Intrinsic-mediated caspase activation is essential for cardiomyocyte hypertrophy

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Cardiomyocyte hypertrophy is the cellular response that mediates pathologic enlargement of the heart. This maladaptation is also characterized by cell behaviors that are typically associated with apoptosis, including cytoskeletal reorganization and disassembly, altered nuclear morphology, and enhanced protein synthesis/translation. Here, we investigated the requirement of apoptotic caspase pathways in mediating cardiomyocyte hypertrophy. Cardiomyocytes treated with hypertrophy agonists displayed rapid and transient activation of the intrinsic-mediated cell death pathway, characterized by elevated levels of caspase 9, followed by caspase 3 protease activity. Disruption of the intrinsic cell death pathway at multiple junctures led to a significant inhibition of cardiomyocyte hypertrophy during agonist stimulation, with a corresponding reduction in the expression of known hypertrophic markers (atrial natriuretic peptide) and transcription factor activity [myocyte enhancer factor-2, nuclear factor kappa B (NF-κB)]. Similarly, in vivo attenuation of caspase activity via adenoviral expression of the biologic effector caspase inhibitor p35 blunted cardiomyocyte hypertrophy in response to agonist stimulation. Treatment of cardiomyocytes with procaspase 3 activating compound 1, a small-molecule activator of caspase 3, resulted in a robust induction of the hypertrophy response in the absence of any agonist stimulation. These results suggest that caspase-dependent signaling is necessary and sufficient to promote cardiomyocyte hypertrophy. These results also confirm that cell death signal pathways behave as active remodeling agents in cardiomyocytes, independent of inducing an apoptosis response.

The vertebrate heart is structurally complex, yet this organ retains a remarkable ability to adjust intrinsic cell properties to alterations in the exterior milieu. A most critical aspect of this phenomenon is hypertrophic growth of individual cardiomyocytes. This form of cell adaptation is a vital feature that matches physiologic enlargement of the heart to the growth of the organism, yet compensatory hypertrophy is also a prominent feature of cardiac disease. Consequently, disease hypertrophy has been intensely studied, resulting in the identification of a consistent cellular pathology. In general, the pathologic condition derives from an agonist or trigger that stimulates key intracellular signaling pathways, which converge on transcription factors to reengage the fetal gene expression program in cardiomyocytes. Prominent examples of this molecular circuit are agonist-induced mitogen-activated protein kinase (MAPK)/CAMK (Ca^{2+}/calmodulin-dependent kinase) activation of myocyte enhancer factor-2 (MEF2) transcription and calcineurin activation of nuclear factor of activated T cells (NFAT) transcription (1–3).

Despite the success in identifying the key transcription control events during cardiomyocyte hypertrophy, the pathways or proteins that couple the fetal gene expression program with the structural reorganization of the cell remain largely unknown. One notable facet of hypertrophy is the degree to which this cell morphology shares overlapping features of programmed cell death or apoptosis. For example, hypertrophy is characterized by standard hallmarks of programmed cell death, including cytoskeletal reorganization and disassembly, altered nuclear morphology, and enhanced protein synthesis/translation (4, 5). Moreover, although cardiomyocyte hypertrophy is initially adaptive, hypertrophy often transits to a myopathic response that results in dilation, a latter event that is concurrent with an increased incidence of caspase-mediated cell death (6, 7). Therefore, a reasonable supposition is that activation of canonical cell death pathways may contribute to the initiation and/or progression of hypertrophy.

In addition to these general features, a number of proapoptotic agonists have been directly implicated in the development of cardiac hypertrophy, including tumor necrosis factor alpha (TNF-α) and the cognate FAS receptor. Antibody blockade of TNF-α leads to diminished overload-induced hypertrophy (8), and mice with genetic deletion of TNF-α display similar attenuation of an overload-induced hypertrophy response (9). Similarly, Fas ligand activation of the Fas receptor has been noted to prompt the induction of cardiomyocyte hypertrophy, and loss of the Fas receptor in vivo was reported to result in a dramatic reduction in compensatory hypertrophy through an as yet undefined signal (10). Interestingly, the apoptotic endonuclease EndoG has recently been shown to play a critical nonapoptotic role in maintaining mitochondrial bioenergetics, and defects in EndoG have directly been linked to the subsequent development of maladaptive hypertrophy (11).

These phenotypic and biochemical intersections raise the provocative hypothesis that a caspase signaling mechanism may propagate or direct the hypertrophy response in cardiomyocytes. This is not an unreasonable premise, as a growing body of literature has demonstrated that caspase-dependent apoptosis pathways act as essential drivers of cell differentiation, including the differentiation of cardiac progenitor cells (12, 13). Canonical cell

Significance

Cardiac hypertrophy is a pathologic enlargement of the heart, an alteration that leads to contractile dysfunction and eventual organ failure. The hypertrophy phenotype originates from concentric growth of heart muscle cells and shares many biochemical features with programmed cell death, implying a common molecular origin. Here, we show cell-autonomous activation of a mitochondrial cell death pathway during initial stages of muscle cell hypertrophy, a signal that is essential and sufficient to promote hypertrophy. Targeting individual cell death proteins may offer an effective means to limit the initial stage of cardiac disease, and forgo the transition to heart failure.

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death pathways derive from an intrinsic source originating from the mitochondria, or an extrinsic source as mediated by prodeath ligand/receptor interactions. Once engaged, these pathways ultimately converge, activating caspase proteases (such as caspase 3), which cleave vital protein substrates that usher in the cell death response. Here, we demonstrate that agonist-induced cardiomyocyte hypertrophy is associated with and critically dependent on the transient activation of the mitochondrial/intrinsic cell death pathway. Inhibition of the intrinsic pathway (at multiple levels) led to a dramatic attenuation of hypertrophy, whereas small-molecule induction of caspase activation alone was sufficient to recapitulate the phenotypic and biochemical characteristics of cell hypertrophy.

