Cell stress-induced phosphorylation of ATF2 and c-Jun transcription factors in rat ventricular myocytes

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Ventricular myocytes are exposed to various pathologically important cell stresses *in vivo*. *In vitro*, extreme stresses (sorbitol-induced hyperosmotic shock in the presence or absence of okadaic acid, and anisomycin) were applied to ventricular myocytes cultured from neonatal rat hearts to induce a robust activation of the 46 and 54 kDa stress-activated protein kinases (SAPKs). These activities were increased in nuclear extracts of cells in the absence of any net import of SAPK protein. Phosphorylation of ATF2 and c-Jun was increased as shown by the appearance of reduced-mobility species on SDS/PAGE, which were sensitive to treatment with protein phosphatase 2A. Hyperosmotic shock and anisomycin had no effect on the abundance of ATF2. In contrast, cell stresses induced a greater than 10-fold increase in

total c-Jun immunoreactivity detected on Western blots with antibody to c-Jun (KM-1). Cycloheximide did not inhibit this increase, which we conclude represents phosphorylation of c-Jun. This conclusion was supported by use of a c-Jun(phospho-Ser-73) antibody. Immunostaining of cells also showed increases in nuclear phospho-c-Jun in response to hyperosmotic stress. Severe stress (hyperosmotic shock + okadaic acid for 2 h) induced proteins (migrating at approx. 51 and 57 kDa) that cross-reacted strongly with KM-1 antibodies in both the nucleus and the cytosol. These may represent forms of c-Jun that had undergone further modification. These studies show that stresses induce phosphorylation of transcription factors in ventricular myocytes and we suggest that this response may be pathologically relevant.

INTRODUCTION

Ischaemic and hypertensive myocardial disease is currently a major cause of mortality and morbidity. In these conditions, the heart is exposed to numerous cell stresses including increased production of reactive oxygen species, osmotic stress, mechanical stress and metabolic deprivation (reviewed in [1,2]). Acute ischaemia/reperfusion or hypertension increases mRNA levels of *c-jun* and other immediate early genes (for example, *c-fos*, *Egr-1*) in the heart *in vivo* [3–5] and in perfused heart preparations *in vitro* [6–8]. Up-regulation of immediate early gene expression has also been detected in ventricular cardiac myocytes in primary culture in response to mechanical stretching [9,10], hypoxia [11], and redox or metabolic stress [12,13]. These interventions are assumed to simulate the pathophysiological conditions in the intact animal.

Transcriptional up-regulation of immediate early genes is brought about by the activation of pre-existing transcription factors (reviewed in [14]), which bind to specific regulatory elements in the promoter regions and interact with the basic transcriptional machinery (reviewed in [15]). The *c-jun* promoter contains several regulatory elements. DNA footprinting in cultured human cells has shown that two AP-1-like sites (*jun1* and *jun2*) and six additional sites (NF-*jun*, two Sp-1 sites, CCAAT, RSRF/MEF-2 and an unidentified element) are constitutively occupied, implying that increased DNA binding is not necessarily an important factor in transcriptional regulation [16–19]. Similar regulatory consensus sequences are present in the rat *c-jun* promoter region [20]. In response to UV stress (as opposed to mitogens, serum or phorbol esters), the two AP-1-like sites (*jun1* and *jun2*) are the dominant participants in the up-regulation of *c-jun* expression [21]. These regions bind heterodimers of the

c-Jun and ATF transcription factors [17,22]. The transactivating activities of both c-Jun and ATF2 are increased by phosphorylation of specific sites in the N-terminal transactivation domain [23–26]. Phosphorylation of prebound transcription factors, rather than increased DNA binding, is thus more likely to be the dominant factor in activation of *c-jun* expression controlled through AP-1-like sites.

The stress-activated protein kinases (SAPKs), alternatively known as c-Jun N-terminal kinases (JNKs), are a subfamily of the mitogen-activated protein kinase (MAPK) superfamily (reviewed in [27]). (Strictly speaking, the JNK terminology applies to the human enzymes whereas the SAPK terminology applies to the rat enzymes.) As in other cells, cellular stresses (osmotic stress, exposure to protein synthesis inhibitors, mechanical stretching) activate SAPKs in isolated ventricular myocytes [28,29]. However, as in other cells, the SAPKs are only poorly activated by mitogens and phorbol esters [28]. SAPKs are also activated by ischaemia/reperfusion in isolated rat hearts [30,31]. Activation of SAPKs/JNKs may thus be involved in the response of the heart to pathological stresses. The transactivation domains of c-Jun [23] and ATF2 [24–26] are substrates for the SAPKs/JNKs. Here, we have studied the phosphorylation of c-Jun and ATF2 in cultured ventricular myocytes in response to cell stresses.

EXPERIMENTAL

Materials

Laminin, sorbitol, okadaic acid, anisomycin, Dulbecco's modified Eagle's medium, Medium 199, Protein A-Sepharose and protease inhibitors were from Sigma Chemical Co. (Poole,

Abbreviations used: DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; β -MHC, β -myosin heavy chain; PP2A, protein phosphatase 2A; SAPK, stress-activated protein kinase.

