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Proteomic profiling of endothelin-1-stimulated hypertrophic cardiomyocytes reveals the increase of four different desmin species and α-B-crystallin

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ABSTRACT

We performed a proteomic investigation on primary cultures of neonatal rat cardiomyocytes after treatment with 10 nM endothelin-1 (ET1) for 48 h, an *in vitro* model for cardiac hypertrophy. Two-dimensional gel electrophoresis profiles of cell lysates were compared after colloidal Coomassie Blue staining. 12 protein spots that significantly changed in density due to ET1 stimulation were selected for in-gel digestion and identified through mass spectrometry. Of these, 8 spots were increased and 4 were decreased. Four of the increased proteins were identified as desmin, the cardiac component of intermediate filaments and one as α-B-crystallin, a molecular chaperone that binds desmin. All the desmins increased 2- to 5-fold, and α-B-crystallin increased 2-fold after ET1 treatment. Desmin cytoskeleton has been implicated in the regulation of mitochondrial activity and distribution, as well as in the formation of amyloid bodies. Mitochondria-specific fluorescent probe MitoTracker indicated mitochondrial redistribution in hypertrophic cells. An increase of amyloid aggregates containing desmin upon treatment with ET1 was detected by filter assay. Of the four proteins that showed decreased abundance after ET1 treatment, the chaperones hsp60 and grp75 were decreased 13- and 9-fold, respectively. In conclusion, proteomic profiling of ET1-stimulated rat neonatal cardiomyocytes reveals specific changes in cardiac molecular phenotype mainly involving intermediate filament and molecular chaperone proteins.

1. Introduction

Various common cardiovascular diseases, such as ischemic heart disease, hypertension and valve defects result in increased cardiac workload and/or impaired mechanical performance, leading to hypertrophy [1,2]. It has become evident that not only mechanical stress but also locally produced (neuro)hormones – such as noradrenaline, angiotensin II, endothelin-1 (ET1) and growth factors – contribute to the initial development of cardiac hypertrophy. Although cardiac hypertrophy at initial stages is considered to be compensatory in nature, the hypertrophied heart eventually decompensates and ultimately goes into failure [1–4]. The molecular processes involved in the transition from hypertrophy to failure are still poorly understood [5]. Changes in cardiac phenotype, i.e. quantitative (amount) and qualitative [isoform and post-translational modifications (PTMs)] variations in the protein expression profile, are believed to be associated with the deterioration of cardiac function. Like other hypertrophic stimuli, such as angiotensin II and α_1-adrenergic agonists, ET1 has potent effects on signaling pathways that depend on both Ca^{2+} and hydrolysis of membrane phospholipids [6]. In addition, ET1 activates tyrosine kinases and mitogen-activated protein kinases, signaling pathways known to regulate cell growth [7]. Increased levels of plasma ET1 have been observed in several cardiovascular diseases such as acute myocardial infarction, chronically increased workload and heart failure [8].

Previously, we and many other groups demonstrated that the primary culture of rat neonatal cardiomyocytes, as far as the signaling reactions and characteristic phenotypic changes are concerned, responds to the various hypertrophic stimuli (angiotensin II, ET1, noradrenaline, leukemia inhibitory factor, etc) in a manner very similar to the *in vivo* situation [3,5,7,9]. Moreover, cell cultures offer different advantages when one tries to dissect phenotype changes through a

Abbreviations: 2DE, 2-dimensional gel electrophoresis; ACN, acetonitrile; CryAB, α-B-crystallin; DCM, dilated cardiomyopathy; DRM, desmin-related cardiomyopathy; ET1, endothelin-1; IEF, isoelectric focusing; IFs, intermediate filaments; IPG, Immobilized pH gradient; LC–ESI-Q-TOF-MS/MS, liquid chromatography–electrospray-ionisation-quadrupole selection/time-of-flight tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser-desorption-ionisation/time-of-flight mass spectrometry; MT, MitoTracker; PTM, post-translational modification; SFM, serum-free medium; TBS, Tris-buffered saline; TCA, trichloroacetic acid; TFA, trifluoroacetic acid

