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Protection against cell damage due to hypoxia and reoxygenation: the role of taurine and the involved mechanisms

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Summary. The effect of taurine on cell viability and metabolism of human colon and porcine renal cells was investigated during and after hypoxia. Taurine administered during hypoxia markedly reduced cellular deterioration due to hypoxia and reoxygenation and led to a significantly greater recovery of cellular function following the hypoxic insult. The responsible mechanisms for the beneficial effects were an improvement in osmotic status and calcium homeostasis and an induction in cellular growth despite oxygen deficiency and reoxygenation. Free oxygen radical generation and lipid membrane peroxidation were not reduced by taurine. Taurine acted as a potent endogenous agent with multifactorial effects against cellular damage due to hypoxia and reoxygenation.

Keywords: Amino acids – Taurine – Hypoxia – Reoxygenation – Free radicals – cell growth – calcium metabolism – monolayer cell culture – LLC PK 1 – HT 29

Introduction

Since the discovery of taurine in 1827 by Tiedemann and Gmelin as a constituent of ox bile, this sulfur containing amino acid has been recognized as a biologically important substance, which exerts multiple actions on cerebral, cardiovascular, pulmonary, neuronal, hepatic and renal tissues (Huxtable, 1992; Michalk et al., 1985; Satoh, 1994; Schuller-Levis et al., 1994). Taurine, 2-aminoethanesulfonic acid, is involved in the regulation of calcium homeostasis and osmotic balance, promotes the energy metabolism of the heart and acts as a protector against the so called "calcium paradox" of the heart. These functions of taurine suggest that it might be an endogenous protective agent against hypoxic and reoxygenation cellular deterioration (Minor et al., 1994; Nakamori et al., 1990; Nakashima et al., 1990; Schaffer et al., 1994; Trachtman, 1991 and 1992; Wingenfeld et al., 1994, 1995).

The aim of this study was to investigate whether taurine is able to protect human colon and porcine renal tubular cell cultures against the cellular deterioration due to hypoxia and reoxygenation and to elucidate the mechanisms underlying this action.

Methods

Cell cultures

Proximal porcine renal tubular cells from the cell strain LLC PK 1 (ICN Biomedical, Meckenheim FRG) and human colon cells of the cell strain HT 29 (American type culture collection, Rockville Maryland, USA) were cultured as monolayers either for 48 hours in M 199 medium (LLC PK 1) or for 72 hours in Mc Coy 5, a medium (HT 29) with an admixture of fetal calf serum (100 ml serum/l, serum diluted 1:4), penicillin (100 I.U./ml), streptomycin (100 μg/ml) and amphotericin B (250 μg/l). The cultures were incubated with 21% oxygen and 5% carbon dioxide. The temperature was kept constant at 37 C and the oxygen partial pressure was about 140 mmHg. The cultures were fed three to four times a week. After an aerobic incubation, each renal cell monolayer contained between 1 and 2.5 million cells. Intestinal cell cultures contained between 4–5.5. million cells.

Hypoxia and reoxygenation

After a period of exposure to aerobic culture medium, the cells were transferred to either 5 ml Euro Collins solution (Glucose 39.27 g/l, KH_2PO_4 2.092 g/l, $KHPO_4$ – 7.548 g/l, KLC 1.14 g/l, $NaHCO_3$ 0.86 g/l) or HKT solution (Custodiol^R, Köhler Chemie, Ansbach FRG, NaCl 15 mmol/l, KCL 9 mmol/l, MgCl· $6H_2$ 4 mmol/l, $CaCl_2$ 0.015 mmol/l, potassium 2-ketoglutaric acid 1 mmol/l, histidine ·HCL·H_20 198 mmol/l, tryptophan 2 mmol/l, mannitol 30 mmol/l). The preservation solution was rendered hypoxic by equilibration with 95% nitrogen and 5% carbon dioxide, resulting in an oxygen partial pressures below 8 mmHg. The culture flasks were plugged with a seal screw cap, described previously (Wingenfeld et al., 1995), which allowed a long term gas flow with oxygen partial pressures less than 20 mmHg in the supernatant of the culture in order to simulate moderate or severe hypoxia. With a constant low gas flow (0.5 l/min) and defined supernatant volume, the flasks were placed in a water bath (4 C) simulating cold ischemia. After 5 hours (EC) or 20 hours (HKT) the hypoxic preservation medium was removed and replaced by a crystalloid perfusion solution (Hank's balanced salt solution without calcium, glucose 600 ml/l, NaCl 80 g/l, KCl 4 g/l, KH_2PO_4 10 g/l, $Na_{2H}HPO_4$ 475 mg/l, Sigma chemicals, St. Louis, USA). The cell cultures were incubated aerobically again for 30 minutes at a temperature of 37 C and an oxygen partial pressure of 140 mmHg. During hypoxic storage, taurine (Sigma chemicals, USA) was added to the preservation solution yielding a final concentration of 0.1, 0.5, 1, 10 or 20 mmol/l. Cultures preserved hypoxically without taurine addition served as controls. The reoxygenation buffer solution remained taurine free. Fig. 1 shows the experimental design.

