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Cyanide inhibition and pyruvate-induced recovery of cytochrome *c* oxidase

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Abstract The mechanism of cyanide's inhibitory effect on the mitochondrial cytochrome *c* oxidase (COX) as well as the conditions for its recovery have not yet been fully explained. We investigated three parameters of COX function, namely electron transport (oxygen consumption), proton transport (mitochondrial membrane potential $\Delta\psi_m$) and the enzyme affinity to oxygen (p_{50} value) with regard to the inhibition by KCN and its reversal by pyruvate. 250 μM KCN completely inhibited both the electron and proton transport function of COX. The inhibition was reversible as demonstrated by washing of mitochondria. The addition of 60 mM pyruvate induced the maximal recovery of both parameters to 60–80% of the original values. When using low KCN concentrations of up to 5 μM, we observed a profound, 30-fold decrease of COX affinity for oxygen. Again, this decrease was completely reversed by washing mitochondria while pyruvate induced only a partial, yet significant recovery of oxygen affinity. Our results demonstrate that the inhibition of COX by cyanide is reversible and that the potential of pyruvate as a cyanide poisoning antidote is limited. Importantly, we also showed that the COX affinity for oxygen is the most sensitive indicator of cyanide toxic effects.

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Introduction

Cytochrome *c* oxidase (COX, EC 1.9.3.1) is an essential member of the cellular ATP-producing system under aerobic conditions. As the terminal enzyme of the respiratory chain in the inner mitochondrial membrane, COX reduces molecular oxygen. Electrons are released from various respiratory substrates by mitochondrial dehydrogenases and transported to the cytochrome *c*, which is reoxidized by COX. Oxygen binds to the fully reduced binuclear center cyt a_3-Cu_B of COX, where it is reduced. Four protons are used for water formation, and four protons are translocated from the matrix into the intermembrane space of mitochondria. COX thus contributes to the formation of electrochemical proton gradient across the inner mitochondrial membrane, which is utilized by the F_oF_1-ATP synthase for the synthesis of ATP (reviewed in (Ludwig et al. 2001; Hosler et al. 2006; Brzezinski and Gennis 2008)).

The catalytic activity of COX is strongly inhibited by cyanide as first demonstrated by Otto Warburg (Warburg et al. 1933). This inhibition has been referred to as reversible although some controversy still remains (Niknahad and Ghelichkhani 2002; Cooper and Brown 2008; Leavesley et al. 2008). Hydrogen cyanide binds to the fully oxidized binuclear center on condition that at least one electron has entered the COX complex (Nicholls et al. 1972; Palmer 1993; Wilson et al. 1994). Therefore, cyanide is a non-competitive inhibitor of COX towards oxygen (Petersen 1977; Isom and Way 1984). However, oxygen was also reported as a cyanide antagonist in some studies (Isom et al. 1982; Delhumeau et al. 1994), probably due to the binding

of cyanide anion to the fully reduced COX (Muramoto et al. 2010).

Several methods have been used to treat cyanide poisoning, but most of them are not very effective and may suffer from severe negative side-effects (Baskin and Brewer 1997; Beasley and Glass 1998; Cummings 2004; Zottola et al. 2009). The best option seems to be cobalamin derivatives (Broderick et al. 2006; Brenner et al. 2010). An alternative treatment might be based on the observation that the cyanide toxicity can be partially reversed by keto acids due to their reaction with cyanide yielding non-toxic cyanohydrins (Marshall and Rosenfeld 1934). The protective effect of keto acids was demonstrated in *in vivo* experiments as the cyanide LD_{50} increase. (Nosek et al. 1957; Cittadini et al. 1972). The most frequently used keto acid, α-ketoglutarate (2-oxoglutarate), has been considered even as a prophylactic agent during chronic sublethal cyanide poisoning (Bhattacharya et al. 2001; Bhattacharya and Tulsawani 2009; Hariharakrishnan et al. 2009).

The antidotal effect of another keto acid—pyruvate, which can be easily distributed to sites of cyanide localization due to a specific carrier, was also investigated *in vivo* (Schwartz et al. 1979). In mitochondria isolated from rat liver, heart and testes, the pyruvate-induced recovery of ATP synthesis as well as the activity of isolated COX were examined (Delhumeau et al. 1994). Other experiments demonstrated the protective effect of pyruvate against cyanide inhibition on intact smooth muscles and also the different sensitivity to cyanide of various tissues (Clark et al. 2000).

