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Commonly used thiol-containing antioxidants reduce cardiac differentiation and alter gene expression ratios of sarcomeric isoforms

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ABSTRACT

Reactive oxygen species (ROS) scavengers such as beta-mercaptoethanol (BME) and monothiol glycerol (MTG) are extensively used in stem cell research to prevent cellular oxidative stress. However, how these antioxidant supplements impact stem cell cardiac differentiation, a process regulated by redox-signaling remains unknown. In this study, we found that removal of BME from the conventional high-glucose, serum-based differentiation medium improved cardiac differentiation efficiency by 2-3 fold. BME and MTG treatments during differentiation significantly reduced mRNA expression of cardiac progenitor markers (*NKX2.5* and *ISL1*) as well as sarcomeric markers (*MLC2A*, *MLC2V*, *TNNI3*, *MYH6* and *MYH7)*, suggesting reduced cardiomyogenesis by BME or MTG. Moreover, BME and MTG altered the expression ratios between the sarcomeric isoforms. In particular, *TNNI3* to *TNNI1* ratio and *MLC2V* to *MLC2A* ratio were significantly lower in BME or MTG treated cells than untreated cells, implying altered cardiomyocyte phenotype and maturity. Lastly, BME and MTG treatments resulted in less frequent beating, slower contraction and relaxation velocities than untreated cells. Interestingly, none of the above-mentioned effects was observed with Trolox, a non-thiol based antioxidant, despite its strong antioxidant activity. This work demonstrates that commonly used antioxidant supplements may cause considerable changes to cellular redox state and the outcome of differentiation.

1. Introduction

Reactive oxygen species (ROS) are byproducts of cellular metabolism, produced primarily during mitochondrial oxidation. Excessive intracellular ROS, or the imbalance between ROS and antioxidants, can cause damage to biomacromolecules such as DNA [1], RNA [2], and proteins [3], a process generally referred to as oxidative stress. Pluripotent stem cells (PSCs) exposed to oxidative stress undergo cell cycle arrest and apoptosis [4,5] as well as loss of pluripotency [5,6]. To prevent oxidative stress, thiol-based ROS scavengers such as beta-mercaptoethanol (BME) and monothioglycerol (MTG) have been extensively used in both human [7–9] and mouse [10–12] pluripotent stem cell culture.

ROS, however, are not merely harmful byproducts of cellular metabolism. Redox signaling plays a critical role in regulating stem cell differentiation [13–15], including cardiac differentiation. Scavenging of cellular ROS, either by exogenous antioxidants (e.g., N-acetyl cysteine, NAC) or by knocking down the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4, the main source of endogenous ROS), effectively inhibits cardiac lineage specification [16–19]. Conversely, adding free radicals (e.g., H_2O_2) or forced expression of NOX4 increases ROS and promotes cardiac differentiation [18,19]. ROS can also serve as a bridge between physical cues and intracellular signaling. Studies have shown that electrical stimulation [20] and mechanical strain [21] promote cardiac differentiation via ROS. Mechanistically, several targets of ROS have been identified as important drivers for cardiac differentiation. ROS directly activate p38 mitogen-activated protein kinase (MAPK), which subsequently facilitates nuclear translocation of myocyte-specific enhancer factor 2C (MEF2C), a critical cardiac transcriptional factor [17]. Additionally, ROS target the redox-sensitive transcriptional factor C-Jun, leading to the upregulation of GATA binding protein 4 (GATA-4), an early cardiac transcription factor [19]. Finally, ROS and antioxidants are able to modulate the cell cycle of mouse embryonic stem cells (mESCs)-derived cardiomyocytes, with H_2O_2 promoting proliferation and N-(2-mercapto-propionyl)-glycine, a thiol-containing antioxidant, repressing it [18].

Despite the accumulating evidence of ROS impacting cardiac differentiation, how BME and MTG, the most commonly used antioxidants in stem cell culture and differentiation, affect cardiac lineage specification remains unknown. In this work, we show that while removal of BME or MTG from differentiation medium led to elevated oxidative

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stress and reduced cell growth, it concurrently enhanced cardiac differentiation efficiency from 15% to over 40% as measured by flow cytometry analysis of α-myosin heavy chain (MHC)-mCherry reporter cell line. On the other hand, Trolox, a water-soluble analog of vitamin E and a none-thiol based antioxidant, showed a stronger ROS-scavenging efficacy than BME or MTG yet did not reduce cardiac differentiation efficiency. The presence of BME or MTG during cardiac differentiation also altered gene expression ratio of myosin heavy chain (*MHC*), troponin I (*TNNI*), and myosin light chain (*MLC*) isoform pairs in resulting cardiac cells. Specifically, the expression ratios of sarcomeric genes *MLC2V* to *MLC2A*, *TNNI3* to *TNNI1*, and *MYH7* to *MYH6* were decreased by BME or MTG but not Trolox treatment. Functionally, mESC-derived cardiac cells differentiated in medium containing BME or MTG exhibited slower beating rates and slower contraction velocity. Together, these data suggest that the commonly used thiol-based antioxidants BME and MTG can reduce cardiac differentiation and alter the resulting cellular phenotype.

