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Static magnetic fields increase cardiomyocyte differentiation of Flk-1$^+$ cells derived from mouse embryonic stem cells via Ca^{2+} influx and ROS production ☆

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ABSTRACT

Aims: To investigate the effects of static magnetic fields (MFs) on cardiomyogenesis of mouse embryonic stem (ES) cell-derived embryoid bodies and Flk-1$^+$ cardiac progenitor cells and to assess the impact of cytosolic calcium $[Ca^{2+}]_c$ and reactive oxygen species (ROS).
Methods and results: Embryoid bodies and ES cell-derived Flk-1$^+$ cardiovascular progenitor cells were exposed to static MFs. The expression of cardiac genes was evaluated by RT-PCR; sarcomeric structures were assessed by immunohistochemistry; intracellular ROS and $[Ca^{2+}]_c$ of ES cells were examined by H_2DCF-DA- and fluo-4-based microfluorometry. Treatment of embryoid bodies with MFs dose-dependent increased the number of contracting foci and cardiac areas as well as mRNA expression of the cardiac genes MLC2a, MLC2v, α-MHC and β-MHC. In Flk-1$^+$ cells MFs (1 mT) elevated both $[Ca^{2+}]_c$ and ROS, increased expression of the cardiogenic transcription factors Nkx-2.5 and GATA-4 as well as cardiac genes. This effect was due to Ca^{2+} influx, since extracellular Ca^{2+} chelation abrogated ROS production and MF-induced cardiomyogenesis. Furthermore absence of extracellular calcium impaired sarcomere structures. Neither the phospholipase C inhibitor U73122 nor thapsigargin inhibited MF-induced increase in $[Ca^{2+}]_c$ excluding involvement of intracellular calcium stores. ROS were generated through NAD(P)H oxidase, since NOX-4 but not NOX-1 and NOX-2 mRNA was upregulated upon MF exposure. Ablation of NOX-4 by sh-RNA and treatment with the NAD(P)H oxidase inhibitor diphenylen iodonium (DPI) totally abolished MF-induced cardiomyogenesis.
Conclusion: The ability of static MFs to enhance cardiomyocyte differentiation of ES cells allows high throughput generation of cardiomyocytes without pharmacological or genetic modification.

1. Introduction

Natural MFs vary over the earth's surface between 0.035 and 0.07 mT, and are perceived by certain animals that use it for orientation [1,2]. Many investigators have tested the effect of exposure to natural MFs and electro-magnetic fields (EMFs) on living organisms [3–6], and it has been estimated that some of their biological system responses may be mediated through free radical reactions [7]. Biological free radicals are highly reactive molecules that have unpaired electrons, leading to the terms "reactive oxygen species (ROS)" or "reactive nitrogen species (RNS)" [7]. Most studies on EMF effects investigated the influence of EMFs on healing of bone, tendon, and skin [8–12], but only few investigations have been undertaken to examine the effects of MFs on cardiac cells and cardiomyogenesis of stem cells [13–16].

Considering the limited regenerative capacity of the heart muscle, renewable sources of cardiomyocytes are highly sought [17]. One of a possible avenue to increase differentiation of stem cells is the application of physical stimuli [18] such as electrical pulses [19], MFs [13], mechanical forces [20] and heat treatment [18]. Indeed, there is increasing evidence indicating that such physical stimuli may play an important role in cardiomyocyte differentiation. Electrical stimulation has been reported to be beneficial to primary cardiomyocytes cultured in vitro [21,22]. Direct current electrical field pulses stimulated cardiomyogenic differentiation of ES cells through intracellular generation of ROS [19]. Application of sinusoidal MFs (50 Hz, 0.8 mT) to ES cells increased the yield of ES cell-derived cardiomyocytes [16]. Furthermore extremely low frequency EMFs (ELF EMFs) tuned at calcium (Ca^{2+}) ion cyclotron energy resonance were used to drive cardiac specific differentiation of adult cardiac progenitor cells [13]. However, the exact mechanism(s) of the observed effects are not yet fully understood.

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Meanwhile, it is generally accepted that Ca^{2+} and ROS are important messengers in the regulation of a variety of signaling pathways [23]. Moreover, several studies have underscored the notion that Ca^{2+} and ROS signaling systems are intimately integrated such that Ca^{2+}-dependent regulation of components of ROS homeostasis might influence intracellular redox balance, and *vice versa* [23–25]. In the past few years, many experiments were performed to study the interaction between organisms and EMFs [26,27]. A rapid increase in the level of $[Ca^{2+}]_c$ is one of the several early response signals in cells triggered by EMFs [28–33]. In contrast, the role of intracellular Ca^{2+} signaling in cardiac cell differentiation and heart development is just emerging and remains largely unexplored [13,34].