Measurement of cellular integrity and energy metabolism

Before each hypoxic preservation and reoxygenation experiment, 2–3 cultures were rinsed from the monolayer surface using trypsin and the cells were then counted in a Neubauer chamber to determine cell number. Viability rates were determined using the trypan blue exclusion test (Jauregul et al., 1981). After centrifugation and resuspension the percentage of remaining viable cells was determined by cell counting and the trypan blue exclusion test. As a measure of the metabolic status of cultured cells after

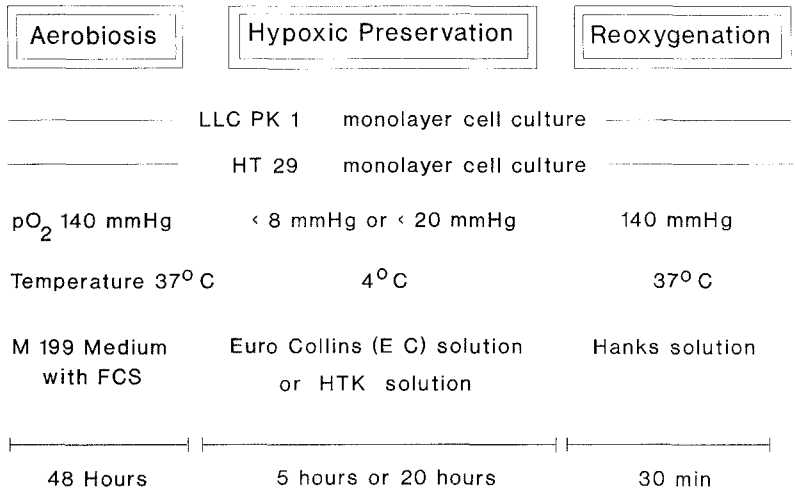

Fig. 1. Experimental design

reoxygenation, the cells were treated with perchloric acid and neutralized extracts were assayed by means of high pressure liquid chromatography (HPLC). Before denaturation of the proteins with perchloric acid (ice bath, 0.25 ml 1M $HC10_4$), the protein content of each culture was measured using the method described by Bradford (1976). After hypoxia and reoxygenation, 1 ml of the supernatant of the cultures was removed for the determination of lactate dehydrogenase (LDH, LDH Monotest, Boehringer Mannheim, Germany).

Measurement of cell volume

For the determination of the mean volume of the cells (LLC-PK1) a blood cell counter with computered volume analysis was used (Cobas Minos STX, Hoffmann La Roche, Switzerland). The data represent the mean cellular volume of 1.2 to 2.5 $\times$ 10^6 cells.

Measurements of cellular calcium homeostasis

Total intracellular calcium content was measured by modified flame photometry (EFOX 5053), Eppendorf, Germany). To eliminate a contribution from extracellular calcium and to prevent contamination of the extracts, all solutions were pretreated with Tritriplex VI (EGTA, Merck, Germany). Furthermore cell cultures were concentrated by centrifugation and cellular membranes were disintegrated by ultrasonic treatment. Calcium content was obtained from 2.5 to 7 $\times$ 10^6 cells.

