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Hypoxic responses of Na^+/K^+ ATPase in trout hepatocytes

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Summary

Reduction in oxygenation induces inhibition of Na^+/K^+ ATPase in a number of cells and tissues, including hepatocytes. When not reversed, decrease in Na^+/K^+ pump activity leads to a gradual Na^+ accumulation, cell swelling and death. However, when accompanied by suppression of dissipative cation pathways, it has also been shown to be a beneficial adaptive strategy used by some hypoxia-tolerant species to reduce ATP consumption during prolonged periods of anoxia. This study aims to investigate acute hypoxic responses of the Na^+/K^+ ATPase in primary cultures of trout hepatocytes. Gradual decrease in oxygenation was followed by an instantaneous transient dose-dependent downregulation of the Na^+/K^+ ATPase transport activity, but was without an effect on hydrolytic function of the enzyme. Hypoxia-induced inhibition of active K^+ influx was reversed spontaneously when hypoxic incubation time exceeded 20 min. The stimulating effect of prolonged hypoxic exposure on the Na^+/K^+ pump is most probably secondary to hypoxia-induced activation of the Na^+/H^+ exchanger with the following Na^+ accumulation leading to Na^+/K^+ ATPase activation. Hypoxia-induced inhibition of the Na^+/K^+ pump was not caused by ATP depletion or global oxidative stress. However, local controlled production of reactive oxygen species seems to play an important role in hypoxia-induced regulation of the Na^+/K^+ ATPase. Treatment of cells with mercaptopropionyl glycine (MPG), a scavenger of OH^-, abolished hypoxia-induced inhibition of the Na^+/K^+ ATPase. Earlier on we have shown that activation of Na^+/H^+ exchanger under hypoxic conditions can be opposed by MPG treatment as well. Taken together our results suggest that regulation of both oxygen-sensitive transporters may be accomplished by local changes in free radical production.

Key words: Na^+-K^+ ATPase, hypoxia, redox state, hepatocytes.

Introduction

The liver cells of different species show a decrease in Na^+/K^+ pump activity in response to deoxygenation. In mammals, hypoxia-induced suppression of Na^+/K^+ pump occurs together with increased passive Na^+ uptake caused by hypoxia-induced stimulation of Na^+/H^+ and later also Na^+/Ca^{2+} exchanger. Together, these responses lead to Na^+ load, cell swelling and necrotic cell death in hypoxia (Carini et al., 2000). In some hypoxia-tolerant species, downregulation of Na^+/K^+ ATPase occurs in parallel with a decrease in passive cation permeability, which is often called 'channel arrest' that contributes to a reduction of ATP consumption. The latter is a key factor helping animals to survive prolonged anoxic periods (Buck and Hochachka, 1993; Hochachka et al., 1997). Notably, however, in hypoxia-tolerant species that remain active during anoxic periods, as crucian carp, the activity of Na^+/K^+ pump does not change substantially in response to deoxygenation nor does the Na^+ and K^+ permeability of excitable tissues change, suggesting that global channel arrest does not occur (Nilsson, 2001).

Although the activity of Na^+/K^+ pump is oxygen sensitive in some species, the mechanisms by which oxygen influences the pump function remain obscure (e.g. Buck and Hochachka, 1993; Angermuller et al., 1995; Krumschnabel et al., 2000b; Lifshitz et al., 1986; Lutz and Nilsson, 1997; Bogdanova et al., 2003a,b; Clausen, 2003; Dada et al., 2003). Hypoxia-induced reduction or cessation of Na^+/K^+ pump activity in rat liver tissue is fast and can be rapidly reversed upon reoxygenation (e.g. Angermuller et al., 1995). As follows from the data we have obtained on trout hepatocytes and mouse erythrocytes, hypoxia-induced inhibition of the pump is independent of the changes in cellular ATP levels (Bogdanova et al., 2003a,b). In addition, hypoxic response of the Na^+/K^+ pump in fish hepatocytes does not require the presence of adenosine, although its release into the circulation in response to hypoxic insult may somewhat decrease the activity of the pump in vivo (Krumschnabel et al., 2000a). However, reduction in oxygen tension may have a pronounced effect on cellular redox state. Acute *in vivo* and *in vitro* hypoxic exposure results in shift in redox balance that is especially pronounced in erythrocytes and myocytes because of high levels of reactive oxygen species (ROS) production in these cells under normoxic conditions (Hermes-Lima and Zenteno-Savin, 2002; Di Meo and Venditti,

2001; Bogdanova et al., 2003b) (A. Bogdanova and O. Ogunshola, unpublished data on *in vivo* hypoxic effects on GSH levels in murine red blood cells). The following increase in cellular reduced glutathione (GSH) levels is the cause of hypoxia-induced suppression of the Na^+/K^+ pump function in these cells (Hermes-Lima and Zenteno-Savin, 2002; Bogdanova et al., 2003b). Suppression of free radical production by NAD(P)H oxidases under hypoxic conditions has also been shown in some cell types (e.g. Porwol et al., 2001; Acker and Acker, 2004). Alternatively, under conditions of severe hypoxia, gradual reduction of mitochondrial electron transduction chain components may result in an uncontrolled formation of ROS with consequent shifts in cellular redox potential (e.g. Rifkind, 1993; Chandel and Schumacker, 2000). In particular, slow build-up in ROS production can be observed after a 1 h incubation of trout hepatocytes at 1% O_2 (Bogdanova et al., 2003a). Recent studies reveal that both upregulation of mitochondrial ROS and suppression of ROS generation by NADPH oxidases in response to hypoxia may occur simultaneously both being important for hypoxic signaling (Aley et al., 2005).