In this study, we investigated the reversibility of COX inhibition by cyanide and, in more detail, the process by which pyruvate recovers both the electron-transporting and proton-pumping activity of COX inhibited by 250 μM KCN. In isolated rat liver mitochondria, we measured the rate of succinate- and ascorbate+TMPD-dependent respiration and the related changes of the mitochondrial membrane potential with the aim of examining whether these parameters of COX function can be recovered after cyanide inhibition. We also calculated the p_{50} value for oxygen to assess the changes of oxygen affinity of partially inhibited and recovered respiration, and compared the sensitivity of three different functional parameters of COX (namely electron transport, proton pumping, and oxygen affinity) to cyanide.

Materials and methods

Animals

Male Wistar rats (Bio-Test Konarovice, Czech Republic) with a body weight of 220–250 g were kept at 23 °C with a free access to the standard laboratory diet (DOS 2B Velaz, Czech Republic) and tap water. Animals were starved overnight before experiments and killed by dislocation of cervical spine in CO_2 narcosis. All animal studies were approved by the Animal Care Committee of the Institute of Physiology and fulfilled the NIH guidelines for the human use of animal subjects.

Materials

All chemicals were of the highest commercially available purity and were purchased from Sigma (Sigma-Aldrich Co., Germany).

Isolation of rat liver mitochondria

The rat liver mitochondria (RLM) were prepared by differential centrifugation as described in (Bustamante et al. 1977) in an isolation medium containing 320 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, 0.5 $g.L^{-1}$ BSA, pH 7.4. The protein content was determined according to the method of Bradford (Bradford 1976) using BSA as standard.

Oxygraphic measurement

The oxygen consumption of mitochondria (0.05–0.30 mg. mL^{-1} of the mitochondrial protein) was measured in 2 mL of incubation medium containing 80 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM KH_2PO_4, pH 7.3. The measurements were performed at 30 °C using the high-resolution respirometry system Oxygraph-2 k (OROBOROS INSTRUMENTS, Austria) (Gnaiger 2001). Datlab4 software was used for data acquisition, analysis and plotting. Oxygen consumption was expressed as pmol $O_2.s^{-1}$.mg $protein^{-1}$.

Succinate (10 mM) and ascorbate (5 mM) + TMPD (N, N,N′,N′-tetramethylphenylenediamine, 1 mM) were used as the respiratory chain substrates throughout the study. In case of succinate-dependent respiration, the mitochondria were preincubated with rotenone (1 μM) to avoid a possible inhibition of succinate dehydrogenase by accumulated oxaloacetate. In addition, the inhibition of the upstream located respiratory chain complexes ensured that the added pyruvate was not metabolized and did not contribute to the respiration. Prior to the measurements of ascorbate+TMPD-dependent respiration, the complex III was inhibited by antimycin A (1 $\mu g.mL^{-1}$). To obtain the most accurate value of ascorbate+TMPD-dependent respiration, the autooxidation of ascorbate+TMPD was subtracted from the overall oxygen consumption as the KCN-insensitive oxygen uptake.

p_{50} calculation

p_{50} of succinate- and ascorbate+TMPD-dependent respiration was calculated as described previously (Steinlechner-Maran et al. 1996; Smolkova et al. 2010). The p_{50} value

was determined on the basis of oxygraphic measurements from the flux while approaching zero oxygen. The flux was corrected for the exponential time constant and the background correction was also applied (the background flux was measured in the incubation medium and in the incubation medium with ascorbate+TMPD for succinate- and ascorbate+TMPD-dependent respiration, respectively). Using DatLab2 software (OROBOROS), a hyperbolic fit was performed to yield the oxygen partial pressure at half-maximum flux (p_{50}), and the maximum flux related to the kinetic oxygen range (J_{max}). A fitting procedure according to the equation derived from the enzyme kinetics $J_{O_2} = (J_{\max} \cdot p_{O_2})/(p_{50} + p_{O_2})$ was used, where p_{O_2} is the oxygen partial pressure and J_{O_2} is the flux at this p_{O_2}.

Measurement of mitochondrial membrane potential

The membrane potential of isolated rat liver mitochondria (0.5 mg.mL^{-1} of the mitochondrial protein) was measured as changes of tetraphenylphosphonium (TPP^+) concentration in the incubation chamber using a TPP^+-selective electrode and an Ag/AgCl reference electrode as described in (Labajova et al. 2006). Measurements were performed in 2 mL of the incubation medium described above at room temperature. Before each measurement, the electrode was calibrated by a stepwise addition of TPP^+ (1–6 μM). The membrane potential was plotted as pTPP, *i.e.* the natural logarithm of TPP^+ concentration (μM). The values were used for data analysis and comparison. The corresponding values of mitochondrial membrane potential ($\Delta\psi_m$) were corrected for TPP^+ binding as described in (Zolkiewska et al. 1989).