Results and Discussion

Activation of the Intrinsic Cell Death Pathway During Agonist-Induced Cardiomyocyte Hypertrophy. To begin to address the role of caspase-mediated signaling in cardiomyocyte adaptation, we used immunofluorescence microscopy to detect caspase activity during agonist-induced hypertrophy (Fig. 1 and Figs. S1–S3). Given the prominent role of caspase 3 as a mediator of cell fate determination, we initially examined the activity kinetics for this effector protease. Treatment of primary cardiomyocytes with the hypertrophy agonist phenylephrine (PE) resulted in rapid formation of active caspase 3 foci in the cytoplasm and within a nuclear/perinuclear compartment (Fig. 1*A*, *c* and *d*). Similar to PE, isoproterenol (ISO) treatment also resulted in nuclear localized active caspase 3 foci (Fig. 1 *A*, *e* and *f*). Caspase 3 activation and localization was also analyzed for additional hypertrophic agonists, including angiotensin II (AngII) and endothelin 1 (ET1), which displayed a similar caspase 3 activation pattern (Fig. S1 *A*, *c* and *d* and *e* and *f*, respectively). Within only 30 min of PE treatment, the number of cardiomyocytes expressing active caspase 3 increased compared with cells not treated with PE (Fig. S2, green, *e* and *f* vs. *g* and *h*; white arrows indicate nuclear and perinuclear localization patterns). Similar caspase 3 activation and localization was observed at 1 h (Fig. 1 *A*, *a* and *b* vs. *c* and *d*) and 3 h (Fig. S2, *i* and *j* vs. *k* and *l*) of PE exposure. Following 24 h of PE treatment, the caspase 3 activation pattern was detectable yet diminished, with limited cytoplasmic and perinuclear/nuclear activity (Fig. S2, green, *m* and *n* vs. *o* and *p*). Caspase 3 localization was quantified based on the number of cardiomyocytes expressing nuclear activated caspase 3 and the number of active caspase 3 foci per nuclei (Fig. 1*A*). A significant increase in the number of nuclei with active caspase 3 as well as total foci number was observed during agonist-induced hypertrophy (Fig. 1*A* and Fig. S1*A*). Furthermore, caspase 3 nuclear specificity was demonstrated by *z*-stack confocal imaging (Fig. S4). The focal activity pattern for caspase 3 suggested that the cardiomyocyte may restrict the protease activity to discrete compartments to engage nonapoptotic cellular functions. Indeed, otherwise lethal caspase activity can be refocused and restrained to effect targeting of particular cellular structures during remodeling and differentiation (14–16). Preliminary experiments indicate that caspase 3 activity in the total soluble protein fraction/lysate does not change during PE-induced hypertrophy (Fig. S5), suggesting that the active protease may be directed to a unique insoluble compartment within the cell.

α-Adrenergic stimulation via PE exposure has been reported to alter mitochondrial calcium leak and membrane potential (17, 18). As such, we reasoned that the source of caspase 3 activation during hypertrophy stimulation may originate from transient activation of the intrinsic/mitochondrial cell death pathway rather than the ligand-associated extrinsic-mediated cell death pathway. Caspase 9 activity represents an immediate downstream proxy for mitochondrial-derived signals whereby, in response to an apoptogen, the mitochondria releases cytochrome *c* (cyt *c*), promoting activation of procaspase 9 within the multiprotein apoptosome complex. Immunofluorescence analysis revealed a caspase 9 activation pattern as early as 15 min post hypertrophic induction (Fig. S3 *A*, *a* and *b* vs. *c* and *d*), with a peak of activation at 1 to 3 h after PE exposure in healthy, intact cardiomyocytes (Fig. 1 *B*, *a* and *b* vs. *c* and *d*; and Fig. S3 *A*, *m* and *n* vs. *o* and *p*). Additional hypertrophic agonists ISO (Fig. 1 *B*, *a* and *b* vs. *e* and *f*; and Fig. S3*A*), AngII (Fig. S1 *B*, *a* and *b* vs. *c* and *d*; and Fig. S3*B*), and ET1 (Fig S1 *B*, *a* and *b* vs. *e* and *f*; and Fig. S3*B*) showed similar caspase 9 activation patterns. With all hypertrophic agonists, caspase 9 activity was dispersed throughout the cell, reminiscent of a typical mitochondrial distribution pattern. Activation of the intrinsic (caspase 9-mediated) pathway was further confirmed by measuring the drop in mitochondrial membrane potential, which is an early essential step in this pathway. PE and ISO treatments led to a similar induction of the intrinsic pathway as early as 5 min after hypertrophic induction (see number symbols in Fig. 1 *C*, *Left* and *Right*, respectively). Together, these data demonstrate that agonist-induced hypertrophy leads to activation of the intrinsic cell death signal pathway in a temporally restricted manner.

It is important to note that this initiation signal (caspase 9 and caspase 3 activation) is not followed by induction of apoptosis per se. Extensive analysis of cardiomyocyte viability during agonist-induced hypertrophy was conducted by annexin V/propidium iodide (PI) labeling, TUNEL labeling of apoptosis-induced DNA damage, and analysis of nuclear integrity via DAPI staining (Fig. 1*D*). These efforts indicate that there is no significant increase in apoptotic cell death during hypertrophic agonist stimulation.

Activation of the Intrinsic Cell Death Pathway Is Required for Cardiomyocyte Hypertrophy. Next, we investigated the requirement of the intrinsic mediated cell death pathway for mediating the hypertrophy response. Presumably, if the agonist-induced hypertrophy response required sequential activation of the intrinsic pathway, inhibition of this signal should reduce or block hypertrophy. Primary cardiomyocytes were treated with PE in the presence or absence of the caspase 9 peptide inhibitor *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (z-LEHD-fmk). A significant attenuation of cardiomyocyte cell size (~39%) was noted after caspase 9 inhibition (Fig. 2 *A*, *b* vs. *c*). In addition to cell size, a significant reduction in the expression of the prohypertrophic marker atrial natriuretic peptide (ANP; ~62%) (19) was also observed following caspase 9 inhibition (Fig. 2 *A*, *e* vs. *f*). Although caspase 9 represents an accurate proxy for intrinsic pathway activation, we sought to confirm the mitochondria as the de facto initiation signal. Here, the intrinsic pathway was inhibited during agonist stimulation by infection with an adenovirus (AdV) expressing the myeloid cell leukemia 1 (Mcl-1) protein. Mcl-1 binds to various proapoptotic Bcl-2 homology 3 (BH3)-only proteins such as Bid and Bim and multiple BH domain proteins Bax and Bak, inhibiting BH3 pore formation in the mitochondrial membrane, squelching cyt *c* release, and effectively blocking activation of the intrinsic apoptotic pathway (20, 21). A significant decrease in cell size (~33%) and ANP levels (~61%) was noted in Mcl-1-AdV–infected cardiomyocytes compared with PE treatment alone (Fig. 2 *B*, *b* and *h* vs. *c* and *i*). ISO treatment (Fig. 2 *C*, *b* and *h* vs. *c* and *i*) and additional hypertrophic agonists, AngII and ET1, yielded similar reductions in cell size and ANP levels (Fig. S6). The decrease in cell size and ANP levels resulting from Mcl-1-AdV infection mirrors the observations with caspase 9 peptide inhibition, suggesting an essential role for the intrinsic cell death pathway in promoting hypertrophic induction.

Effector Caspase/Caspase 3 Activation Is Essential for Cardiomyocyte Hypertrophy in Vitro and in Vivo. We next examined the requirement of caspase 3 activity in the development of the hypertrophy response. Primary cardiomyocytes were treated with the caspase 3-specific peptide inhibitor N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), followed by cell size and ANP quantifications. The PE-induced hypertrophic pheno-