Hypoxic response of the Na^+/K^+ pump in mouse red blood cells resembles that observed in other cell types, including liver. However, the presence of high concentrations of heme iron functioning as a catalyst of the Fenton reaction (in which the most active ROS, hydroxyl radicals, are produced) and the absence of mitochondria in erythrocytes does not allow any direct comparisons between the red blood cells and other cell types. Therefore, in this study we have focused on the mechanisms of the oxygen sensitivity of the Na^+/K^+ pump, using primary cultures of rainbow trout hepatocytes. Earlier, we have shown that active K^+ influx into these cells decreases substantially in response to a 15 min exposure to a medium equilibrated with 1% oxygen (Bogdanova et al., 2003a). This observation indicated oxygen sensitivity of the Na^+/K^+ pump in trout liver cells. Notably, most studies using liver cells have compared anoxia to 'normoxia' corresponding to 21% O_2 (Krumschnabel et al., 2000b). For primary cultures of liver cells these conditions are non-physiological. Oxygen concentration measured under normoxic conditions *in vivo* in mammalian liver tissue ranges between 6 and 3 kPa, and short-term anoxia does not result in complete tissue deoxygenation (e.g. Brooks et al., 2004; El Desoky et al., 1999). We have monitored transport and hydrolytic activities of the Na^+/K^+ ATPase as a function of partial O_2 pressure (P_{O_2}) in the incubation medium in the range of 21–0.5 kPa. Kinetics of hypoxic response were monitored and the obtained data related to the changes in cellular ATP levels, ROS formation and redox state. Responses of the pump to changes in oxygenation were also studied in cells with manipulated redox state.

Materials and methods

Preparation of hepatocytes primary cultures

Rainbow trout *Oncorhynchus mykiss* (Walbaum) were obtained from the Finnish Institute for Fisheries and Environment (Parainen, Finland) and maintained at 13°C in the aquarium facilities of the Department of Biology of the University of Turku. The isolation of hepatocytes was carried out according to Rabergh (1995). In short, the fish were stunned with a blow on the head, opened and the portal vein cannulated in the direction of the liver. The liver was perfused free of blood using a Ca^{2+}-free buffer (137 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 8.06 mmol l^{-1} Na_2HPO_4, 1.47 mmol l^{-1} KH_2PO_4, 0.5 mmol l^{-1} EGTA and 25 mmol l^{-1} tricine, pH 7.6). Thereafter, the perfusion was continued with a collagenase-containing buffer (137 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 8.06 mmol l^{-1} Na_2HPO_4, 1.47 mmol l^{-1} KH_2PO_4, 50 mmol l^{-1} Hepes, 0.5 mmol l^{-1} $CaCl_2$ and 0.05% collagenase, pH 7.4) until the liver looked swollen and soft. It was then excised and placed in a sterile Petri dish. The gall bladder was removed and liver cells dispersed using a steel comb. The cells were filtered through a 75 μm nylon mesh and washed three times in cold Leibowitz's L-15 medium containing penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) (cells for microfluorometric imaging of ROS production) or with non-sterile incubation medium containing 133 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 3 mmol l^{-1} Na_2HPO_4, 10 mmol l^{-1} Hepes, 1.6 mmol l^{-1} $CaCl_2$, 0.9 mmol l^{-1} $MgSO_4$ and 20 mmol l^{-1} glucose, pH 7.6 (cells for other studies). After the washes, the viability of the cells was checked using 0.4% Trypan Blue and cells used for experiments only when it exceeded 85%. Cell suspensions were left 1–1.5 h in the incubation solution at 4°C for restitution before using them. For microfluorometric imaging, cells were cultured for 1–3 days in Leibowitz L-15 medium at 18°C on 2.4×4.0 cm coverslips coated with poly-L-lysine.

Manipulation of the cellular redox state

To shift cytosolic redox state to more oxidized, cells were treated with a conjugating agent chloro-dinitrobenzene (CDNB). CDNB penetrates both plasma and mitochondrial membranes, and when in the cells selectively interacts with GSH in a reaction catalyzed by glutathione S-transferase, stable adduct 2,4-dinitrophenyl-s-glutathione is formed that is actively transported out of the cell (Awasthi et al., 1981; Scott et al., 1990). Depending on the concentration of CDNB (0.1 to 3.0 mmol l^{-1}), slight or almost complete GSH depletion can be achieved within minutes of incubation with conjugating agent (Han et al., 2003; Lauf et al., 1995; Bogdanova et al., 2003b). Treatment of cells with permeable thiols such as permeable diethyl ester of GSH (et-GSH) or *N*-acetyl cysteine (NAC) shifts the redox balance in the cells towards reduced. These compounds are usually used at concentrations comparable with intracellular GSH concentration (1–10 mmol l^{-1}) to achieve significant shift from the physiological reduced thiol levels. Finally, exposure of cells to virtually impermeable GSH may be used to study the effects of extracellular reductants on ion transport and other cellular functions.

