Myocardial Impairment in Chronic Hypoxia Is Abolished by Short Aeration Episodes: Involvement of K^+_{ATP} Channels

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***In vivo* exposure to chronic hypoxia is considered to be a cause of myocardial dysfunction, thereby representing a deleterious condition, but repeated aeration episodes may exert some cardioprotection. We investigated the possible role of ATP-sensitive potassium channels in these mechanisms. First, rats (n = 8/group) were exposed for 14 days to either chronic hypoxia (CH; 10% O_2) or chronic hypoxia with one episode/day of 1-hr normoxic aeration (CH+A), with normoxia (N) as the control. Second, isolated hearts were Langendorff perfused under hypoxia (10% O_2, 30 min) and reoxygenated (94% O_2, 30 min) with or without 3 μM glibenclamide (nonselective K^+_{ATP} channel-blocker) or 100 μM diazoxide (selective mitochondrial K^+_{ATP} channel-opener). Blood gasses, hemoglobin concentration, and plasma malondialdehyde were similar in CH and CH+A and in both different from normoxic ($P < 0.01$), body weight gain and plasma nitrate/nitrite were higher in CH+A than CH ($P < 0.01$), whereas apoptosis (number of TUNEL-positive nuclei) was less in CH+A than CH ($P < 0.05$). During *in vitro* hypoxia, the efficiency (ratio of ATP production/pressure × rate product) was the same in all groups and diazoxide had no measurable effects on myocardial performance, whereas glibenclamide increased end-diastolic pressure more in N and CH than in CH+A hearts ($P < 0.05$). During reoxgenation, efficiency was markedly less in CH with respect to N and CH+A ($P < 0.0001$), and rate × pressure product remained lower in CH than N and CH+A hearts ($P < 0.001$), but glibenclamide or diazoxide abolished this difference. Glibenclamide, but not diazoxide, decreased vascular resistance in N and CH ($P < 0.005$ and < 0.001) without changes in CH+A. We hypothesize that cardioprotection in chronically hypoxic hearts derive from cell depolarization by sarcolemmal K^+_{ATP} blockade or from preservation of oxidative phosphorylation efficiency (ATP turnover/myocardial performance) by mitochondrial K^+_{ATP} opening. Therefore K^+_{ATP} channels are involved in the deleterious effects of chronic hypoxia and in the cardioprotection elicited when chronic hypoxia is interrupted with short normoxic aeration episodes.** Exp Biol Med 229:1196–1205, 2004

Key words: hypoxia; glibenclamide; diazoxide; reoxygenation; apoptosis

Introduction

Chronic myocardial hypoxia is a common feature in several diseases including stroke, infarction, anemia, obstructive lung diseases, and cyanotic congenital heart defects. In clinical settings, chronic hypoxia is perceived as a hazardous condition, but many experimental studies demonstrated that the chronically hypoxic heart is more tolerant to ischemia than the normoxic heart (1–7). It has been shown that the increased tolerance exhibited by hypoxic hearts is associated with the activation of the ATP-sensitive potassium (K^+_{ATP}) channels (4, 8). The hypothesized mechanism involves hypoxia-driven ATP depletion, K^+_{ATP} channel activation, and reduced Ca^{2+} overload. Cardiomyocytes contain two distinct isoforms of the K^+_{ATP} channel: sarcolemmal ($sarcK^+_{ATP}$) and mitochondrial ($mitoK^+_{ATP}$). Although there is strong evidence for a role of both channels as triggers in ischemic preconditioning (9), hypoxia-driven protection is more controversial. On the one hand, cardioprotection in chronically hypoxic hearts was shown to need the contribution of both types of channels (10). On the other hand, activation of $mitoK^+_{ATP}$ specifically increases protection in intermittently hypoxic hearts (11). In addition, in a Kir6.2-knockout mice model, protection is induced by activating $sarcK^+_{ATP}$ rather than $mitoK^+_{ATP}$ (12).

Recently, we developed a model whereby hearts from chronically hypoxic rats (2 weeks at 10% O_2) display impaired performance recovery after reoxygenation (13,

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14), in agreement with the dominant clinical observations (15). In contrast, hearts from hypoxic rats exposed to periodical normoxic aeration episodes (1-hr daily exposure to room air) become tolerant against the same episode of reoxygenation (13). This indicates that repeated, albeit brief, episodes of normoxic aeration in hypoxic hearts induce cardioprotection, but the underlying mechanism is still elusive, although a role for reactive O_2 species (ROS) was envisaged (13). Therefore, the aim of this study is to investigate the roles that sarcK^+_{ATP} or mitoK^+_{ATP} channels could play in myocardial deterioration in chronic hypoxia or protection in chronic hypoxia with short periodic normoxic aeration episodes.

This study is, therefore, composed of two phases (Fig. 1). In the *in vivo* phase, rats were exposed to either chronic hypoxia (CH) or chronic hypoxia with normoxic aeration (CH+A), with a group of normoxic rats (N) as control. To assess oxidative stress and myocardial response to hypoxia, we measured plasma malondialdehyde (MDA) and nitrates–nitrites (NOx), as well as myocardial apoptosis. In the *in vitro* phase, all hearts (including those from the N group) were subjected to hypoxic perfusion followed by oxygenation (CH) or reoxygenation (CH+A and N) while observing performance with or without either glibenclamide, a nonselective sarcK^+_{ATP} channel blocker, or diazoxide, a mitoK^+_{ATP} channel opener, with vehicle-perfused hearts as control. The goal of the hypoxic perfusion is to assess the response of CH hearts to K^+ channel manipulation during their first (after 2 weeks) oxygenation as compared to that of CH+A hearts during their sixteenth reoxygenation episode.

