSA) increases cardiomyocyte cell size in vivo, since this might hamper the clinical use of inhibitors.

There is some evidence that the hypertrophic response, at least in the initial stages, can enhance the resistance of cardiomyocytes to ischemia/reperfusion injury. For example, overexpression of calcineurin, which induces myocyte hypertrophy and ANF expression, also protects against apoptosis induced by 2-deoxyglucose or staurosporine.[38] Furthermore, induction of hypertrophy in vitro, can sometimes confer resistance to apoptosis. For example, treatment of cardiomyocytes with PE protects against apoptosis induced by 2-deoxyglucose or a membrane-permeable cAMP analogue.[39,40] Conversely, some peptides known to confer resistance to ischemic injury also

stimulate hypertrophy. UCN and cardiotrophin 1 are two such peptides that might prove efficacious in reducing damage after myocardial ischemia, if their protective effect could be separated from their induction of hypertrophy.[2] We demonstrate here that the protection mediated by PE and UCN was equally effective against both apoptotic and necrotic pathways of cell death after simulated ischemia and reperfusion. Interestingly, in response to PE, the hypertrophic and protective pathways overlap at the level of p300. In contrast, in response to UCN, p300 is involved only in the hypertrophic and not the protective pathway (Figure 7B). Interestingly, based on results with SpA, certain HDACs also appear to be required for PE-mediated, but not UCN-mediated, protection.

UCN is known to protect cardiac myocytes by activation of the p42/44 MAPK-dependent pathway, while the hypertrophic effect of UCN is independent of MAPK.[4] We have previously shown that p300 is activated by PE through MAPK.[8] Taken together, this suggests that there are at least two possible scenarios for activation of p300 by different agonists—one in which MAPK directly leads to p300 phosphorylation and another in which MAPK activation and p300 phosphorylation are independent events (Figure 7B). The protective pathway activated by UCN might permit a more rapid response, since UCN is protective even when added at the time of reperfusion.[3]

It is not know how p300 activation leads to protection, but there is evidence that PE can mediate protection by altering the phosphorylation of proteins such as Bad.[40] Transgenic mice overexpressing p300 in the heart are more resistant to doxorubicin-induced apoptosis and heart failure,[41] and these mice have increased levels of BCL2 and MDM2 protein; this might also explain, at least in part, how p300 confers resistance to IR in our system. However, after 20 weeks of age, these mice tend to develop heart failure, with an increase in myocardial cross-sectional area.[26] This might reflect the hypertrophic response we also saw in cardiomyoctes after p300 overexpression in our system.

In summary, we have used two new chemical inhibitors to show that both HDAC activity and the HAT activity of p300/PCAF are required for the induction of cardiomyoycte hypertrophy in response to either UCN or PE. However, the protection from ischemia/reoxygenation injury conferred by UCN appears to be independent of p300 activity, while that conferred by PE is blocked by an inhibitor of p300 HAT activity. These results indicate that inhibitors such as these might find application in the treatment of cardiac disease, in light of their ability to prevent hypertrophy while permitting the development of protection.

Experimental Section

Culture and transfection of primary cardiomyocytes. Neonatal rat cardiomyocytes were prepared as previously described[25] and cultured in gelatin-coated 24-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) with fetal calf serum (15%) for 24 h prior to treatment. Most cells could be seen to beat spontaneously in a confluent monolayer 24–48 h after plating. After 24 h the medium was replaced with DMEM supplemented with fetal calf serum (1%). Cells were transfected with plasmid DNA by the standard calcium phosphate method as described previously.[42] The following morning, the medium was changed, with the addition of the chemicals indicated, at the following concentrations: PE (50 μM); UCN (10 nM); TSA (100 nM); spiruchostatin A (2.5 nM); *epi*-SpA (2.5 nM); anacardic acid (15 μM). Cells were generally left for 24 h before harvesting.