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Dorset, U.K.). Protein phosphatase 2A (PP2A) was from Calbiochem (Beeston, Notts., U.K.). Lab-Tek tissue culture chamber slides were from Life Technologies (Paisley, Scotland, U.K.). Prestained protein molecular-mass markers, enhanced chemiluminescence Western-blotting reagents, autoradiography film and streptavidin/Texas Red were from Amersham International (Amersham, Bucks., U.K.). [γ - 32 P]ATP was from Dupont–New England Nuclear (Brussels, Belgium). SDS/PAGE reagents and Bradford [32] protein assay reagent were from Bio-Rad (Hemel Hempstead, Herts., U.K.). Nitrocellulose was from Schleicher & Schuell [supplied by Anderman & Co. (Kingston-upon-Thames, Surrey, U.K.)]. cAMP-dependent protein kinase inhibitory peptide (sequence TTYADFIASGRTGRRNAIHD) was from Bachem (Saffron Walden, Essex, U.K.). Antibodies to JNK1, ATF2 and c-Jun were from Santa Cruz Biotechnology Inc. [supplied by Autogen Bioclear (Devizes, Wilts., U.K.)]. The JNK1 antibody (sc-474) was a rabbit polyclonal antibody raised to a C-terminal region of human JNK1 (amino acids 368–384). The ATF2 antibody (sc-187) was a rabbit polyclonal antibody raised to amino acids 487–505 of the human sequence. The antibody to c-Jun (KM-1, sc-822) was a mouse monoclonal antibody to amino acids 56–69 of the human sequence. The kit for the detection of c-Jun(phospho-Ser-63) or c-Jun(phospho-Ser-73) was from New England Biolabs (U.K.) Ltd. (Hitchin, Herts., U.K.). A mouse monoclonal antibody to β -myosin heavy chain (β -MHC) was from Novocastra (Newcastle-upon-Tyne, Tyne and Wear, U.K.). Secondary antibodies (horseradish peroxidase-linked, FITC-conjugated and biotinylated anti-mouse and anti-rabbit IgG antibodies) and fluorescent mounting medium were from Dako (High Wycombe, Bucks, U.K.). The N-terminal transactivation domain of c-Jun (amino acid residues 1–135) was expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* and purified by glutathione–Sephadex (Pharmacia, St. Albans, Herts., U.K.) chromatography [33].

Primary culture of ventricular myocytes

Myocytes were dissociated from the ventricles of neonatal Sprague–Dawley rat hearts by a previously described adaptation [34] of the method of Iwaki et al. [35]. To deplete the myocytes of fibroblasts, the cells were preplated in Dulbecco's modified Eagle's medium/Medium 199 (4:1, v/v) supplemented with 10% (v/v) horse serum, 5% (v/v) foetal calf serum and 100 units/ml each penicillin and streptomycin. The (non-adherent) myocytes were then plated on 60 mm culture dishes precoated with 1% (w/v) gelatin at a density of 1.4×10^3 cells/mm². Myocytes were confluent and beating within 18 h. Serum was withdrawn for 24 h before use.

Cell extraction and preparation of nuclear extracts

Myocytes were exposed to 0.5 M sorbitol, 0.2 μ M okadaic acid, 0.5 M sorbitol + 0.2 μ M okadaic acid or 50 ng/ml anisomycin in serum-free medium. Nuclear extracts were prepared essentially by the method of Dignam et al. [36]. Cells from one 60 mm dish were washed in ice-cold Ca²⁺/Mg²⁺-free Dulbecco's PBS and harvested into 150 μ l of buffer A [10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.3 mM Na₃VO₄, 200 μ M leupeptin, 10 μ M E-64, 5 mM dithiothreitol (DTT), 300 μ M PMSF]. Samples were centrifuged (Eppendorf centrifuge 5403; 10000 g; 5 min; 4 °C) and the supernatant (cytosolic) fractions boiled with 50 μ l of sample buffer [0.33 M Tris/HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 133 mM DTT, 0.2 mg/ml Bromophenol Blue]. The pellets were resuspended in 150 μ l of buffer A containing 0.1% (v/v) Nonidet P40, vortex-mixed and incubated on ice for

10 min. After being mixed again, the samples were centrifuged (10000 g; 5 min; 4 °C) and the supernatant fractions discarded. The pellets were resuspended in 50 μ l of buffer B [20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 0.3 mM Na₃VO₄, 200 μ M leupeptin, 10 μ M E-64, 5 mM DTT, 300 μ M PMSF] and extracted on ice for 1 h with occasional vortex-mixing. The samples were centrifuged (10000 g; 5 min; 4 °C) and the supernatant nuclear extracts boiled with 20 μ l of sample buffer.

Immunoprecipitation of SAPK from nuclear extracts

Nuclear extracts from one 60 mm dish (50 μ l in buffer B) were diluted tenfold in buffer C (10 mM Tris/HCl, 5 mM EDTA, 50 mM NaF, 50 mM NaCl, 2 mM Na₃VO₄, 0.1% fatty acid-free BSA, 20 μ g/ml aprotinin, 300 μ M PMSF, pH 7.4). JNK1 antibody (1 μ g) was added and samples were rotated at 4 °C for 2 h. Protein A–Sephadex (20 μ l of a 50% slurry in buffer C) was added and samples were rotated for a further 1 h. After centrifugation (10000 g; 1 min; 4 °C), the precipitates were washed with 3×150 μ l of buffer C. Precipitates were resuspended in 50 μ l of buffer C and boiled with 20 μ l of sample buffer.

In-gel SAPK assays

SAPKs were assayed by an in-gel kinase method as we have previously described [28,31]. Proteins (measured by the Bradford method [32]) from 0.66×10^6 cells (50 μ g of protein; cytosolic fractions) or 2×10^6 cells (100 μ g of protein; nuclear extracts) were separated by SDS/PAGE on 10% polyacrylamide gels containing 0.5 mg/ml GST–c-Jun(1–135). For JNK1 immunoprecipitates, protein from 1.5×10^6 cells was loaded. After electrophoresis, SDS was first removed from the gels by washing in 20% (v/v) propan-2-ol in 50 mM Tris/HCl, pH 8.0 (3×20 min). The propan-2-ol was removed by washing in 50 mM Tris/HCl, pH 8.0, containing 5 mM 2-mercaptoethanol (3×20 min). Proteins were denatured in 6 M guanidinium chloride, 50 mM Tris/HCl, pH 8.0, and 5 mM 2-mercaptoethanol (2×30 min), then renatured in 50 mM Tris/HCl, pH 8.0, containing 5 mM 2-mercaptoethanol and 0.04% (v/v) Tween 40 (1×30 min, 2×1 h, 1×18 h, 1×30 min; 4 °C). The gels were equilibrated to room temperature with 40 mM Hepes, pH 8.0, containing 2 mM DTT and 10 mM MgCl₂ (2×30 min) and incubated for 3 h with 12.5 μ Ci/gel [γ - 32 P]ATP in 5 ml of 40 mM Hepes, pH 8.0, containing 0.5 mM EGTA, 10 mM MgCl₂, 50 mM ATP and 0.1 μ M cAMP-dependent protein kinase inhibitory peptide. The reaction was stopped and gels were washed with 1% (w/v) disodium pyrophosphate/5% (w/v) trichloroacetic acid. The gels were dried on to 3 MM Whatman chromatography paper and autoradiographed.