Evaluation of the changes in cellular ROS production

To start the experiments, a chamber (fluid volume ~200 μl) was attached to the cover slip, which served as bottom for the

chamber, and the cells were loaded in darkness with 20 μmol l^{-1} 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) for 20 min at room temperature. The experimental chamber was attached to the perfusion system (Ismatec Reglo perfusion pump, Glattbrugg, Switzerland; 10 ml min^{-1}; fluid volume of the experimental chamber was 200 μl, therefore a 95% change of the perfusion fluid was achieved within 5 s) on an inverted microscope (Nikon Diaphot 200; 20× fluorescence objective). Fluorescence intensity measurements were made using Photon Technology International Imagescan setup (Lawrenceville, NJ, USA) with Deltascan monochromator unit and IC-100 CCD video camera. For H_2-DCFDA the excitation and emission wavelengths of 502 and 530 nm respectively were used. Recordings were started 3 min after the onset of perfusion with a standard incubation saline containing (in mmol l^{-1}) 133 NaCl, 5 KCl, 3 Na_2HPO_4, 10 Hepes, 1.6 $CaCl_2$, 0.9 $MgSO_4$ and 10 glucose, pH 7.6 at room temperature (20°C). The medium was not recirculating. Within the first 20 min of perfusion, the intensity of fluorescent signal increased linearly with time but reached a steady-state plateau after 30 min of perfusion. This initial linear period could be used for observations of acute changes in redox state, with changes in the slope of the linear curves corresponding to a decrease or increase in oxidation rate. To avoid artifacts caused by increased leakage of the oxidized dye, viability of cells was controlled using Trypan Blue staining. Increase in fluorescent intensity monitored over the first 10 min of perfusion was taken as 'control', thereafter perfusion was continued for another 10 min either with air-equilibrated incubation saline containing 1 mmol l^{-1} CDNB or with the standard incubation saline pre-equilibrated with gas mixture containing 1% O_2 and 99% N_2. Photon Technology International Image Master software was used to analyze the data obtained from the changes in fluorescent intensity for single cells. Data from 35 to 56 cells were pooled and changes in the rate of oxidation of the fluorescent dye were detected as changes in the slope of the curves during the linear period (see Fig. 1).

Evaluation of hydrolytic and transport activity of Na^+/K^+ ATPase

Hydrolytic activity of the Na^+/K^+ pump at optimal substrate and ligand concentration was quantified as ouabain-sensitive inorganic phosphate production in cell homogenates. Intact cells were incubated at room temperature in the standard incubation saline with or without 5 mmol l^{-1} GSH, or NAC, or 1 mmol l^{-1} CDNB for 15 min. Incubation at different P_{O_2} values was performed in the Cameron (Port Aransas, TX, USA) DEQ-1 tonometers equilibrated with gas mixtures of fixed oxygen concentrations generated from air and N_2 by the Cameron gas mixing flowmeter. Thereafter, cells were destroyed by repeated freeze-thaw cycles and cell homogenates or microsomes were incubated with or without 1 mmol l^{-1} ouabain in media containing (in mmol l^{-1}) 130 NaCl, 20 KCl, and 3 $MgCl_2$ for 10 min. After binding of inhibitor was complete, ATP hydrolysis was measured in the presence of 3 mmol l^{-1} ATP as the rate of production of inorganic phosphate. The amount of inorganic phosphate was determined using the method of Rathbun and Betlach (1969).

Transport activity of the pump was evaluated in intact cells using ^{86}Rb as a radioactive tracer for K^+. To measure unidirectional K^+ (^{86}Rb) influx hepatocytes were suspended in the standard incubation saline. Cells were preincubated for 15 min with or without 5 mmol l^{-1} GSH, *N*-acetyl cysteine (NAC), MPG or 1 mmol l^{-1} CDNB. To distinguish between active, Na^+/K^+ pump-mediated, and passive K^+ influx, a selective inhibitor of the Na^+/K^+ pump (ouabain) at the concentration of 100 μmol l^{-1} was added to a half of the samples 15 min prior to the addition of the radioactive tracer. Flux measurements were started by adding of 5 μl ^{86}Rb (~0.1 mCi ml l^{-1} stock on distilled water, Perkin Elmer, Boston, MA, USA) per ml cell suspension. Aliquots of suspension were collected after 3, 5 and 10 min of incubation with the radioactive tracer, and the flux was stopped by immediate dilution of 0.9 ml aliquot with 10 ml cold washing medium [100 mmol l^{-1} $Mg(NO_3)_2$ and 10 mmol l^{-1} imidazole (buffered at pH 7.4)]. Cells were washed twice to eliminate external tracer and lysed in 5% TCA. Radioactivity of cells (A_c) and incubation medium (A_m) was measured using Microbeta Wallac liquid scintillation counter (Perkin-Elmer Wallac, Finland) in water phase (Cherenkov effect). Accumulation of ^{86}Rb in heaptocytes was linear for the 10 min incubation time. Unidirectional fluxes J were calculated from the following equation:

$$J = (A_c/A_m)[K^+]_e / mt ,$$

where A_c and A_m are radioactivity of cells in 1 ml suspension and 1 ml medium, respectively; m is amount of protein (mg ml^{-1} of cell suspension), $[K^+]_e$ is K^+ concentration in the incubation medium, and t is the equilibration time with the tracer.

Measurements of cellular ATP and GSH levels

Quantification of total non-protein reduced thiol levels, of which GSH is the most abundant, was performed spectrophotometrically. After 15 min of incubation in a tonometer equilibrated at 21, 5 or 1% O_2 in the standard incubation medium with or without 1 mmol l^{-1} CDNB, 5 mmol l^{-1} GSH or NAC, cells were lysed in 5% trichloroacetic acid and protein removed by centrifugation. Reduced thiol levels were evaluated in supernatants using Ellmann's technique. The optical density of colored complexes which thiols formed with Ellmann's reagent (5,5′-dithiobis(2-nitrobenzoic acid) was determined at 412 nm using Lambda 25 spectrophotometer (Perkin-Elmer). Details of the analytical protocol are described by Tietze (1969). Cellular GSH was depleted by treatment of cells with 1 mmol l^{-1} CDNB.

Cellular ATP levels were measured using ATP bioluminescent assay kit (Sigma, St Louis, MO, USA). Chemiluminescence measurements were carried out on a Sirius Luminometer (Berthold Detection Systems, Pforzheim, Germany). Measurements were performed in protein-free cell lysates prepared by mixing cell suspensions with equal amount of 5% TCA as described in the standard kit protocol.