Materials and Methods

***In vivo* Phase: Exposure to Chronic and Chronic Hypoxia with Normoxic Aeration.** The design of the hypoxic chamber, which prevents exposure of the animals to room air during feeding, cleaning operations, and killing has been described in detail elsewhere (13, 14). Briefly, the chamber is equipped with a window with a plastic sleeve, whereas the compensation chamber has two windows in the facing walls. Both chambers are flushed with hypoxic gas. Before opening the hypoxic chamber, the compensation chamber is placed over the hypoxic one with the windows adjacent one to the other. The operator inserts her or his arm through the sleeves in the compensation chamber and in the hypoxic chamber. For killing, the animal is transferred into the compensation chamber, the windows are closed, and the compensation chamber is moved near the perfusion apparatus. The anesthetized rat is immobilized over the dissection table, the chest is opened, a ligature is passed around the aorta, and the heart is excised and dropped into a beaker, which is rapidly taken outside the chamber. The aorta is immediately mounted on the apparatus, and the heart is perfused with the hypoxic medium. With the described technique, the animals remain continuously exposed to 10% O_2, avoiding any unwanted exposure to room air.

We used male, 5-week-old Sprague-Dawley rats (weight = 257 ± 2 g). N rats breathed room air (21% O_2) for 2 weeks. CH rats breathed a normobaric hypoxic (10% O_2) mixture. CH+A rats were subjected to the same treatment as CH rats but were exposed to room air for 1 hr/day. All rats had free access to water and a conventional laboratory diet containing 90 mg/kg α-tocopherol until 24 hr before death. Every other day, CH rats were transferred into the compensation chamber (allowing for a continuous exposure to 10% O_2) for weighing and chamber cleaning, whereas CH+A rats remained in their chamber, opened for 1 hr/day.

At the end of the *in vivo* phase, all rats were killed, the normoxic (N) were exposed to room air, while the hypoxic (CH and CH+A) were killed under 10% O_2 by intraperitoneal injection of Na-thiopental (10 mg/100 g body weight) plus heparin and randomly assigned either to one of

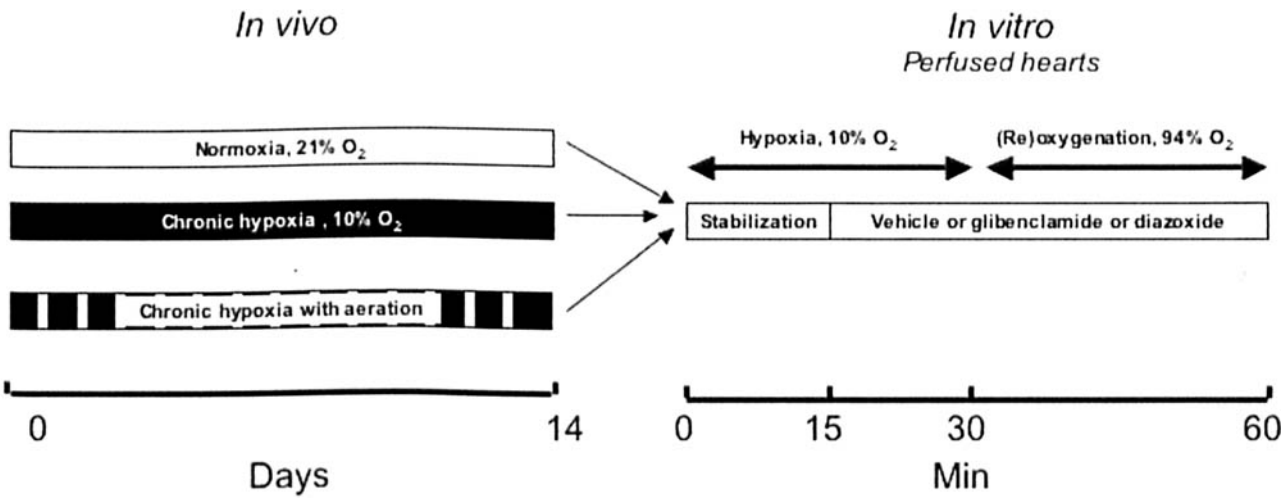

Figure 1. The *in vivo* and *in vitro* phases of the experimental protocol.

the *in vitro* phase groups (see following) or to heart freeze-clamp. The rats in the group CH+A were killed 24 hr following last normoxic aeration. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996).

In vitro Phase: Myocardial Performance. After anesthesia, the animal was dissected under 10% O_2, and the heart was excised and immersed in a beaker containing de-aerated isotonic saline at 25°C. The beaker was taken out of the chamber, the aorta immediately cannulated on the perfusion system, and the heart perfused at 37°C with hypoxic (10% O_2) Krebs–Henseleit buffer. The time interval between heart excision and mounting on the Langendorff perfusion system was less than 90 sec.

The fixed-flow (15 ml/min) perfusion apparatus is described elsewhere (13). The gas (Carbagas, Lausanne, Switzerland) flowing through the membrane microoxygenator (Dideco, Mirandola, Italy) contained O_2, CO_2, and N_2, in the proportions 10/6/84 or 94/6/0, for the hypoxic perfusion and (re)oxygenation, respectively (nominal accuracy, ±0.1). The temperature of the medium (Krebs–Henseleit buffer with 2.0 m*M* free Ca^{++} and 11 m*M* glucose, pH 7.33 ± 0.01) and heart was 37°C. A latex balloon in the left ventricle was connected to a pressure transducer (MPC-500, Millar Instruments Inc., Houston, TX) to monitor performance. An additional transducer above the aortic cannula monitored the coronary perfusion pressure (CPP). The venous return was collected through a cannula in the pulmonary artery to measure venous P_{O_2} (model 5300 Oxygen Monitor, Yellow Springs Inc., Yellow Springs, OH) and lactate.