Assessment of cellular growth and protein synthesis

Cellular growth and DNA synthesis was determined by the use of the 5-bromo-2-deoxyuridine test (5-BrdU Labelling and Detection Kit III, Boehringer Diagnostics, Mannheim Germany), a nonradioactive alternative for the [^{3}H] thymidine incorporation assay. 5-BrdU, a pyrimidine analogue, is incorporated in place of thymidine into the DNA of proliferating cells. Cell cultures were incubated with 5-BrdU during 7 hours of hypoxia in

the presence or absence of 10 mmol taurine/l preservation solution. The reoxygenation period in this experimental group lasted 1 hour. The reoxygenation buffer solution contained no taurine.

Measurement of malondialdehyde production as a parameter of oxidative cell injury

Oxygen free radical induced cellular deterioration was approximated by HPLC separation and the measurement of malondialdehyde-thiobarbituric acid adducts according to the method of Wong et al. (1987).

Statistics

Analyses of 5 to 8 cell cultures per group were used for the determination of each parameter. Statistical analyses were calculated parametrically by Students t-test in comparison to the controls with different levels of significance (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$).

Results

Protective effects of taurine

Addition of taurine to the culture medium prevented the loss of cell viability following 5 hours of hypoxia (Table 1). Taurine treatment also led to a significant reduction in enzyme leakage during hypoxia and a marked improvement in ATP content following reoxygenation. Similar results were obtained with longer periods of hypoxia, as well as different human cell culture lines (intestinal cells) and a different preservation solution (HTK). In addition, cell viability after combined hypoxia and reoxygenation were significantly increased when taurine was added to the preservation fluid during hypoxia. Comparable to the experiments with LLC PK 1 monolayer cultures, enzyme leakage during hypoxia was significantly reduced (Table 2). Thus taurine administered during hypoxia protected the cells against hypoxia cell damage and improved posthypoxic recovery.

Mechanisms of protection

Osmoregulation

In comparison to the aerobic controls, cellular volume increased during the period of oxygen deficiency. Addition of taurine to the medium evoked a dose dependent reduction in cell swelling. Surprisingly, taurine at concentrations as low as 0.5 mmol/l reduced mean cellular volume. Higher concentrations of taurine caused a more marked reduction in the extent of osmotic disruption (Fig. 2). Taurine supplementation approaching the concentration of 10 mmol/l completely prevented hypoxic-induced osmoregulatory deterioration. A similar effect was obtained by the addition of a supraphysiological concentration of 10 mmol/l glucose.

Table 1. Viability rates, leakage of lactate dehydrogenase (LDH) and energy metabolism of porcine kidney cell cultures after aerobic incubation, 5 hours hypoxic preservation (pO_2 < 8 mmHg) with Euro Collins solution and 30 min reoxygenation, with different taurine concentration in preservation medium

Medium taurine (mmol/l)	Cell viability (%)	LDH Leakage (U/l)
	Hypoxia	
0	61 ± 5	24 ± 8.6
0.1	62 ± 5	26.9 ± 3.1
0.5	76 ± 7[a]	11.1 ± 1.0[b]
1	65 ± 2	13.5 ± 2.1[b]
20	81 ± 3[c]	11.6 ± 3.1[b]
Taurine (mmol/l)	**Viability (%)**	**ATP content (nmol/mg protein)**
	Hypoxia and reogygenation	
0	63 ± 3	38 ± 17
0.1	95 ± 1[c]	115 ± 24[c]
0.5	93 ± 2[c]	34 ± 7
1	96 ± 1[c]	57 ± 9[a]
20	93 ± 3[c]	65 ± 12[a]

n = 5–8 cultures per experiment, mean values ± SD; Students t-test.
vs. untreated group: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Table 2. Viability and LDH-leakage of human intestinal (Ht 29) cell cultures after 20 hours of cold hypoxia and 30 min. of reoxygenation in HKT preservation solutions with different taurine concentration