Statistics

Data are presented as mean ± S.E.M. The statistical significance of the obtained data was analyzed with Mann–Whitney *U*-test or Wilcoxon matched-pairs, signed-rank test, or paired and unpaired *t*-test (depending on the normality of the data) provided by GraphPad Instat (version 3) program.

Results

Cellular redox state at different oxygen concentrations

Manipulation of cellular redox state

We have used the fluorescent probe H_2-DCFDA to monitor changes in ROS formation in primary cultures of trout hepatocytes by means of micro fluorescence imaging. Our previous findings reveal a gradual increase in ROS production in trout hepatocytes as oxygen concentration changed from 1 to 50% (Bogdanova et al., 2003a). Increase in fluorescence intensity, as the oxidized form of the dye accumulated, was proportional to the free radical production rate and linear during the first 20 min of incubation, corresponding to a current ROS steady-state level (Fig. 1A). Shifts from this steady state caused by treatment with agents affecting cellular redox state resulted in a change of the rate of dye oxidation, allowing us to monitor both the increase and the decrease in ROS production as shifts in the rate of oxidation of the dye. As shown in Fig. 1B, treatment of hepatocytes with the GSH-conjugating agent CDNB resulted in upregulation of ROS production, which could be partially suppressed in the presence of the hydroxyl radical scavenger MPG. Treatment of cells with the scavenger alone did not affect the rate of oxidation of the fluorescent dye, revealing low basal level of $OH^{\cdot-}$ in cells. Acute decrease in P_{O_2} in the perfusion medium from 20 to ~1 kPa corresponding to 21 and 1% O_2 in the gas phase was followed by a decrease in oxidation rate of the fluorescent dye throughout the first 10 min of hypoxic exposure (Fig. 1A,B).

To establish if the changes in ROS production caused by oxygen deprivation affected bulk cellular redox state significantly, we have measured the level of reduced non-protein thiols, most of which are represented by GSH, in hepatocytes equilibrated with 21, 5 or 1% oxygen for 15 min. As can be seen from Fig. 2, a slight increase in cellular GSH content was observed in cells incubated at 5% O_2 when compared with 21% O_2, whereas no difference of GSH levels in cells incubated at 21% and 1% O_2 could be detected (Fig. 2). As expected, GSH depletion caused by treatment of cells with 1 mmol l^{-1} CDNB was oxygen independent. Incubation of cells at 1% O_2 up to 30 min was without an effect on cellular GSH levels (data not shown).

Cellular ATP levels as a function of oxygen concentration and redox state

Cellular ATP levels were monitored as a function of oxygen concentration and cellular redox state. No significant differences in ATP levels could be seen between cells incubated at 21, 5 and 1% for 15 min (Fig. 3). GSH depletion as well as treatment of cells with reducing agents such as NAC or GSH had no effect on ATP levels in trout hepatocytes.

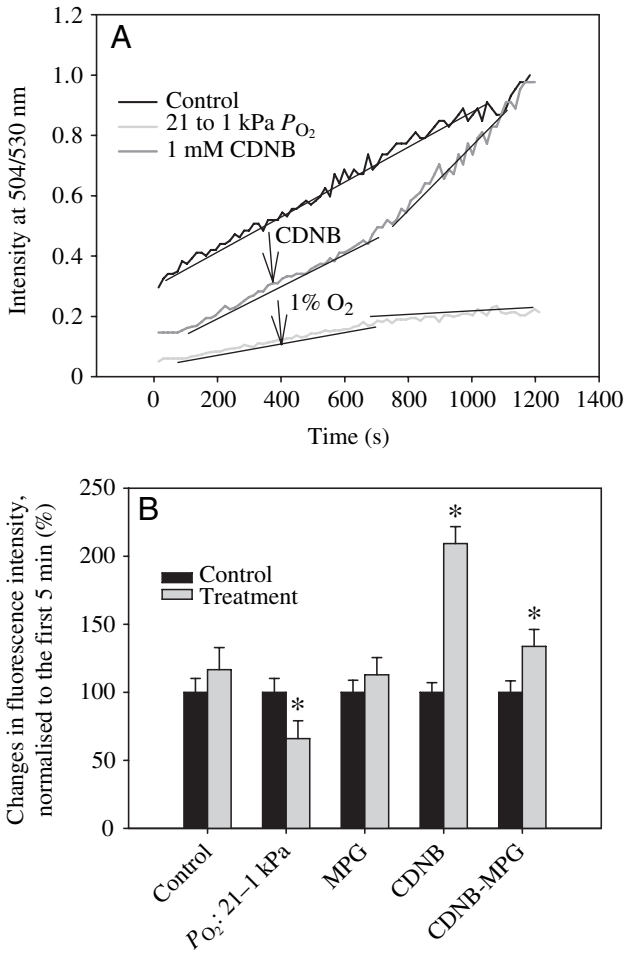

Fig. 1. Changes in H_2O_2 levels in trout hepatocytes in response to hypoxia, MPG and CDNB. (A) Original traces of the changes in intensity of DCF fluorescence from individual cells over time. (B) Average changes in the rate of increase in fluorescence during the first 300 s and an interval of 800 to 1100 s of recording. Onset of hypoxic exposure or addition of 5 mmol l^{-1} MPG or 1 mmol l^{-1} CDNB corresponded to the 400 s time point. When analyzed, data for the corresponding intervals of time were fitted using linear regression and slope of each curve used to characterize increase in fluorescence for single cells with time. Presented values are means ± S.E.M. for 28–35 individual cells. * indicates $P<0.05$.