Hearts were stabilized for 15 min at P_{O_2} = 67 mmHg. During this period, the balloon volume was set to end-diastolic pressure (EDP) = 10 mmHg. Afterward, the balloon volume was kept constant. At the end of the hypoxic stabilization, with stable performance, the medium was switched to vehicle (Krebs–Henseleit plus 0.01% dimethylsulfoxide, DMSO), glibenclamide (3 μ*M* glibenclamide in 0.01% DMSO), or diazoxide (100 μ*M* diazoxide in 0.01% DMSO, *n* = 8/subgroup). Hearts underwent further 15-min hypoxic perfusion and were then subjected to 30 min reoxygenation (N and CH+A) or oxygenation (CH) at P_{O_2} = 670 mmHg. Performance was monitored continuously, but for clarity we report data for every 5 min.

We measured EDP, heart rate (HR), left-ventricle developed pressure (LVDP = difference between peak systolic pressure and EDP), maximal rate of pressure development ($+dP/dt_{max}$) and relaxation ($-dP/dt_{max}$), CPP, and O_2 consumption (V_{O_2}). MV_{O_2} was calculated with the following formula: (arterial P_{O_2} − venous P_{O_2}) × 0.0014 (solubility coefficient of O_2 in H_2O at 37°C) × coronary flow. The rate × pressure product (RPP; i.e., LVDP × HR) was used as a performance index. The coronary vascular resistance (CVR) was calculated as (CPP − EDP)/flow/(ventricle weight) (16), thereby accounting for different degrees of cardiac hypertrophy in normoxic and hypoxic hearts (17). Samples of the venous effluent were taken at *t* = 15, 30, and 60 min and were frozen at −80°C for lactate assay (COBAS FARA II, Hoffman-La Roche, Basel, Switzerland). The lactate release (J_{lac}) was calculated from lactate concentration and coronary flow. When assuming glucose as the only oxidizable substrate and negligible contribution from glycogen, the anaerobic and aerobic contributions to ATP production (J_{ATP}) are calculated as (J_{lac} × 1) and (V_{O_2} × 6), respectively (18).

Other Measurements. In additional experiments (*n* = 4/group), a blood sample was collected anaerobically from the femoral artery into heparinized syringes. After immediate gas analysis measurement (ABL700 Analyzer, Radiometer, Denmark), an aliquot was taken for hemoglobin concentration determination (Abbott Cell-dyn 3500 R System, Baar, Switzerland). The remaining part was centrifuged, and plasma was frozen for analysis of MDA and NOx.

Plasma MDA concentration was determined according to (19). Thawed plasma (100 μl) was mixed with 0.25 *M* NaOH (25 μl), and the mixture was vortexed, incubated at 60°C for 30 min, cooled, and centrifuged (14,000 rpm for 5 min). The supernatant (100 μl) was mixed with 10 *M* HCl (10 μl), 2.5 m*M* 2,4 di-nitrophenyl-hydrazine dissolved in 1 *M* HCl (50 μl) and the internal standard 2-nitroresorcinol (0.967 m*M* in 0.01 *M* HCl, 20 μl). The mixture was vortexed and incubated for 1 hr at room temperature in the dark. After centrifugation (14,000 rpm for 5 min), the supernatant was filtered (0.22 μm GV-Cat SJGVL04NS, Millipore, Bedford, MA) and injected (20 μl) in a high-performance liquid chromatography apparatus (Kontron Instruments, Milan, Italy) composed of a pump and a ultraviolet/Vis spectrophotometer set at λ = 310 nm. The column (250 × 4.6 mm; Hypersyl ODS-5, Sigma-Aldrich, St. Louis, MO) was eluted with a mixture containing acetonitrile and 0.01 *M* HCl (45:55) at flow = 1.5 ml/min. The internal standard and the adduct formed in the reaction of 2,4 dinitrophenyl-hydrazine, with MDA eluted 3.1 and 5.9 min after the solvent peak, respectively. MDA concentration was calculated from the ratio between the areas of the corresponding peak and that of the internal standard. The calibration curve was linear (R^2 = 0.992, SE = 0.0387) until 0.07 μ*M*.

NOx was measured by enzymatic catalysis coupled with the Griess reaction (20). Nitrate reductase converts nitrates into nitrites, which were reacted with 0.1% naphthylethylenediamine, which yields a colored dye monitored by spectrophotometry at λ = 540 nm. NOx concentration was calculated against a calibration curve.

The degree of apoptosis was measured in the left ventricle of hearts (*n* = 4/group) that were not subjected to perfusion but were rapidly excised, frozen in liquid N_2, and stored at −80°C. Five-micrometer-thick sections (four to six per each sample) were obtained (Cryomicrotome Leica CM1510, Nussloch, Germany), placed on glass slides, and

fixed in 4% formalin for 45 min at 4°C. DNA fragmentation was measured by the terminal deoxynucleotidyl transferase–mediated dUTP-rhodamine nick end labeling (TUNEL) by ApopTag Red In Situ Apoptosis detection kit (Intergen, Oxford, UK). The slides were examined in an inverted fluorescence microscope (Axiovert 25 CFL, Carl Zeiss, Göttingen, Germany), equipped with 40× objective and filters for detection of rhodamine (filter set 15, excitation BP 546/12, emission LP 590). The images were acquired by a color video camera (AxioCam czv CD 4.0, Zeiss, Göttingen, Germany) and stored in a PC. To quantify TUNEL-positive nuclei, the image was analyzed (NIH Image) by selecting five random fields (0.2 mm^2 each) for each section and averaging the results. The red slice was obtained, the number of red spots was counted, and the procedure was checked against a manual method. Data are reported as number of TUNEL-positive nuclei per unit section area after subtracting the negative control background count obtained for each slide.

Statistics. Data are expressed as mean ± SEM. Treatment effects were considered significant where significance level was $P = 0.05$ (two-tailed). To detect significant differences among the three groups, we first performed one-way ANOVA. If they were significant, the differences between selected pairs of data were tested using the Fischer comparison procedure (StatView, Abacus Concepts, Berkeley, CA).