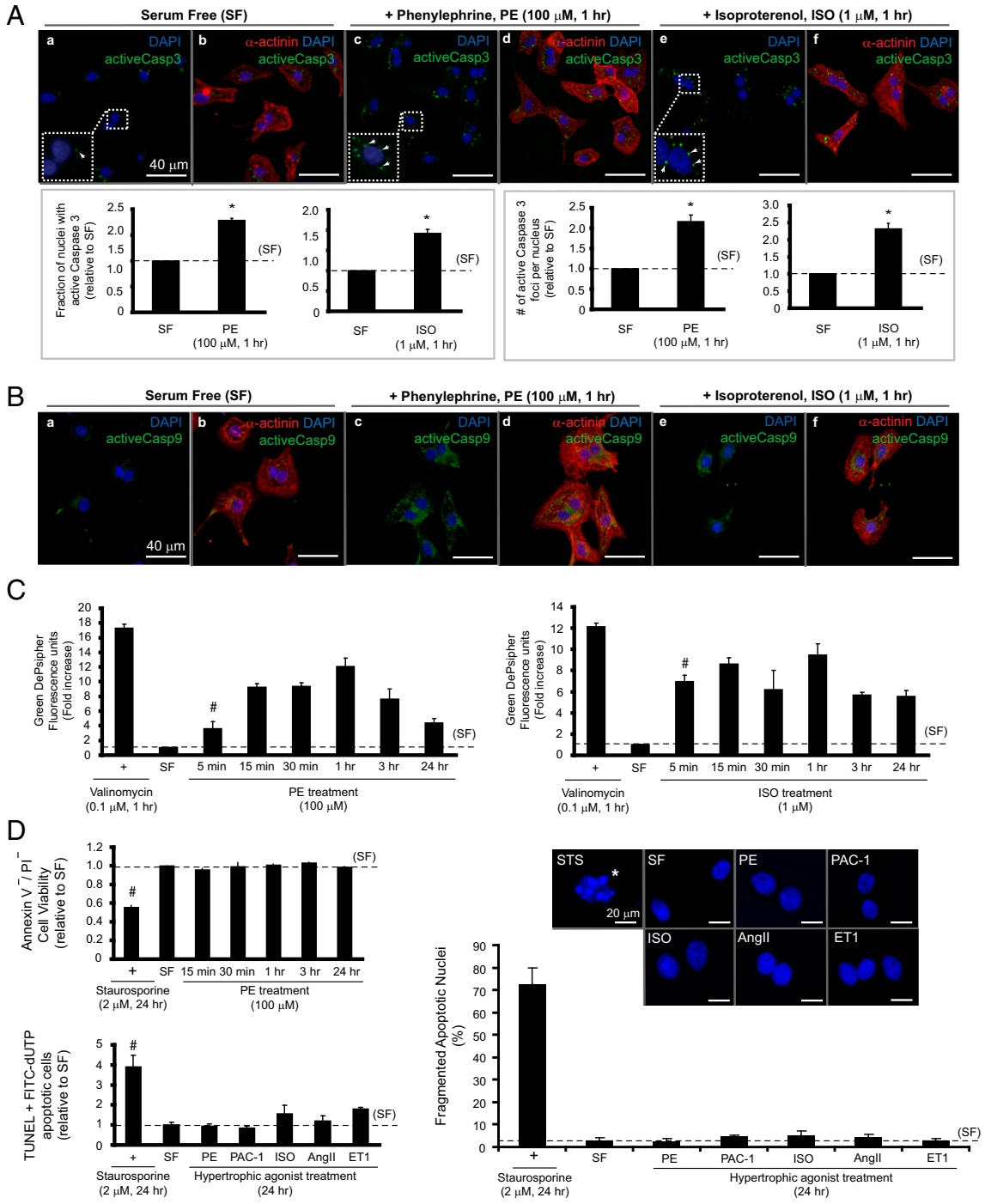

Fig. 1. Increased caspase 3 and 9 activation during early stages of PE- and ISO-induced cardiomyocyte hypertrophy. (*A*) Cardiomyocytes were treated with hypertrophic agonists PE (100 μM; *c* and *d*) or ISO (1 μM; *e* and *f*) to induce hypertrophy for 1 h. Immunofluorescence was used to detect activated caspase 3 (green), α-actinin (red), and DAPI (blue). Exposure to PE or ISO for only 1 h resulted in a significant increase in the number of cardiomyocytes expressing activated caspase 3 (green; PE, *c* and *d*; ISO, *e* and *f*) compared with SF media-treated cells (*a* and *b*). Activated caspase 3 displayed localization to the perinuclear and nuclear regions during hypertrophic induction (compare *c* and *d* and *e* and *f* vs. *a* and *b*). Magnified views are shown in boxed regions (white arrows; *a* and *c* vs *a* and *e*). The fraction of nuclei with active caspase 3 and the fraction of active caspase 3 foci per nucleus during PE and ISO treatment significantly increased after 1 h compared with SF controls ($n = 3$; $^{*}P < 0.05$; Figs. S1*A* and S2). (*B*) Cardiomyocytes were treated as in *A* and stained for activated caspase 9 (green). Caspase 9 activation was readily detected during PE and ISO treatment (green; *c* and *d* and *e* and *f*) compared with SF media-treated control cells (*a* and *b*; Figs. S1*B* and S3). (Scale bars: 40 μm.) (*C*) Activation of the intrinsic (caspase 9-mediated) pathway was also evaluated by use of the DePsipher kit. Green DePsipher fluorescence was quantified during PE- and ISO-induced hypertrophy from 5 min to 24 h. A significant increase in green fluorescence was observed after only 5 min of hypertrophic induction, suggesting early intrinsic pathway activation ($n = 3$; $^{\#}P < 0.05$). (*D*) Caspase activation during agonist-induced hypertrophy occurs in viable cardiomyocytes. Cardiomyocyte viability was evaluated by analysis of annexin V/PI double-negative cell population, fragmented apoptotic nuclei quantifications (asterisk), and TUNEL assay. Staurosporine treatment led to a significant reduction in cardiomyocyte viability ($n = 3$; $^{\#}P < 0.05$). Cardiomyocyte viability remained intact after 24 h hypertrophic induction with PE (100 μM), PAC-1 (25 μM), ISO (1 μM), AngII (100 nM), and ET1 (20 nM). (Scale bars: 20 μm.)

type was abrogated by caspase 3 inhibition, as demonstrated by reduced cell size (~36%) and ANP levels (~42%; Fig. 3 *A*, *b* and *e* vs. *c* and *f*). Caspase 3 inhibition was also accomplished by infection of primary cardiomyocytes with an AdV expressing the baculoviral protein p35 (22). p35 acts to competitively inhibit effector caspases by an irreversible chemical process. The caspase recognition sequence of p35 is targeted by the effector caspase (3, 6, 7), forming a thioester bond between the active cysteine and the P_1 residue, resulting in a completely disabled enzyme (23). p35 is the most potent inhibitor of caspases identified to date, providing an irreversible inhibition of all caspase enzymes except caspase 9 (23, 24). Importantly, the infection of cardiomyocytes with p35-AdV led to a similar reduction in the hypertrophic response following exposure to PE and ISO (decreased ANP levels in Fig. 3*B*), as well AngII and ET1 (decreased ANP and cell size in Fig. S7 *A–C*). Finally, we demonstrate that, within the same cardiomyocyte culture, cells that are infected with caspase signaling inhibitors (p35-AdV) do not respond to hypertrophy agonists, whereas the uninfected cardiomyocytes in the same culture undergo hypertrophy (Fig. S8). Taken together, these data confirm that secondary paracrine effects of caspase activity in adjacent apoptotic cells do not significantly impact the hypertrophy response.