Hydrolytic and transport activity of the Na^+/K^+ ATPase

Hydrolytic activity of the Na^+/K^+ ATPase was not altered by hypoxia in the range of concentrations of 21–1% O_2 (Fig. 4) and after incubation for 40 min at 1% O_2 (data not shown). Reducing agents did not affect the hydrolytic function of the Na^+/K^+ ATPase whereas GSH depletion was followed by a twofold decrease in hydrolytic activity independent of the oxygen concentration used (Fig. 4).

In contrast to hydrolytic activity, transport function of the Na^+/K^+ ATPase was strikingly dependent on the oxygen

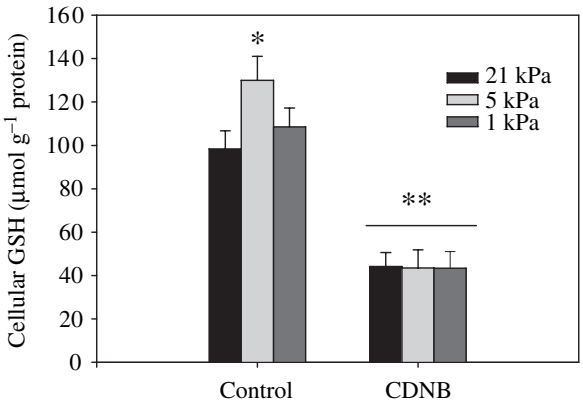

Fig. 2. Cellular reduced glutathione levels as a function of oxygen concentration and treatment with the conjugating agent CDNB. GSH levels in cells incubated for 15 min at 21, 5 or 1% O_2 in the presence or in the absence of 1 mm CDNB. Data are means ± S.E.M. of four independent experiments. *P<0.05 and **P<0.01.

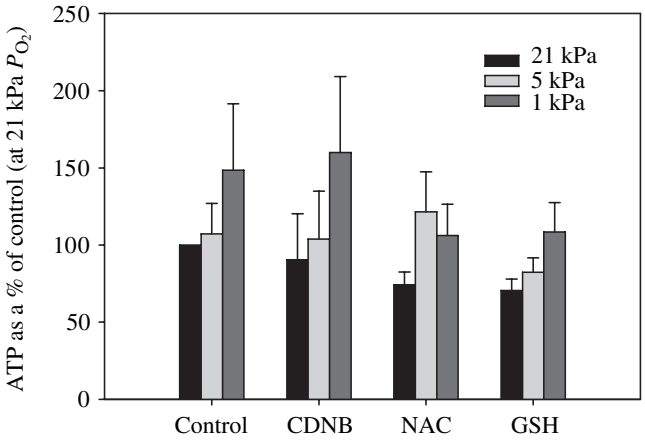

Fig. 3. ATP levels in cells incubated for 15 min at 21, 5 or 1% O_2 alone or in the presence of 1 mmol l^{-1} CDNB, 5 mmol l^{-1} *N*-acetyl cysteine or 5 mmol l^{-1} GSH. Data are means ± S.E.M. of five independent experiments.

concentrations. Active K^+ influx decreased after 15 min of exposure to 10% O_2 in comparison with 21% O_2 (Fig. 5A). Passive K^+ influx was slightly increased at P_{O_2} of 1 kPa, remaining constant at any other oxygen concentration tested. The lowest values of active transport were observed in cells incubated at 1% oxygen and consequently the kinetics of the pump inhibition was studied at 1% oxygen level. As can be seen in Fig. 5B, the active K^+ transport component responded to hypoxic treatment in a time-dependent manner. Ouabain-resistant K^+ influx decreased transiently immediately after the onset of hypoxic exposure but recovered within 10 min of hypoxic treatment. Active K^+ influx was also transiently suppressed by hypoxic exposure. Hypoxia-induced inhibition of the Na^+/K^+ ATPase could be observed over 15 min incubation at 1% O_2. However, transport activity of the pump was restored if hypoxic exposure lasted for 20 min. Moreover, 30 min treatment was followed by a two- to threefold

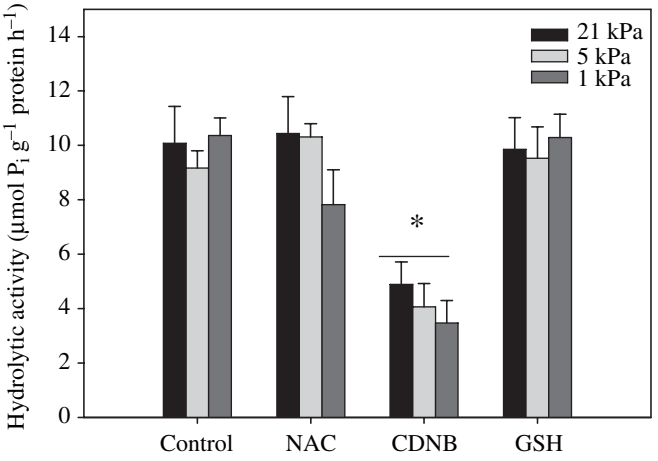

Fig. 4. Hydrolytic activity of Na^+/K^+ ATPase after exposure to different oxygen concentrations and manipulation of cellular redox state. Cells were exposed for 15 min to 21, 5 or 1% O_2 in the presence or in the absence of 1 mmol l^{-1} CDNB, 5 mmol l^{-1} *N*-acetyl cysteine or GSH. After incubation, the cells were immediately destroyed by repetitive freezing–thawing and ouabain-sensitive production of P_i measured in cell homogenate in the presence of 3 mmol l^{-1} ATP. Data are means ± S.E.M. of five independent experiments. *P<0.05.

stimulation of active K^+ influx compared with normoxic control (Fig. 5B). Measurements of cellular ATP under identical conditions revealed no correlations with changes in the Na^+/K^+ ATPase transport activity.

