Angiotsenin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes

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Submitted 29 November 2010; accepted in final form 6 April 2011

Lunde IG, Kvaløy H, Austbø B, Christensen G, Carlson CR. Angiotsenin II and norepinephrine activate specific calcineurin-dependent NFAT transcription factor isoforms in cardiomyocytes. J Appl Physiol 111: 1278–1289, 2011. First published April 7, 2011; doi:10.1152/japplphysiol.01383.2010.—Norepinephrine (NE) and angiotensin II (ANG II) are primary effectors of the sympathetic adrenergic and the renin-angiotensin-aldosterone systems, mediating hypertrophic, apoptotic, and fibrotic events in the myocardium. As NE and ANG II have been shown to affect intracellular calcium in cardiomyocytes, we hypothesized that they activate the calcium-sensitive, prohypertrophic calcineurin-nuclear factor of activated T-cell (NFATc) signaling pathway. More specifically, we have investigated isoform-specific activation of NFAT in NE- and ANG II-stimulated cardiomyocytes, as it is likely that each of the four calcineurin-dependent isoforms, c1-c4, play specific roles. We have stimulated neonatal ventriculoocytes from C57/B6 and NFAT-luciferase reporter mice with ANG II or NE and quantified NFAT activity by luciferase activity and phospho-immunoblotting. ANG II and NE increased calcineurin-dependent NFAT activity 2.4- and 1.9-fold, measured as luciferase activity after 24 h of stimulation, and induced protein synthesis, measured by radioactive leucine incorporation after 24 and 72 h. To optimize measurements of NFAT isoforms, we examined the specificity of NFAT antibodies on peptide arrays and by immunoblotting with designed blocking peptides. Western analyses showed that both effectors activate NFATc1 and c4, while NFATc2 activity was regulated by NE only, as measured by phospho-NFAT levels. Neither ANG II nor NE activated NFATc3. As today’s main therapies for heart failure aim at antagonizing the adrenergic and renin-angiotensin-aldosterone systems, mediating hypertrophic, apoptotic, and fibrotic events in the heart (reviewed in Ref. 3), it is likely that each of the four calcineurin-dependent NFATc isoforms in cardiomyocytes, understanding the molecular mechanisms of ANG II and NE signaling in cardiomyocytes is important.

At the cardiomyocyte level, NE and ANG II bind to seven-transmembrane domain, G-protein-coupled receptors (GPCRs; Refs. 25, 44, 45). Activated GPCRs, in turn, induce several intracellular signaling pathways involved in the pathophysiology of heart failure (2, 4, 12, 16, 21, 22, 26, 32, 36). NE and ANG II have previously been shown to affect hypertrophic remodeling and to increase intracellular calcium in cardiomyocytes (13–15, 30, 37, 38, 49). Calcineurin, a calcium-sensitive phosphatase regulating the activity of a nuclear factor of activated T-cell (NFATc) transcription factor family, was first shown to be important for cardiac hypertrophy by Molkentin et al. (37), who also showed that the calcineurin inhibitor cyclosporine A (CsA; Ref. 31) prevented ANG II-induced hypertrophy in cultured neonatal rat cardiomyocytes, indicating that ANG II may activate one or more of the NFAT isoforms. Later, calcineurin was established as a critical intracellular signal transducer of cardiomyocyte hypertrophy (7, 8, 34, 35).

There are four calcineurin-dependent NFAT isoforms known to be expressed in cardiomyocytes (6, 39, 48), termed c1-c4, and their activity is regulated by dephosphorylation of several N-terminal serine (Ser) and threonine (Thr) residues by calcineurin, leading to nuclear translocation and activation of NFATc target genes (5, 29, 41). Calcineurin-NFATc signaling has previously been shown to be important for regulation of pathological hypertrophy and apoptosis in cardiomyocytes (26, 39, 50, 52), and it is likely that each of the four isoforms play specific roles. However, measuring activation of NFAT isoforms in cardiomyocytes has been difficult (6, 48), and accordingly, little is known about the endogenous cardiac NFAT isoform protein expression, differential regulation, or their possible connection to ANG II or NE hypertrophic signaling.

The aim of the present study was to determine if ANG II and NE activate specific calcineurin-dependent NFAT isoforms in cardiomyocytes. We used peptide arrays and immunoblotting with blocking peptides to validate phospho- and nonphospho-specific NFATc1-c4 antibodies and analyzed NFAT activation by level of phosphorylation and in an NFAT-luciferase reporter assay.

METHODS

Genotyping, isolation, and stimulation of primary neonatal cardiomyocyte cultures. Experiments on neonatal C57/B6 and NFAT-luciferase reporter mice were approved by the Norwegian National Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996).

