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Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells

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Abstract Cardiotypic development in embryonic stem cell-derived embryoid bodies may be regulated by reactive oxygen species (ROS). ROS were generated by a NADPH oxidase-like enzyme which was transiently expressed during the time course of embryoid body development. Incubation with either H_2O_2 or menadione enhanced cardiomyogenesis, whereas the radical scavengers trolox, pyrrolidinedithiocarbamate and *N*-acetylcysteine exerted inhibitory effects. The phosphatidylinositol 3-kinase (PI-3-kinase) inhibitors LY294002 and wortmannin abolished cardiac commitment and downregulated ROS in embryoid bodies. Coadministration of LY294002 with prooxidants resumed cardiomyocyte differentiation, indicating a role for PI-3-kinase in the regulation of the intracellular redox state.

Key words: Reactive oxygen species; NADPH oxidase; Phosphatidylinositol 3-kinase; Embryonic stem cell; Embryoid body; Cardiomyocyte differentiation

1. Introduction

Several transcription factors including Nkx2.5, GATA4/5/6 and MEF2C have been described to be involved in the transcription of cardiac specifying genes [1,2]. Furthermore, cardiomyocyte differentiation is promoted by several growth factors and hormones, such as the fibroblast growth factors FGF-2, FGF-4, insulin, insulin-like growth factor I (IGF-I), and activin [3,4]. However, the signal transduction pathways that coordinate the action of growth factors and hormones on plasma membrane-linked receptors with the transcription factors present in the cytoplasm and cell nucleus are virtually unknown.

Recently, it has been demonstrated that phosphatidylinositol 3-kinase (PI-3-kinase) is involved in cardiac commitment, since the specific inhibitor LY294002 inhibited cardiomyogenesis in embryoid bodies cultivated from pluripotent murine embryonic stem (ES) cells [5]. The molecular mechanism by which PI-3-kinase promotes cardiomyocyte differentiation has, however, not yet been investigated.

The present study elucidates the signal transduction pathway involving PI-3-kinase. It is demonstrated that reactive oxygen species (ROS), which have been recently shown to play a significant role in signal transduction pathways following the binding of several growth factors to their cognate receptors [6,7], are involved in cardiomyogenesis. Since it has been demonstrated that PI-3-kinase activates the small GTPase Rac [22], which is a component of the NADPH oxidase of neutrophils, it is assumed that PI-3-kinase may regulate ROS levels in embryoid bodies.

2. Materials and methods

2.1. Cell culture

The permanent ES cell line D3 [8], cultivated in undifferentiated state on primary cultures of mouse embryonic fibroblasts, was used throughout the study. Cells were cultivated on feeder layers in Iscove's modified Eagle's medium (Gibco BRL, Life Technologies, Germany) supplemented with 15% fetal calf serum (Boehringer, Mannheim, Germany), L-glutamine (2 mM) (Gibco BRL), β-mercaptoethanol (final concentration 5×10^{-5} M) (Sigma, Deisenhofen, Germany), non-essential amino acids (NAA; Gibco BRL; stock solution diluted 1:100), 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin (Gibco BRL) in a humidified atmosphere containing 5% CO_2. The cell culture medium was supplemented with leukemia inhibitory factor (10 μg/ml) which keeps ES cells in the undifferentiated state. At day 0 of differentiation, adherent cells were enzymatically dissociated using 0.2% trypsin and 0.05% EDTA in phosphate buffered saline (PBS) and seeded at a density of 1×10^7 cells/ml in 250 ml siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 100 ml Iscove's medium supplemented with 20% fetal calf serum (Boehringer, Mannheim, Germany), L-glutamine (2 mM) (Gibco BRL), β-mercaptoethanol (final concentration 5×10^{-5} M) (Sigma, Deisenhofen, Germany), NAA (Gibco BRL; stock solution diluted 1:100), 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin (Gibco). After 24 h, 150 ml medium was added for a final volume of 250 ml. The spinner flask medium was stirred at 20 rpm using a stirrer system (Integra Biosciences) and was partly changed every day.