Results

Hypoxia in vivo. In rats exposed to either CH or CH+A, the body weight gain was always slower than in N ($P < 0.0001$; Fig. 2A). However, CH rats experienced an even slower weight gain than CH+A ($P < .0001$). Arterial Po_2 was less in both hypoxic groups than in N ($P < 0.0001$, Figure 2B), whereas blood hemoglobin concentration ($P < 0.0001$) and plasma MDA ($P < 0.004$) were higher in both CH and CH+A than in N, without differences between the two hypoxic groups. In contrast, NOx, although higher in both hypoxic groups than in N ($P < 0.0001$), was markedly increased in CH+A with respect to CH ($P = 0.035$).

Apoptosis. The number of TUNEL-positive nuclei (Fig. 2B) increased in CH with respect to N ($P = 0.002$). This increase had a borderline significance ($P = 0.06$) for CH+A hearts, but the difference between CH and CH+A was always significant ($P = 0.05$).

Functional Parameters of Untreated Hearts during Hypoxia. To test the stability of the preparation, the changes in LVDP, HR, and EDP in normoxic hearts ($n = 6$) perfused for 2 hr under aerobic conditions was less than 8.3 ± 4.3 mmHg, less than 22 ± 10 min^{-1}, and less than 1.2 ± 0.9 mmHg, respectively. As none of the hearts died during the experiments, all hearts ($n = 8$/subgroup) could be used for statistic analysis. In this preparation, increases in EDP mark the onset of diastolic stiffness because the volume of the intraventricular balloon is kept constant throughout the experiment, RPP is an integrated index of the contractility, and the changes in CPP indicate alterations in CVR, as the flow through the coronary bed was kept constant by a pump.

Table 1 shows performance during hypoxic stabilization before manipulating K^+ channels. There were no detectable differences in EDP, HR, and MVo_2. Higher HR compensated for the slight LVDP depression in CH, so RPP was the same in all groups. CVR ranked N > CH > CH+A (ANOVA $P < 0.0001$). During hypoxic perfusion, venous Po_2 was always very low, indicating that hearts use up all available O_2. However, venous lactate was markedly depressed in CH and CH+A. As a consequence, J_{ATP} was less in both CH and CH+A.

Effect of K^+ Channel Modulators on Myocardial Performance during Hypoxia. Figure 3 shows the time courses of EDP, RPP, CVR, O_2 consumption, and lactate production during either $sarcK^+_{ATP}$ channel blocking by glibenclamide or $mitoK^+_{ATP}$ channel opening by diazoxide.

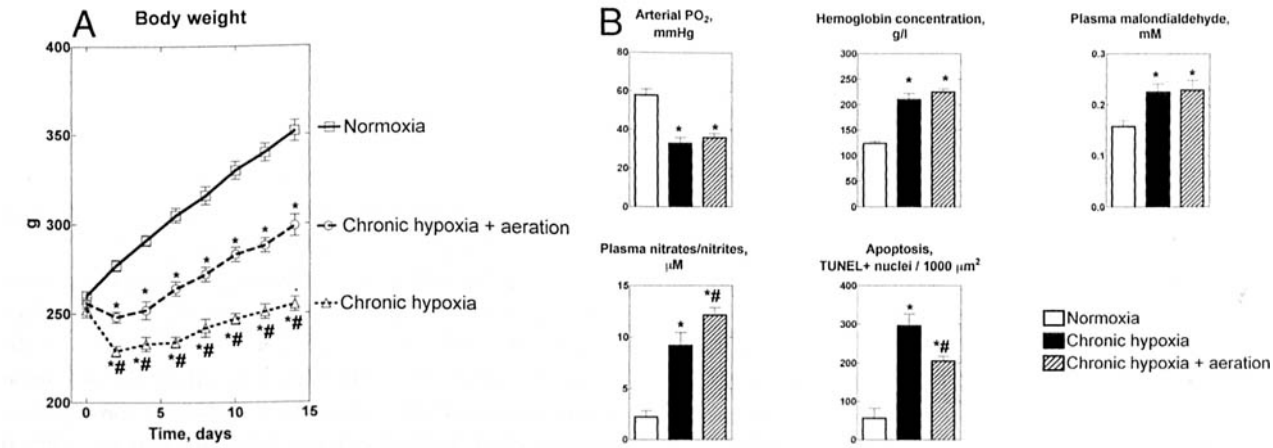

Figure 2. (A) Body weight (mean ± SEM) in rats exposed to normoxia, chronic hypoxia, and chronic hypoxia with aeration ($n = 8$/group). One-way ANOVA: * = $P < 0.001$ vs. normoxia, # = $P < 0.001$ vs. chronic hypoxia. (B) Arterial O_2 tension, blood hemoglobin concentration, plasma malondialdehyde, nitrates + nitrites, and TUNEL-positive cells in left ventricle ($n = 4$/group). One-way ANOVA: $P < 0.0001$, $P < 0.0001$, $P = 0.0034$, $P < 0.0001$, and $P = 0.006$, respectively; * = $P < 0.05$ vs. normoxia; # = $P < 0.05$ vs. chronic hypoxia (Fisher's posttest).