Cyanide determination

The method described in (Afkhami et al. 2007) based on the König reaction was used to determine cyanide concentration in the incubation medium. The formation of the final colored product was determined in the Shimadzu UV-1601 spectrophotometer by measuring the increase of absorbance at 578 nm. The measurements were performed in 20 mM acetate buffer (pH 4.0) containing 0.025% (w/v) chloramine-T, 1% (v/v) pyridine and 15 mM barbituric acid at 30 °C. The samples were first incubated with chloramine-T in the acidic buffer for 3 min. After the addition of pyridine and barbituric acid, the kinetics of the reaction was analyzed. The final volume of the reaction mixture was 1 mL. Concentration dependence was linear in the range of 0–40 μM KCN with the detection limit of 0.1 μM KCN.

Statistics

The data are presented as mean values±standard error of the mean (SE) from 3–5 measurements of individual mitochondrial preparations. The statistical significance was evaluated with the standard two-sample *t*-test using Origin (OriginLab, Northampton, MA).

Results

Effect of cyanide on succinate- and ascorbate+TMPD -dependent respiration

TMPD ensures a direct transfer of electrons from external donors (ascorbate) to COX, whereas the electrons from succinate are transported to COX through the redox components of the respiratory chain. The flux of ascorbate+TMPD-fuelled respiration thus reflects the maximal COX capacity while the succinate-fuelled respiration makes it possible to analyze the COX activity supplied with electrons from the respiratory chain, namely by succinate dehydrogenase.

We first studied the inhibitory effect of different KCN concentrations on state 3 (ADP-stimulated) respiration. For a complete inhibition of respiration in experiments with cells or isolated mitochondria, 250–330 μM cyanide is used (Pecina et al. 2003). Our measurements in freshly isolated rat liver mitochondria confirmed that both the succinate- and ascorbate+TMPD-dependent respiration (1,541±90 and 2,542±91 pmol O_2.s^{-1}.mg protein^{-1}, respectively) are completely inhibited at 250 μM cyanide (Fig. 1). Within the concentration range of 20–200 μM KCN, a lower sensitivity of the succinate-dependent respiration was observed.

We investigated the reversibility of cyanide inhibition of COX by washing the mitochondria. Freshly isolated intact

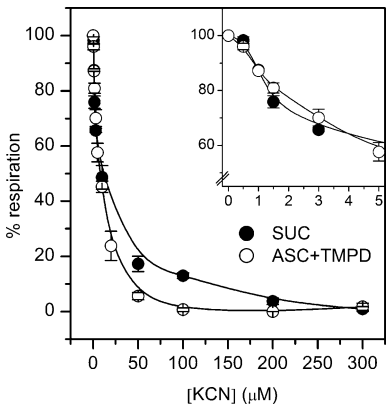

Fig. 1 Cyanide inhibition of succinate- and ascorbate+TMPD-dependent respiration. State 3 (ADP-stimulated) succinate-dependent respiration (●) of isolated rat liver mitochondria (0.09–0.15 mg.mL^{-1} of mitochondrial protein) was measured in the presence of 1 μM rotenone, 10 mM succinate, 1.5 mM ADP; state 3 (ADP-stimulated) ascorbate+TMPD-dependent respiration (○) of isolated rat liver mitochondria (0.05–0.07 mg.mL^{-1} of mitochondrial protein) was measured in the presence of 1 μg.mL^{-1} antimycin A, 5 mM ascorbate, 1 mM TMPD, 1.5 mM ADP. The inset shows data within the low cyanide concentration range

mitochondria were incubated with 250 μM KCN for 10 min and then washed twice with the incubation medium by centrifugation. In the presence of KCN, neither succinate- nor ascorbate+TMPD-dependent respiration could be observed (Fig. 2a) but after washing (Fig. 2b), the rates of both respiration types returned to the control values, indicating that under the used experimental conditions the cyanide inhibition of respiration is reversible. Further addition of KCN during the measurement was again completely inhibitory.

To investigate the recovery of KCN-inhibited respiration by pyruvate, we measured the effect of increasing pyruvate concentrations on the rates of succinate- and ascorbate+TMPD-dependent respiration of mitochondria treated with 250 μM KCN as shown in Fig. 3a and b. As demonstrated in Fig. 3c, the maximum effect was obtained at 60 mM pyruvate, which recovered 80% and 70% of the original succinate- and ascorbate+TMPD-dependent respiration, respectively. Higher concentrations of pyruvate were not effective any longer.

