Impact of Modulators of Mitochondrial ATP-Sensitive Potassium Channel (mitoK$_{ATP}$) on Hypoxic Pulmonary Vasoconstriction

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Abstract Previously, we demonstrated that hypoxic pulmonary vasoconstriction (HPV) of intra-acinar arteries (IAA) requires mitochondrial complex II (= succinate dehydrogenase, SDH) activity (Paddenberg et al., Respir Res, 7:93, 2006). Interestingly, SDH subunits A and B have recently been described as components of a multiprotein mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$), together with mitochondrial ATP-binding cassette protein-1, adenine nucleotide translocator (ANT), ATP synthase, and phosphate carrier (Ardehali et al., Proc Natl Acad Sci USA, 101(32):11880–5, 2004). Hence, we tested the hypothesis that such an SDH-containing mitoK$_{ATP}$ is involved in HPV. For this purpose, the impact of modulators of mitoK$_{ATP}$ on HPV of IAA was studied videomorphometrically in precision cut murine lung slices. Inhibitors of mitoK$_{ATP}$ (glibenclamide, 5-hydroxydecanoate) completely suppressed HPV, mitoK$_{ATP}$ activators (pinacidil, diazoxide) even induced vasodilatation, and ANT inhibitors (bongkrekic acid, atractyloside) attenuated HPV. This pharmacological profile differs clearly from that described for mitoK$_{ATP}$. Accordingly, co-immunoprecipitation experiments provided no evidence for association of complex II subunits SDH-A, -B and -C with ANT, ATP synthase or cytochrome c oxidase in murine heart mitochondria. Hence, it is likely that the inhibitory effects on HPV that we observed in our experiments result from modulation of several mitochondrial protein complexes independently involved in the signalling cascade such as ROS-producing complex II and ANT-regulated mitochondrial permeability transition pore.

Keywords Complex II · Hypoxia · Hypoxic pulmonary vasoconstriction HPV · Intra-acinar arteries · Mitochondrial ATP-sensitive potassium channel mitoK$_{ATP}$ · Respiratory chain · Succinate dehydrogenase SDH · Oxygen sensing

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1 Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a local reflex directing the blood flow from poorly to well oxygenated regions of the lung thereby matching perfusion to ventilation. In search of the underlying molecular mechanisms of oxygen sensing we observed an essential role of complex II (= succinate dehydrogenase, SDH) of the mitochondrial respiratory chain (Paddenberg et al. 2006). According to work published by Ardehali et al. (2004), SDH is part of a macromolecular supercomplex located in the inner mitochondrial membrane which possesses potassium channel activity. Based on its pharmacological profile, it has been identified as mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) (Ardehali et al. 2004). Interestingly, this channel also participates in another oxygen-regulated process, i.e. hypoxic preconditioning (Gross and Fryer 1999). Ardehali et al. co-immunoprecipitated at least 5 proteins from highly purified fractions of inner membranes of rat liver mitochondria: Adenine nucleotide translocator (ANT), ATP synthase, phosphate carrier, ATP-binding cassette protein-1 and SDH subunits A and B. In proteoliposomes containing this multiprotein complex, potassium transport was increased by mitoK_{ATP} activators (diazoxide, pinacidil), and reduced by its blockers (5-hydroxydecanoate, glibenclamide), SDH-inhibitors (malonate, 3-nitropropionic acid) and ATP. Inhibitors of ANT (atractyloside, bongkrekic acid) had no impact on channel activity.

Here, we analysed whether SDH is also a component of such a multiprotein mitoK_{ATP} complex in cardiovascular mitochondria, and whether its pharmacological modulation interferes with HPV.

2 Methods

2.1 Videomorphometry of IAA

HPV of IAA was estimated by videomorphometric analysis of murine precision cut lung slices (PCLS) as described earlier (Paddenberg et al. 2006). The hypoxic response of individual arteries with inner diameters between 20 and 30 μm was recorded as changes in the luminal area. The values obtained at the beginning of the experiments were set as 100%, and vasoreactivity was expressed as relative decrease or increase of these areas. Data are presented as means $\pm$ standard error of the mean (SEM). A single vessel per PCLS was analysed.

