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Heat output as a bio-marker of the dimethyl sulfoxide-induced decrease in rat hepatoma cell metabolism in vitro

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Abstract

Dimethyl sulfoxide (DMSO) is widely and routinely used as a vehicle in various investigations, especially within the pharmaceutical industry. It has been used for the evaluation of the effects of hydrophobic xenobiotics on cells, as well as for the cryopreservation of biological material. Isothermal microcalorimetry is a powerful tool for monitoring heat production, which is a function of general cellular metabolic activity. Employing this microcalorimetric technique, a low concentration of DMSO routinely used for the addition of hydrophobic substances to, e.g., cell cultures, was shown to decrease heat production (per unit DNA) by the rat hepatoma cell lines FAO, Morris 7800C1 and H4IIE by 32–38%. However, such low concentrations of DMSO did not influence the cell cycle or the degree of apoptosis in these cell populations. Caution is thus advisable when utilizing DMSO as a vehicle in cell culture experiments.

Keywords: Microcalorimetry; DMSO; Vehicle; Heat production

1. Introduction

The planar–polar compound dimethyl sulfoxide (DMSO) is generally used in the cryopreservation of cells, as well as a vehicle for dissolving hydrophobic substances for addition to aqueous solutions, e.g., cell culture media. However, a number of investigations have demonstrated that DMSO can influence various cellular processes, including differentiation and apoptosis, as well as the cell cycle [1–6]. Therefore, when DMSO was chosen as a vehicle for our studies with rat hepatoma cell lines, we wished to determine the effects of this vehicle on cellular metabolic activity.

Microcalorimetry has proven to be a suitable procedure for the assessment of overall metabolic activity in living cells [7]. Heat is released by a large number of processes in plants, animals and microorganisms. In animal cells, ATP and other forms of energy are utilized in numerous exothermic processes, including protein synthesis, maintenance of electrolyte gradients and muscle contraction. None of these cellular energy-converting or -utilizing processes is 100% efficient and the energy is converted into heat according to the first and second laws of thermodynamics. This heat production is the basis for the rapid development of isothermal microcalorimetry as a powerful technique for monitoring general cellular metabolism [7].

Abbreviations: DMSO, dimethyl sulfoxide

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In the present study, we have employed a microcalorimetric technique involving an improvement developed in our laboratory previously [8,9]. In this procedure micro-plates serve as a carrier for adherent cells, to mimic the conditions employed for normal cell culturing. Alterations in the rate of heat production in response to exposure of the cells to DMSO were monitored.

2. Materials and methods

2.1. Chemicals

Dulbecco's MEM/Nutrient Mixture F-12 (1:1) medium, Dulbecco's minimal essential medium with Earle's salts and L-glutamine, Ham's F10 medium, fetal bovine serum, horse serum, penicillin/streptomycin, fungizone, anti-PPLO and trypsin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). RNAse (type I-A) was obtained from Sigma Chemical (St. Louis, MO). Cell culture flasks and dishes were procured from Falcon (Becton Dickinson Labware, USA). All other chemicals were at least of reagent grade and also purchased from common commercial sources.

2.2. Cells

Three different established rat hepatoma cell lines, e.g., FAO (European Collection of Cell Culture, Wiltshire, UK), H4IIE (ATCC, USA), and Morris hepatoma 7800C1 cells [10] were the model systems employed here.

FAO cells were cultured in 75 cm^2 plastic culture flasks with Dulbecco's MEM/Nutrient Mixture F-12 (1:1) medium supplemented with 5% fetal bovine serum and penicillin/streptomycin (125 U/ml). H4IIE cells were cultured in 75 cm^2 plastic culture flasks with Dulbecco's minimal essential medium (containing Earle's salts and L-glutamine) supplemented with 10% fetal bovine serum and penicillin/streptomycin (50 U/ml). Morris 7800C1 cells were cultured as described in an earlier report from our laboratory [10]. Upon reaching confluence, the cells were harvested by treatment with 0.025% trypsin for about 5 min. When the cells had detached, they were washed with fresh medium to remove the trypsin.