PP2A treatment

Nuclear extracts were prepared as described except that Na₃VO₄ was omitted from the extraction buffers and the nuclei were extracted with 25 μ l of buffer B. To this was added an equal volume of 100 mM Tris/HCl, pH 6.0, containing 1 mM CaCl₂ and 80 mM MgCl₂, giving a final pH of 7.2. Samples were incubated with 0.5 units of PP2A (1 h; 37 °C) in the presence or absence of 1 μ M okadaic acid. The reaction was stopped by the addition of 20 μ l of sample buffer and the samples were boiled.

Western-blot analysis

Proteins from 0.66×10^6 (50 μ g of protein; cytosolic fractions) or

Figure 1 Stimulation of SAPK activities by cellular stresses

(A) The time course of activation of SAPKs in ventricular myocytes exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid at 37 °C. Cytosolic and nuclear extracts were prepared and assayed for SAPK activities using the in-gel kinase assay with GST-c-Jun(1-135) as substrate. The two gels were autoradiographed for the same period of time (24 h). The molecular masses in kDa of the two most prominent SAPK activities are indicated on the right. (B) The order of potency of SAPK activation was studied by the in-gel kinase assay. Cells were exposed to 0.5 M sorbitol (S), 0.5 M sorbitol + 0.2 μ M okadaic acid (SOA), 0.2 μ M okadaic acid (OA) or 50 ng/ml anisomycin (A) for 30 min at 37 °C. The two gels were autoradiographed for the same period of time (24 h). The molecular masses in kDa of the two most prominent SAPK activities are indicated on the right. (C) SAPK activities were immunoprecipitated (using a JNK1 antibody) from nuclear extracts of cells exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid at 37 °C for the times indicated. The molecular masses in kDa of the two most prominent SAPK activities are indicated on the right. (D) Western blot using a JNK1 antibody which principally detected a protein migrating at 46 kDa. Cells were exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid at 37 °C for the times indicated. All results are representative of three individual preparations of myocytes.

2×10^6 cells (100 μ g of protein; nuclear extracts) were separated by SDS/PAGE on 10% polyacrylamide gels for c-Jun, and JNK1 immunoblots, or 8% polyacrylamide gels for ATF2 immunoblots. For c-Jun(phospho-Ser-73) immunoblots, nuclear extracts from 4×10^6 cells (200 μ g of protein) were separated on 10% polyacrylamide gels. The proteins were transferred to nitrocellulose using Towbin [37] transfer buffer [25 mM Tris base, 192 mM glycine, 20% (v/v) methanol] for c-Jun (KM-1), c-Jun(phospho-Ser-73) and JNK1 immunoblots or SDS transfer buffer (48 mM Tris base, 39 mM glycine, 1.3 mM SDS) for ATF2 immunoblots.

For c-Jun (KM-1), JNK1 and ATF2 immunoblots, non-specific binding was blocked with 5% (w/v) non-fat milk in buffer D [20 mM NaH_2PO_4 , 80 mM Na_2HPO_4 , 100 mM NaCl, 0.05% (v/v) Tween 20]. Primary antibodies were diluted in the blocking buffer (1:100) and incubated with the blots (18 h; 4 °C). The blots were washed with buffer D (3 $\times$ 5 min; 20 °C) and incubated with horseradish peroxidase-linked secondary antibodies in buffer D containing 1% (w/v) non-fat milk powder (1:5000 dilution; 90 min; 20 °C). The blots were again washed (3 $\times$ 5 min; 20 °C), and the bands detected using enhanced chemiluminescence. Blots were exposed to Hyperfilm MP for 2–15 min and bands were quantified by laser densitometry.

When c-Jun(phospho-Ser-73) antibody was used, immunoblots were probed according to the manufacturer's instructions.

Immunocytochemical staining

Cells were cultured in eight-well Lab-Tek tissue culture chamber slides precoated with laminin (20 μ g/ml in PBS) and 1% (w/v) gelatin at a density of 5×10^5 cells/well for 18 h. Serum was withdrawn for 24 h and the cells were treated with 0.5 M sorbitol or 0.5 M sorbitol + 0.2 μ M okadaic acid in serum-free medium. The medium was removed and the cells washed with ice-cold PBS (3 $\times$ 0.5 ml). The cells were fixed for 10 min with 4% (w/v) formaldehyde and permeabilized with 0.3% (v/v) Triton X-100 in PBS, and non-specific antibody binding was blocked with 1% (w/v) BSA in PBS containing 0.3% (v/v) Triton X-100. c-Jun was detected with KM-1 antibody (1:300 dilution; 1 h; 37 °C), using a biotinylated anti-mouse IgG secondary antibody (1:200 dilution; 30 min; 37 °C) and streptavidin/Texas Red (1:200 dilution; 15 min; 37 °C). Myocytes were identified by double immunostaining with anti- β -MHC antibody (1:40 dilution; 1 h; 37 °C) and FITC-linked anti-mouse IgG (1:200 dilution; 30 min; 37 °C). All antibody dilutions were in PBS and wells were washed

with PBS (3×0.5 ml) between each incubation. When c-Jun (phospho-Ser-63) antibody was used, detection was carried out according to the manufacturer's instructions. Slides were mounted and examined using a Zeiss Axioskop microscope with a $100 \times$ oil immersion objective. Cells were photographed using Tmax 400 film with set exposure times (30 s for Texas Red, 15 s for FITC).

