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Bupivacaine uncouples the mitochondrial oxidative phosphorylation, inhibits respiratory chain complexes I and III and enhances ROS production: Results of a study on cell cultures

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ABSTRACT

This study aimed to validate, in situ, proposed mechanisms of bupivacaine citotoxicity pointing to impairment of the mitochondrial oxidative metabolism. High resolution oxymetry, carried out on a panel of cell cultures, revealed a dual dose- and time-dependent effect of bupivacaine consisting of uncoupling of the mt$\Delta\mu_{H+}$-controlled respiratory rates in a cyclosporine A-insensitive manner and further inhibition of the respiratory rates. Intriguingly, a relatively small decrease on the mt$\Delta\Psi$ (about 20 mV) was sufficient to account for both the bupivacaine- and the FCCP-mediated impairment of the oxidative phosphorylation coupling thereby supporting a common protonophoric mechanism of action. The bupivacaine-induced depression of the cell respiration related to specific inhibition of complexes I and III and accompanied with production of reactive oxygen species. Importantly, inhibition of the respiratory chain complexes was prevented by antioxidant treatment and reversed following removal of the anaesthetic thereby suggesting an oxidant-mediated feed-back mechanism reinforcing the primary inhibitory action of the anaesthetic.

1. Introduction

Bupivacaine (BPV) is a sodium channel blocker administrated for local anaesthesia including infiltration, nerve block, epidural, and intrathecal anaesthesia. Compared to other local anaesthetics, bupivacaine is markedly cytotoxic (Cox et al., 2003; Leone et al., 2008). Systemic exposure to excessive quantities of BPV mainly results in central nervous system (CNS) (Perez-Castro et al., 2009) and cardiovascular effects (Zink and Graf, 2004; Albright, 1979). CNS effects usually occur at lower blood plasma concentrations and additional cardiovascular effects present at higher concentrations, though cardiovascular collapse may also occur with low concentrations.

Increasing number of studies has shown that BPV is either an apoptosis- and a necrosis-inducer depending on the cellular context (Unami et al., 2003). Different cytotoxic mechanisms have been proposed but all converge on BPV-mediated interference with the mitochondrial energy transduction. Indeed BPV induces a decrease in the aerobic ATP synthesis (Sztark et al., 1994, 1998; Nouette-Gaulain et al., 2002) by at least two major proposed actions: (i) uncoupling of the oxidative phosphorylation (OXPHOS) (Dabadie et al., 1987; Sztark et al., 1997, 2000) and (ii) inhibition of the complex I of the respiratory chain (Sztark et al., 1998, 2000). Other recently suggested mechanisms include inhibition of the carnitine–acylcarnitine translocase (Weinberg et al., 2000), activation of the mitochondrial permeability transition pore (MPTP) (Irwin et al., 2002), enhanced reactive oxygen species (ROS) production (Park et al., 2005).

It must be pointed out, however, that the aforementioned BPV-related toxic mechanisms have been largely inferred from studies carried out on isolated mitochondria with only a few limited exceptions (Pulselli et al., 1996; Grouselle et al., 1990). The relevance of these investigations to the physiology of in situ mitochondria might be questioned considering that: (i) the intracellular network architecture of mitochondria is lost following organelle isolation and recent evidences clearly suggest that the degree of

Abbreviations: BPV, Bupivacaine; L-BPV, Levogire S(−) bupivacaine; mt$\Delta\Psi$, Mitochondrial transmembrane electrical potential; mt$\Delta\mu_{H+}$, Mitochondrial transmembrane electrochemical proton gradient; RCR, Respiratory control ratio; OXPHOS, Oxidative phosphorylation; FCCP, Carbonylcyanide p-trifluoromethoxyphenylhydrazone; TMRE, Tetramethylrhodamine ethyl ester; DCF, Dichlorofluorescein; ROS, Reactive oxygen species; MPTP, Mitochondrial permeability transition pore; dUQ, Decyl ubiquinone; dUQH$_2$, Decyl ubiquinol.

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the inter-organelle connection is related to different bioenergetic tasks (Chen and Chan, 2005; Liesa et al., 2009); (ii) in situ mitochondria establish physical and functional links with components of the cytoskeleton as well as with other membranous cellular compartment like the endo(sarco)plasmic reticulum (Hayashi et al., 2009); (iii) the lipophylic feature of BPV leads to distribution of the drug within all the intracellular membranous compartments with effects that are hidden in suspensions of pure mitochondrial membrane; (iv) the pKa of the BPV tertiary amine group is 8.1 whereby the $BPVH^+$/BPV ratio changes in intracellular compartments with different local pH (Lee and Tannock, 1998); (v) the aerobic metabolic flux, driving OXPHOS within the cell, encompasses many steps which are by-passed when the respiratory activity is elicited in isolated mitochondria by exogenous-substrates thus altering, in principle, the impact of physiologically relevant committing steps (Villani and Attardi, 2000; Cortassa et al., 2009).

The aim of this study was to re-assess the effects of BPV on the in situ mitochondrial bioenergetics using a panel of phenotipically different cell lines. Since in the latest anesthesiological protocols the enantiomer S(−) of bupivacaine is largely used (Sztark et al., 2000; Foster and Markham, 2000), the levogire isoform of the drug (i.e. L-BPV) was tested throughout the present study.

2. Materials and methods

2.1. Cell samples and chemicals

Human hepatoma (HepG2), murine cardiomyoblasts (H9c2), murine skeletal myoblasts (L6) cell lines and primary normal dermal human fibroblasts (NDHF) were from ATCC. HepG2, NDHF and H9c2 were cultured in DMEM whereas L6 was cultured in MEM; both media were supplemented with 10% fetal bovine serum. Cell lines were grown to 70–80% of confluence and the cell viability assessed by the Tripan Blue exclusion assay. S(−) bupivacaine chlorohydrate was from Abbott and prepared as 23 mM of stock aqueous solutions. All the other chemicals, unless indicated, were from Sigma–Aldrich or of analytical grade.