Relation between redox-and oxygen-sensitivity of the Na^+/K^+ ATPase

Depletion of GSH in cells incubated at 21% O_2 resulted in suppression of Na^+/K^+ pump transport activity (Fig. 6A). Active K^+ influx did not differ for CDNB-treated cells exposed to 21 or to 1% oxygen for 15 min. Addition of hydroxyl radical scavenger MPG at concentration of 5 mmol l^{-1} 15 min prior to the onset of hypoxia abolished hypoxia-induced inhibition of the pump function (Fig. 6A).

Passive K^+ influx decreased significantly (from 0.052±0.019 to 0.014±0.002 mmol mg^{-1} protein h^{-1}) during the first 3–5 min of exposure to 1% O_2 and, thereafter, showed slightly higher values than those at 21% O_2. CDNB treatment resulted in upregulation of ouabain-resistant K^+ influx at P_{O_2} of 21 kPa but not at 1 kPa, which was in agreement with the data in Fig. 1 showing reduction in free radical production under hypoxic conditions as compared to air-equilibrated 'normoxic' control. MPG had no effect on passive K^+ influx either at 21 or at 1% O_2 (Fig. 6B).

Discussion

The obtained data indicate that the oxygen-sensitivity of Na^+/K^+ ATPase activity in trout hepatocytes is mainly due to the effects of oxygen on the transport function of the pump. By contrast, ATP hydrolysis by the Na/K ATPase is not affected by oxygen level at the range 1–21 kPa. Our results

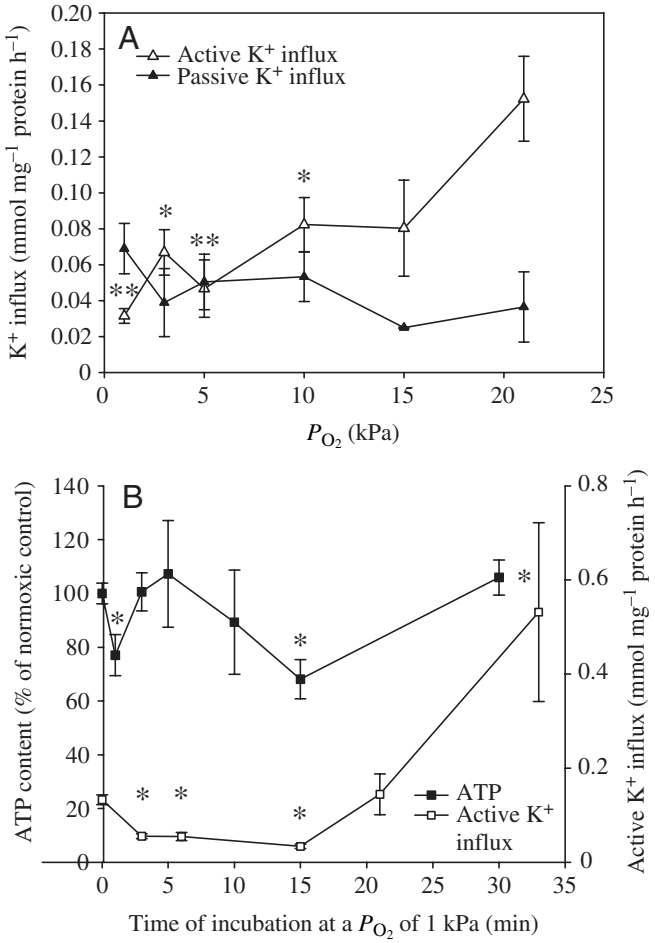

Fig. 5. K^+ influx into trout hepatocytes as a function of oxygen concentration. (A) Active and passive components of K influx as a function of oxygen concentration. Active K^+ influx was calculated as difference in $K^+(^{86}Rb)$ uptake in the presence or in the absence of 100 μmol l^{-1} ouabain. Cells were incubated at different oxygen concentrations for 15 min. Data are means of five independent experiments ± S.E.M. According to the results of Dunnett multiple comparison test (one-way ANOVA), *P<0.05 and **P<0.01 when compared with 21% O_2 'control'. (B) Kinetics of the changes in active K^+ influx and cellular ATP levels in trout hepatocytes exposed to 1% O_2. Data are means ± S.E.M. of four or five independent experiments. *P<0.05.