Table 1. Myocardial Parameters after Stabilization under Hypoxic Perfusion (Mean ± SEM) in the Absence of Drugs in Hearts from Normoxic, Chronic Hypoxic, and Chronic Hypoxic with Aeration Animals (coronary flow = 15 ml/min, Pao_2 = 67 mmHg)

	Hearts from group		
	Normoxia	Chronic hypoxia	Chronic hypoxia with aeration
N	24	24	24
Heart rate, min^{-1}	146 ± 9	144 ± 10	128 ± 11
End-diastolic pressure, mmHg	9.9 ± 0.2	9.8 ± 0.1	9.6 ± 0.1
$+dp/dt_{max}$, mmHg/s	1926 ± 87	1630 ± 134	2114 ± 98[b]
$-dp/dt_{max}$, mmHg/s	737 ± 45	612 ± 40[a]	783 ± 45[b]
Coronary perfusion pressure, mmHg	59 ± 1	43 ± 1[a]	43 ± 1[a]
Developed pressure, mmHg	62 ± 4	51 ± 4	65 ± 4[b]
Rate • pressure product, (mmHg/min) • 10^{-3}	8.0 ± 0.3	6.9 ± 0.5	7.9 ± 0.3
Venous Po_2, mmHg	2 ± 1	3 ± 1	2 ± 1
O_2 consumption, μmoles/min	1.37 ± 0.02	1.35 ± 0.02	1.36 ± 0.02
Resistance, mmHg • min/ml • g_{ww}[c]	2.37 ± 0.12	1.89 ± 0.11[a]	1.67 ± 0.07[ab]
Venous (lactate),m*M*	0.70 ± 0.02	0.46 ± 0.04[a]	0.53 ± 0.03[a]
Lactate production, μmoles/min/g_{ww}	7.97 ± 0.26	5.40 ± 0.43[a]	5.74 ± 0.41[a]
ATP production, μmoles/min/g_{ww}	14.28 ± 0.35	11.91 ± 0.66[a]	11.71 ± 0.66[a]

[a] $P < 0.05$ versus normoxia.
[b] $P < 0.05$ versus chronic hypoxia (one-way ANOVA and Fisher's posttest).
[c] WW = wet weight.

Whereas diazoxide did not affect EDP in any group during the hypoxic perfusion, glibenclamide increased EDP in all groups. The increase ranked N ~ CH > CH+A (28.8 ± 3.6, 23.8 ± 2.7 and 16.5 ± 1.1 mmHg, respectively; N vs. CH, P = NS; N vs. CH+A, P = 0.004; and CH+A vs. CH, P = 0.05). Therefore, $sarcK^+_{ATP}$ channel blockade during hypoxia impaired diastolic function, especially in N and CH. By contrast, $mitoK^+_{ATP}$ channel opening was ineffective. RPP during hypoxic perfusion was affected by neither glibenclamide nor diazoxide. CVR was not affected by diazoxide (P = NS) but was decreased by glibenclamide in N and CH hearts (P = 0.002 and 0.001 in N, respectively) with no change in CH+A.

Effect of K^+ Channel Modulators on Myocardial Performance during (Re)oxygenation. The (re)oxygenation decreased EDP in all groups, with the decrease being more marked in CH+A. In the presence of glibenclamide, (re)oxygenation quickly restored EDP values close to those of vehicle-perfused hearts (P = NS). In the presence of diazoxide, EDP in CH hearts decreased to a value comparable to that observed in CH+A vehicle-perfused hearts (P = NS). Therefore, the $mitoK^+_{ATP}$ channel opening, although ineffective in the other groups, is protective during the first oxygenation in CH hearts.

The (re)oxygenation increased RPP in all groups. In vehicle-perfused hearts, RPP increased less in CH than CH+A (P = 0.0005) and N (P = 0.001). RPP markedly improved in the presence of either glibenclamide (P = 0.0001) or diazoxide (P = 0.002). The drugs did not affect RPP in either CH+A or N hearts. Therefore, both $sarcK^+_{ATP}$ channel blockade and $mitoK^+_{ATP}$ channel opening improved RPP in CH hearts during their first oxygenation challenge.

In vehicle-perfused hearts, the (re)oxygenation increased CVR in all groups. The increase ranked N ~ CH > CH+A (ANOVA $P < 0.0001$). Diazoxide prevented CVR increase in all groups, including N, but with glibenclamide, the protection afforded in CH+A was blunted. Therefore, during the (re)oxygenation, $mitoK^+_{ATP}$ channel opening prevented CVR increases in all hearts.

Aerobic and Anaerobic Metabolism during Hypoxia and (Re)oxygenation. In hypoxic hearts during stabilization, MVo_2 was the same in all groups (Fig. 3D), but J_{lac} was higher in N than CH and CH+A (Fig. 3E). Whereas glibenclamide and diazoxide did not affect MVO_2, glibenclamide decreased ($P < 0.001$) J_{lac} in N (Fig. 3D and 3E).

At the end of the (re)oxygenation, the venous lactate concentration was below the sensitivity limits, indicating that $J_{lac} \sim 0$, and that J_{ATP} was entirely dependent on aerobic mechanisms, with no measurable effect of either glibenclamide or diazoxide.

Discussion

The Chronic Hypoxia Model. We previously showed that the aeration episodes that occur during routine chamber opening for cleaning and animal weighing improve the myocardial tolerance to (re)oxygenation (13), whereas blood gasses, hemoglobin concentration, and ventricular hypertrophy are not affected (17). The compensation chamber allows the performance of all operations related to animal maintenance and death, including anesthesia and heart excision, without any contact with room air. Thus, the CH protocol mimics the physiological (high-altitude) and pathological (congenital heart disease, pulmonary obstruction, anemia and disrupted microcirculation) settings in