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proteomic approach, such as: homogenous cell population, enrichment of cardiac myocyte-specific protein, improved reproducibility, etc. Therefore we performed a global two-dimensional gel electrophoresis (2DE)-based proteomic analysis of ET1-stimulated rat neonatal cardiomyocytes to track changes in protein expression and their PTMs during the development of cardiomyocyte hypertrophy. Here we show that a cluster of post-translationally modified desmin species and α-B-crystallin (CryAB) are among the cardiomyocyte proteins found to be increased upon ET1 stimulation. Recent literature highlights the concerted role of intermediate filament and chaperone proteins, and mitochondria in the etiopathology of diverse cardiomyopathies. [10–12]. Therefore the cellular localization of desmin and mitochondria in control and ET1-stimulated cardiomyocytes using a monoclonal antibody against desmin and the specific mitochondrial fluorescent probe, MitoTracker (MT), respectively, is also presented. Finally, the presence of desmin-immunopositive amyloid-like aggregates is also quantified since it may indicate the induction of a maladaptive phenotype already at the early stages of hypertrophy.

2. Materials and methods

2.1. Reagents

ET-1 (human/porcine sequence), and DE-U-10 mouse anti-desmin antibody, were obtained from Sigma (St Louis, MO, USA), and MitoTracker Red CM-H_2XRos from Molecular Probes (Eugene, OR, USA). Culture dishes were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Culture medium M199 and DMEM were obtained from Gibco BRL (UK) while fetal calf and horse serum (FCS and HS respectively) from Boehringer Ingelheim (D) were used. 30% Acrylamide/Bis Solution, 37.5:1 (2.6% C), mineral oil, iodoacetamide (IAA) and Biolytes were from Bio-Rad Laboratories (Hercules, CA, USA). Immobiline DryStrip pH 3–10 NL, 18 cm and 24 cm, electrode paper wicks and L-[4,5-^{3}H]-leucine (54 Ci/mmol) were from Amersham (UK). Complete protease inhibitor cocktail tablets were purchased from Roche Diagnostic (IN, USA). All other reagents were from Sigma (St Louis, MO, USA).

2.2. Cell culture

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No 85–23, revised 1996). Primary cultures of neonatal cardiomyocytes were prepared as described previously [6,7,13–15] with slight protocol modifications. Briefly, cultures were obtained from 1–2 day-old Wistar rats. Cardiomyocyte to non-cardiomyocyte ratio was increased by preplating. Cells were seeded in 6 cm diameter dishes ($1{,}25\times10^5$ cells/cm^2 for 2-DE analysis) and 4-well multiplates (3×10^4 cells/cm^2 for immunocytochemistry). For the latter assay sterile glass coverslips were placed inside each well prior to cell seeding. Cells were cultured in complete HEPES buffered medium (DMEM/M199) (4:1) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5% FCS and 5% HS and maintained at 37 °C and 5% CO_2. After 24 h serum content was decreased to 4% HS. Cardiomyocytes formed a confluent monolayer of spontaneously contracting cells 24 h after plating. The day prior to ET1 treatment cells were cultured in serum-free medium (SFM) to prevent the effect of serum growth factors. ET1 was diluted in SFM (10^{-6} M), sterilized with a 0.22 μm porosity filter and kept at −20 °C (stock solution). ET1 stock solution was diluted 100-fold (final concentration 10 nM) in SFM immediately before addition to the cell cultures and incubation was continued for 48 h.

2.3. Biochemical parameters for hypertrophy

^{3}H-leucine incorporation and protein/DNA ratio were measured as previously described [6,7]. Briefly, after washing the cells with phosphate-buffered saline (PBS), protein and DNA content of cardiomyocytes were determined in lysates prepared by dissolving the cells in 1 N NaOH for 16 h at 4 °C. Protein concentration was determined using the Bradford assay, while DNA concentration was measured fluorimetrically with 4,6-diamine-2-phenylindol-dihydrochloride [6,7]. Protein synthesis was quantified by pulse-labelling of cardiomyocytes with 2 μCi/ml ^{3}H-leucine for the last 2 h of the 48 h incubation either in the absence or presence of ET1. After washing with PBS to remove extracellular free label, the cells were lysed and the protein was precipitated and