RESULTS

Activation of SAPKs in cytoplasmic and nuclear extracts of ventricular myocytes

The nucleus represents an important site of action of SAPKs where they are involved in phosphorylation of specific transcription factors (reviewed in [27]). Hyperosmotic stress (0.5 M sorbitol) in combination with protein Ser/Thr phosphatase inhibition ($0.2 \mu\text{M}$ okadaic acid) induced a prolonged activation of 46 and 54 kDa SAPKs in cytosolic extracts of ventricular myocytes (Figure 1A). Maximum activation was attained at about 90 min with little loss of activity even at 180 min (Figure 1A). In nuclear extracts, maximum activation occurred at about 30 min and was then sustained for up to 120 min. However, since the nuclear extracts used for the in-gel assay were derived from approximately three times the number of cells as the cytosolic fractions, the total levels of SAPK activity were considerably lower on a per cell basis in the nuclear extracts compared with the cytosol.

Figure 2 Western blots of ATF2 in nuclear extracts

(A) Nuclear extracts were prepared from ventricular myocytes exposed to 0.5 M sorbitol + $0.2 \mu\text{M}$ okadaic acid (SOA) for 30 min at 37°C . The nuclear extracts were subsequently treated with PP2A (1 h; 37°C) in the presence or absence of $1 \mu\text{M}$ okadaic acid (OA) and then blotted. The results are representative of two individual preparations of myocytes. (B) Nuclear extracts were prepared from cells exposed to 0.5 M sorbitol + $0.2 \mu\text{M}$ okadaic acid, 0.5 M sorbitol or 50 ng/ml anisomycin at 37°C for the times indicated. Results are representative of three individual preparations of myocytes.

Figure 3 Quantification of ATF2 phosphorylation in response to cellular stress

Data (means $\pm$ S.E.M.) were derived from Western blots (examples shown in Figure 2B) for three separate preparations of myocytes. Cells were treated with (A) 0.5 M sorbitol + $0.2 \mu\text{M}$ okadaic acid, (B) 0.5 M sorbitol or (C) 50 ng/ml anisomycin. $\bullet$, Band 1; $\circ$, bands 2/3; $\triangle$, band 4.

The order of potency of stresses in terms of their ability to activate cytosolic and nuclear SAPKs was examined after 30 min (Figure 1B). Okadaic acid ($0.2 \mu\text{M}$) alone caused only a marginal stimulation but enhanced the response to sorbitol. The 46 and 54 kDa species were the major c-Jun(1-135) kinase activities detected. In the cytosol, minor bands (approx. 60 and 98 kDa) were detected, but these activities were largely unchanged in response to any treatment. An activity at approx. 40 kDa was also present but could not be readily resolved from the 46 kDa SAPK activity after stimulation. Additional bands were detected in nuclear extracts (129, 95 and 59 kDa) which were more prominent relative to p54 and p46 SAPKs than in cytosolic fractions and also appeared to be stimulated by cell stress.

Importantly, SAPK activity was immunoprecipitated from nuclear extracts of cells exposed to 0.5 M sorbitol + $0.2 \mu\text{M}$ okadaic acid using a JNK1 antibody (Figure 1C). The major activity immunoprecipitated had a molecular mass of 46 kDa,

Figure 4 Western blots of c-Jun in nuclear extracts

(A) Nuclear extracts were prepared from ventricular myocytes exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid (SOA) for 30 min at 37 °C. The nuclear extracts were subsequently treated with PP2A (1 h; 37 °C) in the presence or absence of 1 μ M okadaic acid (OA) and then blotted with KM-1 antibody. The results are representative of two individual preparations of myocytes. (B) Nuclear extracts of cells exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid, 0.5 M sorbitol or 50 ng/ml anisomycin at 37 °C for the times indicated were blotted with KM-1 antibody. Results are representative of three individual preparations of myocytes.

although weak activity was also seen at 54 kDa. We have previously observed that this antibody immunoprecipitates mainly a 46 kDa activity [28]. p46 SAPK was detected by Western blotting with a JNK1 antibody in cytosolic and nuclear fractions, but protein levels were considerably higher in cytosolic fractions than in nuclear extracts (Figure 1D). Sorbitol + okadaic acid induced the appearance of a reduced-mobility p46 SAPK band from 15 min in cytosolic extracts. By analogy with ERK2 [38], this probably represented the phosphorylated form of the enzyme. Because of the much smaller amounts of p46 SAPK protein in nuclear extracts, the reduction in mobility was not as readily apparent. However, there were no detectable increases in p46 SAPK protein levels in cytosolic or nuclear extracts, indicating that there is no net import of p46 SAPK protein into the nucleus when myocytes are stressed.

Phosphorylation of ATF2

Using Western-blot analysis, ATF2 was detected as a single band of approx. 69 kDa in nuclear extracts of unstimulated ventricular myocytes (Figures 2A and 2B, band 1). Only trace amounts of ATF2 were detected in the cytoplasm (results not shown). Sorbitol + okadaic acid caused the appearance of at least three reduced-mobility bands: two closely migrating bands of apparent molecular mass approx. 76 kDa (bands 2/3) and one band of approx. 87 kDa (band 4) (Figures 2A and 2B). To demonstrate that bands 2, 3 and 4 resulted from phosphorylation of ATF2,

Figure 5 Quantification of c-Jun phosphorylated in response to cellular stress

Data (means $\pm$ S.E.M.) were derived from Western blots (examples shown in Figure 4B) using KM-1 antibody for three separate preparations of myocytes. Cells were treated with (A) 0.5 M sorbitol + 0.2 μ M okadaic acid, (B) 0.5 M sorbitol or (C) 50 ng/ml anisomycin. ●, Band 1; ○, band 2.

Figure 6 Effects of cycloheximide treatment on c-Jun immunoreactivity in nuclear extracts from cells exposed to cellular stress

Nuclear extracts were prepared from control cells (C) or cells exposed to 0.5 M sorbitol (S), 0.5 M sorbitol + 0.2 μ M okadaic acid (SOA), or 50 ng/ml anisomycin (A) for 30 min at 37 °C. Where applicable, cells were pretreated with 20 μ M cycloheximide (CHX) for 10 min and cycloheximide was present throughout the incubation. c-Jun phosphorylation was detected using KM-1 antibody.