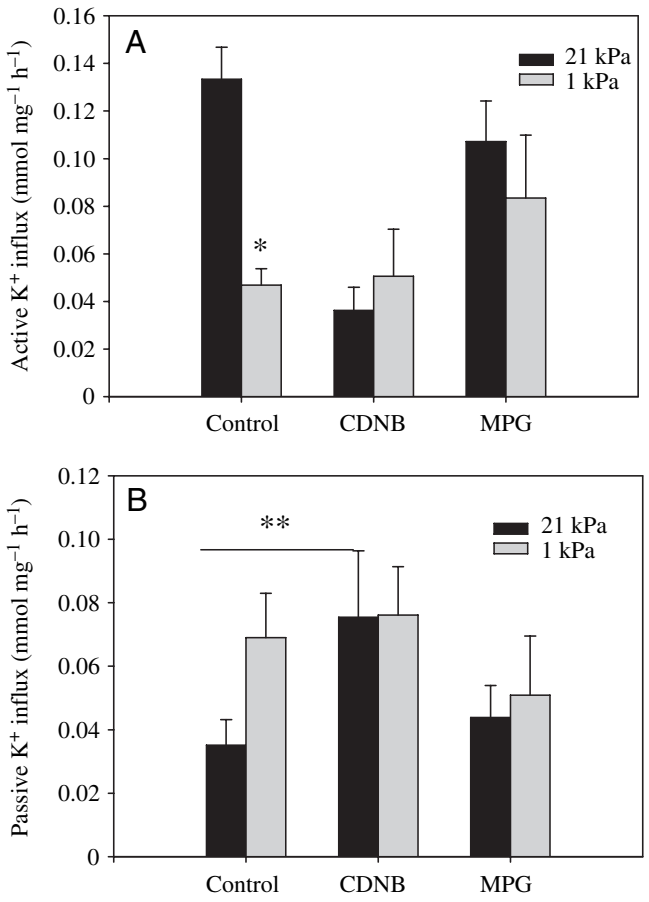

Fig. 6. Redox-sensitivity of active (A) and passive (B) K^+ influx in cells incubated at 21 or 1% O_2. Cells were exposed to hypoxic or normoxic conditions in the presence or in the absence of 1 mmol l^{-1} CDNB or 5 mmol l^{-1} MPG for 15 min. Data are means ± S.E.M. of four independent experiments. *P<0.05; **P<0.01.

furthermore suggest that ROS play a role in modulating the activity of the transporter when oxygen levels change.

It is known that mammalian hepatocytes respond to hypoxic challenge (1 kPa and lower) almost instantaneously by reduction in cellular ATP levels, increase in cellular Na^+ concentrations and cell death (Aw and Jones, 1985; Carini et al., 1997). Compared with this extreme oxygen sensitivity, trout hepatocytes seem to be rather hypoxia tolerant, although the species is known to be oxygen sensitive among fish. However, oxygen consumption rates for mammalian and trout hepatocytes are strikingly different, making 20–40 nmol oxygen per 10^6 cells min^{-1}) for rat cells compared with 0.6–0.7 nmol oxygen per 10^6 cells min^{-1}) for trout liver cells (Rissanen et al., 2003; Aw et al., 1987). Environmental temperature range is also different, being 15–20°C for the trout and 37°C for the rat liver cells. Therefore, lack of ATP depletion in trout hepatocytes exposed for 30 min or less to low oxygen concentration is not an unexpected finding.

Decrease in transport activity of the Na^+/K^+ pump in response to 15 min hypoxic exposure can be seen already at 10% O_2 with a most pronounced effect at 1% O_2. Unfortunately, no data are available on the in vivo oxygen partial pressure in rainbow trout liver tissue. In mammalian liver tissue, oxygen levels detected are in the range of 4–6 kPa, whereas at concentrations of 1.5–1.7 kPa, oxygen availability no longer matches consumption (Soller et al., 2001; Brooks et al., 2004). 'Physiologically normoxic' and 'critical' oxygen tensions correspond with 5–3% and 1% O_2 levels in gas phase, respectively. Our data shows that Na^+/K^+ pump transport function in this range of oxygen concentrations is significantly lower than at 21% O_2. Reversible inhibition of the active K^+ uptake was shown for trout hepatocytes in response to hypoxia (1% O_2) and reoxygenation (Krumschnabel et al., 2000b). As a possible mechanism of hypoxia-induced inhibition of active

K^+ influx, Krumschnabel et al. suggest downregulation of ATP production, postulating existence of transport-metabolic coupling (Krumschnabel et al., 2000b). Our data do not support this hypothesis as hypoxia-induced inhibition of the pump is almost instantaneous and occurs at oxygen concentrations of 10–5%, at which no changes in ATP content occur.

Notably, the hypoxic inhibition of the pump at P_{O_2} of 1 kPa is transient with activity recovering within 25–30 min of incubation under hypoxic conditions. Data we have obtained previously on oxygen sensitivity of Na^+/H^+ exchanger reveal that this transporter is activated by hypoxic treatment (Tuominen et al., 2003). If this were the case, hypoxic conditions should result in gradual Na^+ accumulation as passive Na^+ uptake is increased and active efflux downregulated. Increases in intracellular Na^+ cause activation of Na^+/K^+ pump in mouse red blood cells that overrides hypoxic deactivation (Bogdanova et al., 2003b). Therefore, re-activation of the Na^+/K^+ ATPase most probably results from intracellular Na^+ accumulation.

Interestingly, the hypoxic inhibition of the transport activity of Na^+/K^+ pump can be abolished by pretreatment of cells with the scavenger of hydroxyl radicals, MPG. The selectivity of MPG to scavenging hydroxyl radicals but not H_2O_2 and superoxide anion has been proven elsewhere (Sekili et al., 1993; Sun et al., 1993). This observation makes it tempting to suggest that this radical species is the second messenger involved in transferring information from so far unknown primary oxygen-binding protein(s) to ion transporters. Notably, treatment of hepatocytes with MPG suppresses proton extrusion mediated by Na^+/H^+ exchanger (Tuominen et al., 2003). The latter finding suggests common regulatory pathways for oxygen-induced regulation of both ion transporters. Interestingly, similar coupling of two oxygen-sensitive ion transport systems, the K^+/Cl^- cotransporter and Na^+/H^+ exchanger, mediated by hydroxyl radicals has been demonstrated in rainbow trout erythrocytes (Bogdanova and Nikinmaa, 2001; Nikinmaa et al., 2003). Earlier, it was shown that the transport function of the Na^+/K^+ pump in ischemic kidneys could be restored by application of MPG (Kato and Kako, 1987).