Changes of mitochondrial membrane potential

In further experiments, we examined the effect of KCN on the mitochondrial membrane potential ($\Delta\psi_m$) generated by substrate oxidation at state 4. Under these conditions, the potential mainly reflects the H^+-pumping activity of the respiratory chain as the synthesis of ATP is prevented by the absence of ADP. The TMPD+ascorbate-dependent $\Delta\psi_m$ (−140±3 mV) represents only the proton pump of COX and is thus lower than the succinate-dependent $\Delta\psi_m$ (−171±4 mV), which also includes the proton pump of the complex III.

The inhibitory effect of KCN on $\Delta\psi_m$ was similar to its effect on the respiration (Fig. 4a). At 250 μM KCN, the values of both succinate- and ascorbate+TMPD-dependent $\Delta\psi_m$ were decreased to the value obtained after the addition of the uncoupler FCCP. The ascorbate+TMPD-dependent $\Delta\psi_m$ was more sensitive to cyanide than the succinate-dependent $\Delta\psi_m$, and this difference was most evident at the lowest cyanide concentrations.

The cyanide-induced inhibition of mitochondrial membrane potential was also shown to be entirely reversible by washing (data not shown). The maximum recovery of the succinate-dependent $\Delta\psi_m$ (Fig. 4b) to 80% of original values was achieved by the addition of 60 mM pyruvate while the ascorbate+TMPD-induced $\Delta\psi_m$ was restored only to 60% (Fig. 4c). In agreement with the oxygraphic data, any further increase of pyruvate concentration did not improve the recovery of $\Delta\psi_m$. Both electron-transporting and proton-pumping activity of COX are thus similarly inhibited by cyanide and largely but not fully recovered by pyruvate.

Effect on the oxygen affinity

The oxygen affinity of the enzyme system involved in mitochondrial respiration can be quantified as the partial pressure of oxygen at which the respiratory flux is 50% of maximum (p_{50}). The oxygen affinity, *i.e.* the reciprocal value of p_{50}, decreases with the rate of oxidative phosphorylation and is a function of the metabolic state (e.g. coupled vs. uncoupled respiration) (Steinlechner-Maran et al. 1996; Gnaiger et al. 1998a, b). A lower oxygen affinity (*i.e.* a higher p_{50} value) is observed at a higher electron flux, which is the case of the ascorbate+TMPD-dependent respiration. This fact is responsible for the huge (about 40-fold) difference in the absolute p_{50} values between the succinate- and ascorbate+TMPD-dependent respiration (Fig. 5).

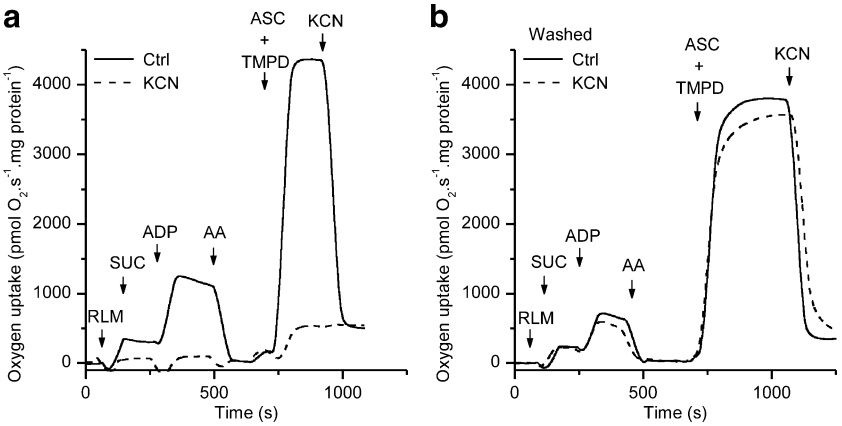

Fig. 2 Reversibility of cyanide inhibition of COX. Respiration of rat liver mitochondria (RLM, 0.6–0.8 mg.mL^{-1} of mitochondrial protein) was measured in the presence of 1 μM rotenone, 10 mM succinate (SUC), 1.5 mM ADP, 1 μg.mL^{-1} antimycin A (AA), 5 mM ascorbate (ASC), 1 mM TMPD, 250 μM KCN. (**a**) Measurements were performed in the presence (KCN) or absence (Ctrl) of 250 μM KCN. (**b**) Mitochondria were incubated with 250 μM KCN or with the medium (Ctrl) for 10 min, washed by centrifugation (10 min at 10,000×*g*) twice and then used for the measurement