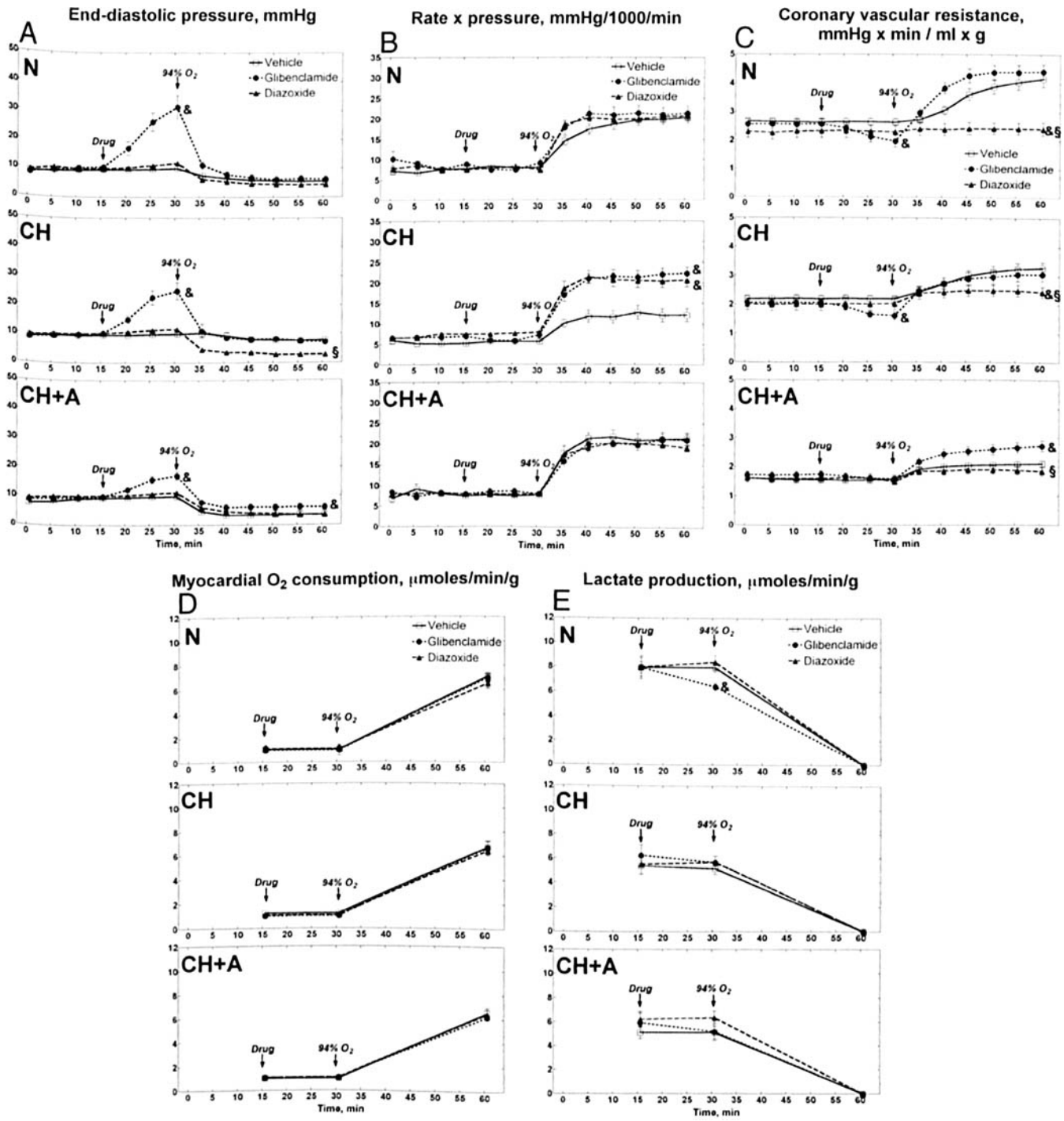

Figure 3. Time course of end-diastolic pressure (A), rate × pressure product (B), coronary vascular resistance (C), myocardial O_2 consumption (D), and lactate production (E) in hearts from normoxic animals and animals previously exposed to chronic and chronic hypoxia with aeration. Hearts were perfused for 30 min under hypoxic conditions (10% O_2) and then reoxygenated (94% O_2). The vertical arrows indicate the times at which the perfusion medium was switched to drug or vehicle (left) or to the oxygenated medium (right). Data are expressed as mean ± SEM; n = 8/group. The statistical analysis refers to parameters measured at the end of the hypoxic perfusion and reoxygenation. One-way ANOVA and Fisher's posttest: & = $P < 0.05$ vs. vehicle, § = $P < 0.05$ vs. glibenclamide.

which hypoxic tissue oxygenation is prevented and allows focusing into the response of chronically hypoxic hearts to the first exposure to high O_2 tensions.

After reviewing the existing literature, we noticed that aeration episodes occurred in virtually all previous reports on the effects of chronic hypoxia, except those in which hypoxia was induced by surgical techniques (21–23). Therefore, CH+A and CH data are comparable, respectively, with "chronic hypoxia" in studies employing standard hypoxic chambers and in studies in which hypoxia was induced by surgical techniques. Thus, it is not surprising that tolerance to (re)oxygenation was improved in CH+A, in

analogy with reports employing standard hypoxic chambers (1–7). In contrast, tolerance to (re)oxygenation was low in CH hearts, pointing to a deleterious effect of chronic hypoxia, in analogy with reports employing surgical techniques. It was already pointed out that signal transduction pathways, including decreased activity of main antioxidants enzymes and stress proteins, might play a critical role in generating adverse effects in true chronic hypoxia, as opposed to protective effects in intermittent hypoxia (24). Whereas these authors were the first to argue that CH causes adverse effects, which may be prevented by CH+A, this report is the first experimental validation of that hypothesis.

The same plasma level of MDA, an index of lipid peroxidation, in CH and CH+A indicates lack of irreversible tissue injury (25). Higher plasma NOx, an index of NO production, in CH+A than CH, together with higher level of CoQ_9, an index of endogenous antioxidant level (13), favors the hypothesis of greater oxidative stress in CH+A rats as a result of ROS generated during the normoxic aeration episodes *in vivo*. ROS are known to act as second messengers to stimulate protective signal transduction pathways through activation of protein-kinase C that, in turn, might activate mitoK^+_{ATP} channels (26, 27). However, it appears that repeated normoxic aeration episodes *in vivo* might have profound effects, yet to be investigated, in cell metabolism and signaling because the degree of apoptosis was reduced in CHA hearts.

