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Nitric oxide affects immune cells bioenergetics
Long-term effects of nitric-oxide derivatives on leukaemic Jurkat cell metabolism

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ABSTRACT

Major advances in dissecting mechanisms of NO-induced down-regulation of the anti-tumour specific T-cell function have been accomplished during the last decade. In this work, we studied the effects of a NO donor (AT38) on leukaemic Jurkat cell bioenergetics. Culturing Jurkat cells in the presence of AT38 triggered irreversible inhibition of cell respiration, led to the depletion of 50% of the intracellular ATP content and induced the arrest of cell proliferation and the loss of cell viability. Although a deterioration of the overall metabolic activity has been observed, glycolysis was stimulated, as revealed by the increase of glucose uptake and lactate accumulation rates as well as by the up-regulation of GLUT-1 and PFK-1 mRNA levels. In the presence of NO, cell ATP was rapidly consumed by energy-requiring apoptosis mechanisms; under a glucose concentration of about 12.7 mM, cell death was switched from apoptosis into necrosis. Exposure of Jurkat cells to DMSO (1%, v/v), SA and AT55, the non-NO releasing moiety of AT38, failed to modulate neither cell proliferation nor bioenergetics. Thus, as for all NSAIDs, beneficial effects of AT38 on tumour regression are accompanied by the suppression of the immune system. We then showed that pre-treating Jurkat cells with low concentration of cyclosporine A, a blocker of the mitochondrial transition pore, attenuates AT38-induced inhibition of cell proliferation and suppresses cell death. Finally, we have studied and compared the effects of nitrite and nitrate on Jurkat cells to those of NO and we are providing evidence that nitrate, which is considered as a biologically inert anion, has a concentration and time-dependent immunosuppressive potential.

Introduction

The metabolic, energetic and biosynthetic demands of immune cells increase dramatically after their activation by antigens and mitogens. Mounting a functional immune response requires rapid and extensive cell growth, proliferation, activation and production of effector proteins (Maciver et al. 2008). Immunoregulatory functions are known to be ATP-dependant and sensitive to disturbances in intracellular nutrient levels (Buttgereit et al. 2000). Co-stimulation of T-cells by T-cell receptor and CD28 was shown to lead in PI3K/Akt-dependent up-regulation of glucose transporter 1 (GLUT-1) gene expression, glucose uptake and oxygen metabolism (Frauwirth et al. 2002).

In aerobic organisms, energy is provided by glycolysis or respiration via oxidative phosphorylation, which has the higher energy production efficiency. However, the tumoural microenvironment is characterized by low concentrations of oxygen and glucose, as well as high levels of lactate and reductive and oxidative species (Raghunand et al. 2003). Specifically, a myeloid-derived suppressor cell population ensures high output of NO via the enzymatic activity of inducible NO synthase (iNOS) (Serafini et al. 2006). NO diffuses freely and reacts rapidly with various biomolecules and intracellular compounds (e.g. O_2^-, H_2O_2) within both NO-generating cells and also target cells to produce reactive nitrogen oxide species (Mocellin et al. 2007). The primary decomposition product of NO in aerobic aqueous solution is nitrite (NO_2^-), which can be further oxidized to generate nitrate (NO_3^-) in the presence of additional oxidizing species such as oxyhemoproteins (Ignarro et al. 1993). NO is known to be responsible for various physiological functions and is implicated in multiple pathologies (Mocellin et al. 2007), but special attention is accorded to its role in cancer pathogenesis and tumour-related immunosuppression

Abbreviations: CsA, cyclosporine A; DMSO, dimethyl sulfoxide; DO, dissolved oxygen; GLUT, glucose transporter; IL-2, interleukin-2; iNOS, inducible nitric oxide synthase; MPTP, mitochondrial permeability transition pore; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; OUR, oxygen uptake rate; PFK, phosphofructokinase; SA, salicylic acid.

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phenomena since effective anti-NO and NO-based anti-cancer drugs showed contradictory findings. In fact, low/intermediate steady-state concentrations (nano/picomolar) promote cancer progression, whereas higher levels (micromolar) lead to tumour regression. Moreover, short-term exposure of T-cells to physiologic concentrations of NO rapidly induces mitochondrial hyperpolarization and inhibits complex IV of the respiratory chain in a reversible manner, while prolonged exposure to NO results in a gradual and persistent inhibition of complex I (Clementi et al. 1998). NO also triggers the release of cytochrome *c* followed by the cleavage, and so the activation, of pro-apoptotic caspases, morphological changes, DNA fragmentation, and, ultimately, cell death (Li et al. 1997; Kuida et al. 1998). Concomitant release and activation of cell death signals and the availability of glycolytic ATP induce apoptosis; and the decrease of ATP turnover is known to switch cell death process into necrosis (Leist et al. 1999a,b).