2.2. Incubation of embryoid bodies with inhibitors of PI-3-kinase, prooxidants and free radical scavengers

The PI-3-kinase inhibitors wortmannin and LY294002 were purchased from Sigma and Calbiochem (Bad Soden, Germany), respectively. Trolox (water-soluble vitamin E) was purchased from Calbiochem, pyrrolidinedithiocarbamate (APDC), menadione, H_2O_2 and *N*-acetylcysteine (NAC) were from Sigma. On day 3 of embryoid body culture embryoid bodies were removed from the spinner flasks. Thirty to forty embryoid bodies were added to each of 6-cm bacteriological Petri dishes (Nunc, Wiesbaden, Germany), containing 6 ml cell culture medium supplemented with either 1 μM wortmannin, 20 μM LY294002, 10 nM H_2O_2, 20 μM menadione, 20 μM APDC, 30 μM trolox, or 2 mM NAC. Cell culture medium supplemented with the agents was completely exchanged every day. On day 7 of embryoid body culture, the PI-3-kinase antagonists and radical scavengers were removed and the embryoid bodies were plated to 24-well cell culture dishes (Nunc). On day 2 after plating (7+2) beating embryoid bodies were counted. The extension of the beating area of cardiomyocytes was evaluated on day 7+5.

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Abbreviations: APDC, pyrrolidinedithiocarbamate; H_2DCF-DA, 2′7′-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium chloride; ES cell, embryonic stem cell; FGF, fibroblast growth factor; IGF-I, insulin-like growth factor I; NAC, *N*-acetylcysteine; PI-3-kinase, phosphatidylinositol 3-kinase; ROS, reactive oxygen species

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2.3. Determination of the intracellular redox state

The intracellular redox state was investigated using the fluorescent dye 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR, USA) which is a non-polar compound that is converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. The membrane-impermeable H_2DCF is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS [9]. Embryoid bodies were incubated for 30 min with 10 μM H_2DCF-DA (dissolved in dimethylsulfoxide) in E1 buffer containing (in mM) NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 10 (pH 7.4 at 37°C). After loading, the embryoid bodies were rinsed three times in E1 buffer and the DCF fluorescence was recorded by confocal laser scanning microscopy. For fluorescence excitation the 488-nm line of the argon ion laser was used. Fluorescence emission recording was performed with a longpass LP515 nm filter set. In the experiments where the time course of H_2DCF oxidation was monitored, embryoid bodies immersed in E1 buffer were incubated with 10 μM H_2DCF-DA and the time-dependent increase of DCF fluorescence was monitored without preincubation. Full frame images (512×512 pixels) were taken every 120 or 180 s. DCF fluorescence was evaluated in 5000 μm^2 regions of interest (ROIs) in the center of embryoid bodies. Data are presented in arbitrary units as percentage of fluorescence variation F with respect to the resting level F_0.

2.4. Immunohistochemistry

Immunohistochemistry was performed with whole-mount embryoid bodies. Embryoid bodies either in suspension culture or plated on coverslips were washed three times in PBS and subsequently fixed in ice-cold 7:3 methanol/acetone for 60 min. After washing for three times with PBS (pH 7.4) containing 0.1% Triton X-100 (PBST) embryoid bodies were incubated for 1 h in PBS containing 10% milk powder to block against unspecific binding. Incubation with primary antibodies was performed for 1 h in PBS containing 10% milk powder. After incubation with the primary antibody embryoid bodies were washed three times with PBST and staining with secondary antibodies was performed in PBS containing 10% milk powder. The primary antibodies used were: monoclonal anti-α-actinin (sarcomeric), clone number EA-53 (Sigma, Deisenhofen, Germany) used in a concentration of 10 μg/ml and monoclonal anti-$p67^{phox}$ (Dianova, Hamburg, Germany) used at a concentration of 12.5 μg/ml. As secondary antibody a Cy5™-conjugated rabbit anti-mouse IgG (H+L) (Dianova, Germany) was used in a 1:150 dilution. Excitation was performed using the 633-nm band of a He–Ne laser of the confocal setup. Emission was recorded using a longpass LP655 nm filter set. For the semiquantitative evaluation of $p67^{phox}$ protein, fluorescence was recorded in 3000 μm^2 ROIs at the periphery of embryoid bodies. The thickness of the optical section (full width half maximum) was adjusted to 20 μm.