Isolated Heart Perfusion. Perfusing hearts with crystalloids rather than blood may be considered a limit in this study, but it allows us to retain accuracy in grading hypoxia and to exclude interference from blood-O_2 affinity changes, red cell spacing within capillaries, hemoglobin-O_2 unloading kinetics, lymphocyte-mediated inflammatory processes, hormones, coagulation factors, and capillary clogging phenomena. Most of these phenomena are altered by chronic hypoxia, which also increases blood viscosity as a result of raised hematocrit and induces hematocrit heterogeneity in the various districts of the myocardium. The lower O_2 solubility in crystalloids than in blood decreases the arterial O_2 content from ~9 m*M* in vivo to 0.94 m*M* in in vitro–perfused hearts during (re)oxygenation, but if the O_2 supply (O_2 content × flow) is considered, the higher coronary flow rate (13.6 vs. ~0.7 ml/min per gram in vivo, at physiological CPP resulting from low medium viscosity) and Po_2 (670 mmHg vs. 95 mmHg) lead to an O_2 supply of 8.2 μmol/min per gram in vivo versus 12.8 μmol/min per gram in in vitro (re)oxygenated, perfused hearts. Therefore, the crystalloid-perfused heart may represent a reasonable approach to the in vivo situation.

Mechanical Efficiency. During the hypoxic perfusion, myocardial contractility was the same in all groups, but during the (re)oxygenation, it was remarkably depressed in CH vehicle-perfused hearts only (Fig. 3B). As MVO_2 was the same in all groups (Fig. 3D), impaired mitochondrial function in CH vehicle-perfused hearts is unlikely. To assess the efficiency of energy conversion into contractile work, we estimated the relationship between J_{ATP} (which includes both the aerobic and anaerobic contributions to ATP synthesis) and RPP either at the end of the hypoxic perfusion or at the end of the (re)oxygenation (Fig. 4). Whereas during the hypoxic perfusion this ratio was the same in all groups, at the end of the (re)oxygenation it was markedly less in CH vehicle-perfused hearts only ($P < 0.0001$). This indicates less contractility at the same J_{ATP} and an "energy-wasting" effect attributable to diverted ATP utilization from contractile work into other energy-consuming mechanisms. Because this effect disappears in hearts perfused with glibenclamide or diazoxide, it is possible that some of these mechanisms are needed to maintain the intracellular distribution of K^+ ions, as discussed below.

Modulation of K^+_{ATP} Channels during Hypoxia. Glibenclamide administration during hypoxic perfusion did not alter contractility with respect to vehicle-perfused hearts but did increase diastolic stiffness and decreased vasoconstriction in N and CH hearts. In a working heart model, unaltered contractility and increased diastolic stiffness translate into depressed function, and a recent paper (28) shows that glibenclamide depresses function in N but not hypoxic, that is, CH+A hearts, in agreement with our data. In contrast, diazoxide did not alter performance. To the best of our knowledge, this is the first

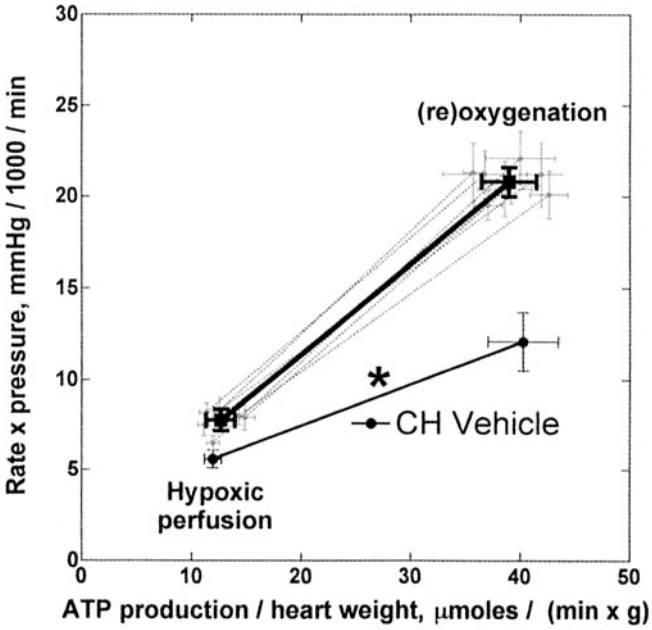

Figure 4. ATP production/heart weight on *x*-axis and rate × pressure product on *y*-axis at the end of the hypoxic perfusion and of the (re)oxygenation in hearts from rats exposed to normoxia, chronic hypoxia, and chronic hypoxia with aeration in the presence of vehicle, glibenclamide, or diazoxide. The midtone lines and symbols represent all the groups except CH vehicle. The filled squares represent mean ± SEM of all the group except the CH-vehicle ($n = 8$/group). Average slope for all data points except the CH-vehicle is 0.435 ± 0.020 mmHg • μmoles/(1000 • g_{ww}). Slope for CH-vehicle only: 0.182 ± 0.041 mmHg • μmoles/(1000 • g_{ww}). *, $P < 0.0001$). WW, wet weight.