For statistical analysis of the impact of various drugs on HPV, the values obtained immediately before exposure to hypoxic gassed medium $\pm$ drug were set as 100% (not shown) and the values of corresponding time points were analyzed using SPSS 15.0. The Kruskal-Wallis- followed by the Mann-Whitney-test was performed to compare the means of the different experimental groups. The threshold for significance was set at $p \leq 0.05$.

2.2 Isolation of Mitochondria from Murine Heart

Isolation of mitochondria from murine hearts was performed by a combination of differential centrifugation and sucrose density gradient centrifugation as described in "Isolation of Mitochondria Manual" from MitoSciences (www.mitosciences.com/PDF/mitos.pdf).

2.3 Co-immunoprecipitation of Mitochondrial Proteins and Western Blotting

Co-immunoprecipitation experiments employing extracts of murine heart mitochondria were performed with the Complex II Immunocapture Kit (MitoSciences, Oregon, USA) consisting of protein G-agarose beads conjugated with complex II specific or unspecific (control) antibodies. Eluates of the beads (= pellets) and corresponding supernatants were analysed by Western blotting. As primary antibodies we used mouse anti-OxPhos Complex II 70 kDa (= SDH-A; Molecular Probes/Invitrogen, Karlsruhe, Germany), mouse anti-OxPhos Complex II 30 kDa (= SDH-B; Molecular Probes/Invitrogen), mouse anti-complex V subunit α (= ATP synthase; Molecular Probes/Invitrogen), mouse anti-cytochrome C oxidase subunit IV (Abcam, Cambridge, UK), goat anti-ANT (Santa Cruz, Heidelberg, Germany), as well as an own rabbit anti-SDH-C antibody obtained by immunization against a peptide consisting of amino acids 29-52 of SDH-C. As secondary antibodies HRP-conjugated anti-mouse IgG, anti-goat IgG or anti-rabbit IgG were used (all from Pierce/Perbio Science Deutschland GmbH, Bonn, Germany). Bound antibodies were visualized by enhanced chemiluminescence.

3 Results

3.1 Impact of mitoK$_{ATP}$ Modulators on HPV

The use of PCLS facilitates the investigation of HPV of small IAA. Exposure of the vessels to hypoxic gassed medium induced a distinct reduction of the luminal area (Fig. 1) whereas in normoxic gassed medium the areas were unchanged (not shown). Application of the SDH inhibitor malonate completely suppressed HPV whereas the U46619-induced contraction was unaffected (Fig. 1A). For analysis of the impact of classical modulators of ATP-sensitive potassium channels on HPV, we applied blockers and openers which were either specific for the mitochondrial channel or non-selective in that they modulate both surface and mitochondrial channels. The mitoK$_{ATP}$-specific blocker 5-hydroxy – decanoate (Fig. 1B) and the non-selective inhibitor glibenclamide (not shown) completely suppressed HPV whereas the response to U46619 was unchanged. Application of the mitochondrial channel