To further confirm the caspase 3-induced hypertrophy response, we measured the transcriptional response of known prohypertrophic markers [MEF2, NF-κB, ANP, (B-type natriuretic peptide (BNP)]. Primary cardiomyocytes were transfected with MEF2, NF-κB, ANP, or BNP plasmids (with a *Renilla* luciferase internal control plasmid) and monitored for the response to agonist and caspase activation (SF plus DMSO, PE plus DMSO, PE plus z-DEVD-fmk or z-LEHD-fmk). PE treatment resulted in a significant increase in all hypertrophic markers compared with cardiomyocytes treated with SF plus DMSO media (Fig. 3*C*), whereas PE treatment followed by caspase 3 or caspase 9 inhibition led to a dramatic reduction in reporter activation compared with PE plus DMSO treatment (Fig. 3*C*).

We next sought to identify a molecular cascade whereby caspase 3 activity engages the hypertrophy response. In this regard, the MEF2 pathway was of considerable interest. The MEF2 family functions as a point of convergence for a variety of hypertrophic signals, including the fetal gene program (3, 25). MEF2 transcriptional activity is suppressed by the binding of histone deacetylases (HDACs), and relief of this repression has been well documented as a key step in the hypertrophic response (3). Of interest, caspase 3 has been shown to cleave HDAC3 and HDAC4, resulting in their cytoplasmic relocalization during apoptotic signaling (26–28). Moreover, cardiac-specific deletion of HDAC3 is synonymous with a pronounced cardiac hypertrophy phenotype (29). To address our supposition that caspase 3 activity is restrained in insoluble foci, we first examined levels of activated caspase 3 in the soluble and insoluble fractions. We observed a significant increase in the level of active caspase 3 within the insoluble fraction of PE-treated cardiomyocytes as early as 30 min, followed by a transient decrease and then a latter-stage increase in activity (Fig. S7*D*). We observed a specific and temporal decline in levels of full-length HDAC3 at early time points following PE-induced hypertrophy in the soluble fraction, whereas HDAC4 protein levels remained unchanged (Fig. S7*D*). Furthermore, infection of cardiomyocytes with p35-AdV blocked the temporal decline in HDAC3 protein levels, suggesting that the HDAC3 decrease was caspase 3-dependent (Fig. S7*D*). Finally, we noted a large concentration of full-length HDAC3 (~48 kDa) within the insoluble protein fraction, which was associated with a cleavage event that was attenuated with p35-AdV (Fig. S7*D*, asterisk). The size of the putative fragment (~44 kDa) corresponds to the predicted size of the caspase 3-mediated large C-terminal cleavage fragment of HDAC3 observed by others (26). Interestingly, a classic caspase 3 cleavage site is present at Asp-391 of the C terminus of HDAC3 that would produce this putative large HDAC3 fragment. Prior observations in the literature also suggest that HDAC proteins do segregate and associate with an insoluble fraction (30, 31). Liu et al. noted that Hos2 (yeast HDAC) and Yca1 (yeast metacaspase) were contained within insoluble stress granules (30). Our group has previously demonstrated that the core molecular function of Yca1 is to limit protein aggregate formation in response to stress (16). As such, a reasonable conjecture is that the colocalization of caspase 3 and HDAC3 (and the caspase-mediated processing of HDAC3) may represent an adaptive stress response in cardiomyocytes, a response that has been conserved from single-celled eukaryotes. This is a topic of considerable interest and will require additional experimental validation to confirm this hypothesis.

Consistent with a model of caspase-mediated derepression of MEF2, we also observed that MEF2 transcriptional activity (via an MEF2-dependent reporter assay) was dramatically attenuated (~80%) in cardiomyocytes with impaired caspase activation via p35-AdV during PE stimulation (Fig. 3*D*). An additional hypertrophic transcription signal that may respond to and translate caspase activity is the NF-κB pathway. NF-κB is a transcription factor that responds to stress signaling to promote or enhance cell survival, yet this factor is also a prominent feature in pathological cardiac hypertrophy (32). Interestingly, caspase proteases have been shown to activate NF-κB signaling in a variety of noncardiac settings, steps that involve cleavage-activation of the inhibitor of kappa B kinase (IKK) kinases, as well as targeted cleavage and removal of the inhibitor of kappa B alpha (IκBα), the NF-κB inhibitor protein (33–35). PE-treated cardiomyocytes displayed a significant increase in NF-κB reporter activity, yet caspase inhibition via p35-AdV infection resulted in complete blockade of the NF-κB–induced response (~84%; Fig. 3*D*). These results suggest that the hypertrophy-associated engagement of NF-κB activity is caspase 3-responsive, yet we have been unable to identify the precise caspase 3 substrate that is targeted in this pathway.

Caspase 3-mediated induction of cardiomyocyte hypertrophy was also examined in the intact myocardium. PE control and experimental groups were subject to ultrasound-guided microinjection of the left ventricle wall with control GFP-AdV or p35-AdV expressing AdVs. After 3 d, osmotic minipumps were implanted in rats containing saline solution (control) or PE (PE control and experimental groups) to induce pathologic hypertrophy. Subsequently, hearts were retrieved at 3 wk after treatment, to coincide with the early stages of adaptive hypertrophy. Hearts treated with PE and infected with p35-AdV displayed a significant reduction in heart weight to body weight (HW/BW) ratio (~12%) and cardiomyocyte cell size (~37%) compared with the PE-treated GFP-AdV–infected group (Fig. 4*A*). Similarly, ISO-induced hypertrophy in vivo (2 wk) was significantly blunted in the presence of p35-AdV, with a decreased HW/BW ratio (~12%) and cardiomyocyte cell size (~42%) compared with the ISO-treated GFP-AdV–infected group (Fig. 4*B*). Therefore, similar to the in vitro experimental observations, inhibition of effector caspase activity (caspase 3) attenuates cardiomyocyte hypertrophy in the intact heart.

Caspase 3 Activation Is Sufficient to Induce Cardiomyocyte Hypertrophy. The combination of peptide and biologic inhibitor experiments demonstrate that intrinsic-mediated cell death signaling is required for the development of cardiomyocyte hypertrophy. As such, we next examined whether caspase activation was sufficient to engage hypertrophy, independent of any agonist stimulation. To test this premise, we used a small molecule that specifically stimulates caspase 3 activation, termed procaspase 3 activating compound 1 (PAC-1). PAC-1 is a potent and specific activator of procaspase 3 that acts via sequestration of the inhibitory zinc ions at the catalytic site on the pro form of caspase 3 (36, 37). As unrestricted caspase 3 activation leads to induction of apoptosis, we conducted a dose range experiment to establish