Figure 7 Western blots of c-Jun(phospho-Ser-73) in nuclear extracts

Nuclear extracts were prepared from ventricular myocytes exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid for the times indicated at 37 °C and were blotted with c-Jun(phospho-Ser-73) antibody. Results are representative of two individual preparations of myocytes.

nuclear extracts from cells stimulated with sorbitol + okadaic acid for 30 min were incubated with PP2A. This resulted in the reappearance of primarily the 69 kDa ATF2 band, with only a trace of band 2 remaining (Figure 2A). The appearance of the 69 kDa ATF2 was inhibited by including okadaic acid in the PP2A incubation. Bands 2, 3 and 4 therefore all result from Ser-/Thr-phosphorylation of the 69 kDa ATF2 protein.

Quantification of the immunoblotted ATF2 bands (Figure 2B) using laser scanning densitometry clearly showed the sequential shift to the more slowly migrating phosphorylated forms (Figures 3A–3C). Because of the lack of clear separation, ATF2 bands 2 and 3 were combined for quantification. Total amounts of ATF2 decreased slightly with sorbitol + okadaic acid (Figure 3A) or sorbitol treatment (Figure 3B) after 120 min but did not change

Figure 8 Immunostaining of c-Jun in cells exposed to sorbitol

Cells exposed to 0.5 M sorbitol at 37 °C for the times indicated were immunostained with antibodies against β -MHC (A, C and E) or c-Jun (B, D and F). Bar = 50 μ m.

Figure 9 Immunostaining of c-Jun in cells exposed to sorbitol + okadaic acid

Cells exposed to 0.5 M sorbitol + 0.2 μ M okadaic acid at 37 °C for the times indicated were immunostained with antibodies against β -MHC (**A**, **C** and **E**) or c-Jun (**B**, **D** and **F**). Bar = 50 μ m.

significantly with anisomycin (Figure 3C). Stimulation with sorbitol + okadaic induced a shift from band 1 to bands 2/3 in approx. 15 min and then a much stronger shift to band 4 (Figures 2B and 3A). Sorbitol (Figures 2B and 3B) and anisomycin (Figures 2B and 3C) both induced a shift from band 1 to 2/3 in approx. 15 min, but whereas this was sustained with sorbitol stimulation, anisomycin treatment also induced low levels of band 4 from 15 to 30 min.

Phosphorylation of c-Jun

c-Jun was detected by Western blotting with antibody KM-1 as a weak 39 kDa band in nuclear extracts of unstimulated myocytes (Figures 4A and 4B) with trace amounts detectable in the

cytoplasm (results not shown). A band of apparent molecular mass approx. 46 kDa (band 2, Figure 4A) was detected after treatment of cells with sorbitol + okadaic acid for 30 min. Occasionally, an additional band was detected between bands 1 and 2 (Figure 4A). Treatment of these extracts with PP2A reduced the levels of c-Jun band 2 and increased 39 kDa c-Jun (Figure 4A). This was inhibited by including okadaic acid in the PP2A reaction mixture. More prolonged exposure to sorbitol + okadaic acid induced the appearance of two further bands, namely band 3, approx. 51 kDa, and band 4, approx. 57 kDa (Figure 4B). However, the migration of bands 3 and 4 was not sensitive to treatment with PP2A (results not shown). These bands may thus not represent Ser-/Thr-phosphorylated c-Jun and have not been analysed further. Sorbitol or anisomycin

Figure 10 Immunostaining of c-Jun(phospho-Ser-63) in cells exposed to sorbitol + okadaic acid

Cells not exposed to agonists (**A**) or exposed to 0.5 M sorbitol (**B**) or 0.5 M sorbitol + 0.2 μ M okadaic acid (**C**) at 37 °C for 30 min were immunostained with antibodies against c-Jun(phospho-Ser-63). Bar = 50 μ m.

induced the appearance of c-Jun bands 1 and 2, but (unlike sorbitol + okadaic acid) neither sorbitol nor anisomycin caused the appearance of bands 3 and 4 (Figure 4B).

Quantification of c-Jun immunoblots using antibody KM-1 showed that there was a large (> 10-fold) apparent increase in total immunoreactivity from as early as 5 min with all treatments, maxima being attained at 15–30 min (Figures 5A–5C). Whereas sorbitol + okadaic acid or anisomycin induced higher relative levels of band 2 from 15 to 30 min (Figures 5A and 5C), the levels of bands 1 and 2 changed in parallel with sorbitol stimulation (Figure 5B). Although this could be interpreted as indicating that there is induction of c-Jun protein in addition to phosphorylation, inhibition of protein synthesis with 20 μ M cycloheximide (a

concentration that inhibited myocyte protein synthesis by more than 90 %; results not shown) had no effect on these increases in total c-Jun (Figure 6). Indeed, cycloheximide increased total levels of c-Jun and induced the appearance of band 2 in the absence of additional treatments. We assume that this is a consequence of cycloheximide-induced activation of SAPKs. These results indicate that the increased levels of c-Jun (bands 1 and 2) seen in Figures 4 and 5 represent phosphorylation and are not the result of *de novo* protein synthesis.

Other antibodies to c-Jun were also used. A polyclonal antibody raised to amino acids 91–105 (Santa Cruz Biotechnology Inc.; sc-45) did not detect band 2 in these samples (results not shown). This sequence contains two Thr-phosphorylation sites [39,40]. Another study using an antibody to the same sequence showed that phosphorylation prevented the recognition of the epitope [40]. This suggests that band 2 is Thr-phosphorylated in the 91–105-amino acid sequence of c-Jun. An antibody that recognizes c-Jun(phospho-Ser-73) showed a similar increase in c-Jun immunoreactive bands 1 and 2 (Figure 7) to that observed with antibody KM-1 (Figure 4B). Bands 3 and 4 were not detected. These results confirm that antibody KM-1 recognizes phospho-c-Jun species.