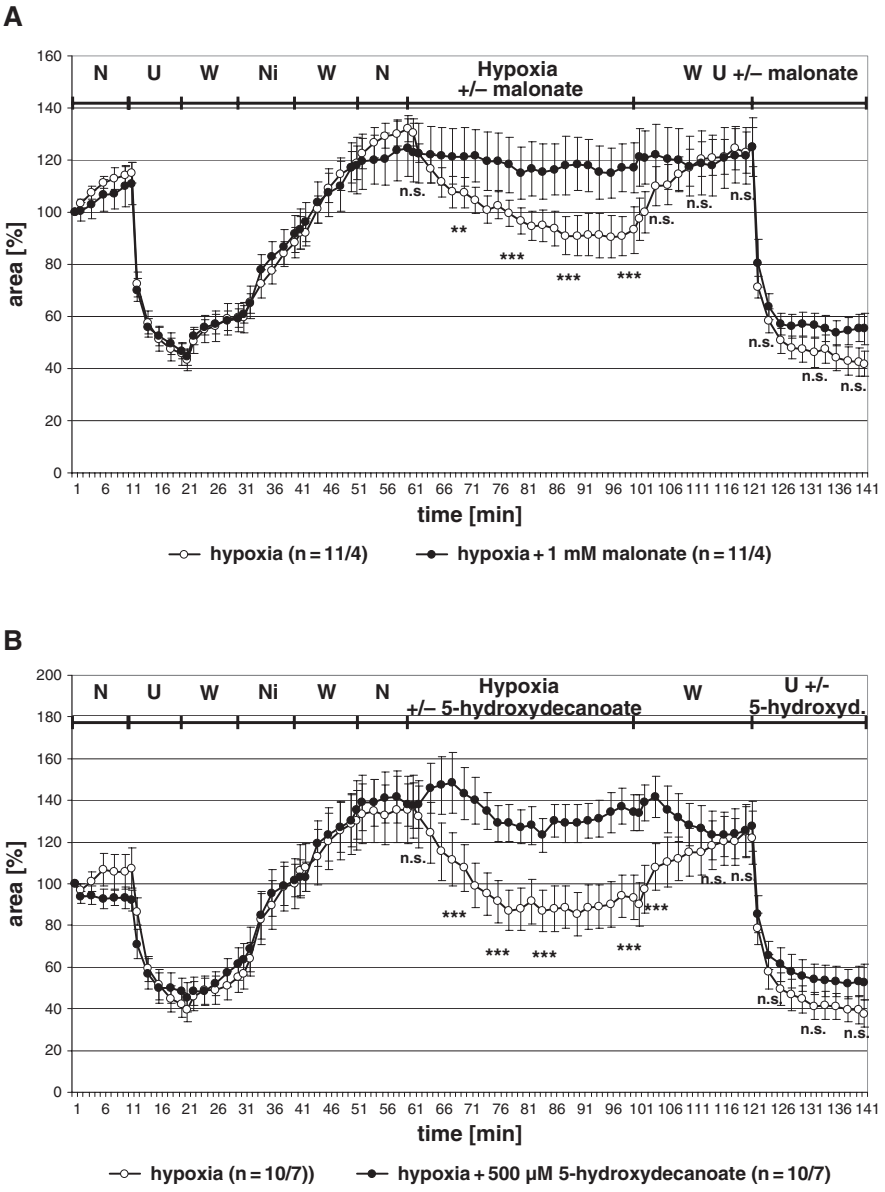

Fig. 1 Videomorphometric analysis of the impact of modulators of mitoK$_{ATP}$ on HPV of IAA. For adaptation to the chamber, PCLS were incubated with normoxic medium (N). The viability of the arteries was assessed by the successive application of the vasoconstrictor U46619 (U) and the vasodilatator nipruss (Ni). After washing out of the drugs (W), PCLS were incubated with hypoxic medium (pregassed with 1% O_2) alone or supplemented with modulators of mitoK$_{ATP}$. At the end of each experiment the specificity of the impact of the drug for HPV was investigated by its simultaneous application with U46619. Drugs were added at the concentrations given in the legends of the individual graphs. At the given time points the differences between both groups were tested for significance. n.s.: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