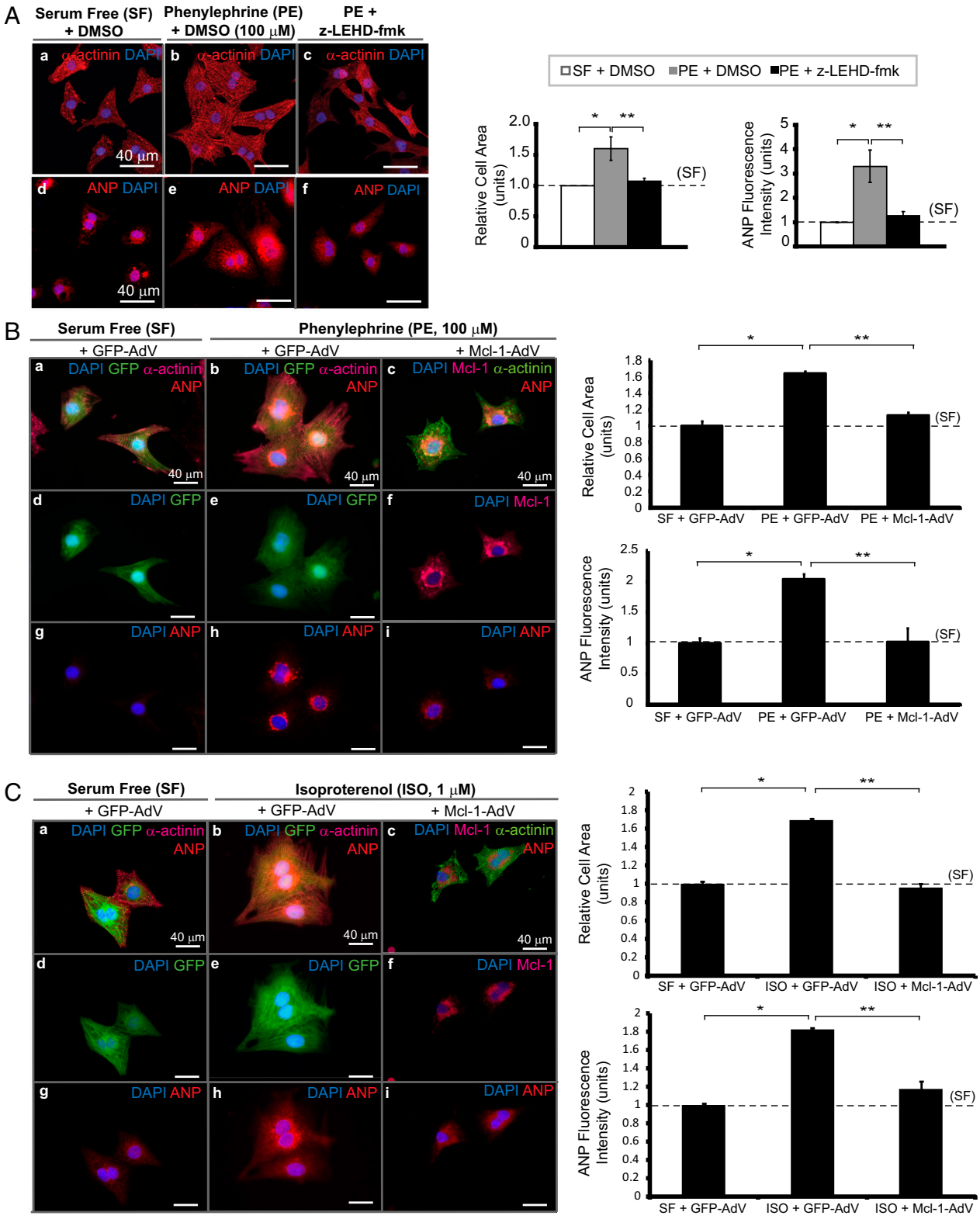

Fig. 2. The intrinsic mitochondrial/caspase 9 cell death pathway is required for PE- and ISO-induced cardiomyocyte hypertrophy. (*A*) Cardiomyocytes were treated for 24 h with SF medium (*a* and *d*), 100 μM PE (*b* and *e*), and PE plus 20 μM caspase 9 inhibitor z-LEHD-fmk (*c* and *f*). PE treatment increased cell size, which was significantly attenuated in the presence of z-LEHD-fmk (*Left*; $n = 3$, $^{**}P < 0.05$). ANP levels were significantly reduced in the presence of z-LEHD-fmk compared with PE-treated cells (*Right*; $n = 3$, $^{**}P < 0.05$). (*B*) Mcl-1-AdV inhibition of the intrinsic caspase 9 pathway, with GFP-AdV used as a control. Infection with Mcl-1-AdV significantly decreased cell size and ANP levels compared with PE plus GFP-AdV–treated cardiomyocytes ($n = 3$; $^{**}P < 0.05$). (*C*) Cardiomyocytes were treated as in *B* except ISO was the hypertrophic agonist (1 μM). Similarly, intrinsic pathway inhibition by Mcl-1-AdV infection led to significantly reduced cell size and ANP levels ($n = 3$; $^{**}P < 0.05$). For *B* and *C*, Mcl-1 expression was detected with anti-myc antibody and an Alexa 647 (pink)-conjugated secondary antibody (Fig. S6). Cell sizes analyzed by using ImageJ software. (Scale bars: 40 μM.)