2.3. Culturing on micro-plates

The plastic foil (Thermanox) was purchased from Tamro Lab AB (Mölndal, Sweden) and sterilized with 75% ethanol and rinsed in distilled water prior to use. Cell culturing on micro-plates has been described previously [9]. Briefly, the cells (about 10^5 cells per 0.2 ml) were seeded on a 1 cm × 2.2 cm piece of plastic foil (Thermanox) lying in a 60 mm × 30 mm Petri dish and allowed to attach overnight, after which the floating cells were removed and 3 ml fresh medium added to the Petri dish. Thereafter, the medium was changed every second day. Cell growth was monitored using an inverted microscopy (Zeiss MC30, Oberkochen, Germany). When the cells had become confluent, the cultures were incubated with 0.1% DMSO (v/v, final concentration) (FAO and H4IIE cells) or 0.5% DMSO (Morris 7800C1 cells) and cultured for another 2 days. In several experiments the DMSO concentration was increased to 2% (v/v) and the incubation period extended to 4 days.

2.4. Microcalorimetry

The Thermanox micro-plate covered with a cell monolayer was introduced into the ampoule and loaded with 3 ml of medium. As demonstrated previously [9], the Thermanox micro-plate alone does not affect heat output in this system. The 3 ml of medium only was loaded into the reference ampoule. The ampoules were introduced step-wise into the measuring position during a 40 min period in order to allow temperature equilibration of the sample.

The rate of cellular heat production was subsequently monitored using a 3-channel microcalorimeter of the heat conduction type (Thermal Activity Monitor 2277, Thermometric AB, Järfälla, Sweden) [11]. Briefly, this instrument is equipped with three separate channels, each consisting of a stainless steel reaction ampoule and a corresponding reference ampoule, each having a total volume of 5 ml. The water bath was maintained at 37 °C. The sample to be measured is loaded into the reaction ampoule and the corresponding volume of medium into the reference ampoule. The heat production in these two ampoules (Φ_{value}) creates a heat flow through the thermopiles and any difference in heat generates a voltage output.

The signal from the microcalorimeter was transferred to a computer via a Pico ADC-16 connector (Pico Technology, United Kingdom). The rate of heat production per cell (Φ_{cell}) or per amount of DNA (Φ_{DNA}) is calculated from the Φ_{value} and the corresponding number of cells or amount of DNA. The instrument demonstrated a baseline variability of less than 1 μW during long-term runs up to 72 h, as described earlier [11]. When an optimal number of cells was loaded, a steady-state power-curve could easily be obtained within 0.5 h.

2.5. Determination of DNA

DNA was extracted and quantitated employing the fluorimetric procedure (Model F-2000 fluorescence spectrophotometer, Hitachi, Tokyo, Japan) described by Shapira et al. [12].

2.6. Quantification of the ratio of hepatoma cells in the G_1 and G_2 phases of the cell cycle by flow cytometry

Approximately 1×10^6 trypsinized and washed cells were centrifuged and the pellet fixed in 2 ml cold 70% ethanol at 4 °C for 60 min. The cells were then centrifuged again, washed in 1 ml PBS buffer and resuspended in 0.5 ml of the same buffer. To 0.5 ml of cell suspension, 0.5 ml RNAse (1 mg/ml) was added, followed by gentle mixing with 1 ml propidium iodide solution (PI, Sigma, 100 μg/ml in PBS). The mixed cells were subsequently incubated in the dark at room temperature for 15 min and maintained thereafter at 4 °C in the dark until their DNA content was analyzed using a flow cytometer (Facscalibur, Becton Dickinson, San Jose, CA, USA). For each sample, 10,000 cells were analyzed. The distinct diploid (2C) and tetraploid (4C) regions present in DNA distribution histograms of hepatoma cells represent the populations in the G_1 and G_2 phases, respectively, of the cell cycle.

3. Results

3.1. Quantitation of heat production by the three different hepatoma cell lines (FAO, Morris 7800C1 and H4IIE) before and after exposure to DMSO

In pilot experiments it was found that use of a micro-plate 1 cm^2 in area resulted in attachment of the optimal number of cells (about 0.5×10^6 cells), leading to a relatively higher heat production and prolonged steady-state power-curves (Fig. 1). Since it is difficult to obtain the same number of cells attached in different micro-plate cultures, cellular DNA content was also determined. Using light microscopy, we observed no morphological differences between cells cultured on our micro-plate and in normal flasks.