2.5. Statistical analysis

Data are given as mean values ± S.E.M. with n denoting the number of experiments performed with different embryoid body cultures. In each experiment at least 30 embryoid bodies were examined unless otherwise indicated. Student's t-test for unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Endogenous generation of ROS in embryoid bodies

To investigate whether ROS are involved in signal transduction pathways resulting in cardiac commitment, the intracellular generation of ROS was investigated in embryoid bodies using the fluorescent ROS indicator H_2DCF-DA. As demonstrated in Fig. 1 embryoid bodies endogenously generated ROS in significant amounts. Maximum ROS generation was observed in 2–3-day-old embryoid bodies. A significant decay in the oxidation kinetics of H_2DCF with increased cultivation time was observed in 4-, 6- and 11-day-old embryoid bodies ($n = 3$ for each experimental condition).

The intracellular level of ROS in cells is determined either by the activity of NADPH oxidase-like enzymes and/or the mitochondrial respiratory chain which generates the superoxide anion which is subsequently dismutated to H_2O_2 by catalase [10]. Hence the amount of ROS generated during embryoid body development may be limited by the expression of a NADPH oxidase-like enzyme. To evaluate the role of a NADPH oxidase-like enzyme in the endogenous ROS generation in embryoid bodies, 2–3-day-old embryoid bodies were incubated with 10 μM diphenyleneiodonium chloride (DPI), which inhibits NADPH oxidase activity [11]. Subsequently DCFH oxidation was monitored. We observed that under these experimental conditions intracellular ROS generation was significantly inhibited and was not significantly different from the ROS generation observed in undifferentiated ES cells cultivated on feeder cells ($n = 3$ for each experimental condition) (see Fig. 1).

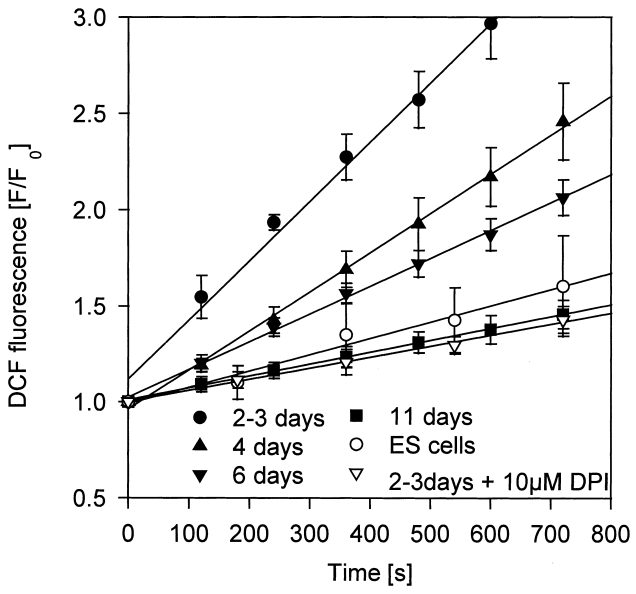

Fig. 1. Endogenous generation of ROS in ES cells and embryoid bodies during different developmental stages. Time course of H_2DCF oxidation in undifferentiated ES cells, in 2–3-, 4-, 6-, and 11-day-old embryoid bodies and in 2–3-day-old embryoid bodies in the presence of 10 μM DPI. Embryoid bodies were incubated with 20 μM H_2DCF-DA and the oxidation of non-fluorescent H_2DCF to fluorescent DCF was monitored. About 10 embryoid bodies in each of three independent experiments were used for the determination of each data point.