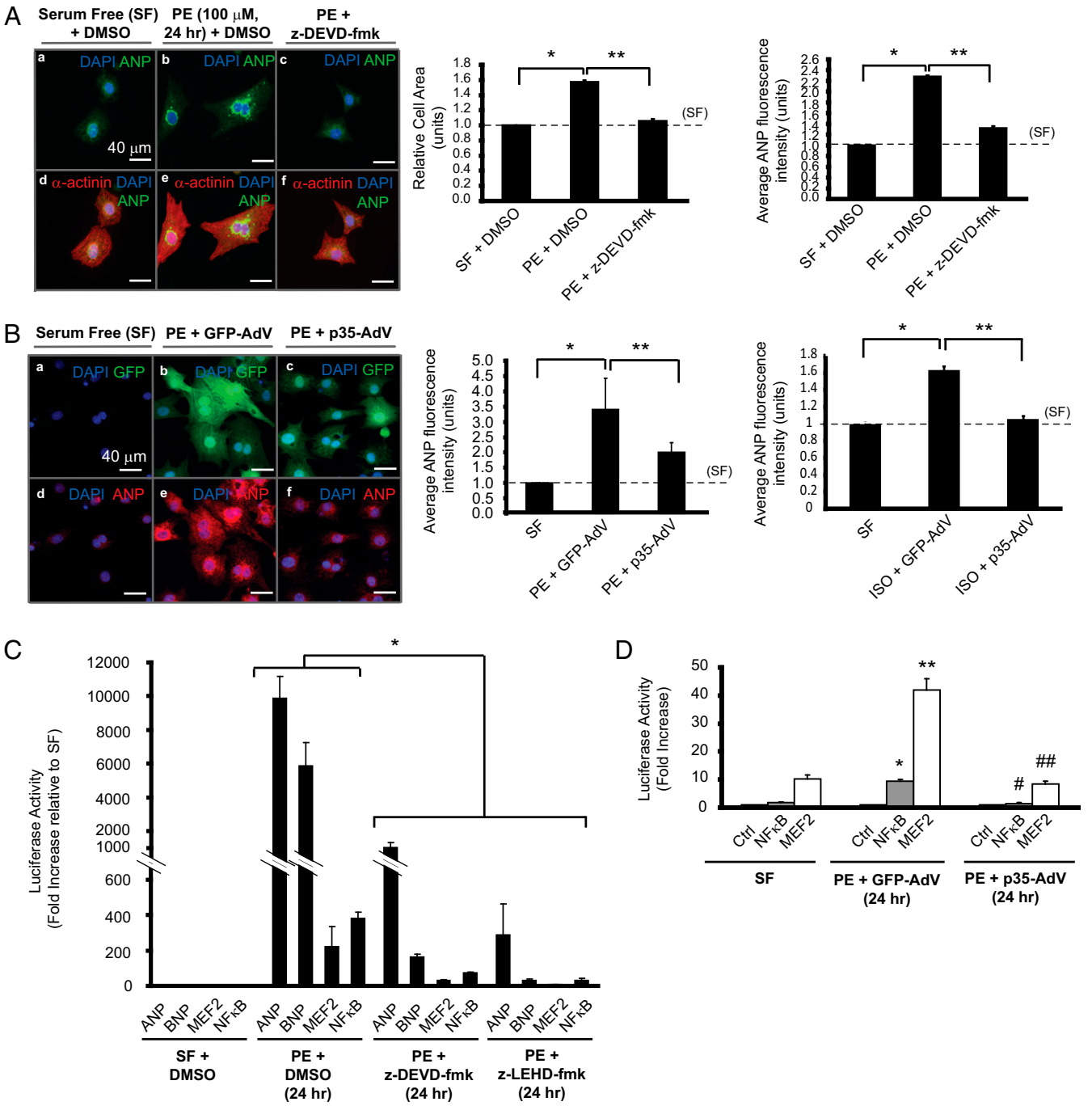

Fig. 3. Caspase 3 activation is required for PE- and ISO-induced cardiomyocyte hypertrophy. (*A*) Cardiomyocytes were treated for 24 h with SF medium (*a* and *d*), 100 μM PE (*b* and *e*), and PE plus 20 μM caspase 3 inhibitor z-DEVD-fmk (*c* and *f*). PE induced a significant increase in cell size, which was inhibited by z-DEVD-fmk (*Left*; $n = 3$; $^{**}P < 0.05$). PE also increased the levels of ANP, whereas the presence of z-DEVD-fmk reduced ANP expression (*Right*; $n = 3$; $^{**}P < 0.05$). (*B*) Cardiomyocytes were treated with SF medium (*a* and *d*), 100 μM PE plus GFP-AdV (*b* and *e*), and PE plus p35-AdV (*c* and *f*). During PE treatment, a significant increase in ANP was detected, whereas p35-AdV decreased ANP levels (*Left*; $n = 3$; $^{**}P < 0.05$). p35-AdV infection also led to significantly reduced ANP levels during ISO-induced hypertrophy (*Right*; $n = 3$, $^{**}P < 0.05$; Fig. S7). (*C*) Cardiomyocytes were transfected with luciferase reporter plasmids for prohypertrophic markers (ANP, BNP, MEF2, and NF-κB), and reporter activity was measured after treatments with SF plus DMSO, PE plus DMSO, and PE plus z-DEVD-fmk or z-LEHD-fmk inhibitors for 24 h. PE plus z-DEVD-fmk or z-LEHD-fmk inhibition led to a significant reduction in PE-induced reporter activation ($n = 3$; $^{*}P < 0.05$). (*D*) Cardiomyocytes were transfected with NF-κB, MEF2, or control luciferase hypertrophic reporter plasmids followed by infection with GFP-AdV or p35-AdV. PE-induced hypertrophy resulted in a significant increase in NF-κB ($n = 3$, $^{*}P < 0.05$) and MEF2 ($n = 3$, $^{**}P < 0.05$) activation compared with SF control. Caspase inhibition with p35-AdV infection suppressed NF-κB ($n = 3$, $\#P < 0.05$) and MEF2 ($n = 3$, $\#\#P < 0.05$) activation. (Scale bars: 40 μm.)

a concentration of PAC-1 that was compatible with maintaining cell viability. Primary cardiomyocytes treated with high doses of PAC-1 (100 μM) displayed decreased viability (i.e., increased cell death) relative to control cells, an outcome that was comparable to a standard apoptotic treatment with staurosporine (Fig. S9). However, low-dose PAC-1 administration (12.5–25 μM) maintained cell viability (i.e., no significant decrease in cell viability) while inducing cardiomyocyte hypertrophy, a response that was

