Biochimie 135 (2017) 28–34

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Exposure to high levels of fumarate and succinate leads to apoptotic cytotoxicity and altered global DNA methylation profiles *in vitro*

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ARTICLE INFO

Article history:
Received 12 December 2016
Accepted 12 January 2017
Available online 16 January 2017

Keywords:
Succinate
Fumarate
Apoptosis
DNA methylation
Methylation sensitive comet assay
Cancer

ABSTRACT

In the Krebs cycle, succinate is oxidized to fumarate by succinate dehydrogenase (SDH), followed by the conversion of fumarate to malate by fumarate hydratase (FH). In cells with defective SDH and FH, the Krebs cycle is congested, respiration impaired and fumarate and succinate accumulates. Several studies have indicated that the accumulation of these substrates are associated with cytotoxicity and oncogenesis. High levels of succinate and fumarate induce hypoxia inducible factor (HIF1A) hydroxylases, leading to the activation of oncogenic HIF pathways. However, the role of HIF as primary inducer of oncogenic change has been questioned, as other non-enzymatic mechanisms have been shown to interfere with cellular metabolism, cell signalling as well as disrupting protein function. Owing to the essential roles that SDH and FH play in cellular energy metabolism, and their associated tumor suppressor capacity, it is vital to understand the biochemical effects resulting from the accumulation of their associated metabolites. Therefore, in this study, we investigated the effect of high concentrations of succinate and fumarate exposure on cell viability, genome integrity and global DNA methylation using a human hepatocellular carcinoma (HepG2) cell culture model. It was found that relatively high concentrations of succinate and fumarate cause a loss of cell viability, which seems to be orchestrated through an apoptotic pathway. Cells exposed to high levels of succinate also presented with elevated caspase 3 and/or caspase 7 levels. In addition, elevated levels of fumarate lead to extensive DNA fragmentation, which may contribute pathophysiologically by inducing chromosomal instability, while succinate demonstrated lower genotoxicity. Furthermore, both succinate and fumarate altered the global DNA methylation patterns via significant DNA hypermethylation. Since numerous studies have reported correlations between aberrant DNA methylation and oncogenesis, hypermethylation may contribute to the oncogenesis observed in cells exposed to high concentrations of these metabolites.

1. Introduction

Cells in the human body are continuously bombarded by various xenobiotics and even the abnormal accumulation of endogenous metabolites can be cytotoxic and/or oncogenic [1]. Nowadays, it is increasingly recognised that altered metabolism may also play a causative role in cancer development [2]. In the Krebs cycle, succinate is oxidized to fumarate by succinate dehydrogenase (SDH), followed by the conversion to malate by fumarate hydratase (FH). SDH and FH not only play an important role in the energy production processes of normal cells, but also act as tumor suppressors [3,4]. Several studies have shown that mutations in the genes encoding for FH and SDH can play a causal role in tumorigenesis [5–7]. In cells with defective FH and SDH, the Krebs cycle is congested, respiration impaired and the substrates fumarate and succinate accumulate. The accumulation of these metabolites inhibit αKG-dependent dioxygenases, resulting in the activation of hypoxia inducible factor (HIF1A) hydroxylases which ultimately leads to pseudohypoxia [8]. Fumarate is an endogenous electrophile and is potentially oncogenic due to its ability to chemically modify cysteine residues resulting in the activation of nuclear factor

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http://dx.doi.org/10.1016/j.biochi.2017.01.004
0300-9084/

(erythroid-derived 2)-like 2 (NFE2L2) [9,10]. Succinate on the other hand has been shown to have the ability to alter gene expression by remodelling the epigenome [11,12]. Succinate can also inhibit HIF-α prolyl hydroxylase, ultimately leading to tumor progression by stimulating the expression of genes that facilitate angiogenesis and glycolysis [13]. Interestingly, the accumulation of succinate in M1 macrophages has been shown to have a direct impact on macrophage cytokine production [14] which may ultimately lead to the inactivation of the electron transport chain [15]. The oncogenic metabolisms of these two metabolites, amongst others, are elegantly summarized by Vazquez et al. [16].

Despite the impact of these observations on the cytotoxic effects of fumarate and succinate, the full scope of the molecular mechanisms by which these metabolites exert their toxic and carcinogenic effects is still subject to debate. Due to the central roles that succinate and fumarate play in cellular energy metabolism, and the associated tumor suppressor capacity of their enzymes, it is vital to understand the biochemical effects resulting from the accumulation of these metabolites. In this study, the effect of high concentrations of succinate and fumarate on cell viability, genome integrity and global DNA methylation of cultured HepG2 cells were investigated.

