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Sodium as the major mediator of NO-induced cell death in cultured hepatocytes

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Abstract

NO has been shown to induce cellular injury via inhibition of the mitochondrial respiratory chain and/or oxidative/nitrosative stress. Here, we studied which mechanism and downstream mediator is responsible for NO toxicity to hepatocytes. When cultured rat hepatocytes were incubated with spermineNONOate (0.01–2 mM) at 2, 5, 21 and 95% O_2 in Krebs-Henseleit buffer (37 °C), spermineNONOate caused concentration-dependent hepatocyte death (lactate dehydrogenase release, propidium iodide uptake) with morphological features of both apoptosis and necrosis. Increasing O_2 concentrations protected hepatocytes from NO-induced injury. Steady-state NO concentrations were lower at higher O_2 concentrations, suggesting formation of reactive nitrogen oxide species. Despite this, the scavenger ascorbic acid was hardly protective. In contrast, at equal NO concentrations loss of viability was higher at lower O_2 concentrations and inhibitors of hypoxic injury, fructose and glycine (10 mM), strongly decreased NO-induced injury. Upon addition of spermineNONOate, the cytosolic Na^+ concentration rapidly increased. The increase in sodium depended on the NO/O_2 ratio and was paralleled by hepatocyte death. Sodium-free Krebs-Henseleit buffer strongly protected from NO-induced injury. SpermineNONOate also increased cytosolic calcium levels but the Ca^{2+} chelator quin-2-AM did not diminish cell injury. These results show that – in analogy to hypoxic injury – a sodium influx largely mediates the NO-induced death of cultured hepatocytes. Oxidative stress and disturbances in calcium homeostasis appear to be of minor importance for NO toxicity to hepatocytes.

Keywords: Nitric oxide; Hypoxia; Hepatocytes; Sodium; Glycine; Reactive nitrogen oxide species

Introduction

Nitric oxide (NO) is a well-known inhibitor of the electron transport chain of mitochondria, binding reversibly – and in competition with O_2 – to cytochrome oxidase, the last enzyme of the mitochondrial electron transport chain (Brown and Borutaite, 2002; Cooper, 2002; Moncada and Erusalimsky, 2002). By inhibition of cytochrome oxidase, NO induces hypoxia-like consequences preferably at high NO/O_2 ratios (Dehne et al., 2004; Swintek et al., 2004). On the other hand, NO, which despite its radical nature only slowly oxidizes biomolecules, can be converted to reactive nitrogen oxide species (RNOS) like peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) in the presence of O_2 and its derivatives (Dehne et al., 2004; Ioannidis et al., 1998; Kirsch and de Groot, 2002; Kirsch et al., 2002; Patel et al., 1999; Petrat et al., 2005; Swintek et al., 2004; Wink and Mitchell, 1998). RNOS have also been shown to inhibit mitochondrial respiration by irreversible inhibition of complex I or II or aconitase (Boyd and Cadenas, 2002; Brown and Borutaite, 2002; Henry and Guissani, 1999), to lead to Ca^{2+}-dependent (or independent) damage of the plasma membrane (Moss and Bates, 2001) and its transporters (Gutierrez-Martin et al., 2005, 2004) and to further promote oxidative stress (Halliwell and Gutteridge, 1999; Ridnour et al., 2004) via consumption of cellular antioxidants like glutathione (Walker et al., 2001) and by inhibition of catalase (Lawler and Song, 2002).

Previously, we demonstrated that at high oxygen partial pressures (pO_2), high concentrations of NO induce cell death in rat liver endothelial cells via the formation of RNOS, whereas at low albeit physiological O_2 concentration and in the absence of glycolytic energy production (i.e. in the absence of glucose)

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pathophysiologically relevant NO concentrations induce hypoxia-like cell death (Dehne et al., 2004; Ioannidis et al., 1998). Compared to liver endothelial cells, hepatocytes have a much higher anti-oxidative capacity and metabolic turnover and their energy metabolism depends almost exclusively on oxidative phosphorylation which cannot be sufficiently compensated by glycolytic energy production (Brecht et al., 1992; Carini et al., 1997; Kawanishi et al., 1991). Therefore, the mechanisms of NO toxicity to hepatocytes and liver endothelial cells should be substantially different. In the present work, our aim was to find out whether NO induces injury to primary cultured rat hepatocytes predominantly via cytochrome oxidase inhibition or RNOS-mediated oxidative stress.