2.2. Measurement of the respiratory activity in intact cells

Cultured cells were gently detached from the dish by tripsinization, washed in PBS, harvested by centrifugation at 500*g* for 5 min and immediately assessed for O_2 consumption with a high resolution oxymeter (Oxygraph-2k, Oroboros Instruments). About 5×10^6 viable cells/ml were assayed in 50 mM KPi, 10 mM Hepes, 1 mM EDTA, pH 7.4 at 37 °C; after attainment of a stationary endogenous substrate-sustained respiratory rate, 2 μg/ml of oligomycin was added. The rates of oxygen consumption were corrected for 2 mM KCN-insensitive respiration. The respiratory control ratio (RCR) was obtained dividing the rates of oxygen consumption achieved before and after the addition of oligomycin.

2.3. Measurement of the mitochondrial respiratory chain complexes activity

The specific activities of NADH: ubiquinone oxidoreductase (complex I), succinate:ubiquinol oxidoreductase (complex II), ubiquinone:cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) were assayed spectrophotometrically on frozen–thawn and ultrasound-treated HepG2 cells in 10 mM Tris, 1 mg/ml serum albumin, pH 8.0. Complex I was assayed (in the presence of 1 μg/ml of antymicin A *plus* 2 mM KCN) by following the initial 2 μg/ml rotenone-sensitive rate of 50 μM NADH oxidation ($\varepsilon_{340nm} = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of 200 μM decylubiquinone (dUQ) as electron acceptor; the NADH-ferricyanide reductase activity of complex I was measured in the presence of rotenone, antimycin A and KCN with 200 μM potassium ferricyanide from the oxidation of NADH. Complex III was assayed (in the presence of rotenone *plus* KCN) by following the initial 1 μg/ml antymicin A-sensitive rate of 50 μM ferri-cytochrome *c* reduction ($\varepsilon_{550nm} = 21.1\ mM^{-1}\ cm^{-1}$) in the presence of 200 μM $dUQH_2$ as electron donor. Complex IV was assayed by following (in the presence of antymicin A) the initial 2 mM KCN-sensitive rate of 20 μM ferro-cytochrome *c* oxidation under aerobic conditions. Complex II was assayed by using 2,6-dichlorophemolindophenol (DCPIP) as final electron acceptor (Barrientos, 2002). The activities were normalized to the initial cell number.

2.4. Laser scanning confocal microscopy imaging of mitochondrial membrane potential and ROS in live cells

Cells cultured on fibronectin coated 35 mm glass bottom dishes were incubated for 10 min at 37 °C with the following probes: 2 μM tetramethylrhodamine, ethyl ester (TMRE), to monitor mitochondrial membrane potential ($mt\Delta\Psi$) or 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) for detection of H_2O_2. Both probes were from Molecular Probes (Eugene, OR). Stained cells were washed with PBS and examined by a Nikon TE 2000 microscope (images collected using a 60X objective (1.4 NA)) coupled to a Radiance 2100 dual laser (four-lines Argon-Krypton, single-line Helium–Neon) confocal laser scanning microscopy system (Biorad). TMRE is a fluorescent lypophilic cations that accumulates electrophoretically in mitochondria. The red fluorescence of TMRE was analysed by exciting the sample with the He–Ne laser beam (λ_{ex} = 543 nm). DCF-DA is a membrane permeant probe which is hydrolysed by intracellular esterases and converted to the ROS (mainly H_2O_2)-reacting product dichlorofluorescein (DCF). The green fluorescence of oxidised DCF was analysed by exciting the sample with the Ar–Kr laser beam (λ_{ex} = 488 nm). Confocal planes (18–20) of 0.2 μm in thickness were examined along the *z*-axes, going from the top to the bottom of the cells. Acquisition, storage and analysis of data were made by using LaserSharp and LaserPix software from Biorad or ImageJ (NIH, USA-http://rsb.info.nih.gov/ij/). Quantification of the emitted fluorescent signal was achieved by producing a xz-intensity profile of the average value of the pixels within the cell contour as a function of each focal plane. Correction was made for minimal background by repeating the procedure in a cell-free field. The integrated value of the xz profile was taken as a measure of the fluorescence intensity per cell and quantified in arbitrary units. At least 20 cells were randomly selected from 8 to 10 different fields for each cell samples under the indicated conditions and statistically analysed.

2.5. Measurement of the mitochondrial transmembrane potential ($mt\Delta\Psi$)

$mt\Delta\Psi$ was assessed spectro-fluorimetrically with safranine O (Akerman and Wikström, 1976; Johnson et al., 1981) in permeabilized HepG2 cells. HepG2 were suspended at 10^6 cell/ml in 0.25 mM sucrose, 10 mM KH_2PO_4, 27 mM KCl, 40 mM Hepes, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.1% BSA, 1.0 mM ADP, pH 7.1 supplemented with 20 μg digitonin/ml/10^6 cells. The optimal concentration of digitonin was tested in pilot titration assays of the respiratory activity. The minimal amount of digitonin suppressing cell respiration (in the presence of oligomycin) but resulting in recovery of the activity following addition of 2 mM pyruvate/2 mM malate was chosen. After 10 min of incubation with digitonin the cell suspension was transferred to the spectrofluorimetric cuvette equipped with a thermostatic control system (T = 37 °C) and a stirring device. The instrumental setting was λ_{ex} = 495 nm, λ_{em} = 596 nm, medium gain (FP-6500, Jasco Analytical Instru-