Subcellular localization of c-Jun

We examined the pattern of c-Jun immunostaining in whole cells exposed to stresses (Figures 8B, 8D, 8F, 9B, 9D and 9F) using antibody KM-1. Cells were co-immunostained with an antibody against β -MHC to identify myocytes (Figures 8A, 8C, 8E, 9A, 9C and 9E). In control myocytes, c-Jun immunostaining was localized to the nucleus, with minimal cytoplasmic staining (Figure 8B). Exposure of cells to sorbitol for 1 h (Figure 8D) increased c-Jun immunostaining in the nuclei of myocytes (and in non-myocytes, which are always present in cultures as a minor population). In comparison with the control cells, the immunostaining became more punctate. After 2 h, c-Jun immunostaining had declined (Figure 8F). Compared with control cells (Figure 9B), nuclear c-Jun immunostaining in cells exposed to sorbitol + okadaic acid for 1 h was also increased and more punctate (Figure 9D). However, after 2 h (Figure 9F), myocytes showed a marked increase in c-Jun immunostaining of both the nucleus and cytoplasm. This was not apparent in non-myocytes (Figure 9F). Cells were also stained with antibody against c-Jun (phospho-Ser-63) (Figures 10A–10C). After exposure to sorbitol (Figure 10B) or sorbitol + okadaic acid (Figure 10C) for 30 min, there was a distinct increase in nuclear staining, indicating phosphorylation of nuclear c-Jun. With both the KM-1 and the c-Jun(phospho-Ser-63) antibodies, there was some increase in cytoplasmic staining after treatment with both sorbitol and sorbitol + okadaic acid for 1 h (Figures 8D, 9D and 10B and 10C), although this was much less than after treatment with sorbitol + okadaic acid for 2 h (Figure 9F). The reasons for this are unclear, but could represent low levels of cytoplasmic c-Jun or other unidentified proteins. This was not investigated further.

DISCUSSION

‘Stress-regulated’ MAPKs in heart

The SAPKs/JNKs were first identified as activities stimulated by cell stresses which preferentially phosphorylated the N-terminal transactivation domain of c-Jun (reviewed in [27]). These activities are the products of at least three alternatively spliced genes [41–45]. SAPK/JNK activities commonly migrate at 46 and 54 kDa on SDS/PAGE, but because of alternative splicing, these may be the products of any of the three genes [45]. A second

separate group of 'stress-regulated' MAPKs, the p38 MAPKs, was subsequently identified (reviewed in [27]). In neonatal rat cultured ventricular myocytes, cell stresses (hyperosmolarity, protein-synthesis inhibitors, mechanical stretching) activate SAPKs [28,29], as does ischaemia/reperfusion stress in perfused rat hearts [30,31]. In contrast with SAPKs, we have been unable to detect activation of p38 MAPK in ventricular myocytes by cell stresses [28], but ischaemia powerfully activates p38 MAPK in perfused heart [31].

Anisomycin and sorbitol activate SAPKs in soluble extracts of ventricular myocytes [28]. The response to sorbitol is slightly greater and more sustained than that obtained with anisomycin [28]. The PP1/PP2A inhibitor, okadaic acid, had a minimal effect on SAPK activation, but considerably enhanced the response to sorbitol (Figure 1B). The most probable explanation is that okadaic acid inhibits SAPK dephosphorylation, but it may generate reactive oxygen intermediates [46] which are activators of SAPKs in heart (A. Clerk and P. H. Sugden, unpublished work).

There is clearly powerful activation of SAPK in the cytosol. As yet, no cytosolic substrates of SAPK have been identified, but this may be merely because evidence of SAPK-mediated phosphorylation of cytoplasmic substrates has not been sought. For SAPK-mediated phosphorylation of transcription factors, the simplest scheme would involve activation of nuclear SAPKs. We detected activation of p46 and p54 SAPKs in the nucleus in response to cell stress (Figures 1A–1C), but the levels of activity were considerably less than in the cytosol. JNK1 antibodies immunoprecipitated p46 SAPK activity from nuclear extracts of cells treated with sorbitol+okadaic acid (Figure 1C). Western blots with JNK1 antibodies detected very low levels of protein in nuclear extracts relative to the cytosol, but there was no net increase in the amount of protein after stimulation with sorbitol+okadaic acid (Figure 1D). Immunocytochemical studies with this antibody gave weak cytoplasmic staining of myocytes, but also showed no accumulation of protein in the nucleus after sorbitol+okadaic acid treatment (results not shown). There is therefore no detectable import of JNK1 into the nucleus. This contrasts with the situation in UV-irradiated HeLa cells, where import of JNK1 into the nucleus has been demonstrated [47]. Activation of a discrete nuclear pool of p46 SAPKs could be involved. Alternatively, there may be exchange of p46 SAPKs between the nuclear and cytoplasmic compartments. We have also detected immunoreactivity with JNK2 antibodies (Santa Cruz Biotechnology Inc.; sc-827-G) on Western blots but the antibodies were not suitable for immunoprecipitation or immunocytochemistry (results not shown). Consequently, activation and distribution of these isoforms could not be assessed.

Phosphorylation of ATF2

ATF2 is phosphorylated by SAPKs/JNKs on three N-terminal residues [24–26]. Phosphorylation of Thr-69 and Thr-71 is necessary for ATF2 transactivation. Ser-90 is also phosphorylated, but this has little effect on transactivating activity. C-terminal residues are also phosphorylated, although these sites are not well characterized and their role in ATF2 activation remains to be determined [24]. ATF2 in nuclear extracts from control myocytes migrates as a single 69 kDa band (Figures 2A and 2B). Phosphorylation of ATF2 by SAPKs/JNKs produces proteins that migrate with reduced relative mobility on SDS/PAGE [24–26]. We detected three reduced mobility ATF2 proteins in myocytes exposed to cell stress (bands 2–4, Figures 2A and 2B). PP2A treatment resulted in the reappearance of the 69 kDa ATF2 band, indicating that bands 2–4 are Ser-/Thr-

phosphorylated forms of ATF2. The progressive shift from band 1 to bands 2/3 and then to band 4 is suggestive of sequential increasing phosphorylation of ATF2 on multiple sites.