2. Materials and methods

2.1. Culture conditions and metabolite treatment

HepG2 cells were cultured in Dulbecco's modified essential medium (D-MEM; Hyclone) containing 10% foetal bovine serum (FBS) (Lonza), 1% penicillin/streptomycin (Lonza), 1% 200 mM L-glutamine (Lonza) and 1% non-essential amino acids (Lonza). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For metabolite treatment, cells were seeded in 0.34 cm^2 wells (96 well plates, TPP) for the cell viability assays and 1.9 cm^2 wells (24 well plate; Nunc™) for the comet assays, and subsequently cultured until 80–90% confluency. A 10 mM stock solution of succinate (Sigma Aldrich) was prepared in 1 x phosphate buffered saline (PBS) and a 10 mM stock solution of fumarate was prepared in 20% ethanol. The cells were subsequently exposed to different concentrations of these metabolites for 24 h diluted in serum free culture medium.

2.2. Cell viability assays

2.2.1. MTT

The 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine *in vitro* cell viability. After 24 h exposure to different concentrations of succinate and fumarate, growth medium was removed and cells were rinsed twice with PBS (100 μl). Directly following the wash step, 100 μl of serum free medium containing 0.5 mg/ml MTT was added to the cells. Cells were then incubated for 4 h at 37 °C, after which the MTT was removed and replaced with 100 μl dimethyl sulfoxide (DMSO) in order to dissolve the purple formazan crystals. After 60 min of incubation at 37 °C, cell viability was determined using a microplate reader (BioTek®, Vermont, USA) by measuring the absorbance at 560 nm and 630 nm with DMSO used as a blank. Blank readings were subtracted from absorbance measurements. Cell viability is expressed as a percentage relative to the untreated control, which was set as being 100% viable using the following equation:

$$Cell\ viability(\%) = \frac{(A630 - A550\ of\ treated\ cells)}{(A630 - A550\ of\ control\ cells)} \times 100$$

2.2.2. Neutral red

The neutral red *in vitro* toxicology assay kit (Promega) was used for visually determining cell viability as prescribed by the manufacturer with minor changes. In short, cultured cells were exposed to different concentrations of succinate and fumarate for 24 h. Following exposure, growth media was carefully removed and the cells were washed twice with PBS (100 μl). Cells were then exposed to a 0.04% (v/v) neutral red reagent solution diluted in PBS and incubated for 2 h at 37 °C. After incubation, the neutral red solution was removed and 100 μl of the fixative agent was added. Cells were fixated for 60 s followed by a wash step with 100 μl PBS. Cells were photographed using a Nikon Eclipse TS100 microscope equipped with a Nikon TV Lens C-0.35X camera and IC Capture Ver. 2.3. capturing software (The Imaging Source Europe GmbH).

2.3. Quantification of DNA damage using the comet assay

The comet assay (single cell gel electrophoresis) was performed under alkaline conditions as described previously [17]. Pictures of the comets were taken with an Olympus IX70 fluorescence microscope (200× magnification) and for each sample a minimum of 100 comets were scored and the percentage tail DNA was determined with the Comet IV computer software (Perceptive Instruments Ltd). DNA damage was quantified based on the amount of tail DNA.

2.4. Detection of apoptosis

The Annexin V FITC apoptosis detection kit (BD Biosciences) was used for the detection of apoptosis using flow cytometry. The assay was performed according to the manufacturer's instructions with minor alterations as described previously [18]. For a positive apoptosis control, HepG2 cells were treated with 1 mM staurosporine (Sigma-Aldrich) for 3 h. Amplification of signals were carried out at logarithmic scale and measurement of events plotted on forward light scatter (FSC), side light scatter (SSC), green fluorescent (FL1) and red fluorescent (FL2). A total of 10 000 events were counted per sample. Stained and unstained negative controls were included in each analysis.

2.5. Detection of caspase-3/7

The CellEvent® caspase-3/7 green detection kit (Molecular Probes) was used for the detection of caspase-3/7 apoptosis using flow cytometry. HepG2 cells were exposed to the metabolites as described above and the assay was performed according to the manufacturer's instructions. The geometric means of fluorescence for all the parameters were calculated from the respective histograms or two parameter fluorescence dot plots.

2.6. Methylation sensitive comet assay

Global DNA methylation was quantified using the medium-throughput methylation sensitive comet assay which makes use of the restriction enzymes *Hpa*II and *Msp*I as described previously [19,20]. The comet images were captured as described earlier for the unmodified comet assay. DNA methylation was calculated using the following equation:

$$DNA\ methylation(\%) = \left(100 - \frac{HpaII}{MspI} \times 100\right) - control$$