Medium taurine (mmol/l)	Cell viability (%)	LDH leakage (U/l)
0	55 ± 12	11.4 ± 2.9
0.5	66 ± 6	19.2 ± 6.5[a]
1	75 ± 8[a]	6.0 ± 2.4
5	78 ± 5[b]	3.8 ± 0.6
10	82 ± 2[c]	5.3 ± 0.8[c]
20	69 ± 7	9.5 ± 2.1

n = 5–8 cultures per experiment, mean ± SD; Students t-test.
cs. untreated group: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Calcium metabolism

During hypoxia with a calcium free solution, total intracellular calcium content declined to levels 25% of the aerobic controls. During reoxygenation using medium containing calcium, a massive increase in cellular calcium

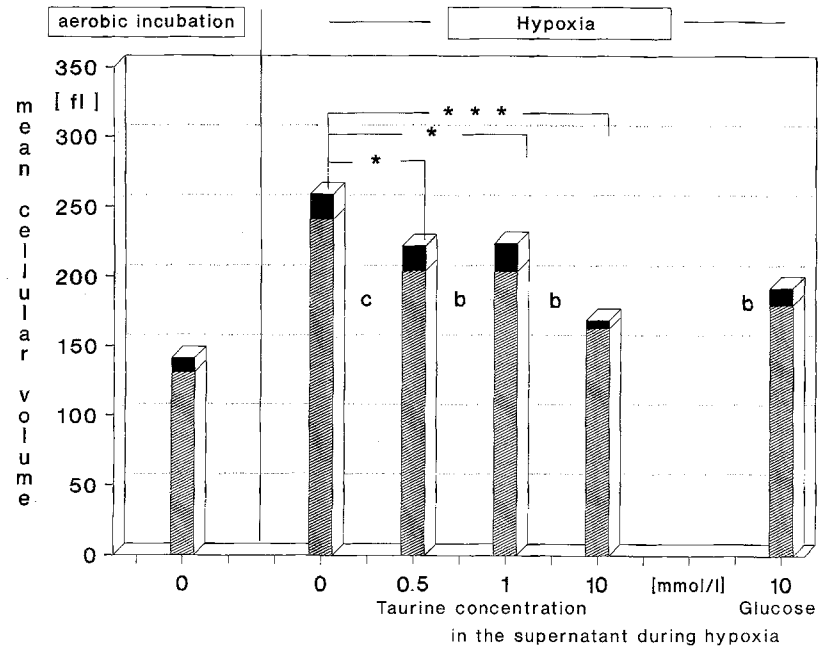

Fig. 2. Mean cellular volume after 5 hours of hypoxia of LLC PK 1 cell cultures in Euro Collins solution. (Student's t-test vs. aerobic control: *b* $p < 0.01$, *c* $p < 0.001$; vs. treated group: *$p < 0.05$, ***$p < 0.001$; solid bars represent standard deviation

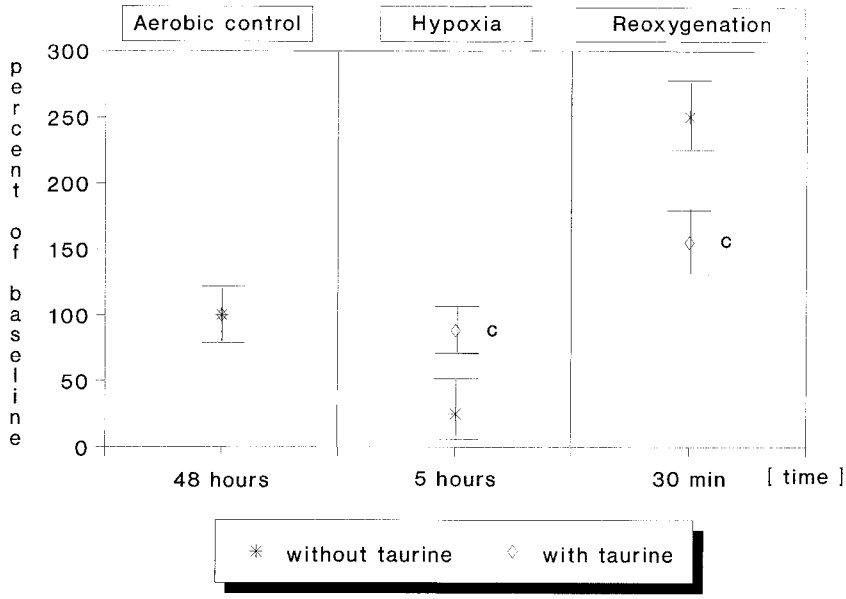

Fig. 3. Effect of taurine supplementation (0.5 mmol/l) during hypoxia on LLC-PKI cellular calcium content after hypoxia and reoxygenation. Data are presented as percentage of the calcium content of the aerobic control level (1.2×10^{-7}M)