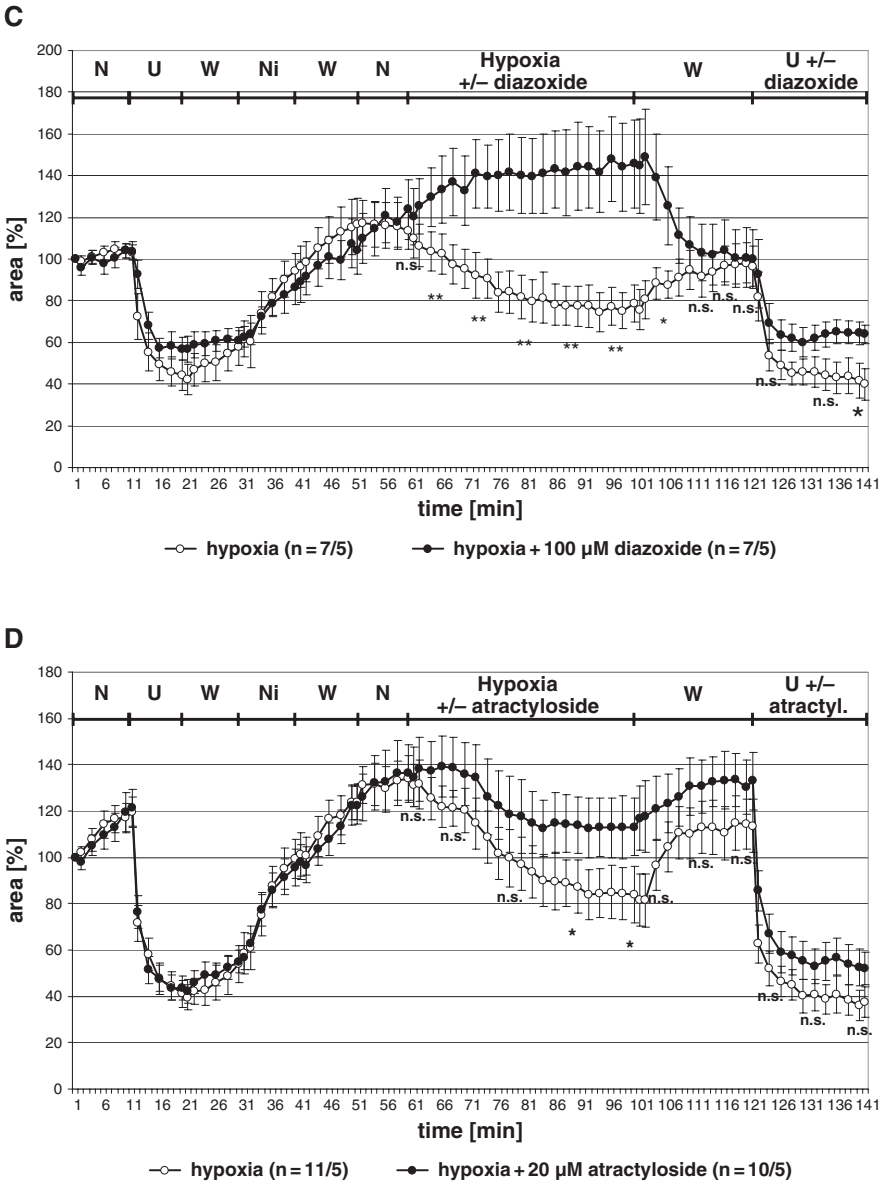

Fig. 1 (continued)

specific activators diazoxide (Fig. 1C) or the non-selective opener pinacidil (not shown) resulted not only in suppression of HPV but even induced vasodilatation. Again, the effects were specific for HPV in that U46619-induced vasoconstriction was unchanged. Finally, we tested the impact of ANT inhibitors on HPV.

Atractyloside (Fig. 1D) and bongkrekic acid (not shown) induced a significant reduction and inhibition of HPV, respectively.

3.2 Co-immunoprecipitation of Mitochondrial Proteins

To clarify whether in the murine cardiopulmonary system SDH is part of a multiprotein complex we performed co-immunoprecipitation experiments on extracts of isolated murine heart mitochondria. Both the precipitates (= pellet) and the corresponding supernatants were analysed by Western blotting (Fig. 2). SDH-A-immunoreactivity was clearly present in the pellet obtained with the beads conjugated with complex II specific antibodies whereas it was absent when beads coupled with unspecific antibodies were used. In addition, both supernatants contained SDH-A (Fig. 2A). Comparable results were obtained for SDH-C (Fig. 2C). Distinct SDH-B-immunoreactivity was precipitated employing specific beads whereas a very weak band was detectable when control beads were used (Fig. 2B). In the corresponding supernatants SDH-B was detectable only after longer exposure of the x-ray films (not shown). Immunoreactivity for cytochrome C oxidase – which is not expected to be part of the multiprotein complex – was detectable exclusively in the supernatants but not in the pellets (Fig. 2D). To evaluate whether the postulated multiprotein complex exists in mouse heart mitochondria we analysed the immunoprecipitates for the presence of ATP synthase and ANT. In both cases we were able to detect the proteins in the supernatants, but not in the pellets (Fig. 2E, F).