A decrease of ROS generation with prolonged cell culture times of embryoid bodies may be correlated to a decay in the expression of the NADPH oxidase complex. Therefore, 2–3-, 6- and 12-day-old embryoid bodies were investigated with respect to the expression of $p67^{phox}$ which is a subunit of the NADPH oxidase involved in the respiratory burst of neutrophils. It was observed that the decay in ROS generation during the development of embryoid bodies correlated well with a decrease in $p67^{phox}$ expression with the highest expression in 2–3-day-old embryoid bodies and a gradual decline within 11–12 days of cell culture ($n = 3$) (Fig. 2).

3.2. Effects of external H_2O_2 and menadione on cardiomyocyte differentiation

If ROS were involved in the signal transduction process leading to cardiomyocyte differentiation, an increase of intracellular ROS should promote cardiomyogenesis, whereas

scavengers of ROS exert inhibitory effects. Therefore, in a first set of experiments, 3-day-old embryoid bodies were incubated with either 10 nM H_2O_2 or 20 μM menadione which is known to generate H_2O_2 [12]. As shown in Fig. 3A incubation with H_2O_2 as well as menadione significantly increased the percentage of embryoid bodies containing beating areas of cardiomyocytes at day 7+2 ($n = 3$ for each experimental condition). Concomitant with the increase of the number of beating embryoid bodies the size of the beating area (evaluated on day 7+5) was significantly augmented in embryoid bodies treated with ROS-generating agents ($n = 3$ for each experimental condition) (Fig. 3B). In a second set of experiments embryoid bodies were incubated with the free radical scavengers trolox