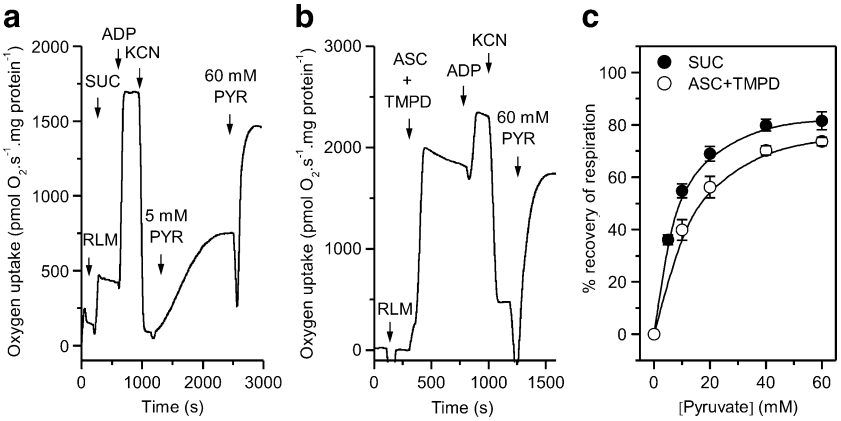

Fig. 3 Reactivation of KCN-inhibited respiration by pyruvate. The recovery of state 3 (ADP-stimulated) respiration of isolated rat liver mitochondria was measured (**a**) as succinate-dependent respiration in the presence of 0.09 mg.mL^{-1} of mitochondrial protein (RLM), 1 μM rotenone (ROT), 10 mM succinate (SUC), 1.5 mM ADP, 250 μM KCN, and pyruvate (5 mM and 60 mM PYR); and (**b**) as ascorbate+TMPD-dependent respiration in the presence of 0.05 mg.mL^{-1} of mitochondrial protein and 1 μg.mL^{-1} antimycin A (RLM), 5 mM ascorbate (ASC), 1 mM TMPD, 1.5 mM ADP, and 60 mM pyruvate (PYR). (**c**) Pyruvate recovery of succinate- (●) and ascorbate+TMPD-dependent respiration (○)

We analyzed the effect of cyanide on the p_{50} values of succinate- and ascorbate+TMPD-dependent respiration and found that p_{50} is highly sensitive to the inhibition of COX by KCN. As shown in Fig. 5a, the COX affinity to oxygen was affected by KCN already at very low concentrations, and that 5 μM KCN increased p_{50} of succinate-dependent respiration more than 30-fold (from 0.021±0.002 kPa to 0.701±0.084 kPa). After the addition of 250 μM KCN, the minute residual succinate-dependent respiration (about 5%) was too low for calculating the p_{50} value. Nevertheless, washing of KCN-treated mitochondria fully restored the original p_{50} and no significant difference was observed with respect to the control mitochondria (Fig. 5b, c). In contrast, only a partial recovery of the affinity to oxygen was found with pyruvate, as illustrated by the p_{50} values of succinate- and ascorbate+TMPD-dependent respiration that increased by a factor of 8.3 and 6.1, respectively.

Determination of free cyanide in the incubation medium

All three parameters used for the analysis of COX function (*i.e.* respiration, mitochondrial membrane potential, and p_{50}) were strongly affected by KCN in a reversible manner but none of them was recovered to 100% by pyruvate, although the pyruvate concentration (60 mM) was much higher than that of cyanide (250 μM). This may be caused