content occurred (calcium overload). Taurine [0.5 mmol/] reduced the loss of calcium during hypoxia and prevented calcium overload during reoxygenation (Fig. 3).

Protein synthesis and cellular growth

During hypoxia and reoxygenation significantly higher rates of BrdU uptake and incorporation into cellular DNA occurred when taurine was added during the period of oxygen deficiency (Fig. 4).

Oxidative cellular injury

Surprisingly, in the presence of taurine the levels of MDA were significantly higher after reoxygenation, indicating elevated oxidation of cellular membranes occurs, presumably due to oxygen derived free radicals (Fig. 5).

Discussion

In the present study, the administration of taurine during the hypoxic period markedly reduced cell damage resulting from both oxygen deficiency and reoxygenation. Although taurine was only added during the hypoxic period, the effect extended into the reoxygenation period leading to a significant improvement in cellular recovery. Taurine was effective during short bouts, as well as long bouts of hypoxia.

Potential mechanisms underlying taurine induced protection of cellular integrity:

One factor influencing recovery from an ischemic-reperfusion insult is improved osmoregulation. Taurine was found to reduce cellular swelling during hypoxia and reoxygenation in a dose-dependent manner. At lower concentrations, taurine could either exert a direct osmoregulatory effect by modulation of the activity of sodium or chloride transporters (Satoh, 1994; Schaffer et al., 1994; Windhager et al., 1996). Furthermore, other transmembraneous electrolyte transporting systems, such as the sodium/potassium or sodium/H^+ transporters could have been influenced by taurine. Higher concentration of taurine were presumed to reduce the osmotic swelling of the cells more directly by increasing osmolality of the culture or preservation medium (Huxtable, 1992; Trachtman, 1991, 1992).

In the absence of extracellular calcium, taurine markedly reduced cellular calcium efflux during hypoxia and impeded calcium influx during reoxygenation. Although it is widely accepted that taurine acts as a modulator of transmembraneous calcium currents (Schaffer et al., 1994), it still remains to be determined whether disturbances in the cellular membrane resulting in increased transmembraneous calcium flux or whether altered calcium transporter activity is responsible for taurine's modulations of calcium flux during hypoxia (Cheung et al., 1986; Greene and Paller, 1994; Kribben et al., 1994; Piper, 1989; Weinberg et al., 1990). Also unclear is the localization (cytosolic,