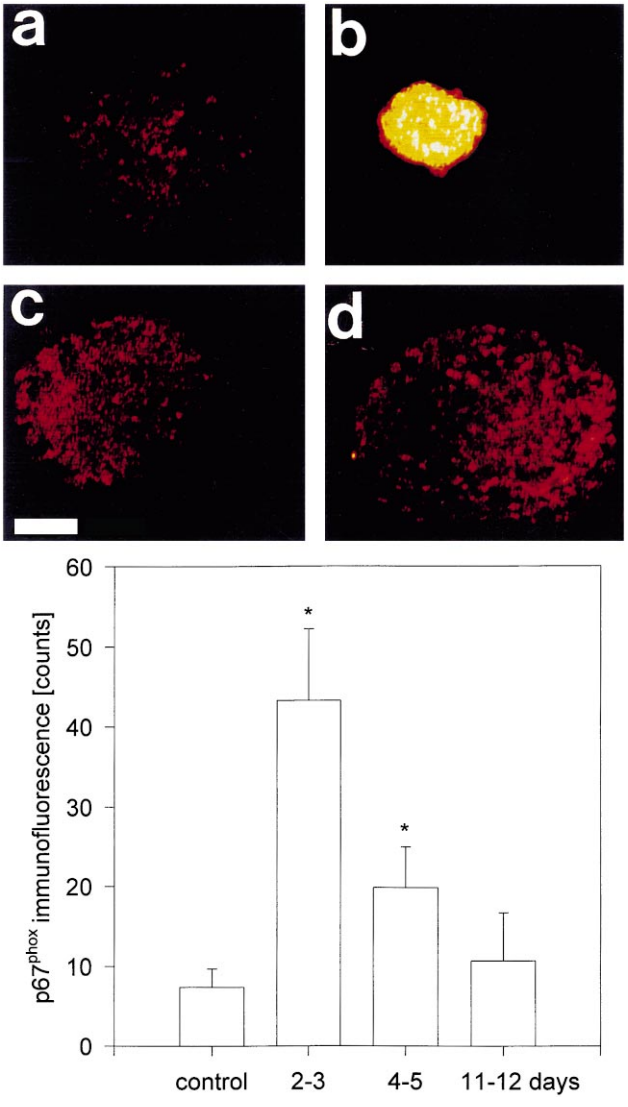

Fig. 2. Expression of a NADPH oxidase-like enzyme during embryoid body development. Top: Immunostaining of representative embryoid bodies with an antibody directed against the $p67^{phox}$ subunit of the neutrophil NADPH oxidase (bar = 50 μm). a: Control (only secondary antibody). b: Two-day-old embryoid body. c: Five-day-old embryoid body. d: Twelve-day-old embryoid body (bar = 50 μm). Bottom: Quantitative immunohistochemistry of $p67^{phox}$ in embryoid bodies of different developmental stages. Data show the means ± S.E.M. of at least 30 embryoid bodies evaluated in one of three experiments with comparable results. $^*P < 0.05$, significantly different from the untreated control.

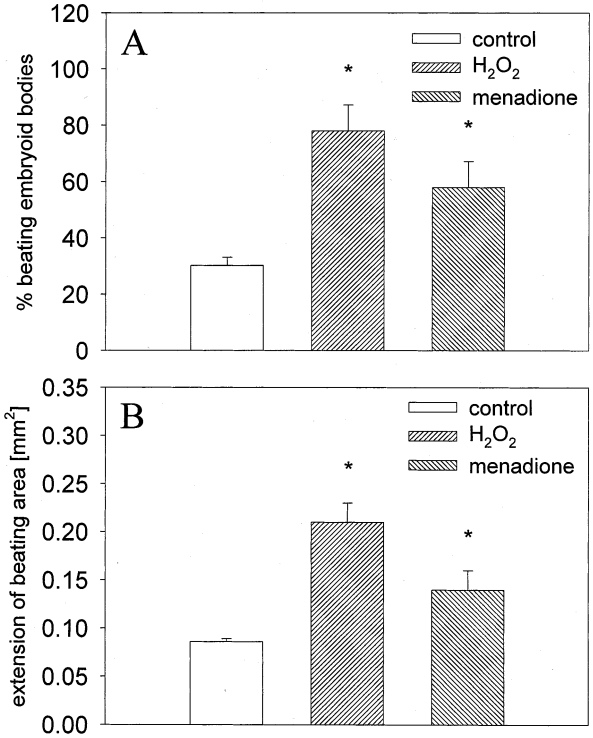

Fig. 3. Effects of the prooxidants H_2O_2 (10 nM) and menadione (20 μM) on the number of embryoid bodies differentiating foci of spontaneously contracting cardiomyocytes (A) and on the area of the beating foci (B). Note that prooxidants significantly enhanced cardiomyocyte differentiation in embryoid bodies. $^*P < 0.05$, significantly different from the untreated control.

(30 μM), NAC (2 mM) as well as APDC (20 μM) which is also known to inhibit apoptosis [13] and oxidize ferrous ions [14]. Following incubation with antioxidants the number of beating embryoid bodies was counted on day 7+2 after plating. Treatment of embryoid bodies with scavengers of free radicals resulted in a significant decrease in the number of beating embryoid bodies by approximately 50%, which underscores our assumption of an involvement of ROS in cardiomyogenesis (Fig. 4) ($n = 3$ for each experimental condition).

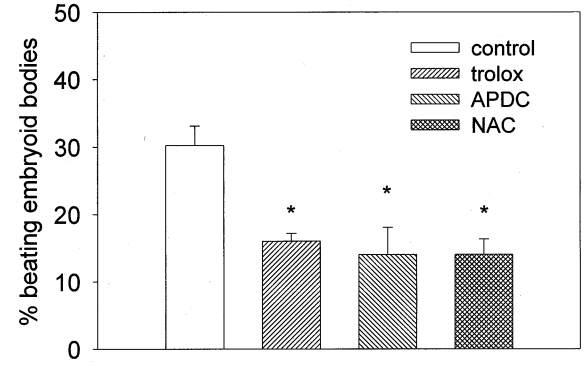

Fig. 4. Effects of the free radical scavengers trolox (30 μM), APDC (20 μM), and NAC (2 mM) on cardiomyocyte differentiation. Embryoid bodies were treated from day 3 to day 7 of cell culture with the compounds. Subsequently they were plated in the absence of the compounds and the number of spontaneously contracting embryoid bodies was counted 2 days later (7+2). $^*P < 0.05$, significantly different from the untreated control.