2. Materials and methods

2.1. mESC culture

To monitor cardiac differentiation, we used two reporter cell lines: NK2 Homeobox 5 (NKX2.5) for cardiac progenitors and αMHC for cardiac cells. The αMHC-mCherry reporter cell line was generated by transfecting wild-type E14JU mESCs (a gift from Dr. Joshua Brickman at University of Copenhagen) with αMHC-mCherry-Rex-Blasticidin lentiviral plasmids, a gift from Dr. Mark Mercola at Stanford University, as previously described [22]. The NKX2.5-green fluorescent protein (GFP) mESC reporter cell line was acquired from the University of California Davis Mouse Biology Program [23]. Growth medium for all cell lines was composed of Glasgow Minimum Essential Medium (GMEM, Sigma-Aldrich), 10% fetal bovine serum (FBS, Invitrogen), 1% non-essential amino acids (NEAA, Thermo Fisher), 1% sodium pyruvate (Thermo Fisher), 1% L-glutamine (Sigma-Aldrich), and 0.05% (v/v) Leukemia inhibitory factor (LIF, gift from Dr. Brickman). For passaging, cells were seeded (0.5 million cells/well) onto 6-well plates coated with 0.1% gelatin (Stem Cell Technologies).

2.2. mESC differentiation

mESCs (passages 15–45) underwent unguided differentiation as embryoid bodies (EB) or as monolayers. For both methods, differentiation medium was composed of high glucose (25 mM) or low glucose (5.5 mM) Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), 15% FBS, 1% L-glutamine, and 1% NEAA. 100 μM BME (Thermo Fisher), 450 μM MTG (Sigma-Aldrich), or 40 μM Trolox (Sigma-Aldrich) was added to the differentiation medium at designated timepoints as indicated in the specific experimental schemes (Figs. 1A and 3A).

Embryoid body (EB) differentiation was performed as previously described [24]. Briefly, mESCs were dissociated into single cells and resuspended in differentiation medium. EBs were generated by forming 20-μL hanging drops with 3000–4000 cells/drop and incubated for 2 days. Each EB was then transferred into a single well of a 96-well ultra-low attachment plate (Sigma-Aldrich) and grown for 2–3 additional days before seeding onto gelatin-coated 48-well tissue culture plates. Differentiation medium was then replaced every other day. Spontaneous beating was first observed between days 6 and 8 of differentiation.

For monolayer differentiation, 25,000 mESCs were seeded onto a gelatin-coated 14-mm glass coverslip. After seeding, each coverslip was transferred into a single well in a 12-well tissue culture plate. This method ensured that differentiating cells received sufficient medium and nutrients, critical for achieving successful cardiac differentiation. Upon seeding, cells were maintained in mESC growth medium for 24 h, which was then replaced with differentiation medium (2 mL/well). Differentiation medium was replaced every other day and supplemented with 66 μg/mL 2-phosphate ascorbic acid (Sigma-Aldrich) to increase cell adhesion and survival.

2.3. Quantitative RT-PCR

Total mRNA was extracted using RNeasy mini kit (QIAGEN) and then reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) according to manufacturers' instructions. Aliquots of cDNA equivalent to 25 ng of total RNA were amplified using standard procedures with PowerUp SYBR green (Thermo Fisher) and StepOne Plus Real-Time PCR System (Applied Biosystems). Relative expression of mRNA was quantified with $\Delta\Delta C_T$ method, using β-actin (*ACTB*) as the housekeeping gene for normalization: $\Delta C_T = Ct_{sample} - Ct_{ACTB}$, $\Delta\Delta C_T = \Delta C_{T\ sample1} - \Delta C_{T\ sample\ 2}$, and relative expression $= 2^{\Delta\Delta CT}$. Melting curves were incorporated to ensure the specificity of the amplification. Primer sequences were obtained from the literature [18] or Harvard PrimerBank and are detailed in Table S1.

2.4. Flow cytometry

αMHC-mCherry mESCs were differentiated using the EB or monolayer differentiation methods as described above. On day 13 or 14 of differentiation, cells were rinsed with Dulbecco's phosphate buffered saline (DPBS), dissociated using Accumax (Innovative Cell Technologies), and filtered through a 0.2-μm cell strainer. mCherry expression was measured with an Accuri C6 (BD Biosciences) fluorescence-activated cell sorter (FACS). Each condition was tested in triplicate with more than 3000 events counted for each sample. Gating and analysis were performed with FlowJo (Tree Star).

2.5. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. After briefly rinsing with PBS, cells were permeabilized with 0.25% Triton in PBS for 15 min, followed by 30 min blocking with 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. Mouse monoclonal cardiac troponin T (cTnT) antibodies (Abcam) were diluted 1:500 with blocking buffer and incubated with the samples overnight at 4 °C. Samples were then rinsed with 0.2% (v/v) Tween 20 in PBS (PBST) 3 times and incubated with donkey anti-mouse secondary antibody (Abcam) diluted 1:200 for 1 h at room temperature. Cell nuclei were stained with 6 μg/mL Hoechst 33342 (Thermo Fisher). Imaging was performed with a Zeiss spinning-disk confocal microscope.

2.6. ROS quantification

Intracellular ROS levels were quantified using CellROX Orange (Thermo Fisher), a non-specific probe that can be oxidized by most free radicals, hence reflecting the overall intracellular ROS level. Briefly, 5 μM of CellROX dye was directly added to cell culture medium, followed by 30 min incubation at 37 °C. Cells were then rinsed with DPBS and dissociated with Accutase (Innovative Cell Technologies).

Mitochondrial superoxide was quantified using MitoSOX (Thermo