NFAT-luciferase mice were genotyped by PCR with primers for luciferase (forward primer: 5’-GCTTACTGGGACGAAGACGAAC-3’ and reverse primer: 5’-GACTTTGTGGCAAGACCGAC-3’).
and reverse primer: 5′-CTTCACTGCTGAGAGCCTCAG-3′ (11) and syndecan-4 as internal control (forward primer: 5′-ACACATGACGTCATGCTGCTCCT-3′ and reverse primer: 5′-AGGGTCGATTCTCAGGTGTTA-3′). Zygosity testing was performed by quantitative PCR by Charles River Laboratories.

Primary left ventricular cardiomyocyte cultures were prepared essentially as described previously (33). In brief, hearts from neonatal C57/B6 and heterozygous NFAT-luciferase reporter mice (line 15.1; Ref. 50), 1- to 3-days-old, were isolated by collagen and pancreatic digestion. Cardiomyocytes were separated from noncardiomyocytes by differential attachment to uncoated culture flasks (90151; Techno Plastic Products). Cardiomyocytes were allowed to attach to 6- or 12-well culture plates (Corning International, Corning, NY) coated with gelatine/fibronectin (G-1890/ F1114; Sigma, St. Louis, MO) overnight in plating medium (2 or 1 ml) at a density of 2.5 × 10^5 cells/ml medium [DMEM (41965; GIBCO-BRL, Invitrogen) supplemented with penicillin/streptomycin/glutamine (G6784; Sigma), medium 199 (31150; GIBCO-BRL), HEPEs (15630; GIBCO-BRL), horse serum (14–703E; Bio-Whittaker, Lonza), and fetal calf serum (14–701E; Bio-Whittaker)]. The cardiomyocytes were maintained in plating medium without serum before being stimulated with 1 μM ANG II (A9525; Sigma), 100 μM NE (A7257; Sigma), 0.41 μM SandImmun Neoral CsA oral solution (586107; Novartis), or vehicle (Cremophore, S5135; Sigma) for 5–30 min, 24, 48, or 72 h, and washed twice with DPBS (BE17-512F; Bio-Whittaker) before being harvested for analyses. Cells from three separate isolations were used for all analyses.

**NFATc2 null hearts.** Hearts from NFATc2 null mice described previously (6, 27), and C57/B6 wild-type controls were generously provided by Dr. Leon J. De Windt, (Maastricht, The Netherlands).

**Quantification of [3H]leucine incorporation.** Five microliters Ci per milliliters of [3H]leucine (American Radiolabeled Chemicals) were added at the same time as ANG II, NE, and/or CsA, and the cells were washed six times in 95% EtOH before being harvested in 0.2 mol/l NaOH 24, 48, or 72 h after stimulation, as previously described (24). Serum-stimulated cells served as positive control. [3H]leucine incorporation quantification was performed essentially as described in the Criterion Bio-Rad protocol, using the PVDF Hybond membrane (RPN303F) from Amersham/GE Healthcare. Blots and membranes were blocked in 8% nonfat dry milk (170–6404; Bio-Rad) or 1% casein (11961700; Sigma) for 1 h at room temperature. For antibody specificity examination, phospho-NFATc1-c4 and NFATc1-c4 antibodies were preincubated in 2% dry milk in TBS-T with 4× and 25× volume of blocking peptide, respectively, for 2 h at room temperature before immunoblotting. For self-association studies of NFATc2, NFATc2 membranes were incubated overnight with or without biotinylated peptides (10 μM). Blots and membranes were developed using the ECL Plus Western blotting detection system (RPN2132; American/GE Healthcare) and visualized in the Las-4000 mini from Fujifilm. Blots and membranes were reprobed after stripping using the Phos-tag Western blot stripping buffer (21059l; Thermo Scientific).

**Quantification of luciferase activity.** For measurements of luciferase activity, cells were harvested according to the luciferase assay system protocol (E1500; Promega). Duplicates of each sample were run on a 96-well plate and luminescence quantified at 10 s at the Victor 3 1420 Multilabel Counter (PerkinElmer).

**Peptide synthesis.** The NFATc1-c4 proteins from mice (NP_490821, NP_035029, NP_035031, and NP_076188, respectively) were synthesized with or without phosphorylation (at Ser 259, Ser 326, and Ser 168/170, respectively) with Fmoc-protection chemistry on cellulose membranes as 20-mer peptides with an offset of three or five amino acids, using a Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments, Köln, Germany), as described previously (23).

