Cardiomyocyte hypertrophy is a prominent feature of the heart’s response to biomechanical strain and neurohumoral stimuli. Initiation of the hypertrophic response involves the activation of multiple signaling pathways, including calcineurin, mitogen-activated protein kinases, Akt, and glycogen synthase kinase (GSK)-3β. Recent studies suggest that nuclear factor (NF)-κB signaling also plays a role in cardiomyocyte hypertrophy, and inhibition of NF-κB signaling can attenuate cardiomyocyte hypertrophy in response to phenylephrine (PE) or endothelin-1 (ET-1) stimulation in vitro. However, inhibition of NF-κB in cardiomyocytes by overexpression of nondegradable IκBα sensitizes cardiomyocytes to apoptosis, raising concerns that NF-κB inhibition may have deleterious effects in the heart.

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In the present study, we describe the regulation and biological activity of A20, a feedback inhibitor of NF-κB signaling, in the heart.

Methods

In Vivo Aortic Banding

Biomechanical stress was achieved by banding the ascending aorta in the mouse as previously described.

Recombinant Adenoviruses

Adenoviral vectors (Ad) were generated, propagated, and characterized as previously described. Ad.GFP contains cytomegalovirus-driven expression cassettes for β-galactosidase and green fluorescent protein (GFP). Ad.dnIKKβ(40) and Ad.A20 are structurally similar but encode a kinase-inactive (K44A) IKKβ mutant (a gift from Dr D. Goeddel, Tularik, South San Francisco, Calif) and Flag-tagged human A20 (a gift from Dr V. Dixit, Genentech, South San Francisco, Calif), respectively, instead of β-galactosidase. Cardiomyocytes were infected with adenoviral vectors at a multiplicity of infection (MOI) of 25 to 200.

Immunoblotting

Protein extraction and immunoblotting were performed as previously described.

Reverse Transcription–Polymerase Chain Reaction and Quantitative Reverse Transcription–Polymerase Chain Reaction

RNA was isolated from hearts or cultured cardiomyocytes as previously described. Reverse transcription–polymerase chain reaction (RT-PCR) was performed with A20 primers (forward: TCGTGGCTCTGAAAACCAATG; reverse: GATGGGTCTTCTGAG-1.1-fold, respectively; P<0.05), again paralleling NF-κB activation. Infection of cardiomyocytes with an adenoviral vector (Ad) encoding A20 inhibited tumor necrosis factor-α–stimulated NF-κB signaling with an efficacy comparable to dominant negative inhibitor of κ-B kinase β (dnIKKβ). Ad.dnIKKβ-infected cardiomyocytes exhibited increased apoptosis when they were serum starved or subjected to hypoxia-reoxygenation, whereas Ad.A20-infected cardiomyocytes did not. Expression of Ad.A20 inhibited the hypertrophic response in cardiomyocytes stimulated with phenylephrine or endothelin-1.

Conclusions—A20 is dynamically regulated during acute biomechanical stress in the heart and functions to attenuate cardiac hypertrophy through the inhibition of NF-κB signaling without sensitizing cardiomyocytes to apoptotic cell death. (Circulation. 2003;108:664-667.)

Key Words: hypertrophy • apoptosis • inflammation
previously described.\textsuperscript{12} Amplified product was detected by SYBR1 (vascular cell adhesion molecule [VCAM]-1 and intercellular adhesion molecule [ICAM]-1 or Taqman probe (A20). Fold-change in gene expression was determined as previously described.\textsuperscript{12} Primer sequences were as follows: VCAM-1 forward, GAA GCCGGT CAT-GTCAAGT; VCAM-1 reverse, GAC GTTACCTTTGGA-CAGTTC; ICAM-1 forward, GAATCCAGCCCCCTAATCTGACC; ICAM-1 reverse, CTC CCGTTCAGACAGTTCACC; A20 forward, GGAGACGGGACTTTGCTACGA; A20 reverse, GTGT-GTCTGCTGAGGGCCATT; and Taqman probe, FAM-CGGAACT-GGAATGACGAATGGGCA-BHQ.

Cell Death ELISA
Apoptotic cell death was determined by ELISA for histone-associates DNA fragments, as previously described.\textsuperscript{9} Cardiomyocytes were subjected to serum deprivation (36 hours) or hypoxia-reoxygenation injury (4 hours hypoxia, 2 hours reoxygenation), as previously described.\textsuperscript{9}

Leucine Incorporation
Cardiomyocytes were incubated with [4,5$^3$H]-leucine (1 $\mu$Ci/mL) in the presence or absence of PE (100 $\mu$mol/L) or ET-1 (100 nmol/L) for 36 hours, and leucine incorporation was determined as previously described.\textsuperscript{13}

Northern Blotting
RNA was extracted from cardiomyocytes using the RNeasy kit (Qiagen). RNA (10 $\mu$g) was separated by formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose membrane, and ultra violet-crosslinked. Atrial natriuretic factor (ANF) expression was detected using an ANF probe, kindly supplied by Dr K. Bloch (Massachusetts General Hospital, Charlestown, Mass).

Statistics
All data are from $\geq$3 independent experiments and represented as the mean±SEM. ANOVA was used to determine statistical significance. The null hypothesis was rejected at $P<0.05$.