3.3. Role of PI-3-kinase in cardiomyogenesis

In a recent study it was demonstrated that PI-3-kinase is involved in cardiac cell differentiation since incubation of embryoid bodies with the PI-3-kinase inhibitor LY294002 significantly inhibited cardiac commitment [5]. However, the mechanism of action of PI-3-kinase was not investigated. It has been previously shown that superoxide generation by NADPH oxidase is inhibited by antagonists of PI-3-kinase [15], which may indicate that the intracellular redox state is regulated by this enzyme. To evaluate whether the involvement of PI-3-kinase in cardiomyogenesis of ES cells is related to its modulatory effect on the generation of ROS, 3-day-old embryoid bodies were preincubated for 24 h either with 20 μM LY294002 or with 1 μM wortmannin which are both specific inhibitors of PI-3-kinase. Subsequently the intracellular generation of ROS was evaluated by recording DCF fluorescence using confocal laser scanning microscopy. Our data demonstrate that LY294002 ($n=4$) as well as wortmannin ($n=6$) significantly reduced the ROS levels present in embryoid bodies as compared to the untreated control (set at 100%) (Fig. 5A). This clearly indicates that PI-3-kinase is involved in the regulation of the intracellular redox state in embryoid bodies.

The data presented so far demonstrate that treatment of embryoid bodies with PI-3-kinase inhibitors resulted in a reduction of ROS in embryoid bodies concomitant with a decrease of the number of beating foci and a reduction of the size of the area of cardiomyocytes. Hence raising the intracellular ROS levels in the presence of inhibitors of PI-3-kinase should restore cardiomyocyte differentiation. We therefore incubated embryoid bodies either with LY294002 alone or in combination with the prooxidants menadione (20 μM) or H_2O_2 (10 nM). On day 7+2 after plating the development of beating area was investigated. The extension of beating areas was analyzed by immunohistochemistry on day 7+5. LY294002 significantly decreased the number of beating embryoid bodies (Fig. 5B) and reduced the extension of the beating area ($n=3$ for each experimental condition) (Fig. 5C). Upon coadministration of LY294002 with prooxidants the number of beating embryoid bodies as well as the extension of the beating area was restored ($n=3$ for each experimental condition) (see Figs. 5C and 6). A comparable effect on the number of beating embryoid bodies was achieved following incubation of embryoid bodies with 1 μM wortmannin (data not shown). Hence our data demonstrate that the role of PI-3-kinase on cardiomyocyte differentiation is related to the regulation of the intracellular redox state.