For the phospho-NFATc1-c4 antibodies, blocking peptides were commercially available (sc-32979P, sc-32994P, and sc-32630P, Santa Cruz Biotechnology, Santa Cruz, CA), while for the NFATc1-c4 antibodies, blocking peptides were designed according to the core epitope derived from the immunoblot analyzes on the peptide membranes and synthesized by Genscript (Piscataway, NJ) at > 80% purity. For NFATc1, two peptides were designed, c1-1: MPSTSF-PVPKFLPGLPPAAV and c1-2: RPAPPSSGGMKAAEEHHSY; for NFATc2, c2-1: CGIPIKDWSTSDPTPVSTA and c2-2: QONIFAPSYRTPSPIMSPR; for NFATc3, c3: PKGLEICSDGGNLSRSET; and for NFATc4, c4-1: PTPDPTSLDETPETWDGS and c4-2: PRDYPPEFGGYREAGGQ. For self-association studies of NFATc2, the c2-1 and c2-2 peptides were synthesized with an N-terminal biotin-tag.

**Immunoblot analysis.** For Western blotting of whole-cell lysates, cells were harvested in a PBS buffer containing 1% Triton X-100 (Sigma), 0.1% Tween 20 (Sigma), and protease inhibitors (Complete EDTA-free tablets; Roche Diagnostics, Germany), and the supernatant was collected after centrifugation. For Western blotting of heart lysates, the left ventricle was homogenized in the same buffer as above using a Polytron homogenizer and the supernatant was collected after centrifugation. For separation of cytoplasmic and nuclear protein fractions, cells were harvested according to the protocol of the compartmental protein extraction kit (2145; Chemicon/Millipore). Protein concentrations were measured using the Micro BCA protein assay kit (Pierce/Thermo Scientific), and 10–15 μg of protein were run on 1.0 mm 4–15% Tris-HCl gels (Criterion; Bio-Rad). Ramos cell lysate (sc-2216; Santa Cruz Biotechnology) was used as an NFATc1-c4 positive control. SDS-PAGE and Western blotting were performed essentially as described in the Criterion Bio-Rad protocol, using the PVDF Hybrid membrane (RPN303F) from Amersham/GE Healthcare. Blots and peptide membranes were blocked in 8% nonfat dry milk (170–6404; Bio-Rad) or 1% casein (11961700; Roche Diagnostics) overnight and incubated with primary antibodies, and subsequently secondary antibodies, diluted in 2% nonfat dry milk or 1% casein for 1 h at room temperature. For antibody specificity examination, phospho-NFATc1-c4 and NFATc1-c4 antibodies were preincubated in 2% dry milk in TBS-T with 4× and 25× volume of blocking peptide, respectively, for 2 h at room temperature before immunoblotting. For self-association studies of NFATc2, NFATc2 membranes were incubated overnight with or without biotinylated peptides (10 μM). Blots and membranes were developed using the ECL Plus Western blotting detection system (RPN2132; American/GE Healthcare) and visualized in the Las-4000 mini from Fujifilm. Blots and membranes were reprobed after stripping using the restore Western blot stripping buffer (21059l; Thermo Scientific).

**Antibodies.** For immunodetection of NFATc1-c4, Santa Cruz Biotechnology antibodies sc-13033 (1:200), sc-7296 (1:500), sc-8321 (1:500), and sc-13036 (1:500) were used; for phospho-NFATc1-c4, sc-32979 (1:200), sc-32994 (1:500), sc-32982 (1:500), and sc-32630 (1:500) were used. Anti-actin (1:500, sc-8432) was used for loading control. Anti-GAPDH (1:500, sc-20357) and anti-histone H4 (1:500, sc-8658R) antibodies were used to control for purity of the cytoplasmic and nuclear fractions, respectively. Horseradish peroxidase-conjugated secondary antibodies from Southern Biotechnology (1:1,750, 1031-05, 4030-05, and 6160-05) were applied to all blots. For the peptide membranes, the same antibodies were applied; primary antibodies were diluted 1:1,000 and secondary antibodies 1:2,000. For biotin detection, anti-biotin-horseradish peroxidase from Sigma (A0185) was used at a 1:2,500 dilution.

**TransAm NFATc1 transcription factor assay kit.** The TransAm NFATc1 transcription factor assay kit (40296; Active Motif Europe) provides an ELISA-based method to measure NFATc1 activity in nuclear protein samples in which oligonucleotides of the NFAT consensus binding site, 5′-AGGAAA-3′, have been immobilized on a 96-well plate. Five micrograms of nuclear protein isolated by the compartmental protein extraction kit from Chemicon/Millipore, as described above, were run according to the protocol. Antibodies for detection were provided and NFATc1 activity was quantified and read at 450 nm at the Victor 3 1420 multilabel counter (PerkinElmer).