Phosphorylation of c-Jun

c-Jun is phosphorylated at multiple N- and C-terminal residues. Phosphorylation of C-terminal Thr-231, Ser-243 and Ser-249 in the DNA-binding domain inhibits DNA binding [48]. In contrast, phosphorylation of Ser-63 and Ser-73 in the N-terminal transactivation domain by SAPKs/JNKs increases c-Jun transactivating activity ([39,49] and reviewed in [23,27]). In addition, phosphorylation of Thr-91 and Thr-93 [39] may be important in inducing a conformational change that allows dephosphorylation of the C-terminal residues [40]. The N-terminal phosphorylations result in the appearance of multiple bands that migrate with reduced mobility on SDS/PAGE [40].

Sorbitol+okadaic acid induced the sequential appearance of at least three reduced-mobility c-Jun immunoreactive bands detectable with KM-1 antibody (Figure 4B). Only band 2, which was also detected after sorbitol or anisomycin stimulation (Figure 4B), was sensitive to PP2A treatment (Figure 4A and results not shown). Band 2 thus represents a Ser-/Thr-phosphorylated form of c-Jun. The identities of bands 3 and 4 are unclear (Figure 4B). Band 4 was particularly prominent on Western blots of cytosolic extracts (results not shown), and increased immunostaining was seen throughout the myocyte after 2 h (Figure 9F). Thus band 4 could be a cross-reactive protein induced by sorbitol+okadaic acid or a form of c-Jun which has undergone a PP2A-insensitive modification.

The site(s) phosphorylated in c-Jun band 2 (Figure 4B) cannot be identified from our experiments with KM-1 antibody. However, a polyclonal antibody raised to amino acids 91–105 (sc-45) did not detect band 2 in these samples (results not shown). Another study using an antibody to the same sequence showed that phosphorylation prevented the recognition of this epitope [40]. Band 2 therefore probably represents a form of c-Jun phosphorylated on Thr-91 and/or Thr-93, possibly in addition to Ser-63 and Ser-73. An antibody to c-Jun(phospho-Ser-73) demonstrated that Ser-73 was phosphorylated in both bands 1 and 2 (Figure 7). The additional band sometimes detected between bands 1 and 2 (it is visible in Figure 4A) presumably represents an intermediate phosphorylation step or conformational change.

Cell stresses induced an apparent rapid increase in the total amount of nuclear c-Jun immunoreactivity detected by KM-1 antibody (Figures 4A and 4B, and Figures 5A–5C). This was not the result of nuclear import of c-Jun because cytosolic c-Jun was barely detectable by Western blotting (results not shown) or immunolocalization (Figures 8 and 9). *De novo* protein synthesis was not involved, because cycloheximide did not inhibit the increase (Figure 6). Indeed, cycloheximide alone increased total c-Jun immunoreactivity and induced the appearance of band 2 (Figure 6). We attribute this to activation of SAPKs [41]. The epitope recognized by KM-1 antibody (amino acid residues 56–69) encompasses the Ser-63 phosphorylation site. A conformational change in the protein, resulting from changes in phosphorylation state, presumably exposes the epitope and results in an apparent increase in total c-Jun. Similar results were obtained with an antibody to c-Jun(phospho-Ser-73) (Figure 7), confirming this hypothesis. This has led to confusion in other studies which have not considered this possibility [50]. Indeed, very recently, Santa Cruz have begun to market KM-1 as a phospho-c-Jun-specific antibody.

Correlation of ATF2 and c-Jun phosphorylation with SAPK activation

Maximal phosphorylation of ATF2 and c-Jun was seen after stimulation with sorbitol + okadaic acid (Figures 2B and 4B), the intervention that gave the greatest activation of SAPKs (Figure 1B). However, anisomycin elicited greater and more sustained phosphorylation of ATF2 and c-Jun than was seen with sorbitol (Figures 2B and 4B), although sorbitol gives a greater and more sustained activation of SAPKs [28]. This could result from the marginally higher level of activity seen in nuclear extracts after anisomycin stimulation compared with sorbitol (Figure 1B). Alternatively, additional pathways may be activated by anisomycin leading to ATF2 and c-Jun phosphorylation. ATF2 and c-Jun are substrates for MAPKs other than the SAPKs/JNKs. The extracellularly responsive kinase (ERK) family of MAPKs phosphorylates c-Jun and ATF2 *in vitro* on the same N-terminal sites as the SAPKs/JNKs, although the significance of this in the regulation of transcription *in vivo* is unclear [25,26,49]. The transactivation domain of ATF2 is also phosphorylated by the p38 MAPKs [51].

Stimulation of SAPKs by sorbitol + okadaic acid in both cytosolic and nuclear extracts was maintained over 120–180 min (Figure 1A). Activation of SAPKs by sorbitol in cytosolic extracts is maintained over 240 min [28]. The abundance of phosphorylated c-Jun in cells exposed to sorbitol (bands 1 and 2, Figure 4B) declined over 60–120 min (Figure 5B). This could represent degradation of c-Jun or dephosphorylation by a phospho-c-Jun phosphatase. Similar effects were observed with sorbitol + okadaic acid (Figure 5A). Although degradation or dephosphorylation could account for this, further modification of c-Jun may occur producing bands 3 and 4 (Figure 2B).