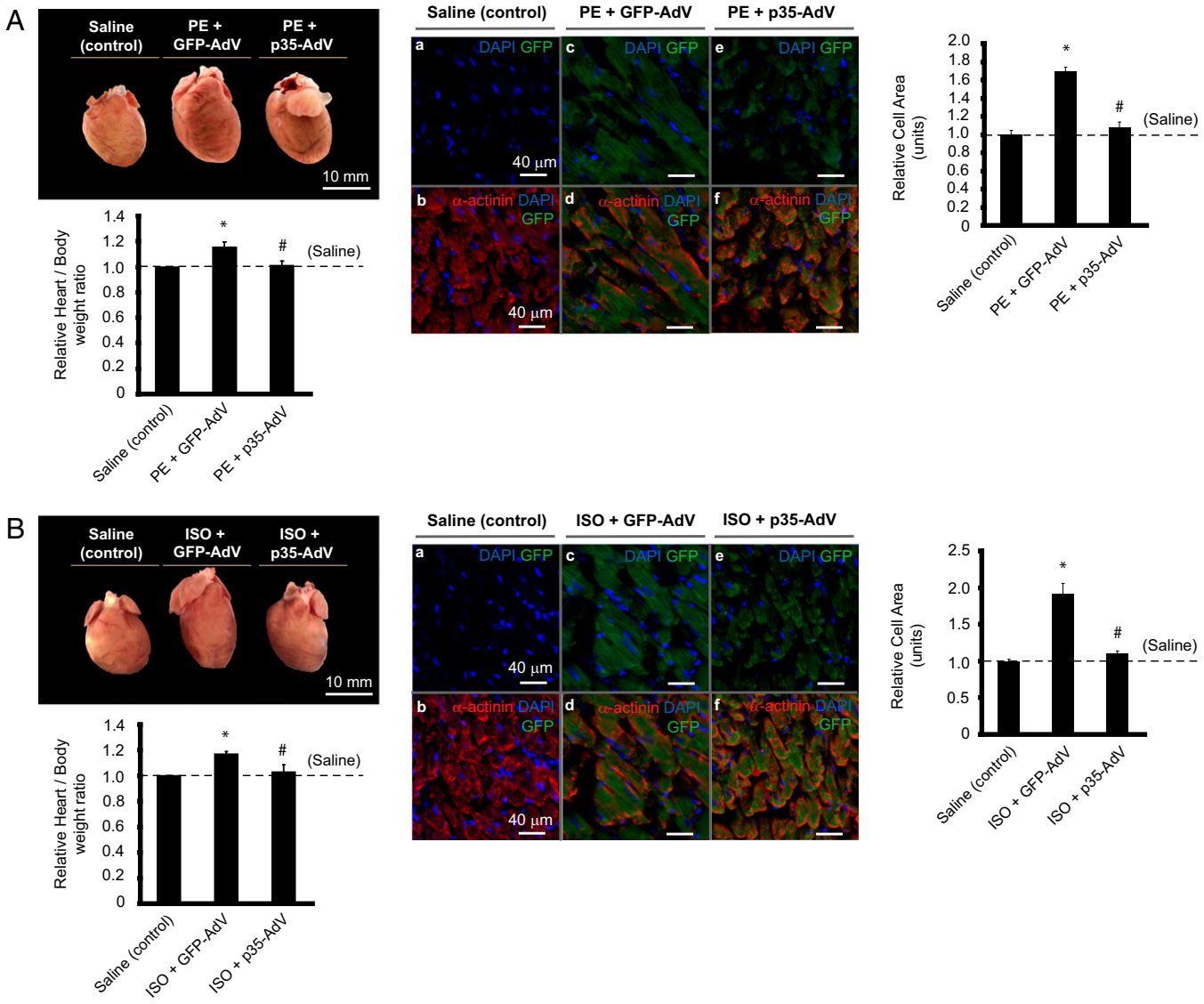

Fig. 4. Inhibition of caspase 3 activity attenuates cardiomyocyte hypertrophy in the intact myocardium. (*A*) Osmotic minipumps were implanted in rats containing saline solution (control) or PE (PE control and experimental) to induce hypertrophy over 3 wk at 10 mg/kg/d. PE control and experimental groups were subjected to GFP-AdV or a GFP tagged p35 expressing AdV (p35-AdV) infection via echo-directed microinjections into the ventricle wall. After 3 wk, whole hearts were isolated, cryosectioned, and stained for α-actinin (red) and DAPI (blue). PE induced an increase in HW/BW ratio of 15% and an increase in cardiomyocyte cell area of 69% compared with control ($n = 3$; $^{*}P < 0.05$) whereas p35-AdV significantly reduced these effects ($n = 3$; $^{\#}P < 0.05$). (*B*) Hypertrophy was induced (as described earlier) using ISO at 1 mg/kg/d for 2 wk. HW/BW ratio and cell size were increased (17% and 90%, respectively) compared with control ($n = 3$; $^{*}P < 0.05$), and p35-AdV treatment led to a significant reduction in these hypertrophic effects ($n = 3$; $^{\#}P < 0.05$). Cell area of GFP (green)-positive cardiomyocytes was evaluated by using ImageJ software. (Scale bars: 40 μm.)

comparable with the hypertrophic agonist PE (Fig. S9 and Fig. 5*A*). Specifically, cardiomyocytes treated with low PAC-1 concentrations (25 μM, 24 h) displayed a significant increase in cell size (~65%) and ANP expression (approximately threefold; Fig. 5 *A*, *a* and *f* vs. *c* and *h*). To further validate the PAC-1/caspase 3-induced hypertrophy response, we measured the transcriptional response of known prohypertrophic markers (MEF2, NF-κB, ANP, BNP) after 1 h, 3 h, and 24 h of PAC-1 (25 μM) treatment. Similar to PE treatment, PAC-1 administration resulted in a significant increase in all hypertrophic markers compared with cardiomyocytes treated with SF plus DMSO media as early as 1 h after exposure to PAC-1 (Fig. 5*B*, *Left*). After 3 h of PAC-1 treatment, significant elevations in reporter activity were observed which were comparable to those following PE exposure (Fig. 5*B*, *Center*). Significant reporter activity after 24 h of PAC-1 treatment was also observed (Fig. 5*B*, *Right*). The differences in the fold activation noted at the 24-h time point in PE treated vs. PAC-1–treated cardiomyocytes may derive from the probability that PE engages additional signaling events, signals that extend beyond the intrinsic cell death pathway and that converge to amplify the hypertrophic gene expression program.

Finally, to confirm specificity of action for PAC-1, we conducted two additional experiments. Assuming PAC-1 stimulates cardiomyocyte hypertrophy through a strict activation of caspase 3, then (*i*) use of an irreversible inhibitor of caspase 3 activity should block the ability of PAC-1 to induce hypertrophy, and (*ii*) limiting activation of the intrinsic pathway should not impact the ability of PAC-1 to promote hypertrophy. Importantly, a significant reduction in cell size (~39%) and ANP expression (~56%) was observed in cardiomyocytes treated with the caspase 3 inhibitor during PAC-1 administration (Fig. 5 *A*, *c* and *h* vs. *d* and *i*). Conversely, caspase 9 inhibition was not able to significantly