**Statistics.** Data are expressed as group means ± SD relative to control (average control value set to 100%). Differences in [3H]leucine incorporation, luciferase activity, and phospho-NFAT short-time levels were tested in GraphPad Prism 5 using repeated-measures one-way ANOVA and Bonferroni posttesting, while differences in phospho-NFAT after 24 h of stimulation, fractionated NFATc2, and NFATc1 activation levels were tested using a paired t-test. Differences were considered significant for P < 0.05.
ANG II AND NE increase protein synthesis in neonatal mouse cardiomyocytes. To confirm the prohypertrophic properties of ANG II and NE in our neonatal ventriculocytes, induction of protein synthesis was chosen as an indicator, as this has been shown to constitute the most robust response of cultured mouse cardiomyocytes to prohypertrophic stimuli (18). Protein synthesis was quantified by radioactive [3H]leucine incorporation after 24–72 h of ANG II, NE, and/or CsA stimulation of cultured cardiomyocytes, with serum stimulation serving as positive control. ANG II stimulation for 24 h resulted in a 50.2% increase in protein synthesis (SD 24.1%; P < 0.001; n = 14), compared with nonstimulated control cells, and the response was blocked by the calcineurin inhibitor CsA (P < 0.05; n = 6; Fig. 2A). While 24 h of NE-stimulation had no effect (Fig. 2A), 72 h of NE stimulation significantly increased [3H]leucine incorporation by 47.1% (SD 20.8%; P < 0.05; n = 18; Fig. 2B). This increase in protein synthesis was blocked by CsA (P < 0.01; n = 6). Of notice, CsA stimulation alone significantly reduced protein synthesis after 72 h (P < 0.05; n = 6). Neonatal cardiomyocytes grown for 24 h in culture showed significantly lower levels of leucine incorporation than at 72 h (22.6 ± 6.9 vs. 100 ± 18.1%; P < 0.001), indicating autonomous hypertrophy as previously shown (18). Thus CsA stimulation for 72 h seemed to reduce the autonomous increase in protein synthesis. These results show that 24 h of ANG II and 72 h of NE stimulation was sufficient to increase protein synthesis in the neonatal mouse ventriculo- cytes, suggesting a hypertrophic response. Furthermore, our results indicate that the hypertrophic response induced by ANG II and NE in neonatal cardiomyocytes, in addition to the autonomous hypertrophic response, is calcineurin-dependent.

Epitope mapping of phospho-NFATc1-c4 antibodies. To examine commercially available antibodies for the detection of phosphorylated NFATc1-c4 isoforms (inactive protein), the NFAT sequences with or without phosphorylation of the relevant serine(s) were synthesized on membranes as 20-mer peptides with an offset of three amino acids. Immunoblotting...
using the Santa Cruz Biotechnology antibodies pNFATc1 (Fig. 3A, sc-32979), pNFATc2 (Fig. 3B, sc-32994), pNFATc3 (Fig. 3C, sc-32982), and pNFATc4 (Fig. 3D, sc-32630; n = 2) showed that all four antibodies had high affinity for its specific phospho-NFAT. Anti-pNFATc1 and anti-pNFATc2 were highly specific for pSer259 and pSer326 (Fig. 3A, top, and B, top, respectively) and hardly detected any nonphosphorylated NFATc1 and c2 (bottom). Anti-pNFATc3 recognized pSer265 (Fig. 3C, top) but also to a certain extent nonphosphorylated NFATc3 (bottom). Anti-pNFATc4 recognized pSer168 and pSer170 more strongly (Fig. 3D, top) than when these sites were not phosphorylated (bottom).

Epitope mapping of NFATc1-c4 antibodies. To examine commercially available antibodies for nonphosphorylated NFATc1-c4, full-length proteins were synthesized on membranes as 20-mer peptides with an offset of 5 amino acids. Immunoblotting showed that each of the four antibodies strongly recognized its specific NFAT isoform. Anti-NFATc1 (sc-13033) recognized amino acids 1–55, and as Ser259 was outside of this area (Fig. 4A), this antibody would theoretically recognize total-NFATc1. Anti-NFATc2 (sc-7296) recognized amino acids 210–240 and 310–345, where the latter region contained Ser265 (Fig. 4B). Further epitope mapping showed that the antibody did not cross-react with pSer326 (Fig. 4E). However, the presence of the second epitope (amino acids 210–240) suggested that the antibody could recognize total-NFATc2. Anti-NFATc3 (sc-8321) recognized amino acids 360–395, a region outside Ser265, suggesting that the antibody would recognize total-NFATc3 (Fig. 4C). At last, anti-NFATc4 (sc-13036) recognized amino acids 115–175, containing Ser168/170 (boxed sequence in Fig. 4F). Underlined amino acid sequences in Fig. 4, A–D, were used to design specific blocking peptides for further immunoblot analyses of NFATc1-c4 in cardiomyocytes.

Identification of nonphosphorylated and phospho-NFATc1-c4 in neonatal mouse cardiomyocytes. To identify the specific NFATc isoforms in cardiomyocytes, immunoblot analyses of neonatal cardiomyocyte lysates in combination with blocking peptides were performed. Ramos cell lysate (Human Burkitt’s lymphoma NFAT rich cell line) was used as a positive control. Antibodies were preincubated with or without its specific blocking peptide before immunoblotting. For phospho-NFATc1-
c4 antibodies, commercially available blocking peptides were used. For NFATc1-c4 antibodies, the designed blocking peptides were used (underlined amino acid sequences in Fig. 4).

