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PKC-δ signalling pathway is involved in H9c2 cells differentiation

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ABSTRACT

H9c2 are rat heart embryonic myoblasts, with skeletal muscle properties, which terminally differentiate by fusing and forming multinucleated myotubes. Here we investigated the possible involvement of Protein Kinases C (PKCs) in H9c2 cell differentiation and explored the interplay of these enzymes both with reactive oxygen species (ROS), upstream physiological mediators of cell differentiation, and with nitric oxide (NO), downstream target of PKC activation, known for being involved in apoptosis induction in differentiated myoblasts. Cells were induced to differentiate (6 days) under low serum culture conditions and assayed for the expression of cell cycle (cyclin A) and differentiation markers (morphology and myogenin). Both ROS and in vivo production of NO were found increased after 6 days of differentiation, when the activation of PKC-δ isoform was 14-fold increased compared with the undifferentiated control cells. The parallel analysis of apoptotic features demonstrated a small increase in Annexin-V+ cells and a concomitant increase in PARP cleavage and Bax expression. Interestingly, a reduced percentage of differentiated cells was obtained both in the presence of Rottlerin, a highly selective PKC-δ pharmacologic inhibitor, and, moreover, with the use of PKC-δ siRNA technology, further supporting the involvement of PKC-δ in switching on the events related to skeletal muscle myoblast differentiation.

1. Introduction

Cell differentiation implies complex mechanisms, such as activation of intrinsic cellular programs by exogenous signalling stimuli, which lead to the activation of a battery of specific genes. Moreover, downstream these signalling events specific morphological features characterizing proliferating and differentiating cells are evidenced. H9c2 are rat heart embryonic myoblasts, with skeletal muscle properties, which terminally differentiate, under low serum culture conditions, by fusing and forming multinucleated myotubes (Kimes and Brandt, 1976). Chemically reactive ROS, molecules derived from oxygen, are generated during muscle differentiation (Piao et al., 2005). When myoblasts undergo terminal differentiation, irreversibly withdraw from the cell cycle, express high level of specific markers, i.e. myogenin, and undergo actin fiber disassembly/reorganization (Sabourin and Rudnick, 2000). Low concentrations of ROS function as physiological intracellular signalling mediators during H9c2 differentiation (Hong et al., 2002) and in cardiovascular differentiation of ES (mouse embryonic stem cells), which can be used for cell replacement therapies of cardiac infarction (Buggish et al., 2007). An intermediate dose of reactive oxygen species (ROS) results in temporary or permanent growth arrest (Zhang et al., 2003; Park et al., 2004). High dose of ROS causes cell death via apoptotic or necrotic mechanisms (Beckman and Ames, 1998). suggesting that cell fate may depend on the intracellular ROS level and on the cell type. Moreover, oxidants can modify cell signalling proteins leading to functional consequences. Among signalling proteins, Protein Kinases C (PKCs) are logical candidates for redox modification by oxidants (Gopalakrishna and Jaken, 2000). PKC isoforms belong to a family of lipid activated enzymes implicated in a wide range of cellular functions, notably proliferation, differentiation, and cell survival (Gould and Newton, 2008; Steinberg, 2008) but are also implicated in cell response to injury as "stress sensors" (Barnett et al., 2007). A role in the physiology of skeletal muscle has been assigned to nitric oxide signalling pathway regulating force production (excitation–contraction coupling), autoregulation of blood flow, myocyte differentiation, respiration, and glucose homeostasis (Kaliman et al., 1999; Stamler and Meissner, 2001). Nitric oxide (NO) is a small, diffusible, multifunctional, highly reactive molecule, which acts as an intracellular messenger with

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dichotomous regulatory roles under physiological and pathological conditions. Apart from its physiological functions, exerted by constitutively liberated NO (neuronal signalling, blood pressure, and clotting regulation), an excessive synthesis of NO could be toxic to cells. In fact, depending on concentration, NO can act as a pro- or an antioxidant or as a pro- or anti-apoptotic agent and can trigger a pro- or an anti-inflammatory response. Pro-apoptotic and pro-inflammatory function seems to be associated with the redox state (Chung et al., 2001). The presence of transition metal complexes within the cells seems to be a consequence of the rate of NO production and the interaction of biological molecules such as iron, thiols, proteins, nucleic acids, lipids, sugar, reactive oxygen species (Brune and Lapetina, 1995; Gow et al., 1997, 1999; Gow and Ischiropoulos, 2001), and expression of survival genes (Kim et al., 1997). NO can directly induce cytochrome c release through mitochondrial membrane potential loss, lastly activating the caspase-dependent apoptotic signalling cascade. NO binds to cytochrome c oxidase in the mitochondrial electron transfer chain. Under this condition, superoxide generated by mitochondria interacts with NO to form peroxynitrite, which induces mitochondrial dysfunction at membrane level, cytochrome c release, and apoptosis. Endogenous nitric oxide is synthesized from L-arginine by a family of NO synthases (NOS) by several distinct NOS isoforms (Nathan, 2002). These include two constitutive Ca^{2+}/CaM^{-}dependent forms of NOS: nNOS (NOS1), whose activity was first identified in neurons and which maps at 12q24.2, and eNOS (NOS3), first identified in endothelial cells and mapping at 7q35–36. Endothelial nitric oxide synthase (eNOS) is an important enzyme in the cardiovascular system (Brunner et al., 2001) since it catalyzes the production of NO, a key regulator of blood pressure, vascular remodeling, and angiogenesis (Shaul, 2002; Di Pietro et al., 2006). The inducible form of NOS, iNOS (NOS2) is Ca^{2+} independent and is expressed in a broad range of cell types, including macrophages and hepatocytes, and in response to a variety of stress, such as inflammatory reactions.