4 Discussion

Here, we demonstrate that HPV of IAA can be inhibited by several modulators of the postulated mitoK_{ATP}. The pharmacological profile of HPV inhibition, however, differs markedly from that of mitoK_{ATP} modulation. For instance, both 5-hydroxydecanoate and diazoxide potently inhibited HPV whereas they have contrasting effects on mitoK_{ATP} (Ardehali et al. 2004). Thus, HPV is not triggered by simple opening or closure of a multiprotein mitoK_{ATP} in the composition suggested by Ardehali et al. (2004). According to these functional data, our co-immunoprecipitation experiments provide no evidence for interaction of SDH-subunits with ANT and ATP synthase in mitochondria isolated from the cardiovascular system. Hence, it is likely that the inhibitory effects on HPV that we observed in our experiments are not caused by targeting one and the same multiprotein complex by all inhibitors. Instead, they may result from modulation of several, independently involved mitochondrial protein complexes such as ROS-producing complex II (Paddenberg et al. 2006; Guzy et al. 2008) and ANT-regulated mitochondrial permeability transition pore (Leung and Halestrap 2008).

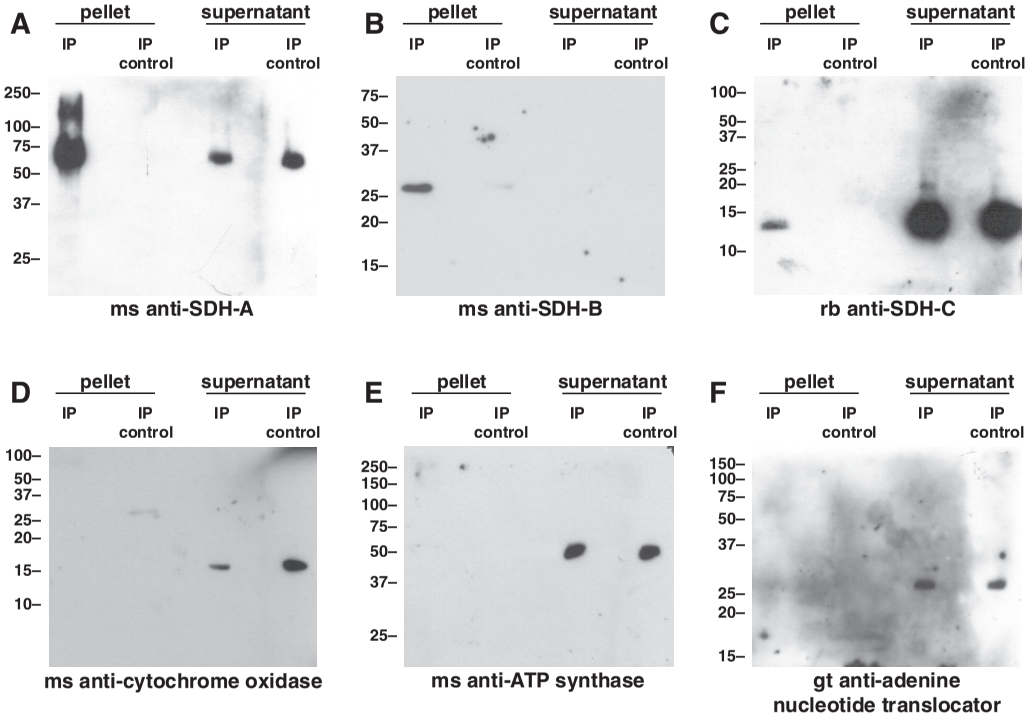

Fig. 2 Co-immunoprecipitation experiments using protein G agarose beads conjugated with complex II specific (IP) or unspecific (control IP) antibodies on mitochondrial extracts. The precipitates (= pellet) and the corresponding supernatants were analysed by Western blotting using antibodies as given in the legends of the individual blots. Molecular weight markers in kDa are given on the left of the blots. ms: mouse, rb: rabbit, gt: goat. Comparable results were obtained in at least 3 independent co-immunoprecipitation experiments

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