Cardiac NFATc1 and phospho-NFATc1 proteins were identified to be 90 kDa (Fig. 5A) and 95 kDa (Fig. 5E), respectively, which is close to the predicted size of 92 kDa for the nonphosphorylated protein (Table 1) and similar in size to that of the positive control. Both protein bands were specifically blocked by peptides, c1-1 and c1-2, and sc-32979P, respectively, confirming the specificity of the two antibodies. Noteworthy, antibody recognition of the proteolytic fragments of NFATc1/phospho-NFATc1 of 75 kDa was blocked as well.

Cardiac NFATc2 and phospho-NFATc2 proteins were identified to be ~100 kDa (Fig. 5B) and 120 kDa (Fig. 5F), respectively, where the former was close to the predicted size of 103 kDa (Table 1). The 100-kDa NFATc2 was the only protein band observed in cardiomyocytes; however, it was not blocked by c2-1 and c2-2; the presence of blocking peptides rather increased the signal from the NFATc2 protein, suggesting one or both peptides bound to NFATc2 with high affinity, which is consistent with the recent finding that the c2 isoform can self-associate (47). Self-association properties of the blocking peptides was confirmed by overlaying biotinylated c2-1 and c2-2 peptides on NFATc2

Fig. 4. Epitope mapping of NFATc1-c4 antibodies. Full-length NFATc1-c4 proteins were synthesized on membranes as 20-mer peptides with an offset of 5 amino acids and immunoblotted with the following antibodies: NFATc1 sc-13033 (A), NFATc2 sc-7296 (B), NFATc3 sc-8321 (C), or NFATc4 sc-13036 (D); n = 2. Amino acid sequences of the stained epitopes are indicated for each antibody and underlined are the sequences used to design blocking peptides for immunoblotting of cardiac NFATs. Twenty-five amino acids in N- and C-terminal direction from the phosphorylatable serine(s) in the NFATc2 and NFATc4 were also synthesized with an offset of 3 amino acids, with phosphorylation of the relevant serine(s) (E and F, top) and without phosphorylation (E and F, bottom), and immunoblotted using the respective antibodies. Phosphorylatable serines Ser326 (E) and Ser168/170 (F) are in bold. Boxes denote sequences containing the phosphorylatable serines in NFATc1-c4: Ser259, Ser326, Ser265, and Ser168/170, respectively.

Fig. 5. Immunoblotting of NFATc1-c4 and phospho-NFATc1-c4 proteins in neonatal mouse cardiomyocytes. Representative blots of NFATc1-c4 (A–D) and phospho-NFATc1-c4 (E–H) proteins isolated from neonatal cardiomyocytes (NCM). Ramos lysate (Human Burkitt’s lymphoma cell line) was used as a positive control, and blocking peptides were used to validate antibody specificity. Actin was used as loading control (bottom).
Table 1. NFATc isoforms in cardiomyocytes, antibodies, and blocking peptides

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<th>Theoretical Size</th>
<th>Observed Size</th>
<th>Antibody</th>
<th>Blocking Peptide (Ref.)</th>
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NFAT, nuclear factor of activated T-cells. *Not able to block.

synthesized as overlapping 20-mer peptides on a membrane (Supplemental Fig. S1A; Supplemental Material for this article is available online at the J Appl Physiol website). Finally, the specificity of the NFATc2 antibody was confirmed using NFATc2 null hearts (n = 2; Supplemental Fig. S1B). The phospho-NFATc2 protein of 120 kDa was specifically blocked by peptide sc-32994P.

Cardiac NFATc3 and phospho-NFATc3 proteins were identified to be ~140 (Fig. 5C) and 160 kDa (Fig. 5G), respectively, close to the predicted protein size of 120 kDa for nonphosphorylated NFATc3 (Table 1). The cardiomyocyte NFATc3 and phospho-NFATc3 proteins were of similar sizes to those in the positive control. The 140-kDa NFATc3 protein band was specifically blocked by the peptide c3. The anti-phospho-NFATc3 antibody detected two protein bands, consistent with the phospho-NFATc3 antibody also recognizing some nonphosphorylated protein as observed by peptide array analysis (Fig. 5C). The cardiomyocyte proteins detected by the antibody were blocked by peptide sc-32982P; however, the Ramos cell NFATc3 was only partially blocked.

