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Original article

Effects of oxidative stress on the cardiac myocyte proteome: modifications to peroxiredoxins and small heat shock proteins

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Abstract

Endogenous oxidative stress is a likely cause of cardiac myocyte death in vivo. We examined the early (0–2 h) changes in the proteome of isolated cardiac myocytes from neonatal rats exposed to H_2O_2 (0.1 mM), focussing on proteins with apparent molecular masses of between 20 and 30 kDa. Proteins were separated by two-dimensional gel electrophoresis (2DGE), located by silver-staining and identified by mass spectrometry. Incorporation of [^{35}S]methionine or ^{32}Pi was also studied. For selected proteins, transcript abundance was examined by reverse transcriptase–polymerase chain reaction. Of the 38 protein spots in the region, 23 were identified. Two families showed changes in 2DGE migration or abundance with H_2O_2 treatment: the peroxiredoxins and two small heat shock protein (Hsp) family members: heat shock 27 kDa protein 1 (Hsp25) and αB-crystallin. Peroxiredoxins shifted to lower pI values and this was probably attributable to 'over-oxidation' of active site Cys-residues. Hsp25 also shifted to lower pI values but this was attributable to phosphorylation. αB-crystallin migration was unchanged but its abundance decreased. Transcripts encoding peroxiredoxins 2 and 5 increased significantly. In addition, 10 further proteins were identified. For two (glutathione S-transferase π, translationally-controlled tumour protein), we could not find any previous references indicating their occurrence in cardiac myocytes. We conclude that exposure of cardiac myocytes to oxidative stress causes post-translational modification in two protein families involved in cytoprotection. These changes may be potentially useful diagnostically. In the short term, oxidative stress causes few detectable changes in global protein abundance as assessed by silver-staining.

Keywords: Oxidative stress; Proteomics; Peroxiredoxins; Small heat shock proteins

1. Introduction

In mammalian cardiac pathologies, a likely prime cause of death of the cardiac myocytes in vivo is the stress resulting from endogenous generation of reactive oxygen species (ROS) [1,2]. Oxidative stress increases in ischaemic hearts, with a further increase occurring within minutes of reperfusion (if indeed this occurs) [3–5]. Because the mammalian cardiac myocyte withdraws from the cell cycle during early postnatal development (3–4 days postnatally in the rat [6]), events that promote cell death such as ischaemia/reperfusion [7] irreversibly reduce the myocyte population and are likely to be deleterious to cardiac function. Moreover, cardiac myocytes may be inherently more susceptible to ROS-mediated damage than other cells such as fibroblasts [8]. Cardiac myocyte death can occur through necrotic and/or apoptotic mechanisms [7], both of which have been observed in various human cardiac diseases, including ischaemic/non-ischaemic heart failure, myocardial infarction and arrhythmias [9,10].

Primary cultures of cardiac myocytes exposed to H_2O_2 provide an in vitro model for the damaging effects of ROS in

Abbreviations: DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; *Gapd*, glyceraldehyde-3-phosphate dehydrogenase gene; GqPCR, Gq protein coupled receptor; Hsp, heat shock protein; IEF, isoelectric focusing; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MS, mass spectrometry; PKC, protein kinase C; *Prdx*, peroxiredoxin gene; Prdx, peroxiredoxin protein; RKIP, Raf-1 kinase inhibitory protein; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SOD2, Mn^{2+}-superoxide dismutase mitochondrial; 2DGE, two-dimensional gel electrophoresis.

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vivo with almost 100% of cells showing signs of apoptosis within 16–24 h exposure to 0.5 mM H_2O_2 [11]. In order to gain insight into the early effects of ROS on cardiac myocytes, we used a proteomic approach involving two-dimensional gel electrophoresis (2DGE) and silver-staining to assess changes in patterns of the cardiac myocyte protein complement, combined with mass spectrometry (MS) to identify such proteins. In addition to identifying 10 proteins that did not change in abundance or 2DGE migration characteristics with oxidative stress, this study revealed changes in the 2DGE migration pattern and/or abundance of two families of proteins putatively involved in cytoprotection, namely the peroxiredoxin (Prdx) family [12–15] and in two members of the small heat shock protein 20 (Hsp20) family, heat shock 27 kDa protein 1 (Hsp25, the rat orthologue of human HSP27) and αB-crystallin [16–18].