We recently reported that low intensity static MFs induced vasculogenesis and promoted maturation and differentiation of chondrosteocytes from ES cells [35]. In the present paper, we demonstrate that cardiomyogenesis of Flk-1^+ cardiac progenitor cells derived from mouse ES cells can be enhanced by application of static MFs. Moreover, the relationship between MF-mediated intracellular ROS generation and $[Ca^{2+}]_c$ as second messengers in signal pathways leading to cardiomyocytes differentiation is unraveled.

2. Materials and methods

2.1. Embryonic stem cell culture

The mouse embryonic stem (ES) cell line CGR8 was established from the inner cell mass of a 3.5 day male pre-implantation mouse embryo (*Mus musculus*, strain 129). The CGR8 cell line (free of mycoplasma contamination) was cultured in Glasgow Minimal Essential Medium (GMEM) (Sigma–Aldrich, Taufkirchen, Germany) supplemented with 16% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS, Sigma, Germany), 2 mM L-glutamine (Biochrom, Berlin, Germany), 45 μM 2-mercaptoethanol (Sigma–Aldrich, Taufkirchen, Germany) and 103 U/ml Leukemia inhibitory factor (LIF) (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO_2 at 37 °C. ES cells were differentiated in vitro as embryo-like aggregates (embryoid bodies) using the spinner flasks (Integra Biosciences, Fernwald, Germany) method [36]. In brief, 1×10^7 cells were seeded in 250 ml siliconized spinner flasks and the cell culture medium was exchanged every day as previously described [36,36].

2.2. Magnetic cell separation (MACS) and FACS analysis for Flk-1^+ cells

CGR8 embryoid bodies were generated and cultivated as described above. 4-day old embryoid bodies were dissociated by incubation with collagenase type II (4 mg/ml) (PAA, Coelbe, Germany) dissolved in IK-buffer containing (in mM) $NaCl_2$ 120, KCl 5,4, MgSO4 5, Na Pyruvate 5, MgCl 21, Glucose 20, taurine 20 and HEPES 10 at 37 °C for 5–10 min. For cell separation MACS separation columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) were used. The separation procedure was carried out using a PE-conjugated rat anti-mouse Flk-1 antibody (dilution 1:10, BD, Franklin Lakes, USA). For labelling, anti-PE micro beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) were used. Purity analysis was carried out using a FacsCalibur (Becton Dickinson, USA) FACS machine with a scanning wave length of 488 nM (PE labelled cells) and a detection wave length of 575 nM. To obtain cardiomyocytes, single sorted cells were plated onto matrigel surface and further cultured. Flk-1^+ cells were exposed to 1mT static MFs for 1 h/day in the presence and absence of the extracellular Ca^{2+} chelator 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA, 50 μM) or the NAD(P)H oxidase inhibitor diphenylene iodonium (DPI, 1 μM) (Sigma–Aldrich, Taufkirchen, Germany) in cell culture medium until day 10. Beating activity was evaluated by microscopic inspection.

2.3. Calcium dye loading and fluorescence imaging

Intracellular Ca^{2+} was measured in cell monolayers loaded with the Ca^{2+} sensitive intracellular probe; fluo-4-AM (Molecular Probes, Eugene, USA) which was dissolved in DMSO to obtain a 1 mM stock solution. Briefly, Flk-1^+ cells seeded on glass coverslips in 24 multiwell tissue culture plates were loaded with 10 μM of fluo-4-AM in serum-free GMEM at 37 °C for 60 min. Coverslips were washed twice with buffer and used immediately in fluorescence microscopy experiments. The buffer for calcium fluorescence measurements contained (in mM) $CaCl_2$ 1, NaCl 135, KCl 6, Na_2HPO_4 0.33, Na pyruvate 5, $MgCl_2$ 1, glucose 10 and HEPES 10. In some experiments Ca^{2+} free conditions were ensured in buffer containing 0 mM $CaCl_2$ and 50 μM BAPTA. All buffers were used at pH 7.4. Fluo-4 fluorescence imaging was performed on a confocal laser scanning microscope (CLSM) LSM 510 (Zeiss; Jena, Germany) equipped with an argon ion laser. To deplete intracellular Ca^{2+} stores, cells were pre-incubated with thapsigargin (200 nM) for 30 min.

2.4. Static MF application

Static MFs were produced as previously reported [35] using a MF exposure system which was manufactured and calibrated in order to obtain a continuous uniform MF within the exposure time. The exposure system consisted of a copper wire coiled onto a polyvinyl chloride (PVC) tube, which completely shielded against emission of electric fields. MF intensity was expressed in milli Tesla (mT) and controlled using a variable FH 51 Gauss-/Teslameter (MAGNET-PHYSIK, model no. 2000510, Cologne, Germany) with the small probe 761. To carry out the Ca^{2+} measurements under MF treatment, the MF exposure system was placed in the immediate vicinity of the coverslip, so that the cells were exposed to a field strength of 1 mT.