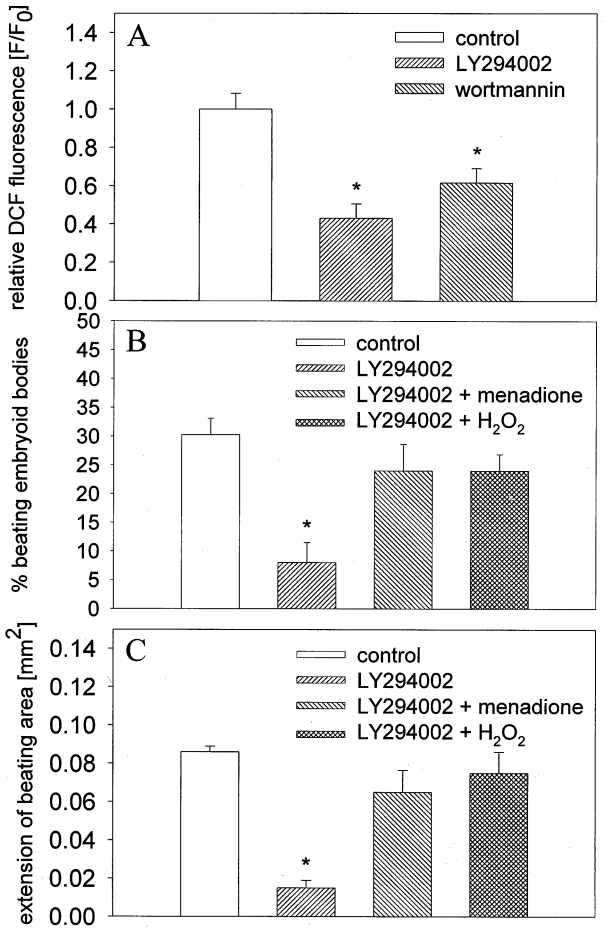

Fig. 5. Effect of the PI-3-kinase inhibitors LY294002 (20 μM) and wortmannin (1 μM) on intracellular ROS levels and cardiomyocyte differentiation. A: ROS levels in embryoid bodies following incubation of 3–4-day-old embryoid bodies for 24 h with PI-3-kinase inhibitors. Note that PI-3-kinase antagonists significantly reduced the ROS levels in embryoid bodies. B, C: Effects of the PI-3-kinase inhibitor LY294002 on the number of embryoid bodies differentiating foci of contracting cardiomyocytes (evaluated on day 7+2) (B) and on the area of the beating foci (evaluated on day 7+5) (C). Note that upon coadministration of LY294002 with the prooxidants H_2O_2 (10 nM) and menadione (20 μM) cardiomyocyte differentiation was restored to the control level, which indicates that PI-3-kinase is involved in the regulation of ROS levels in embryoid bodies. *$P<0.05$, significantly different from the untreated control.

4. Discussion

Although a number of transcription factors have been described to be essential for the commitment of the cardiac cell lineage, the involved signal transduction pathways are not well defined. It is well documented that angiotensin II [16] as well as IGF-I [17], b-FGF [18] and interleukin-1β [19] raise intracellular ROS in cells, which points towards the direction that hormones and cytokines involved in cardiac cell differentiation use the common route of ROS generation.

In embryoid bodies ROS were generated predominantly via a NADPH oxidase-like enzyme, since positive immunostaining was observed with an antibody raised against the $p67^{phox}$ unit of the neutrophil NADPH oxidase and ROS generation was significantly inhibited after preincubation of embryoid bodies with DPI which is an inhibitor of flavoproteins including the superoxide-generating NADPH oxidase [11]. Experimental evidence for the involvement of an NADPH oxidase-like enzyme and the intracellular redox state in cardiomyogenesis has not yet been provided. Recent studies suggest a role for PI-3-kinase in signaling pathways which induce cardiomyogenesis. It was shown that cardiomyogenesis in embryoid bodies was significantly inhibited in the presence of LY294002 [5] which is a specific inhibitor of PI-3-kinase. Furthermore, wortmannin, another PI-3-kinase antagonist, inhibited the chamber growth and maturation observed for the combined treatment of murine embryos with neuregulin 1 and IGF-I, suggesting that these growth factors act through convergent activation of the PI-3-kinase [20]. In the latter study comparable effects were observed for the adenoviral