2. Materials and methods

2.1. Cell culture

Myocytes were dissociated from neonatal 1–3-day-old Sprague–Dawley rat heart ventricles and cultured as described previously in [19]. Following preplating to remove non-cardiac myocytes, cells were plated in 60 mm Primaria plastic culture dishes (Becton Dickenson) in 4 ml per dish of DMEM/M199 (4:1) with 10% (v/v) horse serum and 5% (v/v) foetal calf serum for 18 h, at a density of 1.4×10^3 cells per mm^2 [19]. After plating, the medium was replaced with serum-free DMEM/M199 medium for a further 24 h or, for radiolabelling experiments, the same medium containing either 50 μCi/ml [^{35}S]methionine or 50 μCi/ml ^{32}Pi (both from Amersham Biosciences) for a further 24 h. Subsequently, myocytes were exposed to 0.1 mM H_2O_2 (30 or 120 min) or remained untreated.

2.2. 2DGE

Following removal of culture medium and washing in ice-cold phosphate-buffered saline, cardiac myocytes were resuspended in 25–50 μl extraction buffer (20 mM L-glycerophosphate pH 7.5, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM benzamidine) containing 1% (v/v) Triton-X100, 200 μM leupeptin, 300 μM phenylmethylsulphonyl fluoride, 10 μM L-*trans*-epoxy-succinyl-L-leucylamido-(4-guanidino)butane (E64) and 2 μM microcystin LR. Because of the low ionic strength of the extraction medium and the absence of ATP, this protocol would not solubilise the majority of the myofibrillar protein. Following microcentrifugation (10,000 × *g*, 10 min, 4 °C), supernatant protein (100–300 μg measured by the Bradford method [20]) in < 35 μl of protein extraction buffer was diluted with rehydration buffer (8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 65 mM dithiothreitol (DTT), 2% IPG Buffer pH 3-10 NL (Amersham Biosciences), trace of bromophenol blue) to a final volume of 350 μl and loaded onto an 18 cm Immobiline DryStrip pH 3-10 NL (Amersham Biosciences). First dimension isoelectric focusing (IEF) was performed as recommended by the supplier (Amersham Biosciences) except that a paper wick, soaked in 65 mM DTT was placed inside the cathodic wick to reduce 'streaking' of basic proteins [21]. IEF was performed (Multiphor II electrophoresis system, Multitemp III and EPS 3501 XL power pack, Amersham Biosciences) in step mode (500 V, 5 h; then 3500 V, 14.5 h; 10 °C). Subsequently, strips were treated with 32 mM DTT (15 min), followed by 0.24 M iodoacetamide (15 min). Second dimension sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) separation was performed (Ettan Dalt II electrophoresis system, Amersham Biosciences) on 12% (w/v) gels (5 W/gel, 30 min; then 160 W until the bromophenol blue had migrated 18 cm; 25 °C). Following electrophoresis, gels were fixed (overnight; 40% (v/v) ethanol/10% (v/v) glacial acetic acid). Molecular mass and pI calibration was performed using two-dimensional molecular mass and pI standards (BioRad, cat. no. 161–0320).

2.3. Silver-staining, autoradiography and densitometry

Silver-staining of analytical gels was conducted automatically (Processor Plus, Amersham Biosciences) as recommended by the supplier. For the preparative gels used in the MS analysis, a modified protocol omitting glutaraldehyde was used [22]. For autoradiography, gels were dried (60 °C, 4 h) on Whatmann 3MM paper and exposed to Hyperfilm MP (Amersham Biosciences) either at 25 °C for 7 days (^{35}S) or at –80 °C with intensifying screens for 2 days (^{32}P).

Each of the 23 spots identified by MS (see below), together with a further 15 unknown spots within the 30–20 kDa area of the gel were analysed densitometrically using Amersham Biosciences ImageMaster 2D software (which is now marketed as Phoretix 2-D Evolution software). Gel background was corrected using the 'mode of non-spot' method. This correction is contained in the gel analysis software and is fully automatic. The default 'margin' setting of 45 was used. The sum of the densitometric values (total spot volume) for all 38 spots was calculated. The intensities of the spots were normalised using total spot volume to correct for differences in loading or staining between gels and expressed as a percentage of total spot volume.

2.4. Protein analysis by MS

Proteins were excised from wet gels using a pipette tip. For dried gels, the autoradiographic spot images were first aligned with the corresponding silver-stained spots. Spots were circumscribed with a glass cutting pen and lifted free using a scalpel blade. The excised spots were destained, reduced, *S*-alkylated with iodoacetamide and digested with trypsin using an Investigator Progest (Genomic Solutions) robotic digestion system, as previously described in [23].