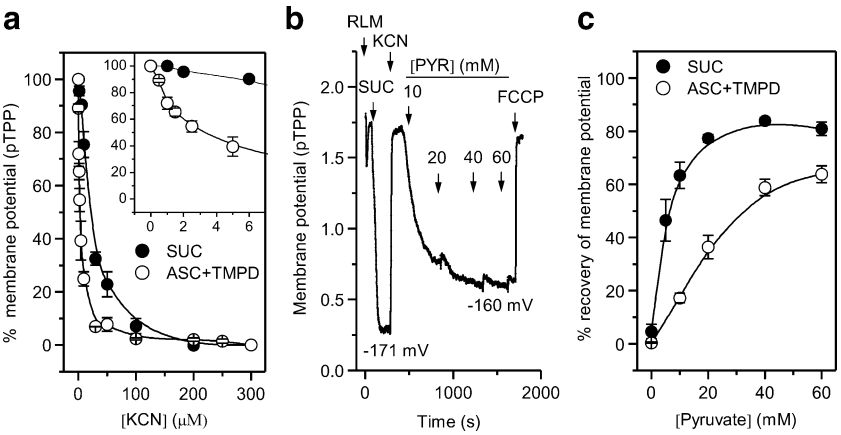

Fig. 4 Cyanide-induced inhibition of succinate- and ascorbate+TMPD-dependent mitochondrial membrane potential ($\Delta\psi_m$) and recovery effect of pyruvate. (**a**) Cyanide inhibitory effect was investigated on succinate-dependent membrane potential (●) (0.5 mg.mL^{-1} of mitochondrial protein, 1 μM rotenone, 10 mM succinate) and on ascorbate+TMPD-dependent membrane potential (○) (0.5 mg.mL^{-1} of mitochondrial protein, 1 μg.mL^{-1} antimycin A, 5 mM ascorbate, 1 mM TMPD). (**b**) Pyruvate-induced recovery of succinate-dependent membrane potential after total deenergization caused by 250 μM KCN was studied in the presence of 0.5 mg.mL^{-1} of mitochondrial protein and 1 μM rotenone (RLM), 10 mM succinate (SUC), and 1 μM FCCP. Membrane potential is plotted as pTPP. (**c**) Pyruvate recovery of succinate- (●) and ascorbate+TMPD-dependent membrane potential (○). In (**a**) and (**c**), data are expressed as the percentage of maximum mitochondrial membrane potential (pTPP) achieved after the energization of mitochondria with indicated substrates

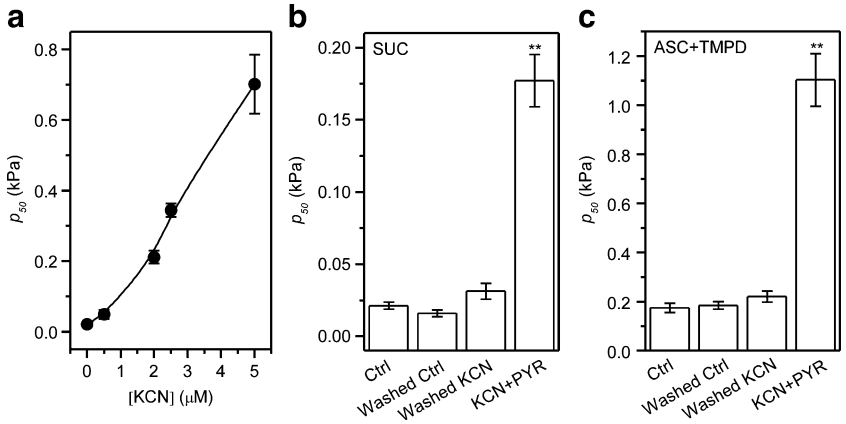

Fig. 5 Cyanide-induced decrease of COX affinity to oxygen demonstrated as an increase of p_{50} values of succinate- and ascorbate+TMPD-dependent respiration and recovery effect of pyruvate. (**a**) KCN-induced increase of p_{50} value, representing a decrease of oxygen affinity (the reciprocal value of p_{50}) of succinate-dependent respiration. The p_{50} values of (**b**) succinate- (0.25–0.30 mg.mL^{-1} of mitochondrial protein, 1 μM rotenone, 10 mM succinate) and (**c**) ascorbate+TMPD-dependent respiration (0.05–0.07 mg.mL^{-1} of mitochondrial protein, 1 μg.mL^{-1} antimycin A, 5 mM ascorbate, 1 mM TMPD) were determined in the untreated mitochondria (Ctrl), in the washed untreated mitochondria (Washed Ctrl), in the mitochondria incubated with 250 μM KCN for 10 min and washed afterwards (Washed KCN) and in the mitochondria treated with 250 μM KCN and 60 mM pyruvate (KCN+PYR). A significant increase of the p_{50} value of KCN+PYR was observed in both succinate- and ascorbate+TMPD-dependent respiration. **, $p<0.01$

by a residual cyanide remaining in the medium and bound to COX. While the reaction of cyanide with pyruvate proceeds rapidly in a basic pH (McMurry 2000), this may not be the case in the neutral incubation medium (pH 7.3, similar to the intracellular pH). To investigate the efficiency of this reaction yielding cyanohydrine, the residual concentration of free cyanide in the incubation medium was determined after oxygraphic measurements. The addition of 60 mM pyruvate decreased the original 250 μM KCN to about 0.8 μM detectable KCN. After washing of mitochondria, the residual KCN concentration was lower than 0.2 μM. Based on the observed effect of KCN on all the functional parameters, such a concentration is too low as to cause any significant decrease of the COX activity in the washed mitochondria (Figs. 2, and 5).