2.5. Immunohistochemistry

Cardiac cells were identified by monoclonal mouse anti-α-actinin (Sigma, Deisenhofen, Germany), monoclonal mouse anti-cardiotroponin T (cTnT), monoclonal mouse anti-myosin heavy chain (MHC) (Abcam, Cambridge, UK), polyclonal goat anti-Nkx-2.5 (Santa Cruz, California, USA) and polyclonal rabbit anti-myosin light chain 2 (MLC-2) antibodies (Acris, San Diego, CA, USA). Cardiac myocytes on matrigel were plated on coverslips for 1 day. They were washed twice with phosphate-buffered saline (PBS) supplemented with 0.1% Triton X-100 and then fixed in methanol/acetone (7:3) for 1 h at −20 °C.

2.6. Stable downregulation of NOX-4 gene expression using shRNA technique

pLKO.1-puro derivative plasmid carrying MISSION™ shRNA MISSION™ shRNA sequence (CCGGGCATTAGTCTTAACCAGACAT-**CTCGAG**-ATGTCTGGTTAAGACTAATGCTTTTTG) targeting NOX-4 (Sigma–Aldrich) was introduced into CGR8 cells by lentiviral particles as previously described [37]. Transduced cells with pLKO.1 vector (containing non-hairpin insert) were used as negative control. Lentiviral particles were generated and added to CGR8 cells as previously described [38,39]. Transduction effectiveness was assessed via GFPTM control vector (Sigma–Aldrich, Taufkirchen, Germany) as previously described [37].

2.7. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

mRNA expression was examined using RT-PCR as described previously [40]. Briefly, total cellular RNA was isolated from pools of 7 to 10 embryoid bodies using an RNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's recommended procedures, followed by genomic DNA digestion using DNaseI (Invitrogen, Karlsruhe, Germany). cDNA was synthesized from 2 μg total RNA using a superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions and using random primer. PCR was performed with thermal cycling conditions of 94 °C for 2 min followed by 38 cycles at 94 °C for 45 s, 55–58 °C for 45 s, and 72 °C for 1 min using Superscript II kit (Invitrogen, Karlsruhe, Germany) and oligonucleotide primers (Sigma, Geonysis, Germany) as shown in Table 1. This was followed by a final

Table 1
Mouse gene-specific primers for PCR.

Gene		Gene
α-MHC	Forward	5′-TGAAAACGGAAAGACGGTGA-3′
	Reverse	5′-TCCTTGAGGTTGTACAGCACA-3′
β-MHC	Forward	5′-CTACAGGCCTGGGCTTACCT-3′
	Reverse	5′-TCTCCTTCTCAGACTTCCGC-3′
β-MHC	Forward	5′-CTCCCAAGGAGAGACGACTG-3′
	Reverse	5′-GAGTTCAGCCCCATGAGGTA-3′
MLC2a	Forward	5′-TCAGCTGCATTGACCAGAAC-3′
	Reverse	5′-AAGACGGTGAAGTTGATGGG-3′
MLC2v	Forward	5′-AAAGAGGCTCCAGGTCCAAT-3′
	Reverse	5′-CCTCTCTGCTTGTGTGGTCA-3′
MLC2v	Forward	5′-CTGCCCTAGGACGAGTGAAC-3′
	Reverse	5′-CCTCTCTGCTTGTGTGGTCA-3′
Nkx-2.5	Forward	5′-CCACTCTCTGCTACCCACCT-3′
	Reverse	5′-CCAGGTTCAGGATGTCTTTGA-3′
GATA4	Forward	5′-ACTCTGGAGGCGAGATGGG-3′
	Reverse	5′-GACACCGCAGCATTACGGCTC-3′
GATA4	Forward	5′-TCTCACTATGGGCACAGCAG-3′
	Reverse	5′-CGAGCAGGAATTTGAAGAGG-3′
NOX-1	Forward	5′-CTGCTCATTTTGCAACCGTA-3′
	Reverse	5′-AGAAGCGAGAGATCCATCCA-3′
NOX-2	Forward	5′-ACTGCGGAGAGTTTGGAAGA-3′
	Reverse	5′-GGTGATGACCACCTTTTGCT-3′
NOX-4	Forward	5′-GATCACAGAAGGTCCCTAGCA-3′
	Reverse	5′-GTTGAGGGCATTCACCAAGT-3′
polymerase II	Forward	5′-GACAAAACTGGCTCCTCTGC-3′
	Reverse	5′-GCTTGCCCTCTACATTCTGC-3′