A20 Is Dynamically Regulated in the Heart and Inhibits the Hypertrophic Response

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**Background**—Nuclear factor (NF)-κB signaling has been implicated in cardiomyocyte hypertrophy. Here, we determine the cardiac regulation and biological activity of A20, an inhibitor of NF-κB signaling.

**Methods and Results**—Mice were subjected to aortic banding, and A20 expression was examined. A20 mRNA upregulation (4.3±1.5-fold; *P*<0.05) was detected 3 hours after banding, coinciding with peak NF-κB activation. A20 was also upregulated in cultured neonatal cardiomyocytes stimulated with phenylephrine or endothelin-1 (2.8±0.6- and 4±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

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, calcium calmodulin–dependent 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.

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.

**Cell Culture**

Primary cardiomyocyte cultures were prepared 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 (dnIKKβ) 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: TCGT-GGCTCTGAAAACCAATG; reverse: GATGGGTCTTCTGAG-GATGTGGC) using the one-step RT-PCR kit (Qiagen) before agarose gel electrophoresis. Quantitative RT-PCR was performed as
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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 IkBα and nuclear translocation of the p65–NF-κB subunit in tumor necrosis factor (TNF)-α–stimulated cardiomyocytes in a manner comparable to dnIKKβ (Figure 2A). As measured by quantitative RT-PCR, Ad.A20 also inhibited TNF-α–induced genes of the NF-κB–dependent genes ICAM-1 and VCAM-1 in a dose-dependent manner and with an efficacy equivalent to Ad.dnIKKβ (data not shown).

A20 Does Not Enhance Cardiomyocyte Apoptosis

Serum starvation is a proapoptotic stimulus in cardiomyocytes.14 Consistent with prior studies,5 downstream inhibition of NF-κB in serum-starved cardiomyocytes by dnIKKβ increased apoptosis as detected by ELISA for histone-associated DNA fragments8 (Figure 2B). A similar phenomenon was observed in cardiomyocytes after hypoxia-reoxygenation, which is a distinct apoptotic stimulus (Figure 2B).15 In contrast, A20 expression did not increase apoptosis in cardiomyocytes under either condition (Figure 2B).

Statistics

All data are from ≥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.
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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.\(^1\)\(^2\)\(^3\) 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 dnnIKKB. However, dnnIKKB 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.\(^3\)\(^4\)\(^5\) After acute pressure overload, A20 was upregulated at a time corresponding with the reported upregulation of two endogenous inhibitors of cardiomyocyte hypertrophy, SOCS-3\(^6\)\(^7\) and iex-1.\(^3\) In the case of A20, it seems likely that its expression is mediated by pressure overload–induced NF-κB activation\(^2\)\(^3\) 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 dnnIKKB increased cardiomyocyte apoptosis. In contrast, A20 expression, although as effective as dnnIKKB 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.

Acknowledgments

We thank Dr V. Dixit (Genentech) for the A20 cDNA, Dr D. Goeddel (Tularik) for the IκKβ cDNA, and Dr K. Bloch (Massachusetts General Hospital) for the ANF probe. This work was supported by the National Institutes of Health (grants HL-59521 and HL-61557 to Dr Rosenzweig and HL-04250 to Dr Matsui) and the Wellcome Trust (to Dr Cook). Dr Rosenzweig is an Established Investigator of the American Heart Association.

References


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Circulation. 2003;108:664-667; originally published online August 4, 2003;
doi: 10.1161/01.CIR.0000086978.95976.41
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/6/664

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