Paradoxically, measurements using the fluorescent dye H_2-DCFDA did not show upregulation but, instead, a modest decrease in total ROS formation immediately after the onset of hypoxic exposure. A small but significant increase in cellular GSH levels could also be observed after a 15 min incubation at a P_{O_2} of 5 kPa, confirming that the bulk level of oxidants decreased upon acute hypoxic treatment. Such a dual response has recently been shown in venous endothelial cells where hypoxia triggers upregulation in free radical production by mitochondria, but reduces ROS levels originating from NADPH oxidase (Kang et al., 2005). Our data suggest that, together with decreased bulk ROS levels under hypoxic conditions, a fraction of $OH^{\cdot -}$ responsible for ion transport modulation appears to be upregulated. Hydroxyl radicals are produced in Fenton reaction from hydrogen peroxide exclusively in the presence of a catalyst (Fe^{2+} or Cu^+) and one could assume that a rate-limiting step in the production of 'signaling' hydroxyl radicals is probably the availability of the catalytic ferrous or cuprous ion, which becomes accessible for H_2O_2 upon delocalization only under hypoxic conditions.

The fluorescent dye used to monitor ROS production is predominantly oxidized by H_2O_2 in the presence of Fe^{2+}-containing enzymes rather than by direct interaction with hydroxyl radicals or superoxide anion (LeBel et al., 1992). However, when formed, hydroxyl radicals cause H_2O_2 production and one cannot rule out the impact of $OH^{\cdot -}$ on the dye oxidation when abnormally high amounts of hydroxyl radicals are produced (as in CDNB-treated cells). As the reduced form of the dye H_2-DCF is negatively charged, it cannot cross the mitochondrial membranes and therefore responds only to bulk changes in H_2O_2 levels in the cytosol. Acute hypoxic exposure decreases bulk H_2O_2 production in the cytosol, suggesting presence of sufficient amounts of enzymes able to bind and reduce oxygen. Longer exposure to low oxygen (e.g. 1 kPa) have been shown to cause a slight increase in cytosolic ROS that may be attributed to leakage of mitochondrial H_2O_2 as uncoupling in electron transduction chain occurs (Bogdanova et al., 2003a; Rifkind, 1993).

Treatment of cells with CDNB results in a rapid depletion of both cytosolic and mitochondrial GSH pools with a consequent massive increase in cytosolic ROS in hepatocytes (Han et al., 2003; Deneke and Fanburg, 1989). This response differs significantly from that we observed in CDNB-treated neurons. The difference can be explained by lower amount of ROS generating systems in neurons where no upregulation in cytosolic ROS production could be observed using H_2-DCFDA in response to CDNB treatment (I. Petrushanko and A. Bogdanova, unpublished). Depletion of both cytosolic and mitochondrial GSH pool with CDNB in neurons did not cause inhibition of hydrolytic activity of the Na^+/K^+ ATPase whereas in CDNB-treated hepatocytes the latter was observed independent on oxygen concentration. Moreover, in neurons CDNB treatment resulted in rapid ATP depletion and burst in mitochondrial free radical production (I. Petrushanko and A. Bogdanova, unpublished). ATP levels in hepatocytes were not affected by CDNB treatment. Since we did not measure mitochondrial ROS production rate in GSH-depleted trout hepatocytes, we can only speculate on two possible reasons for this difference. Either for some reasons antioxidant defense is more powerful in mitochondria of hepatocytes than in neurons, or other metabolic pathways compensate for suppressed mitochondrial function. Slight acidification of the cytosol observed in CDNB-treated hepatocytes (the pH decreases to 7.25±0.03 from 7.40±0.04 in non-treated control) suggests that lactate production may be upregulated. The latter suggests a general susceptibility of the Na^+/K^+ ATPase in trout hepatocytes to oxidative stress, which does not occur in response to acute hypoxic insult, despite local increase in hydroxyl radical production.

Oxidative stress induced by GSH depletion or treatment with oxidants was shown to cause rapid suppression of the transport and hydrolytic Na^+/K^+ ATPase function in different tissues,

including neurons, cardiac myocytes and erythrocytes (e.g. (Haddock et al., 1995a,b; Bilgin et al., 1999; Cheng et al., 1984; Boldyrev and Bulygina, 1997; Bogdanova et al., 2003b) (I. Petrushanko and A. Bogdanova, unpublished). The only study in which no effect of CDNB treatment on active K^+ transport in human red cells was reported is the work of Muzyamba and Gibson (Muzyamba and Gibson, 2003). The reasons of particular resistance of the Na^+/K^+ ATPase to oxidation remains unclear, especially as CDNB treatment of human red blood cells resulted total disappearance of intracellular GSH, which has never been the case in other studies where 1 mmol l^{-1} CDNB was used to deplete GSH in mammalian erythrocytes (Lauf et al., 1995; Bogdanova et al., 2003b).

In conclusion, the data we have obtained for hypoxia-induced changes in K^+ transport across hepatocyte plasma membrane reveal that active transport is oxygen sensitive. Acute hypoxic exposure resulted in a rapid transient decrease in the transport function of the Na^+/K^+ ATPase without affecting its hydrolytic activity. The reduction of active K^+ uptake could be abolished by treatment of cells with hydroxyl radical scavenger, revealing the importance of this radical species in acute hypoxic signaling and coordination of two ion transport systems: the Na^+/K^+ pump and Na^+/H^+ exchanger. Oxygen-induced responses in the transport function of the Na^+/K^+ ATPase did not directly correlate with changes of cellular ATP or pH. By contrast, oxidative stress induced by treatment of trout hepatocytes with the GSH-conjugating agent CDNB was followed by a marked increase in cytosolic ROS production and resulted in inhibition of both transport and hydrolytic activity of the Na^+/K^+ ATPase without any significant changes in cellular ATP levels.

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