Luciferase Assay. At the end of the experiment, cells were washed in PBS and scraped into cell lysis buffer (50 μL) (Promega). An aliquot (20 μL) was taken and assayed by using the dual luciferase kit (Promega) and a TD-20e luminometer. Where pCMV-βgal was used as a control plasmid, it was assayed by using Galacto-light (Tropix, MA) and a luminometer. Values for firefly luciferase were divided by their corresponding *Renilla* luciferase values to obtain relative luciferase units (RLU). Reporter vector RLU was set at one. **Vectors used:** Gal4–p300 full length, 5xGal4 E1B TATA-luciferase, and the ANF reporter construct (−3003 to +62) of the ANF rat gene have been described previously.[8] The β-MHC reporter plasmid (nucleotides −667 to +38) has been described before[43] and was provided courtesy of Prof. Michael D. Schneider. ptk-*Renilla* and pSV40-*Renilla* control vectors were from Clontech. **RNA extraction and RT-PCR.** After 48 h treatment of cells with hypertrophic stimuli and/or inhibitors, they were rinsed in cold PBS and RNA was extracted in Trizol (Invitrogen) according to the manufacturer's instructions. An aliquot of RNA was reverse transcribed with hexanucleotide primers (500 ng) (Promega) and MMLV RT enzyme (Promega) at 45 °C for 50 min. A portion of this cDNA was used in a PCR reaction with Taq polymerase (Promega) according to the manufacturer's instructions under the following conditions: 95 °C 5 min; 30 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 60 s; then 72 °C 7 min. The product was separated on a agarose gel (2%). The primers used for PCR were against rat beta-myosin heavy chain (β-MHC): forward 5′-GCC AAC ACC AAC CTG TCC AAG TTC-3′; reverse 5′-TGC AAA GGC TCC AGG TCT GAG GGC-3′; and GAPDH: forward 5′-TGG AAA GCT GTG GCG TGA TG-3′; reverse 5′-TCC ACC ACC CTG TTG CTG TAG C-3′.

Simulated ischemia/reoxygenation and cell death analysis. For simulation of ischemia, the culture medium was replaced with modified Esumi ischemic buffer containing NaCl (137 mM), KCl (12 mM), $MgCl_2$ (0.49 mM), $CaCl{\cdot}2H_2O$ (0.9 mM), HEPES (4 mM), deoxyglucose (10 mM), and sodium lactate (20 mM, pH 6.2). This buffer has a high potassium content and a low acidic pH and hence mimics the conditions of cells within the heart when exposed to oxygen deprivation. The cells were transferred to a sealed chamber in a humidified atmosphere of CO_2 (5%), argon (95%) for induction of hypoxia and maintained at 37 °C for 4 h. Untreated cells were cultured in Esumi control buffer containing NaCl (137 mM), KCl (3.8 mM), $MgCl_2$ (0.49 mM), $CaCl{\cdot}2H_2O$ (0.9 mM), HEPES (4 mM), and glucose (10 mM, pH 7.4) in a humidified atmosphere of CO_2 (5%) and O_2 (21%) at 37 °C for 4 h. After completion of the hypoxic treatment, the cells were washed with PBS, normal medium added and the cells returned to a standard incubator overnight.[25]

Apoptotic nuclei were labeled with fluorescein-dUTP by TUNEL and simultaneously counter-stained with Hoechst 33258 as described.[44] By using a fluorescent microscope, the percentage of cells that were apoptotic (i.e., condensed nuclei and TUNEL positive) or necrotic (i.e., condensed nuclei but no TUNEL positivity) was scored. The mean percentage of necrotic and apoptotic cells was calculated.

Planimetric measurement of cell size. After treating the cells for 24 h with the indicated chemicals, the cells were fixed in paraformaldehyde (4%) for 10 min then rinsed in PBS and mounted under a

coverslip. Transfected cells were photographed by using a fluorescent microscope fitted with a CCD camera, and the area and length was measured with the Axiovision software (Carl Zeiss). More than fifty cells were measured and averaged, per sample. **Sarcomeric staining.** Cardiomyocytes were plated on gelatin-coated coverslips overnight before treatment for 24 h with PE (50 μM) or UCN (10 nM) in combination with the indicated inhibitors. Cells were then rinsed in PBS and fixed in paraformaldehyde (4% in PBS) at room temperature for 10 min before permeabilization in NP40 (0.1% in PBS) for 10 min. Coverslips were blocked for 1 h in BSA (3% in PBS), then 1 h in BSA (1% in PBS) with antidesmin antibody (1:400) followed by 3 washes of 5 min in PBS. Next, coverslips were incubated 30 min in BSA (1% in PBS) with Alexa-576 antigoat secondary antibody (1:1000) followed by 3 washes of 5 min in PBS. Coverslips were mounted on slides by using Fluorescent Mounting Medium (Dako) and examined under a microscope. Images were captured with Carl Zeiss Axiovision software. **Statistics.** Values are expressed as the mean $\pm$S.E.M. Statistical analysis was performed by using the one-tailed Student's t test for unpaired data, with P values <0.05 considered statistically significant.

Acknowledgements

This work was supported by a grant from the British Heart Foundation and Cancer Research UK. We thank members of the laboratory for helpful discussions.

Keywords: cardiomyocytes · hypertrophy · inhibitors · medicinal chemistry · natural products

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Received: July 16, 2004

Early View Article
Published online on December 9, 2004