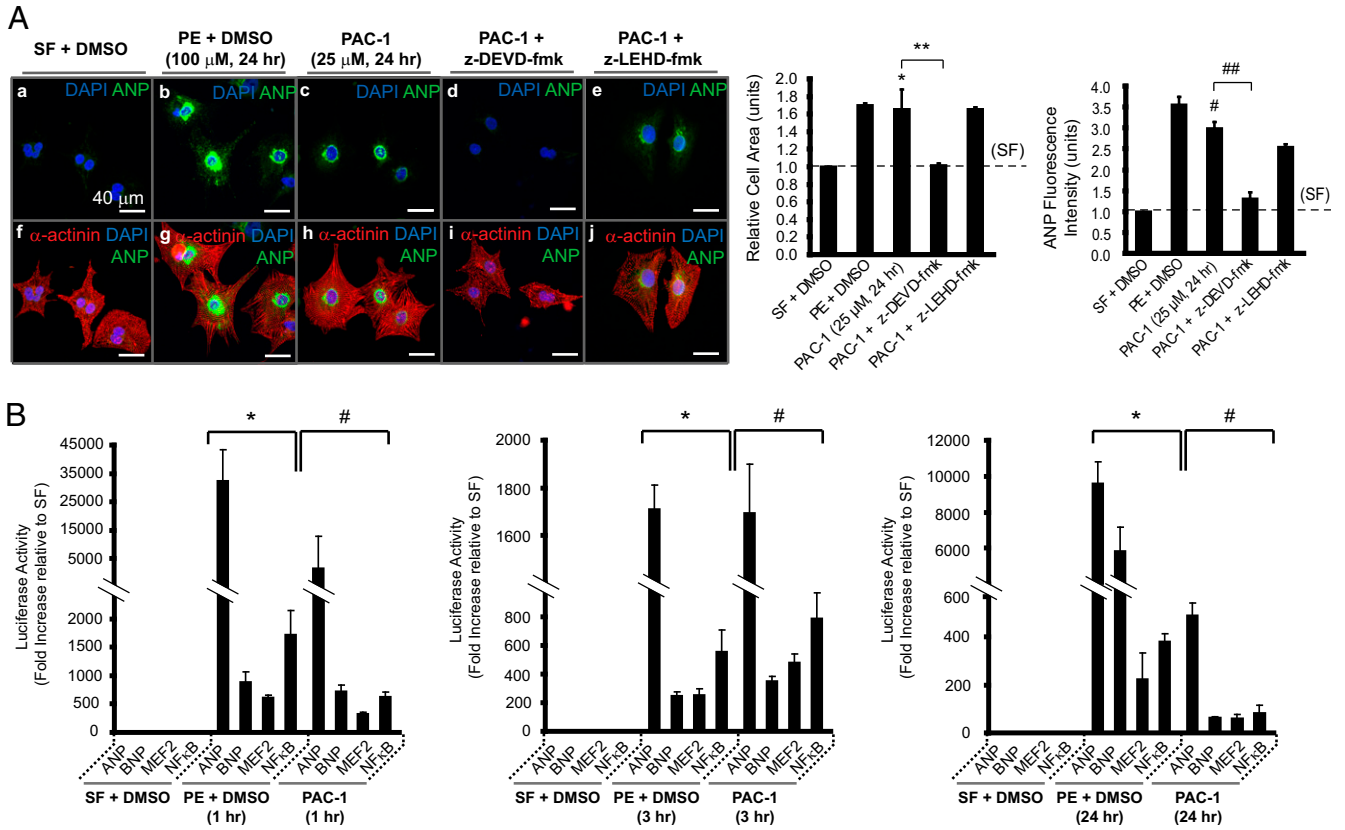

Fig. 5. Caspase 3 activation is sufficient to induce cardiomyocyte hypertrophy. (*A*) Cardiomyocytes were treated with SF media (*a* and *f*), PE (100 μM; 24 h; *b* and *g*), PAC-1 (25 μM; 24 h; *c* and *h*), PAC-1 and caspase 3 inhibitor z-DEVD-fmk (*d* and *i*), or PAC-1 and caspase 9 inhibitor z-LEHD-fmk (*e* and *j*). Cells were stained for α-actinin (red), ANP (green), and DAPI (blue). PAC-1 treatment resulted in a significant increase in cell area and ANP levels, whereas inhibition with z-DEVD-fmk resulted in a significant reduction in cell size ($n = 3$, $^{**}P < 0.05$) and ANP levels ($n = 3$, $^{\#\#}P < 0.05$). Conversely, caspase 9 inhibition was not able to significantly reduce the hypertrophic phenotype induced by PAC-1 treatment (compare *c* and *h* vs. *e* and *j*). (Scale bars: 40 μm.) (*B*) The transcriptional response of known prohypertrophic markers was evaluated following 1, 3, and 24 h of PAC-1 treatment. Cardiomyocytes were transfected with luciferase reporter plasmids for prohypertrophic markers (MEF2, NF-κB, ANP, and BNP), and reporter activity was measured after treatments with SF, PE, and PAC-1. PAC-1 treatment resulted in a significant increase ($n = 3$; $^{\#}P < 0.05$) in all hypertrophic markers compared with SF media during early (1–3 h) and late (24 h) time points.

reduce the hypertrophic phenotype induced by PAC-1 treatment, demonstrating that PAC-1 activates caspase 3 downstream of caspase 9 (Fig. 5 *A*, *c* and *h* vs. *e* and *j*). These results establish that small molecule induction of caspase 3 activation is sufficient to recapitulate the phenotypic and biochemical characteristics of cardiomyocyte hypertrophy, independent of any agonist costimulation. To address the involvement of nonspecific protease activity or other calcium activated proteases as probable contributors to caspase induction of hypertrophy, we analyzed calpain activity during PE- and PAC-1–induced hypertrophy in the presence of caspase and calpain inhibitors (p35-AdV and calpastatin, respectively; Fig. S7*E*). Neither PE stimulation nor PAC-1 treatment of primary cardiomyocytes leads to any appreciable increase in calpain activity at later time points in the hypertrophy cycle (Fig. S7*E*). Although our observations do not preclude a role for calpains in the hypertrophic remodeling process, the data and reagents used in this study demonstrate a caspase specific role that cannot be attributed directly to calpain activity.

The present study establishes the intrinsic/mitochondrial cell death pathway as a central conduit for adrenergic-induced cardiomyocyte hypertrophy. To date, cell death signal pathways have been associated with the transition to end-stage heart failure. For example, long-term caspase inhibition has been reported to reduce cardiomyocyte apoptosis, attenuate cardiac remodeling, while preserving myocardial function in models of cardiomyopathy and pressure overload (6, 38–40). Here, our observations indicate that elevated caspase 3 activity is an early and essential step in the promotion of cardiomyocyte hypertrophy, a cellular response that is not associated with the apoptosis program per se. The ability of caspase signaling to induce hypertrophy or apoptosis may appear to be an incompatible feature for a single pathway, yet may simply reflect alterations in signal intensity or duration. In such a model, transient and low-level activation of intrinsic signaling would be predicted to promote the hypertrophic adaptation, whereas sustained and high level activation would result in apoptosis. Indeed, a similar dichotomy has been reported for many neurohormonal agonists that trigger adrenergic receptor signaling events in the myocardium (e.g., PE, norepinephrine, AngII, and ET1). AdV expression of WT Gαq has been reported to induce compensatory/adaptive hypertrophic growth in cultured cardiomyocytes at 24 h; however, expression of a constitutively active Gαq mutant produced hypertrophy, which rapidly progressed to apoptotic cell death (41). These observations were supported by in vivo studies where transgenic mice overexpressing WT Gαq developed compensatory hypertrophy that rapidly transitioned to progressive heart failure (41, 42). Although these studies did not investigate the requirement for caspase activity, adrenergic stimulation of intrinsic cell death signaling may explain the accelerated progression from hypertrophy to failure.

We favor the hypothesis that caspase 3 cleavage inactivation of MEF2 and NF-κB inhibitory proteins releases the respective transcription factors to induce the cardiomyocyte hypertrophy program. Nevertheless, it is reasonable to conclude that other caspase-related signaling events augment the hypertrophy tran-

sition. Caspase activity can be localized to promote dismantling of specific subcellular structures during cellular differentiation and remodeling (13, 14). In the context of prohypertrophic remodeling, localized/restricted caspase activity may trigger the dynamic reorganization of cytoskeletal and contractile protein components, thus allowing for the subsequent addition to and growth of sarcomeres (43). Communal et al. (43) have reported that β-adrenergic stimulation via norepinephrine exposure in adult rat ventricular cardiomyocytes triggered a caspase 3 targeting of α-actin, α-actinin, and cardiac troponin T before the onset of apoptosis. More recently, other sarcomeric proteins have been shown to be direct caspase targets in apoptotic models including, myosin heavy chain and ventricular myosin light chain (44–46). The ability of caspase 3 to directly modify cellular structure while altering the activity of key gene expression programs implies that this protease (and the intrinsic pathway) may serve as a fulcrum for agonist-induced hypertrophy in general.

In summary, we have provided clear evidence that the mitochondrial/intrinsic caspase-mediated pathway is essential for cardiomyocyte hypertrophy. Currently, antagonists of the upstream adrenergic receptors (e.g., β-blockers) are one of several mainstay treatments of heart failure (47). Based on our results, targeting downstream components of the intrinsic pathway, specifically caspase 3 or 9 protease function, may allow for therapeutic intervention in patients at the earlier compensatory stage of the disease process, limiting cardiomyocyte cell size and forgoing the maladaptive transition to heart failure.

Materials and Methods

Primary neonatal rat cardiomyocytes were isolated from hearts of 2-d-old Sprague–Dawley rats, and ventricles were digested with collagenase II. Subsequently, cardiomyocytes were resuspended in DMEM. Cells were allowed to recover in DMEM culture media for 24 h, followed by a change to serum-free (SF) media for another 24 h before cardiomyocyte treatments with hypertrophic agonists: PE (100 μM), ISO (1 μM), AngII (100 nM), or ET1 (20 nM). Caspase 3 and 9 signaling was inhibited by using 20 μM of a chemical peptide inhibitor (z-DEVD-fmk and z-LEHD-fmk, respectively). Inhibition of the intrinsic and effector caspase pathways was accomplished by infecting primary cardiomyocytes with AdVs expressing the biological caspase inhibitors (Mcl-1-AdV and p35-AdV, respectively).

Additional details regarding materials and methods are provided in *SI Materials and Methods*.

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