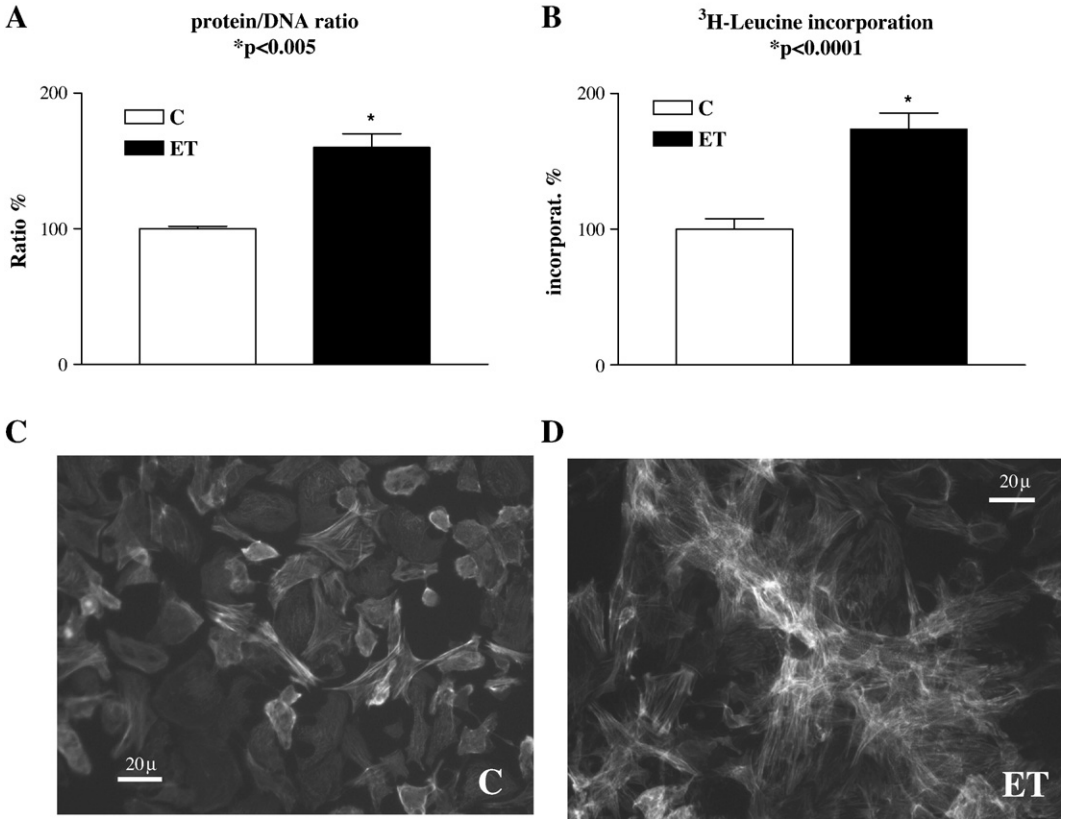

Fig. 1. Hypertrophic response of cardiomyocytes to ET-1. Protein/DNA ratio (A), ^{3}H-leucine incorporation (B), and morphology of the cardiomyocytes after 48 h incubation without (C) or with ET-1 (D) were determined. Data plotted in A and B were obtained from various experiments ($n\geq3$), the values normalized as percentages of average control values (±SEM). A significant increase of protein/DNA ratio (60±10%, mean±SEM) by ET1 was observed (A). A similar increase in ^{3}H-leucine incorporation (74±14%, mean±SEM) was seen by ET1 (B). Photographs of fixed cells stained with FITC–phalloidin were taken by means of an epifluorescence microscope (Olympus IX50) using a FITC filter. Control cells are shown in panel C whereas cells stimulated with 10 nM ET1 are shown in panel D. After ET1 stimulation cells appeared to show a slight increase in cell size together with different "star-shaped" arrangement of cell clusters.