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REFERENCES

- Lip, G. Y., Gammage, M. D. and Beevers, D. G. (1994) *Br. Med. Bull.* **50**, 299–321
- Jennings, R. B., Steenbergen, Jr., C. and Reimer, K. A. (1995) *Monogr. Pathol.* **37**, 47–80
- Izumo, S., Nadal-Ginard, B. and Mahdavi, V. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 339–343
- Brand, T., Sharma, H. S., Fleischmann, K. E., Duncker, D. J., McFalls, E. O., Verdouw, P. D. and Schaper, W. (1992) *Circ. Res.* **71**, 1351–1360
- Rozich, J. D., Barnes, M. A., Schmid, P. G., Zile, M. R., McDermott, P. J. and Cooper, G., IV. (1995) *J. Mol. Cell. Cardiol.* **27**, 485–499
- Bauters, C., Moalic, J. M., Bercovici, J., Mouas, C., Emanoil Ravier, R., Schiaffino, S. and Swynghedauw, B. (1988) *J. Mol. Cell. Cardiol.* **20**, 97–101
- Kolbeck-Ruhmkorff, C. and Zimmer, H. G. (1995) *J. Mol. Cell. Cardiol.* **27**, 501–511
- Plumier, J.-C. L., Robertson, H. A. and Currie, R. W. (1996) *J. Mol. Cell. Cardiol.* **28**, 1251–1260
- Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F. and Yazaki, Y. (1990) *J. Biol. Chem.* **265**, 3595–3598
- Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T. J. and Izumo, S. (1992) *J. Biol. Chem.* **267**, 10551–10560
- Webster, K. A., Discher, D. J. and Bishopric, N. H. (1993) *J. Biol. Chem.* **268**, 16852–16858
- Webster, K. A., Discher, D. J. and Bishopric, N. H. (1994) *Circ. Res.* **74**, 679–686
- Yao, A., Takahashi, T., Aoyagi, T., Kinugawa, K., Kohmoto, O., Sugiura, S. and Serizawa, T. (1995) *J. Clin. Invest.* **96**, 69–77
- McMahon, S. B. and Monroe, J. G. (1992) *FASEB J.* **6**, 2707–2715
- Nordheim, A. (1994) *Nature (London)* **370**, 177–178
- Rozek, D. and Pfeifer, G. P. (1993) *Mol. Cell. Biol.* **13**, 5490–5499
- Herr, I., van Dam, H. and Angel, P. (1994) *Carcinogenesis* **15**, 1105–1113
- Han, T. H. and Prywes, R. (1995) *Mol. Cell. Biol.* **15**, 2907–2915
- Tornaletti, S. and Pfeifer, G. P. (1995) *J. Mol. Biol.* **249**, 714–728
- Kitabayashi, I., Saka, F., Gachelin, G. and Yokoyama, K. (1990) *Nucleic Acids Res.* **18**, 3400
- Stein, B., Angel, P., van Dam, H., Ponta, H., Herrlich, P., van der Eb, A. and Rahmsdorf, H. J. (1992) *Photochem. Photobiol.* **55**, 409–415
- Smith, S. E., Papavassiliou, A. G. and Bohmann, D. (1993) *Nucleic Acids Res.* **21**, 1581–1585
- Karin, M. (1995) *J. Biol. Chem.* **270**, 16483–16486
- Gupta, S., Campbell, D., Dérjard, B. and Davis, R. J. (1995) *Science* **267**, 389–393
- Livingstone, C., Patel, G. and Jones, N. (1995) *EMBO J.* **14**, 1785–1797
- van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P. and Angel, P. (1995) *EMBO J.* **14**, 1798–1811
- Kyriakis, J. M. and Avruch, J. (1996) *J. Biol. Chem.* **271**, 24313–24316
- Bogoyevitch, M. A., Ketterman, A. J. and Sugden, P. H. (1995) *J. Biol. Chem.* **270**, 29710–29717
- Komuro, I., Kudo, S., Yamazaki, T., Zou, Y., Shiojima, I. and Yazaki, Y. (1996) *FASEB J.* **10**, 631–636
- Knight, R. J. and Buxton, D. B. (1996) *Biochem. Biophys. Res. Commun.* **218**, 83–88
- Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J. and Sugden, P. H. (1996) *Circ. Res.* **79**, 162–173
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Smith, S. B. and Johnson, K. S. (1988) *Gene* **67**, 31–40
- Bogoyevitch, M. A., Clerk, A. and Sugden, P. H. (1995) *Biochem. J.* **309**, 437–443
- Iwaki, K., Sukhatme, V. P., Shubeita, H. E. and Chien, K. R. (1990) *J. Biol. Chem.* **265**, 13809–13817
- Dignam, J. D., Lebowitz, R. M. and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Clerk, A., Bogoyevitch, M. A., Andersson, M. B. and Sugden, P. H. (1994) *J. Biol. Chem.* **269**, 32848–32857
- Hibi, M., Lin, A., Minden, A. and Karin, M. (1993) *Genes Dev.* **7**, 2135–2148
- Papavassiliou, A. G., Treier, M. and Bohmann, D. (1995) *EMBO J.* **14**, 2014–2019
- Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) *Nature (London)* **369**, 156–160
- Dérjard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R. J. (1994) *Cell* **76**, 1025–1037
- Kallunki, T., Su, B., Tsigelny, I., Sluss, H. K., Dérjard, B., Moore, G., Davis, R. and Karin, M. (1994) *Genes Dev.* **8**, 2996–3007
- Sluss, H. K., Barrett, T., Dérjard, B. and Davis, R. J. (1994) *Mol. Cell. Biol.* **14**, 8376–8384
- Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Dérjard, B. and Davis, R. J. (1996) *EMBO J.* **15**, 2760–2770
- Schmidt, K. N., Traencker, E. B.-M., Meier, B. and Baeuerle, P. A. (1995) *J. Biol. Chem.* **270**, 27136–27142
- Cavigelli, M., Dolfi, F., Claret, F.-X. and Karin, M. (1995) *EMBO J.* **14**, 5957–5964
- Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. and Hunter, T. (1991) *Cell* **64**, 573–584
- Minden, A., Lin, A., Smeal, T., Dérjard, B., Cobb, M., Davis, R. and Karin, M. (1994) *Mol. Cell. Biol.* **14**, 6683–6688
- Beltman, J., McCormick, F. and Cook, S. J. (1996) *J. Biol. Chem.* **271**, 27018–27024
- Raingaud, J., Gupta, S., Rogers, J. S., Dickens, M., Ulevitch, R. J. and Davis, R. J. (1995) *J. Biol. Chem.* **270**, 7420–7426