NO-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs), which represent a novel and promising class of cancer chemopreventive compounds (Nath et al. 2005), were reported to modulate respiration and bioenergetics of immune cells and to induce their death (Fiorucci et al. 2004). In this study, we investigated the long-term effects of AT38 on leukaemic Jurkat cells. The AT38 used herein is a NSAID consisting of a salicylic acid (SA)-like moiety linked to a furoxan moiety (details for AT38 structure, formulation and release mechanisms will be fully described in another submitted manuscript). These furoxan based-NO donors are now emerging for the selective inhibition of cyclooxygenase, including their use for the treatment of certain cancer types and neurological disorders (Del Grosso et al. 2005). We also compared the effects of NO donors to nitrite and nitrate donors ($NaNO_2$ and $NaNO_3$, respectively) on Jurkat cell bioenergetics and showed a time- and concentration-dependent immunosuppressive potential of nitrate. As for all other NO-donors, AT38 inhibits Jurkat cell bioenergetics, and so, *in vivo*, the AT38-mediated tumour regression will be accompanied by the dysfunction of immune system. Because cyclosporine A (CsA) at low doses (0.1–5 μM) is known to inhibit most of the mitochondrial changes caused by NO, such as the loss of mitochondrial transmembrane potential, the opening of the mitochondrial permeability transition pore (MPTP) and consequently the release of cytochrome *c* (Roy et al. 2006), we demonstrated that pre-treating Jurkat cells with CsA attenuate AT38 effects on cell growth and viability.

Materials and methods

Materials

RPMI1640, Foetal Bovine Serum, Penicillin/Streptomycin, Cyclosporine A, IL-2 ELISA kit, haemoglobin and Jurkat cells (Clone E6-1) were purchased from Cedarlane (Ontario, Canada). AT38 and AT55 were synthesized in Prof. Bronte's laboratory (manuscript under submission). Dimethyl sulfoxide (DMSO), sodium nitrate and sodium nitrate, salicylic acid (SA), perchloric acid, potassium bicarbonate, concanavalin A (ConA), nucleotides standards and all other reagents were from Sigma–Aldrich (St. Louis, MO).

Cell culture and treatment

Jurkat cells were cultured at an initial density of 0.2×10^6 cells mL^{-1} in RPMI1640 culture medium supplemented with 10% (v/v) of FBS, 100 U mL^{-1} Penicillin and 150 U mL^{-1} Streptomycin in 75-cm^2 untreated T-flasks (VWR, Ontario, Canada). Cells were incubated at 37 °C under a humidified 5% CO_2 atmosphere for 2 days.

When required, Jurkat cells were cultured for 36 h in complete culture medium containing $NaNO_2$, $NaNO_3$ and AT38 at final concentrations of 25 μM, 50 μM and 25 μM, respectively. AT38, AT55 and SA are dissolved in DMSO (1%, v/v of final volume) prior to addition to cell suspension.

CsA dissolved in 0.1% Eth (1 mM) was used for cell treatment. In specific experiments, Jurkat cells were pre-treated for 30 min with 5 μM of CsA.

Respirometry and oxygen uptake rate

20×10^6 cells were cultured in a 250 mL glass baffled spinner flask containing 100 mL of complete culture medium in the presence of 25 μM of AT38 or 25 μM of $NaNO_2$ or 50 μM $NaNO_3$. To investigate whether NO-mediated inhibition of Jurkat cell respiration is reversible, 10 μM of haemoglobin (Hb) were added in the medium after 3 h of culture in the presence of AT38. Humidified gas mixture (400 mL/min, 95% air/5% CO_2) was fed by surface aeration and medium was magnetically agitated at 60 rpm. Culture medium was circulated in an in-house 316 stainless steel chamber containing a dissolved oxygen (DO) probe (Ingold, Urdorf, Switzerland) and a pH probe (Fisher) by a peristaltic pump (Cole Parmer, Montreal, Quebec, Canada) at a constant flow rate of 3.0 mL/min. Data were recorded by an acquisition system (Virgo, Longueuil, Quebec, Canada) and a mass balance on oxygen was performed to calculate the oxygen uptake rate (OUR).

Assays

Glucose and lactate concentrations in supernatants were measured using a 2700 SELECT biochemistry analyser (YSI Inc., Ohio). IL-2 concentration was assessed according to manufacturer recommendations.

Nucleotides determinations

The extraction protocol was based on the method developed by Ryll and Wagner (1991). Briefly, 4×10^6 viable cells were extracted twice with 0.5 mL (each) of 0.5 M perchloric acid solution and neutralized by 0.3 mL of a 2 M $KHCO_3$ solution. Nucleotides concentrations were then determined by high performance ion-pairing liquid chromatography–electrospray ionization mass spectrometry using a HPLC–MS (Waters, Milford, MA). Nucleotides were separated on a Symmetry C_{18} column (150 × 2.1 mm, 3.5 μm) (Waters) and a Security C_{18} guard-column (Phenomenex, Torrance, CA). Mobile phase consisted in Buffer A: 10 mM ammonium acetate, 15 mM DMHA, pH 7.00 and Buffer B: 40% (v/v) acetonitrile in water. Flow rate was set at 0.3 mL/min using the following gradient: 0–10 min at 15% B, 10–11 min linear gradient from 15 to 30% B, 11–30 min at 30% B, 30–35 min linear gradient from 30 to 70% B, 35–40 min at 70% B, 40–41 min linear gradient from 70 to 15% B and 41–50 min at 15% B.

Detection of apoptotic versus necrotic cells

The cell death process was distinguished using a PromoKine Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell). Briefly, healthy, apoptotic and necrotic cells were stained with Hoechst 33342, Fluorescein (FITC) and Ethidium Homodimer I, respectively. Fluorescence was detected by an inverted fluorescence microscope Axiovert S100TV (Carl Zeiss Canada, North York, Ontario, Canada) equipped with a mercury lamp and an adequate set of filters. Images were taken with a QICAM Fast 1394 camera (QImaging) and acquired with the NORTHERN ECLIPSE image acquisition software (Empix imaging).