Sensitivity of COX to cyanide

As revealed by our measurements, the functional parameters of COX display different sensitivity to cyanide. In Fig. 6, we compared the cyanide-induced inhibition of the ADP-stimulated respiration, the mitochondrial membrane potential ($\Delta\psi_m$) at state 4 and the oxygen affinity (the reciprocal value of p_{50}). To estimate the possible effects of KCN *in vivo*, we used the succinate-fuelled respiration, which reflects the physiological function of mitochondrial respiratory chain much better than the respiration with the artificial electron donors ascorbate+TMPD. The oxygen affinity decreased tenfold at 2 μM KCN, whereas $\Delta\psi_m$ and electron flux decreased only by 5% and 30%, respectively. Apparently, the oxygen affinity is the most sensitive parameter of COX to the inhibition by cyanide while the proton-pumping activity of COX appears to be the least sensitive.

Discussion

In the last decades, the structural organization of the mammalian COX complex has been described in detail, similarly as its biogenesis and assembly, functional kinetics and regulation (Kadenbach et al. 2000; Ludwig et al. 2001; Hosler et al. 2006; Brzezinski and Gennis 2008). COX was also intensively studied at pathological conditions, in particular in mitochondrial diseases, which are often caused

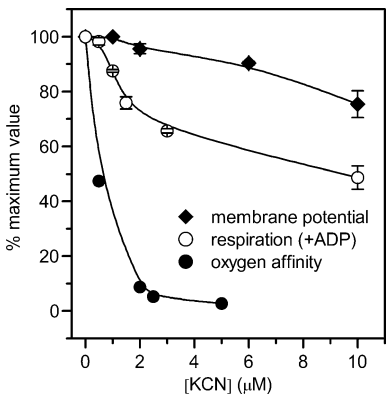

Fig. 6 Sensitivity of COX to low cyanide concentrations. Three parameters of COX function were compared regarding the sensitivity to KCN inhibition—inhibition of electron-transporting function (ADP-stimulated succinate-dependent respiration) (○), proton-pumping function (succinate-dependent membrane potential, pTPP) (♦), and oxygen affinity of succinate-dependent respiration (without ADP stimulation), which is shown as $1/p_{50}$ ratio (●)

by a defective COX due to the mutations of genes encoding its subunits or assembly factors (Shoubridge 2001; Pecina et al. 2004).

The function of COX may be affected by many environmental toxic substances, e.g. cyanide, azide, sulphide, and CO (Cooper and Brown 2008). All these compounds react directly with the COX complex, however, by different mechanisms. The most thoroughly studied inhibitor is cyanide (NaCN or KCN), representing the highest potential risk for humans due to its massive applications in industrial technologies.

The mechanism of KCN interaction with COX is not as simple and clear as proposed originally. For example, a highly complex interaction between cyanide, NO and O_2 was described (Pearce et al. 2003; Leavesley et al. 2008; Pearce et al. 2008) as well as the ability of COX to catalyze the one-electron oxidation of cyanide to cyanyl radical (Chen et al. 1999) and produce nitric oxide under hypoxic conditions (Castello et al. 2006). Better understanding of the mechanisms of COX function and inhibition is essential to design an efficient strategy to protect COX against environmental toxic substances and to recover the inhibited enzyme. The cyanide intoxication in the living organism can be prevented to some extent by several chemical reactions, such as interactions with methemoglobin, thiocyanate, nitrite or keto acids (Baskin and Brewer 1997; Beasley and Glass 1998; Cummings 2004).

In the present study, we analyzed the cyanide inhibition of COX in isolated rat liver mitochondria and the ability of pyruvate to abolish the inhibitory effect of KCN. We used a complex approach that enabled us to investigate in detail the functional consequences of KCN poisoning, by means of two types of model measurements and several functional parameters of COX. Measurements with two different substrates (succinate and ascorbate+TMPD) made it possible to assess the effect of KCN with respect to (i) the function of the complete respiratory chain, as it operates *in situ*, and to (ii) the sole COX, which is the KCN target. As for functional parameters, we followed the electron-transporting activity measured in terms of oxygen consumption, the proton-pumping activity observed via the mitochondrial membrane potential ($\Delta\psi_m$) and the COX affinity to oxygen estimated by as p_{50} for oxygen.