Cardiac NFATc4 and phospho-NFATc4 proteins were identified to be ~100 (Fig. 5D) and 120 kDa (Fig. 5H), respectively, close to the predicted size of 100 kDa of nonphosphorylated NFATc4 (Table 1). Both protein forms were of similar size to that of the positive control and were specifically blocked by peptides, c4-1 and c4-2, and sc-32630P, respectively.

Conclusively, immunoblotting in combination with blocking peptides was used to identify nonphosphorylated and phospho-NFATc1-c4 proteins in neonatal ventricular cardiomyocytes. The examined phospho-NFATc antibodies were specific, except for the phospho-NFATc3 antibody, which also recognized nonphosphorylated NFATc3. Of the NFATc antibodies, the NFATc4 antibody also recognized phospho-NFATc4, while the NFATc2 antibody detected nonphosphorylated NFATc2 specifically.

**ANG II and NE activate NFATc1.** To analyze isoform-specific activation of NFATs by ANG II and NE, the phosphorylation level (inactive, cytoplasmic NFAT) was quantitated by immunoblotting using the antibodies examined in Figs. 3–5. The level of phospho-NFATc1 was significantly reduced by 11% (SD 4.4%; P = 0.02) after 24 h of ANG II stimulation, while NE stimulation reduced the level of phospho-NFATc1 by 16% (SD 6.3%; P = 0.02) compared with nonstimulated control cells (n = 3; Fig. 6, A, top, and B).

Consistently, NFATc1 activity, as measured by the amount of protein binding to the DNA target sequence using a NFATc1 transcription factor assay, was found to be increased by 17% (SD 6.2%; P = 0.04) and 25% (SD 8.7%; P = 0.04) in nuclear fractions isolated from ANG II- and NE-stimulated cardiomyocytes, respectively, compared with controls (n = 3; Fig. 6C).

To examine if NFATc activity also was increased at earlier time points and before the induction of protein synthesis (Fig. 2, A and B), cardiomyocytes were stimulated with the two agonists for 5–30 min. ANG II stimulation for 30 min reduced the level of phospho-NFATc1 by 41% (SD 12.6%; P < 0.05; n = 3) compared with control cells (Fig. 6, D and E), while 30 min of NE stimulation reduced phospho-NFATc1 by 61% (SD 22.8%; P < 0.05; n = 3; Fig. 6, F and G). Conclusively, our data suggest that NFATc1 is activated 0.5–24 h after ANG II and NE stimulation of cardiomyocytes.

**NE activates NFATc2.** Immunoblotting revealed that the level of phospho-NFATc2 was reduced by 49% (SD 12.5%; P = 0.02; n = 4) after 24 h of NE stimulation compared with nonstimulated control cells, while ANG II had no effect (Fig. 7, A, top, and B). In accordance, protein fractionation showed that the cytoplasmic level of NFATc2 was reduced by 65% (SD 14.7%; P < 0.05) while the nuclear level was increased by 50% (SD 17.9%; P < 0.05) after 24 h of NE stimulation, compared with nonstimulated control cells (n = 3; Fig. 7, C and D). Short-time (5–30 min) ANG II stimulation had no effect on the level of phospho-NFATc2 (n = 3; Fig. 7, E and F), while NE stimulation resulted in reduced phospho-NFATc2 levels; by 28% (SD 8.4%; P < 0.05) after 5 min and 25% (SD 1.7%; P < 0.05) after 15 min compared with nonstimulated control cells (n = 3; Fig. 7, G and H). Conclusively, these data suggest that NFATc2 is activated by NE in cardiomyocytes 5 min–24 h after stimulation but not by ANG II.

**ANG II and NE do not activate NFATc3.** The level of phospho-NFATc3 in the neonatal cardiomyocytes was not influenced by 24 h of ANG II nor NE stimulation (n = 3; Fig. 8, A, top, and B). Similarly, 5–30 min of ANG II (Fig. 8, C and D) and NE (Fig. 8, E and F) stimulation did not affect the level of phospho-NFATc3 in cardiomyocytes (n = 3), suggesting that NFATc3 was not activated by these two neurohumoral factors.

**ANG II and NE activate NFATc4.** The level of phospho-NFATc4 was reduced by 31% after 24 h of ANG II stimulation (SD 16.5%; P = 0.03), while NE stimulation reduced phospho-NFATc4 by 19% (SD 6.7%; P = 0.01), compared with nonstimulated control cells (n = 4; Fig. 9, A, top, and B). Short-time ANG II stimulation showed that the level of phospho-NFATc4 was significantly reduced by 43% (SD 15.7%; P < 0.05), 53% (SD 9.5%; P < 0.05), and 52% (SD 18.0%; P < 0.05) after 10, 15, and 30 min of stimulation, respectively.
while NE stimulation for 30 min reduced phospho-NFATc4 by 22% (SD 6.7%; \( P < 0.05; n = 3 \); Fig. 9, E and F) compared with controls (\( n = 3 \)). Conclusively, our data suggest that NFATc4 is activated in cardiomyocytes 10 min to 24 h after ANG II stimulation and 30 min to 24 h after NE stimulation.