Materials and methods

Materials

SpermineNONOate (SpNO) was purchased from Situs (Düsseldorf, Germany), glycine from Merck (Darmstadt, Germany), gentamicin from Gibco (Eggenstein, Germany) and Leibovitz L-15 medium from Invitrogen (Karlsruhe, Germany). Nigericin, pluronic F-127, the acetoxymethyl esters of sodium-binding benzofuran isophthalate (SBFI-AM), quin-2 (quin-2-AM), coroNa green (coroNa green-AM) and fura red (fura red-AM) were obtained from Molecular Probes (Eugene, OR, USA), fetal calf serum, ascorbic acid, choline chloride, choline-free base, choline bicarbonate salt, gramicidin, lactobionic acid, monensin, probenecid, bisbenzimide (H33342) and propidium iodide from Sigma (Deisenhofen, Germany) and collagen (Type R), bovine serum albumin, dexamethasone, collagenase Hep Plus and spermine from Serva (Heidelberg, Germany). Falcon cell culture flasks and Falcon 6-well cell culture plates were from Becton Dickinson Labware (Le Pont De Claix, France), glass coverslips from Menzel (Braunschweig, Germany) and gas mixtures from Messer Griesheim (Krefeld, Germany).

Animals

Male Wistar rats (200–280 g) were obtained from the Zentrales Tierlaboratorium (Universitätsklinikum Essen, Germany). Animals were kept under standard conditions with free access to food and water. All animals received humane care in compliance with the institutional guidelines.

Isolation and culture of hepatocytes

Hepatocytes were isolated and cultured as described previously (de Groot and Brecht, 1991).

Experimental procedures

Experiments were started 20–24 h after isolation of the cells. At the beginning of the experiments, cells were washed three times with Hanks' balanced salt solution (HBSS; 137.0 mM NaCl, 5.4 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM KH_2PO_4, 0.4 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 25.0 mM HEPES, pH 7.4) and then covered with Krebs-Henseleit buffer (KH; 115 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 20 mM HEPES, pH 7.4) or a modification of the buffer without sodium (substituted by choline) at 37 °C. Normoxic incubations were performed in a humidified atmosphere of 95% air and 5% CO_2. Hyperoxic conditions were established by saturating the incubation solution with 95% O_2/5% CO_2, conditions with low O_2 concentrations by saturating the incubation solution with 2% or 5% O_2, 5% CO_2, and 93% and 90% N_2 respectively, before adding it to the cells, followed by gentle flushing of the culture flasks with the respective gas mixtures through cannulae piercing the rubber stoppers of the flasks, as described previously (Brecht and de Groot, 1994). The flasks were again flushed with the respective gas mixtures each time a sample was taken. The experiments were started by the addition of 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM, or 2.0 mM SpNO, from fresh stock solutions prepared with 10 mM NaOH. In some experiments, the effects of fructose (10 mM), ascorbic acid (100 μM, 1 h pre-incubation in cell culture medium), glycine (10 mM), the acetoxymethyl ester of quin-2 (quin-2-AM, 10 μM, 1 h pre-incubation in KH) or spermine (0.5, 1 and 2 mM) were studied; solvent controls were included.

For fluorescence microscopy, coverslips with cells were placed in air-tight incubation chambers that were gassed using copper tubing and rubber stoppers equipped with cannulae (Dehne et al., 2004; Frank et al., 2000; Fuckert et al., 2000). The incubation conditions were the same as described above.

Determination of the cytosolic Na^+ concentration

The cytosolic Na^+ concentration was measured by digital fluorescence microscopy using the sodium-sensitive fluorescent dyes SBFI (Frank et al., 2000; Fuckert et al., 2000) and coroNa green. SBFI was loaded into the cells and fluorescence measured on an inverted microscope as described previously (Frank et al., 2000; Fuckert et al., 2000). Loading with coroNa green was performed by incubating the hepatocytes for 60 min in KH buffer (37 °C) containing 10 μM coroNa green-AM followed by incubation for 10 min in a dye-free KH buffer. Intracellular coroNa green was excited at 488±10 nm; emission was monitored at 520±20 nm at 60- or 120-second intervals. In situ calibration of the Na^+ concentration was performed by the method of Kawanishi et al. (1991) with minor modifications (Frank et al., 2000; Fuckert et al., 2000).

Assessment of cytosolic calcium levels

Cytosolic calcium was assessed by ratio imaging of the fluorescence of the calcium-sensitive fluorescent dye fura red using digital fluorescence microscopy ($\lambda_{exc.}$=405±5 nm and 488± 10 nm, $\lambda_{em.} \geq 590$ nm). For loading with fura red, cells were incubated with fura red-AM (5 μM in KH buffer for 60 min at 37 °C, then 15 min incubation in dye-free buffer); retention of the dye was improved by the addition of probenecid (1.2 mM).

Cytosolic sodium and calcium levels were evaluated on the single cell level until the intracellular indicator fluorescence