Our results clearly show that all three functional parameters are negatively affected by KCN. The affinity for oxygen displayed the highest sensitivity to the added KCN. It was drastically reduced already at micromolar concentrations of KCN, while the electron transport and $\Delta\psi_m$ were less severely affected under these conditions. This is an important finding because the intracellular and particularly intramitochondrial concentration of oxygen is physiologically very low (Erecinska and Silver 2001), as observed in extensive measurements performed in brain and other tissues. The measurements of true p_{O_2} and its gradients at different functional states of mitochondria seem to be accompanied by technical difficulties. Nevertheless, values of p_{O_2} below 1 kPa were observed in tissues (Cater et al. 1961; Nair et al. 1975), and even lower p_{O_2} values are expected in actively respiring mitochondria where oxygen is rapidly consumed (Wittenberg and Wittenberg 1985; Erecinska and Silver 2001). In connection with the KCN-induced decrease of oxygen affinity (p_{50} of 0.2–0.7 kPa at 2–5 μM KCN (Fig. 5a)), this implies that KCN poisoning is very dangerous already at very low concentrations.

According to Gnaiger et al. (Gnaiger et al. 1998a), the "catalytic efficiency" (J_{max}/p_{50}) is a more relevant parameter than p_{50} itself because cells can compensate a large decrease in oxygen affinity in the active state by an increase in J_{max}, so that J_{max}/p_{50} does not change markedly. In our measurements, the low KCN concentrations did not affect the electron flux profoundly indicating that the "catalytic efficiency" was decreased similarly to the oxygen affinity ($1/p_{50}$). The high sensitivity of p_{50} also means that it would be the best parameter to determine the effects of KCN and p_{50} may be of practical use for measurements of KCN poisoning.

The lower sensitivity of electron transport and $\Delta\psi_m$ as compared with oxygen affinity indicates that these parameters could be affected consequently within the mechanism of COX inhibition by KCN. Although $\Delta\psi_m$ was the least affected, it does not necessarily mean that the H^+-transporting activity is less sensitive than the electron transport. The mitochondrial membrane potential is a parameter that reflects the H^+ pumping as well as the "leak" of the mitochondrial membrane and is also affected by mitochondrial transporters and various ligands. In addition, the relationship between $\Delta\psi_m$ and H^+ transport across the inner mitochondrial membrane is nonlinear (Nicholls 1977).

Our measurements included extensive washing, which removed practically all added KCN. Here, the effect of KCN on all measured parameters showed it to be fully reversible, and even the affinity to oxygen, represented by p_{50}, was entirely restored. In contrast, the reactivation of COX function by the added pyruvate was efficient but not complete, which was most apparent in the case of p_{50}. Despite reversibility of the KCN effect, pyruvate could not fully abolish the effects of KCN, although more than a 250-fold excess of pyruvate over KCN was used. It can be concluded that the interaction of keto acids with KCN resulting in the cyanohydrine formation cannot fully eliminate the COX inhibition. Cyanohydrine may have an inhibitory effect on COX as well, but no difference in the respiration rate was observed after the addition of mitochondria to the mixture of 60 mM pyruvate and 250 μM KCN (data not shown). Therefore, the reaction of pyruvate

with KCN added to the incubation medium containing isolated mitochondria cannot be completed and some residual KCN bound to COX is apparently responsible for the persisting decrease of COX functions. The inhibition constant K_i for cyanide binding to isolated COX has been shown to vary between 0.1 and 1.0 μM (Nicholls et al. 1972), in agreement with the observed residual KCN after pyruvate addition.

The results demonstrate a lower KCN sensitivity of all measured parameters when using succinate as the respiratory substrate, compared to ascorbate+TMPD. And, the other way round, the reactivation effect of pyruvate was more pronounced when the activity of the whole respiratory chain was compared with the sole activity of COX. This generally lower sensitivity to KCN of the respiratory chain function compared to the COX function is most likely caused by the threshold effect of COX. An excess capacity of COX was found in comparison to the other complexes of the mitochondrial respiratory chain (Gnaiger et al. 1998a; Rossignol et al. 2000; Piccoli et al. 2006) indicating that a decreased COX activity is still sufficient for a high rate of succinate-dependent respiration.

The excess capacity of COX in the mitochondrial respiratory chain may thus to some extent compensate for the toxic effects of KCN *in vivo* and hence appears as a natural means of defense of the most important energetic pathway of mammalian cells against toxic agents acting at the level of COX. Nevertheless, even the combination of this mechanism and the effect of pyruvate (observed at its almost 250-fold excess over the added KCN) are insufficient to recover the COX function entirely.

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