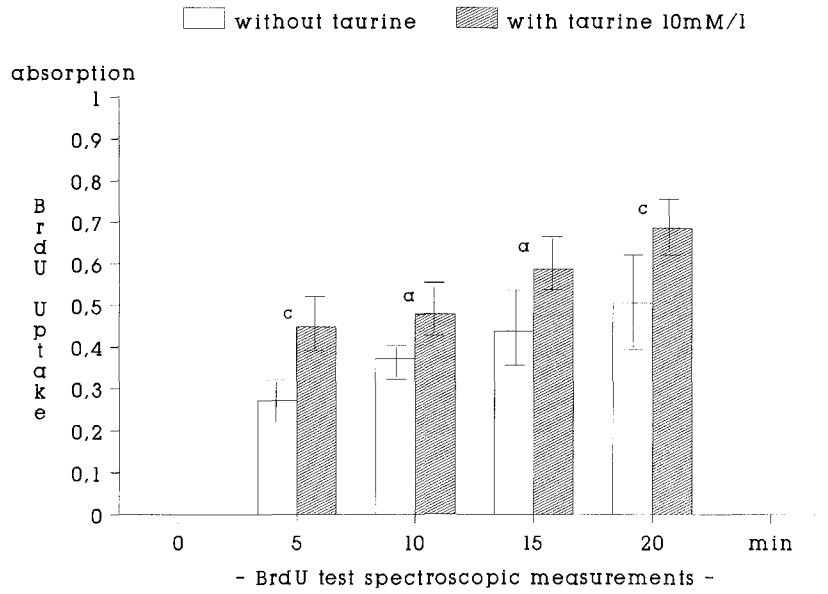

Fig. 4. Effect of taurine (10 mmol/l) addition to hypoxic medium on LLC PKI cellular BrdU uptake during 7 hours of hypoxia and 1 hour of reoxygenation. (Student's t-test vs. control group: *a* $p < 0.05$, *c* $p < 0.001$; present data as mean values ± standard deviation)

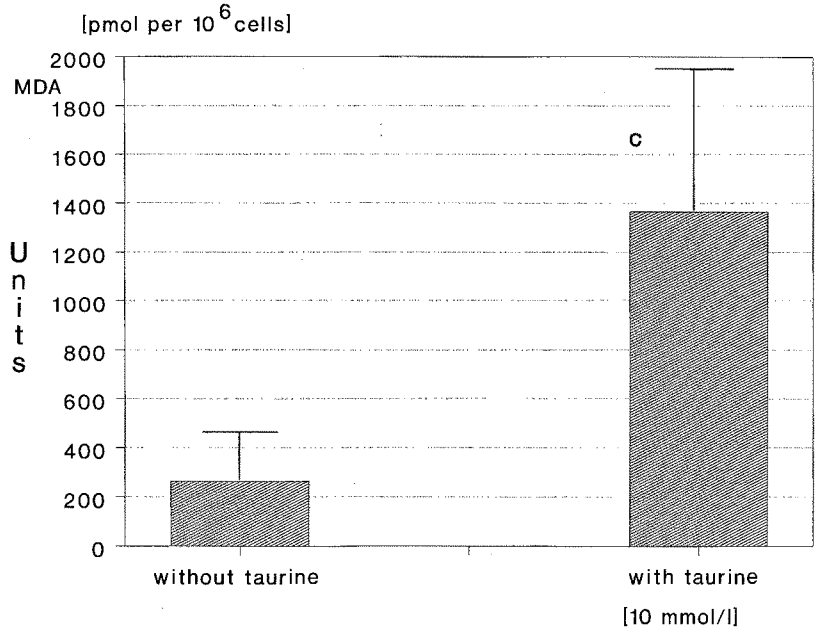

Fig. 5. Production of malondialdehyde (*MDA*) – thiobarbituric acid reactive substances in LLC PK 1 monolayer cell cultures following 5 hours of hypoxia and 30 min of reoxygenation. (Student's t-test vs. control: *c* $p < 0.001$; present data as mean + standard deviation)

mitochondrial, membrane-associated or nuclear) of the excess calcium (Silverman, 1993). Yet, the cytoprotective effect of taurine in the cell culture system seemed to be only partly related to its action on calcium transport since the effect was only detectable in the presence of a physiological calcium concentration.

One of the primary cellular protective mechanisms of taurine appeared to be acceleration of cellular growth. The analyses of BrdU uptake during hypoxia and reoxygenation indicated that taurine stimulated protein synthesis and cellular growth. It is not yet evident if taurine administered during hypoxia, improved cell generation by itself or if different mediators, e.g. cAMP, cGMP or IGF, are involved. The data suggest a role of taurine as a factor for cell growth.

In contrast to these protective actions, taurine exerted a deleterious effect on the cellular membrane, since MDA content was elevated after hypoxia and reoxygenation. But it should be kept in mind that cellular growth was significantly accelerated and that apparently more cells were probably affected by oxidative stress and more membranes were influenced by free oxygen derived radicals. Nevertheless this adverse effect of taurine was counteracted by the other cytoprotective mechanisms.

In conclusion the phylogenetically ancient amino acid, taurine, seems to be a potent physiological protective agent responsible for cellular homeostasis or enantiostasis during and after oxygen deficiency.

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