study examining the acute (15 min) effects of K^+_{ATP} channels blockade or opening during hypoxic perfusion. Our observations basically support the postulated mechanism of action of glibenclamide: By blocking the sarcK^+_{ATP} channel, glibenclamide depolarizes the cell and enhances the opening the Ca^{2+} voltage-dependent channels, thereby increasing intracellular Ca^{2+} and diastolic stiffness. The lack of effects of glibenclamide on the systolic function as opposed to marked effects on the diastolic function is a result of the higher sensitivity of the latter to intracellular Ca^{++} ion changes. It remains to be assessed why this effect is progressively less pronounced in CH and CH+A hearts, but a possible role for NO can be envisaged, because plasma NOx levels *in vivo* are inversely related to the changes in EDP and CVR on glibenclamide administration. Both the endothelial (29) and the cardiac inducible (30) isoforms of NO synthase were shown to increase after either acute (2 hr) hypoxia or CH+A, and eNOS was found to be increased by about 50% in hypoxic, that is, CH+A hearts exposed to 10.5% O_2 for 14 days (28). NO is known to activate the sarcK^+_{ATP} channel via a cGMP-dependent mechanism (31). However, in CH+A hearts accustomed to high NO levels *in vivo*, the EDP response to glibenclamide was less, thereby indicating possible antagonism between glibenclamide and NO, as glibenclamide was shown to block the hypoxia-induced vasodilatation by inactivating sarcK^+_{ATP} channels (32). Higher plasma NOx in CH+A thus CH justifies lower CVR in CH+A with respect to N (5). The latter finding is consistent with the higher coronary flow observed in intermittently hypoxic hearts perfused in a constant pressure model (8). However, the same authors did not observe any effect of glibenclamide in either normoxic or intermittently hypoxic hearts, despite the higher dosage of the drug (10 μM vs. 3 μM in our study). In addition, our finding is in apparent contrast with other reports supporting vasoconstrictor mechanism of action of glibenclamide (33, 34).

Previous studies demonstrated that "chronic hypoxia," that is, CH+A, increases mitoK^+_{ATP} channel activity, thereby improving mitochondrial bioenergetics and cardioprotection (6). Adaptation to high-altitude hypoxia decreased the susceptibility of intermittently hypoxic hearts to ischemia-induced arrhythmias, implying that mitoK^+_{ATP} channels are more involved than sarcK^+_{ATP} channels (1). However, both mitoK^+_{ATP} and sarcK^+_{ATP} channels contribute to cardioprotection in hypoxic hearts (10), though a recent study demonstrated that cardioprotection is mediated by sarcK^+_{ATP} rather than mitoK^+_{ATP} channels (12). These conflicting results prevent agreement on the role of the two channels in hypoxia-induced cardioprotection.

K^+_{ATP} Channels during (Re)oxygenation. At the end of the (re)oxygenation and in the absence of drugs, the contractility of CH hearts was more compromised than that of CH+A and N hearts. When given to CH hearts before the first oxygenation, both glibenclamide and diazoxide conferred protection similar to that afforded by repeated aeration episodes *in vivo*. Thus, sarcK^+_{ATP} channel blockade and mitoK^+_{ATP} opening independently improve contractility in CH, whereas the other groups were unaffected. Diazoxide also improved the diastolic function and prevented vasoconstriction, thereby indicating that decreased cytoplasmic $[K^+]$, increased mitochondrial $[K^+]$, or both afford protection in CH hearts.

K^+_{ATP} channels have been implicated in cardioprotection induced by both chronic hypoxia or by ischemic preconditioning, a phenomenon whereby one or more brief episodes of ischemia protect the heart against a subsequent lethal ischemic insult (35). Our results in CH+A can thus be compared with data obtained in "chronic hypoxia" models as well as those relative to the second window of protection elicited by ischemic preconditioning. Activation of K^+_{ATP} channels during ischemia or hypoxia may lead to beneficial effects on myocardial function.

However, mitoK^+_{ATP} channel activation is known to increase protection (11). In perfused hearts, mitoK^+_{ATP} opening by diazoxide during ischemia maintains the structure of the intermembrane space, thereby preventing cytochrome c loss and preserving the electron transport capability and mitochondrial membrane permeability to ADP and ATP (36). In addition, K^+ influx into the mitochondria probably causes ROS production, which in turn triggers cardioprotection (27). Although this effect is blocked by glibenclamide, cardioprotection may still partly occur via sarcK^+_{ATP} (37). In perfused hearts, diazoxide enhances the postischemic recovery of RPP by preserving mitochondrial function during ischemia (38). In contrast, in a Kir6.2-knockout mice model, diazoxide induces protection by activating sarcK^+_{ATP} rather than mitoK^+_{ATP} (12).

Possibly, repeated ROS bursts during normoxic aeration episodes in CH+A might produce an effect analogous to that of H_2O_2 that is known to open mitoK^+_{ATP} channels and protects cardiomyocytes against ischemia (39). To support this hypothesis, a cell viability test in cultured cardiomyocytes showed that ROS and NO activate mitoK^+_{ATP} channels, which in turn generates more ROS and NO required to trigger ischemic preconditioning (26). As CH apparently induces less ROS and NO production than IH, this might explain greater the protection afforded by normoxic aeration episodes *in vivo* and by both glibenclamide and diazoxide administration *in vitro*.

Conclusions

Compared with continuous chronic hypoxia, repeated normoxic aeration episodes during chronic hypoxia seem to improve body-weight gain, increase plasma NOx, reduce vasoconstriction, and reduce the degree of apoptosis. Other parameters are not affected, including blood gasses, hematology, plasma MDA level, and diastolic and systolic myocardial function. Whereas mitoK^+_{ATP} channel opening by diazoxide during hypoxia has no measurable effect on myocardial performance, sarcK^+_{ATP} and mitoK^+_{ATP} channels blockade by glibenclamide induce diastolic contracture

and have a slight vasodilator action. On (re)oxygenation, the performance of the hearts previously exposed to chronic hypoxia was less than in all other groups. As either glibenclamide or diazoxide are able to reverse the contractile impairment, it is possible that in chronic hypoxic hearts, cardioprotection might stem from increased Ca^{2+} entry, resulting from depolarization induced by sarcK^+_{ATP} blockade or from the preservation of oxidative phosphorylation efficiency by mitoK^+_{ATP} opening. Therefore, both sarcK^+_{ATP} and mitoK^+_{ATP} channels are involved in the deleterious effects of chronic hypoxia and in the cardioprotection elicited when chronic hypoxia is interrupted with short normoxic aeration episodes.

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