Exposure to DMSO caused a significant decrease in heat production (32–38%) by the three cell lines, compared to untreated cells (Table 1). Within the concentration range tested (0.1–2% of DMSO), inhibition of the heat production of hepatoma cells was neither dose- nor time-dependent. Cells treated with

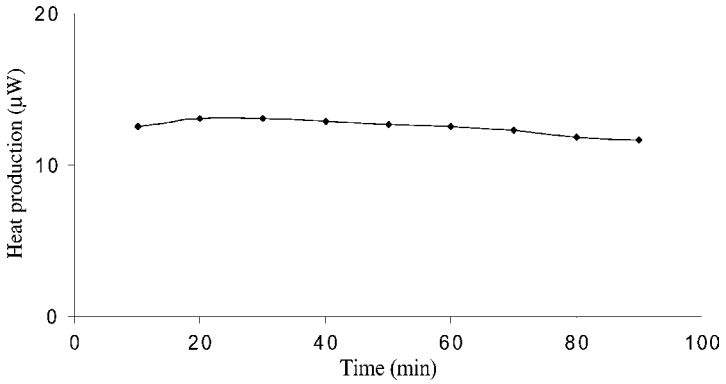

Fig. 1. Recording of a typical power-curve produced by approximately 0.5×10^6 FAO hepatoma cells cultured on 1 cm × 1 cm Thermanox plate and pretreated with 0.1% DMSO for 2 days.

Table 1
Effects of DMSO on heat production by rat hepatoma cell lines

Treatment	Heat production (μW/μg DNA) by		
	FAO cells (%)	Morris 7800C1 cells (%)	H4IIE cells (%)
Control	1.87 ± 0.20 ($n = 11$) (100)	1.60 ± 0.07 ($n = 5$) (100)	0.47 ± 0.08 ($n = 5$) (100)
DMSO (0.1%, v/v)	1.16 ± 0.07*** ($n = 6$) (62)	1.09 ± 0.09*** ($n = 6$)[a] (68)	0.29 ± 0.07** ($n = 5$) (62)

[a] This cell line was incubated with 0.5% DMSO (v/v, final concentration). *n*: the number of independent experiments. All values are means ± S.D.
** $P < 0.01$ compared with the corresponding control.
*** $P < 0.001$ compared with the corresponding control.

2% DMSO for as long as 4 days demonstrated the same reduction in heat production as cells incubated with a lower concentration (0.1%) and for a shorter period of time (2 days). For instance, in the case of FAO cells the highest concentration and longest period of incubation resulted in a decrease in heat output to 1.11 ± 0.13 μW/μg DNA, i.e., not statistically significantly different than that observed with a lower concentration of DMSO and shorter time (Table 1).

3.2. Effect of DMSO on the ratio of diploid and tetraploid FAO cells

In order to determine whether the lowered heat production in the presence of DMSO reflects changes in the cell cycle, the DNA content of FAO cells was characterized. We compared the ratio of diploid and tetraploid cells in both control and DMSO-treated (0.1%) cultures employing flow cytometry after PI staining and observed no significant difference (Table 2). However, exposure to 2% DMSO caused a somewhat larger number of cells to enter the G_2 phase. Fig. 2 also reveals that there is no change in the subdiploid G_2 population, indicating that cells do not undergo apoptosis upon exposure to 0.1% DMSO.

Table 2
Effects of DMSO on the cell cycle of FAO cells treatment

Parameter	Treatment		
	None (control)	0.5% DMSO	2% DMSO
2C cells (G_1 phase) (%)	75	74	70
4C cells (G_2 phase) (%)	25	26	30

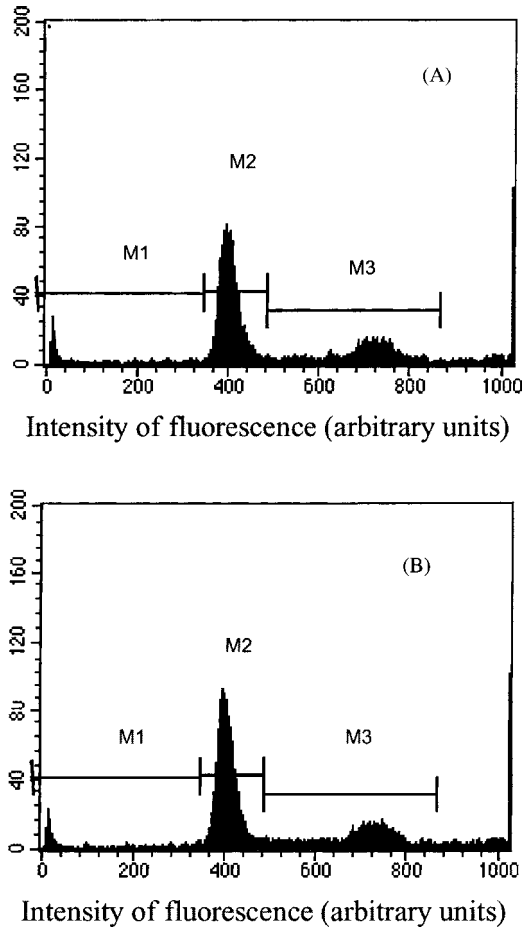

Fig. 2. Flow cytometric diagram of DMSO-treated and untreated FAO cells stained with propidium iodide. There was no significant difference on the ratio of diploid to tetraploid cells in: (A) treated (2.8:1) and (B) untreated (2.9:1) cultures. Nor is any pronounced apoptosis observed. These histograms are representative of at least three separate experiments which gave very similar results.