**DISCUSSION**

In the present study, we show that ANG II and NE activate specific calcineurin-dependent NFAT isoforms in ventricular cardiomyocytes. First, they both activate calcineurin-dependent NFAT signaling as measured by reporter luciferase activity in neonatal cardiomyocytes isolated from transgenic NFAT-luciferase mice, stimulated with either of the two neurohumoral factors. Second, by immunoblotting with antibodies validated on peptide arrays and blocking peptides designed to identify specific NFAT isoforms, we show that ANG II activated NFATc1 and c4 while NE activated NFATc1, c2, and c4, as measured by level of phosphorylation. Neither ANG II nor NE activated NFATc3.

Primary cultures of mouse neonatal cardiomyocytes, such as used in our study, constitute a widely used in vitro tool, accompanying in vivo models of genetically modified mice, for elucidating the mechanisms underlying the development of ventricular hypertrophy. With the use of these cardiomyocyte cultures, the direct effects of various prohypertrophic stimuli can be studied, e.g., of ANG II and NE, and the systemic, indirect effects of in vivo stimulation are avoided. Despite the obvious shortcomings of this model, one of its strengths is that many of the molecules found to be involved in hypertrophy in vitro in fact have been confirmed in vivo (10).

Studies of NFAT isoform-specific knockout and transgenic models have implicated the importance of analyzing NFAT isoforms separately in hypertrophic remodeling and not in a one-represents-all manner. Although the NFATc1-c4 binding site in target gene promoters is similar for all isoforms (28, 29), the impact on cardiac hypertrophy and heart failure (6). Although transgenic overexpression of NFATc4 resulted in massive cardiac hypertrophy (37), loss of the c4 isoform did not compromise the ability of the myocardium to undergo hypertrophic growth (48, 51), while NFATc3-null mice demonstrated a significant reduction in cardiac hypertrophy (51). Recently, NFAT isoforms were also found to deter-
mine fast or slow phenotype specification in skeletal muscle fibers (9). In that study, it was shown that, depending on the electrical stimulation pattern applied, specific NFATc isoforms translocate to nucleus and contribute to transcription of fiber type-specific genes. Collectively, these results imply that NFAT isoforms play essential, but separate, roles in the development of cardiac hypertrophy.

Our results show that the effectors of the sympathetic adrenergic and RAAS systems so central in development of pathological hypertrophy and heart failure induce dephosphorylation and thereby activation of specific NFAT isoforms within the ventricular cardiomyocytes. Consistent with NFAT activation after 24 h and onwards in a pressure-overload model of hypertrophic ventricular remodeling (50), ANG II- and NE-induced NFAT activation was present at this time point, although activation was detected as early as minutes after stimulation and proceeded the remodeling response (24–72 h), as indicated by protein synthesis measurements. Both ANG II and NE activated NFATc1, the NFAT isoform that in the knockout model was shown to be important for cardiac morphology and valve formation (17, 40). NE, in contrast to ANG II, activated NFATc2, the NFAT isoform that has been shown to be essential for cardiac hypertrophy in knockout studies (6), indicating that NFATc2 is an effector of the sympathetic system. NFATc3, an isoform necessary for hypertrophy (51), was not activated by neither ANG II nor NE. NFATc4, which is activated in the myocardium of heart failure patients (19, 20) and induces substantial hypertrophy when overexpressed (37), was activated by both ANG II and NE, suggesting that it is an important signaling molecule for ANG II and NE in cardiomyocytes. Taken together, our results indicate that the prohypertrophic NFAT signaling of ANG II and NE in cardiomyocytes is mediated by the c1 and c4 isoforms, while NE also mediated its effects through NFATc2. Consistent with our findings, ANG II has previously been shown to activate NFATc1 (41) and to induce translocation of exogenous NFATc4-GFP from the cytoplasm to the nucleus.
Measurements of endogenous NFAT isoform activation by immunoblotting have been difficult especially due to low antibody specificity (6, 48). In the present study, we identified the NFATc proteins in cardiomyocytes by validating antibodies on peptide arrays and by immunoblotting, using the NFAT-rich Human Burkitt’s lymphoma cell line as a positive control and blocking peptides designed to discern the NFAT-specific proteins. The identified NFATc proteins were similar to the theoretical size calculated from the amino acid sequence (see Table 1). Except for NFATc2, the phospho- and NFATc proteins expressed in cardiomyocytes were similar in size to the lymphoma cells proteins. Some considerations, however, were necessary when interpreting cardiomyocyte NFATc immunoblotting. All examined phospho-NFATc antibodies were found to be specific, although the phospho-NFATc3 antibody also recognized nonphosphorylated NFATc3 as a separate protein band. Of the NFATc antibodies, the NFATc4 antibody also recognized phospho-NFATc4 as a distinct band, while the NFATc2 antibody recognized nonphosphorylated protein only and thus could be used to measure active NFATc2.