Thus, since a regulation is exerted by various Protein Kinase C isoforms on NOS activation, as elsewhere reported (Barreiro et al., 2006; Ramzy et al., 2006; Karimi et al., 2006; Rashid et al., 2007), the aim of this work was to investigate the involvement of PKC isoforms and the possible interplay with upstream (ROS) and downstream (NO) signalling molecules in the pathway leading to H9c2 differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

H9c2 cells, rat DB1X heart myoblasts, derived from embryonic ventricle (ECACC, Porton Down, Salisbury, UK) were chosen as they represent an "in vitro" model of cardiac and skeletal muscle cells. H9c2 were seeded at a density of 6×10^5 in a 75 cm^2 flask and cultured at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% foetal bovine serum (FBS). When, after 3–4 days, 70–80% of the cells reached a sub-confluence state, they were seeded in DMEM supplemented with 1% horse serum (HS), as elsewhere reported (Chun et al., 2000), for the myocytes/myotubes differentiation induction (Pagano et al., 2004). When required, 3 μM Rottlerin, a highly selective inhibitor of PKC-δ (Gschwendt et al., 1994; Soltoff, 2007), was added to the medium 1 h before 10% FBS or 1% HS DMEM supplementation. Cultured H9c2 cells of passage 6–12 were used for our experiments. Each parameter was assessed by means of three different consistent experiments.

2.2. Cell cycle analysis

Approximately 1.5×10^5 cells per experimental condition were harvested, fixed in 70 °C cold ethanol and kept at 4 °C overnight. Cells were then resuspended in 20 μg/ml PI and 100 μg/ml RNAse final concentrations. Cell cycle profiles (1×10^4 cells) were analyzed with an EPICS-XL flow cytometer with an FL2 detector in a linear mode using the EXPO32 software (Beckmann Coulter, FL, USA). Data were analyzed with Multicycle software (Phoenix Flow Systems, CA, USA).

2.3. Light microscopy

H9c2 cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 min, washed in phosphate buffered saline (PBS) and counterstained with hematoxylin-eosin solution. For myogenin and PKC-δ immune labeling, cells were incubated in 5% donkey serum in PBS for 20 min at room temperature followed by a 45 min incubation in the presence of 5 μg/ml mouse monoclonal myogenin or PKC-δ antibody (Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted in PBS, 5% Tween-20, 2% bovine serum albumin (BSA) for 1 h at 37 °C. Slides were washed in PBS and reacted for 45 min with fluorescein-isothiocyanate (FITC)—conjugated anti-rabbit IgG (immunoglobulin) antibody (Boheringer Mannheim, Germany) diluted 1:50 in PBS, 5% Tween-20, and 2% BSA for 45 min at 37 °C. After several washes in PBS, slides were mounted in glycerol-DABCO (1–4-diazabicyclo[2-2-2]octane) containing 5 μg/ml DAPI (4–6,diamidino-2-phenyl-indol) to counterstain nuclei. Internal controls, performed omitting the primary antibody, did not disclose any FITC staining. The labelled slides were examined under a Leica Light Microscope (Heidelberg, Germany) equipped with a Coolsnap videocamera (RS Photometrics, Tucson, AZ, USA) to acquire computerized images.

2.4. Flow cytometry analysis of ROS production

Reactive oxygen species (ROS) production was determined by monitoring by flow cytometry the increase of green fluorescence after labeling the cells (5×105) with 1 μM dihydrorhodamine 123 (Eugene, Oregon, USA) for 1 h at room temperature. Dead cells were excluded from the analysis by propidium iodide (PI) staining (5 μg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA). The analysis was performed on live cells with an EPICS-XL cytometer with an FL2 detector in a linear mode using the Expo32 software (Beckmann Coulter, FL, USA). At least 15,000 events for each sample were acquired.

2.5. Flow cytometry analysis of NO production

For the flow cytometry measurement of intracellular NO production, cells were washed twice with PBS and incubated in 2% BSA/PBS for 30 min at 37 °C in the presence of the NO-reactive dye DAF-2DA (4,5-diaminofluorescein diacetate) (Calbiochem, La Jolla, CA) at the final concentration of 10 μM. DAF-2DA fluorescence emissions were detected and analyzed with an EPICS Coulter Flow Cytometer using the Expo 32 analysis software. At least 15,000 events for each sample were acquired (Di Pietro et al., 2006).

2.6. Nitric oxide synthase activities

Nitric oxide formation by nitric oxide synthases was monitored by the hemoglobin capture assay. NOS activity was detected in the supernatant derived from $11000 \times g$ centrifuged tissue homogenates. The reaction mixture for NOS contained