4. Discussion

Microcalorimetry has been proven to be a rapid and sensitive technique for measuring heat outputs as low as to 0.1 μW [11]. When samples are loaded into the ampoule as cell suspensions, "crowding" is observed. This "crowding phenomenon" leads to a decrease in the rate of heat production per cell, most likely due to rate-limiting diffusion of nutrients and oxygen, together with accumulation of carbon dioxide, lactic acid and/or other waste products in the presence of large number of cells [13].

Application of this technique to various biological and metabolic investigations has increased since micro-plates were introduced as a carrier for the cells [8,9]. This approach reduces the "crowding" phenomenon and allows cells to be measured under the same conditions as in ordinary cell cultures. Thus, after obtaining a steady-state power-curve, the total heat production divided by the number of cells (or their total DNA content) reflects the average metabolic activity of each cell.

The much lower heat production by H4IIE hepatoma cells compared to the other two cell lines might reflect the high DNA content of this rapidly growing cell line and/or their low peroxisomal acyl-CoA oxidase activity [14,15]. It has been proposed that the peroxisomal acyl-CoA oxidase produces H_2O_2 and heat, instead of conserving energy as in the corresponding mitochondrial acyl-CoA dehydrogenase reaction [16].

The polar solvent DMSO, which is widely used for the cryopreservation of cells, is also routinely used as a vehicle for the addition of hydrophobic substances to aqueous solutions. However, we demonstrate here that even relatively low concentrations of DMSO cause a significant decrease (by 1/3) in cellular heat production. A similar observation has been reported for DMSO-treated lymphocytes [2]. However, in the latter case the size of the effect was dependent on DMSO concentration. The lack of such dependence in our experiments probably reflects the longer incubation time employed, allowing saturation of the effect even with the lowest concentrations of DMSO.

The mechanism underlying the decrease in heat production observed in DMSO-treated cell populations remains to be elucidated. Teraoka et al. [4] reported that 2% DMSO can induce reversible arrest of human lymphoid cell lines in the G_1 phase of the cell cycle, which alters the average DNA content per cell. However, in the case of the studied hepatoma cells studied here, as much as 2% DMSO had no significant effect on cellular DNA. Obviously then, this cannot explain the decreased heat production. In addition, no difference in mitochondrial membrane potential was observed upon such treatment (not shown).

It has also been reported that DMSO can induce both differentiation and apoptosis in several cell types, including leukemic U937 cells [5] and v-myc-immortalized mouse macrophages [6] depending on the concentration of DMSO used. Usually, apoptosis occurred in the presence of 2–10% DMSO. In our observation, 0.5% DMSO induced no apoptosis in FAO cells. Furthermore, it has been demonstrated that DMSO is a potent hydroxyl radical scavenger [3] and, consequently, can inhibit the release of specific granule constituent from polymorphonuclear leukocytes [1].

Heat production reflects, of course, the net sum of all exo- and endothermic processes in a cell. Possibly, DMSO simply slows down the overall metabolic activity of hepatoma cells, rather than affecting a specific reaction(s). This study thus illustrates the importance of keeping in mind that even low concentrations of a vehicle may disturb the experimental model being studied. Such effects have also been observed for other vehicles. Zhao et al. [17] found that ethanol affects the cellular protein kinase, whereas, cremophor, a vehicle for the immunosuppressive drug cyclosporine was demonstrated to inhibit mitochondrial ATP production [18].

Production of heat at a lower rate by a cell population indicates that the individual cells may not be functioning optimally at that given moment from a metabolic point of view. Cells with altered metabolism may also exhibit altered responses to various stimuli and treatments. Before initiating a set of experiments involving the use of a vehicle, the possible influence of this vehicle on the system under study should thus be ascertained. The established microcalorimetric technique applied here to hepatoma cell lines might also be useful for investigating adverse drug affects on human liver cells.

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