In addition to phosphorylation status, there are other methods that can be used to measure cardiomyocyte NFAT activity. As the commercially available NFATc1 activation kit applies the consensus NFAT binding site recognized by all isoforms (28), we used the examined antibodies to test for the other three isoforms. Although we have successfully applied the kit to measure NFATc4 activation in heart lysates, the kit was not compatible with the small amount of nuclear protein we managed to extract from the neonatal cardiomyocytes in combination with NFATc2, c3, and c4 antibodies. Furthermore, only the NFATc2 antibody was sensitive enough to detect NFAT protein in isolated nuclear fractions; however, this could also be explained by NFATc2 being the most abundant NFAT isoform in cardiomyocytes (6).

Our results indicating that NFATs are activated in an isoform-specific manner by ANG II and NE in cardiomyocytes raise questions to how this differential activation is regulated. It is possible that NFAT isoforms may be localized to different cytoplasmic pools where calcineurin is activated by local Ca2+/H100 signals or there may be isoform-specific differences in Ca2+/H100/calcineurin sensitivity. Alternatively, the activation may be dependent on other factors regulating for instance nuclear import/export in an isoform-specific manner. Our results show that neither NE nor ANG II activated NFATc3 in the cardiomyocytes, while both activated NFATc1. This is in line with results from skeletal muscle fibers showing strong nuclear translocation of NFATc1 after electrical stimulation but only transient and weak nuclear localization of NFATc3 (46). In the same study, the nuclear export inhibitor leptomycin B caused nuclear accumulation of the NFATc1 isoform independently of calcineurin, but not of NFATc3, indicating that nuclear transport of NFAT isoforms is indeed differentially regulated. Moreover, in recent studies by Rinne and et al. (42, 43), nuclear export was also shown to regulate cellular localization and thereby activation of NFATc1 and c3 in adult rabbit and cat ventriculocytes. Under basal conditions, NFATc1 was found in the nucleus while NFATc3 was localized to the cytoplasm in both atrial and ventricular cardiomyocytes. In the
atrial cells, both ANG II and phenylephrine could induce additional calcineurin-dependent nuclear translocation of NFATc1-GFP, supporting our finding that both NE and ANG II were able to activate NFATc1 in ventricular myocytes. When stimulated with ANG II, NFATc3 also translocated to the nucleus in atrial cells, but not in ventriculocytes, supporting our finding that neither NE nor ANG II were able to activate NFATc3 in ventricular myocytes, and that NFAT isoforms are activated in a tissue-specific manner. Furthermore, inhibition of kinases responsible for inactivation of NFAT by phosphorylation was sufficient for inducing accumulation of NFATc1 in the nucleus, while the same treatment had no effect on NFATc3, suggesting differential mechanisms of activity regulation for these two isoforms. Nevertheless, nuclear accumulation of NFATc3 was induced by inhibition of nuclear export in ventriculocytes. This indicates that nuclear localization of the c3 isoform is prevented by nuclear transport mechanisms and thus that its activation is controlled by other mechanisms in addition to phosphorylation. In failing ventriculocytes, however, nuclear localization of NFATc3 was found to be enhanced, in line with the finding that NFATc3-null mice demonstrate reduced cardiac hypertrophy (51). Phenylephrine stimulation further increased its nuclear accumulation, while ANG II had no effect, supporting a role for sympathetically induced activation of NFATc3 in cardiac hypertrophy and failure, although not under basal conditions, such as seen in our cardiomyocytes. Of notice, NFATc3 seemed to actually accumulate around the nucleus in nonstimulated cells (43), which might support that this isoform is activated by local Ca^{2+} pools or proteins.

In conclusion, immunoblotting of phospho-NFATs showed that ANG II and NE activated NFATc1 and c4 in ventricular cardiomyocytes, while NFATc2 activity was regulated by NE only. As today’s main therapies for heart failure aim at antagonizing the adrenergic system and RAAS, understanding their intracellular actions is of importance, and our data, through validating a method for measuring cardiomyocyte NFATs, indicate that ANG II and NE activate NFATs in an isoform-specific manner.

ACKNOWLEDGMENTS

We thank Dr. Jeffery D. Molkentin for the NFAT-luciferase reporter mouse and the CsA protocol, and Kristin Brevik Andersson for designing the genotyping of neonatal NFAT-luciferase mice.

GRANTS

This work was supported by EMBIO grants, University of Oslo, Anders Jahr’s Fund for the Promotion of Science, Norway, the South-Eastern Norway Regional Health Authority, and the Research Council of Norway.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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