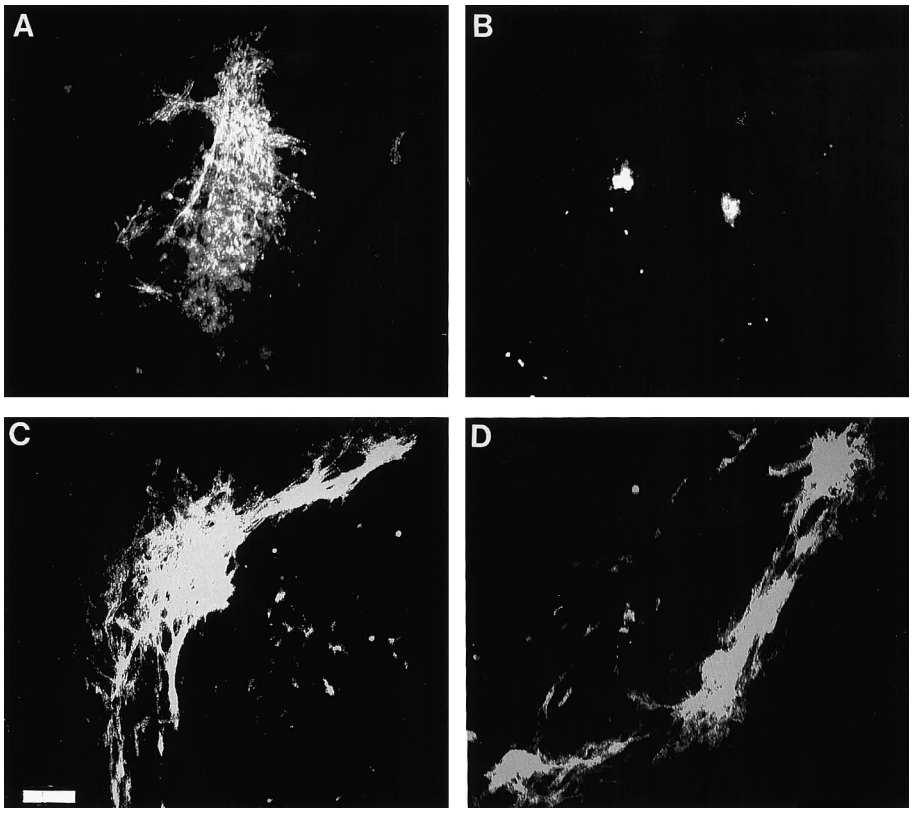

Fig. 6. Reversal of LY294002-mediated inhibition of cardiomyocyte differentiation in embryoid bodies by the prooxidants H_2O_2 (10 nM) and menadione (20 μM). Embryoid bodies were treated from day 3 to day 7 with the compounds and were subsequently plated. They were stained for sarcomeric α-actinin on day 7+5. Shown are representative foci of cardiac cells. A: Control. B: LY294002. C: H_2O_2+LY294002. D: Menadione+LY294002. The bar represents 150 μm.

delivery of dominant negative Rac1, which acts downstream of PI-3-kinase. Rac1 and Rac2 interact with the NADPH oxidase subunit $p67^{phox}$ thereby regulating enzyme activity and superoxide generation [21]. The activation of Rac2 has been recently shown to be inhibited by wortmannin and LY294002 [22].

In the present study cardiomyogenesis was enhanced upon treatment of embryoid bodies with prooxidants. On the other hand scavengers of ROS inhibited cardiomyocyte differentiation. These data clearly indicate that cardiac cell differentiation in embryoid bodies is critically dependent on the intracellular redox state and substantiate our previous experiments which demonstrated that electrical fields enhanced cardiomyocyte differentiation in embryoid bodies via an elevation of the intracellular redox state [23].

The data of the present study furthermore demonstrate that both wortmannin and LY294002 significantly reduced ROS levels in embryoid bodies which corroborates the results of others which have demonstrated that inhibitors of PI-3-kinase inhibit the respiratory burst in phagocytic cells [24–27]. The role of PI-3-kinase in cardiomyocyte differentiation of ES cells is apparently related to the regulation of intracellular ROS levels since in our experiments coadministration of the PI-3-kinase inhibitor LY294002 with prooxidants restored cardiomyocyte differentiation to the control level.

To our knowledge, our study for the first time indicates that cardiomyogenesis in embryoid bodies is regulated by the intracellular redox state. It has been previously shown that skeletal muscle differentiation induced by IGFs involves nuclear factor κB and nitric oxide synthase activities downstream of PI-3-kinase [28]. Since both cardiac cells and skeletal muscle cells originate from the mesodermal cell lineage, common signaling pathways may be involved in cardiomyogenesis and skeletal muscle differentiation.

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