Results
A20 Is Dynamically Regulated in the Heart In Vivo and in Cardiomyocytes In Vitro
A20 was dramatically upreglated after 3 hours of acute pressure overload but was not detected by RT-PCR in normal hearts or at other time points (Figure 1A). For more accurate quantification, we developed a quantitative RT-PCR assay using an A20-specific Taqman probe, which revealed that A20 mRNA increased 4.3±1.5-fold ($P<0.05$) at 3 hours after banding (relative to sham-operated animals) and returned to near-basal levels by 6 hours. A20 is an NF-$\kappa$B–dependent gene, and its upregulation after pressure overload correlated with phosphorylation and degradation of the inhibitory I$\kappa$B$\alpha$ subunit (Figure 1B). Correspondingly, restoration of I$\kappa$B$\alpha$ expression at 6 hours was associated with a return of A20 to basal levels of expression.

A20 was upregulated in vitro by the hypertrophic agonists PE and ET-1 (2.8±0.6- and 4±1.1-fold, respectively, at 1 hour; $P<0.05$). As observed in vivo, A20 induction corresponded with NF-$\kappa$B activation by PE or ET-1, evident as degradation of I$\kappa$B$\alpha$ and nuclear translocation of the p65–NF-$\kappa$B subunit (Figure 1C).

A20 Inhibits NF-$\kappa$B Signaling in Cardiomyocytes
To examine the biological effects of A20 in cardiomyocytes, we generated a recombinant adenoviral vector expressing A20. Ad.A20 mediated expression of full-length A20 protein of the expected molecular weight in cardiomyocytes (data not shown). A20 expression inhibited degradation of cytosolic I$\kappa$B$\alpha$ and nuclear translocation of the p65–NF-$\kappa$B subunit in tumor necrosis factor (TNF)-$\alpha$–stimulated cardiomyocytes in a manner comparable to dnIKK$\beta$ (Figure 2A). As measured by quantitative RT-PCR, Ad.A20 also inhibited TNF-$\alpha$–induced transcript levels for the NF-$\kappa$B–dependent genes ICAM-1 and VCAM-1 in a dose-dependent manner and with an efficacy equivalent to Ad.dnIKK$\beta$ (data not shown).

A20 Does Not Enhance Cardiomyocyte Apoptosis
Serum starvation is a proapoptotic stimulus in cardiomyocytes.\textsuperscript{14} Consistent with prior studies,\textsuperscript{5} downstream inhibition of NF-$\kappa$B in serum-starved cardiomyocytes by dnIKK$\beta$ increased apoptosis as detected by ELISA for histone-associates DNA fragments\textsuperscript{9} (Figure 2B). A similar phenomenon was observed in cardiomyocytes after hypoxia-reoxygenation, which is a distinct apoptotic stimulus (Figure 2B).\textsuperscript{15} In contrast, A20 expression did not increase apoptosis in cardiomyocytes under either condition (Figure 2B).
A20 Inhibits the Hypertrophic Response

Cardiomyocytes infected with Ad.A20 exhibited an attenuated increase in protein synthesis after stimulation with either PE or ET-1 in vitro (P<0.01 versus GFP; Figure 2C). In addition, Ad.A20 inhibited ET-1–stimulated upregulation of ANF (Figure 2D).

Discussion

Recent evidence suggests that NF-κB may play a role in hypertrophic signaling in cardiomyocytes.1,3 We examined the cardiac regulation and functional effects of A20, an NF-κB–regulated gene that plays an important role in feedback inhibition of NF-κB signaling.7 We found that A20 is dynamically regulated in the heart and is significantly induced by acute pressure overload at a time point corresponding with peak NF-κB activation. Similarly, A20 expression was induced in cardiomyocytes stimulated with PE or ET-1, coincident with NF-κB activation. A20 expression in cardiomyocytes in vitro inhibited NF-κB activation in response to TNF-α, as did expression of the downstream inhibitor dnIKKβ. However, dnIKKβ expression substantially increased cardiomyocyte apoptosis after serum deprivation or hypoxia-reoxygenation, whereas A20 expression did not. A20 also inhibited the hypertrophic response of cardiomyocytes in vitro to pharmacological stimulation with either PE or ET-1. Together, these data suggest that A20 may be part of an endogenous feedback mechanism that limits NF-κB signaling in the heart and modulates the hypertrophic response.

Growing evidence suggests cardiomyocytes have developed negative feedback mechanisms to limit the hypertrophic response.3,16 After acute pressure overload, A20 was upregulated at a time corresponding with the reported upregulation of two endogenous inhibitors of cardiomyocyte hypertrophy, SOCS-316 and iex-1.3 In the case of A20, it seems likely that its expression is mediated by pressure overload–induced NF-κB activation2,3 and that it plays a role in inhibiting NF-κB signaling. Although direct evidence for this hypothesis is not presented here, the central importance of NF-κB–mediated, A20-dependent feedback inhibition on NF-κB signaling has been conclusively demonstrated in mice.17

Previous studies have shown that inhibition of NF-κB activation by a nondegradable form of IκBα predisposes cardiomyocytes to apoptosis, suggesting that, at least under some circumstances, cardiomyocytes require NF-κB signaling for survival.5 Consistent with this observation, we found that NF-κB inhibition by dnIKKβ increased cardiomyocyte apoptosis. In contrast, A20 expression, although as effective as dnIKKβ in inhibiting NF-κB activation, did not increase apoptosis. It is not clear how this differential effect is achieved, but it may relate to the more proximal level at which A20 inhibits NF-κB signaling and is consistent with observations in other systems in which A20 expression may actually inhibit apoptosis.7 Whatever the underlying mechanism, it suggests that the consequences of NF-κB inhibition in cardiomyocytes can differ substantially according to the nature of the inhibition and that A20 expression may have important strategic advantages as a therapeutic approach to NF-κB inhibition in the heart.

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References


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