Reversibility of electrophysiological changes induced by chronic high-altitude hypoxia in adult rat heart

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Recent evidence in animal models of left ventricular (LV) hypertrophy (LVH) suggests that LVH regression is associated with normalization of ventricular electrophysiology (18, 19, 28). Chronic hypoxia is the main pathophysiological factor in severe disturbances of the cardiovascular system, represented by pulmonary, ischemic, and congenital heart disease and in cardiopulmonary changes induced by exposure to a high-altitude environment (17). Development of right ventricular (RV) hypertrophy (RVH) is a common consequence of increased pulmonary vascular resistance in most mammals (including humans) living at high altitudes (25). This phenomenon also occurs in experimental animal models during exposure to either normobaric or hypobaric high-altitude hypoxia simulated under laboratory conditions (21). One of the most typical characteristics of phenotypical adaptations is their reversible nature. It has been shown that even severe chronic hypoxia-induced changes, such as pulmonary hypertension and RVH, are completely reversible after removal of the animals from the hypoxic atmosphere for a sufficiently long period of time (15). Recently, we demonstrated that chronic hypobaric high-altitude hypoxia induced true RV myocyte hypertrophy in rats and that hypertrophied cells showed prolongation of their action potential duration (APD) compared with control cells (4, 5). Moreover, we showed that the decrease of the transient outward current (Ito1) and the increase of the Na/Ca exchange current (INa/Ca) densities might account for the lengthening of the action potential (4, 7). However, no data are available about the reversibility of the electrophysiological changes, especially membrane current changes, during the course of RVH regression.

In the present study, we examined the reversibility of morphological and electrophysiological changes induced by chronic high-altitude hypoxia in the adult rat heart when the animals were returned to normoxic conditions. The results show a differential normalization of electrophysiological and morphological changes; the normalization of electrophysiological changes occurs before the normalization of morphological changes.

MATERIALS AND METHODS

Animal Model

RVH was induced in adult male Sprague-Dawley rats weighing 350–400 g (11 wk old upon delivery from the breeder, Ifa Credo; L’Arbresle, France) as previously described (4, 5). Briefly, rats were placed for 20 days in a hypobaric chamber, allowing exposure to a simulated altitude (~4,500 m; Po2 = 12 kPa). Control rats were maintained under normoxic conditions. Chronic congestive heart failure never occurred in this model. This was confirmed by anatomic examination (n = 187), which did not show the presence of hypertrophy; right ventricular myocytes; fibrosis; action potential; ionic currents


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ence of pleural fluid, hepatic congestion, and ascites, which are criteria of failure in RVH (23). Animals in both groups had free access to food and water. The regression of hypertrophy was assessed 20 and 40 days after the end of hypoxic exposure, which was assigned day 0. Data obtained from the exposed group were compared with those of age-matched control group.

**Histological Studies**

Heart extraction, perfusion, and chemical treatment. Animals were anesthetized with pentobarbital. The heart was excised and immersed in ice-cold Ca-free Tyrode solution. The aorta was cannulated, and a retrograde perfusion was performed, first with Ca-free Tyrode solution at 37°C for 5–10 min and then with fixative solution (0.5% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer; pH 7.4) for 1 h. After fixation, the heart was transversally cut from the atrioventricular level to the ventricular top into 2-mm-thick slices with a vibratome. Slices were observed overnight in fixative solution, washed in buffer, osmium postfixed (0.25% in buffer), dehydrated, and embedded in a methacrylate histological resin. The morphometric study was made from an identical slice level for each heart. From the 2-mm-thick slices, thin 5-μm sections were realized.

Ventricular wall thickness measurements. Morphometric analysis was performed both on thick and thin sections, the latter being stained with toluidine blue. Each of them was macrophotographed. On uniformly magnified printings, ventricular walls were manually outlined. Image analysis and quantification were then performed with a Leica image analyzer (Performance Q 5001 W). After calibration, the RV wall, LV wall, and septum surfaces were manually outlined on the screen, and their surfaces were automatically deduced.

Myocyte size and total collagenic mass measurements. The ventricular transversal sections present multiple cardiomyocyte orientations, but for the reliability of measurements we always selected the large transversal cellular fields of trabecules protruding in the ventricular luminae (Fig. 1). The Gordon Sweet silver staining method was used to underscore the total collagenic mass: black staining for collagen 3 and 4 which makes an overall analysis of extracellular fibrous elements to evaluate the effects of hypertrophy on the collagenic mass.

For cellular size analysis, outlinings of 25 cardiomyocytes were automatically measured in five different areas of trabecules for each ventricle.

Analysis of the collagenic mass was performed on similar areas. Fifteen values of the black-stained intercellular spaces were compiled from the RV wall and septum for control and treated samples. However, in the window format selected by the computer program, the number of hypertrophied cells was lower compared with control (as cardiomyocyte areas increase) and the comparison was distorted. To compensate for this defect and compare a similar number of cells, we used a correction factor (coefficient of cellular hypertrophy) obtained by making the ratio between the mean of cell circumferences in each treated sample and the mean of cell circumferences in its control. This value was applied to the collagenic mass measurements of treated samples before the comparative analysis was done.

**Heart Weight Measurements and Myocyte Isolation**

These procedures were made as previously described (4, 5). The dimensions of freshly dissociated myocytes was assessed by measuring length and width with a graticule mounted on the lens of a microscope.

**Electrophysiological Techniques and Solutions**

The electrophysiological experiments were carried out at room temperature (20–23°C) on isolated RV cells with the conventional whole cell patch-clamp method in current-clamp or in voltage-clamp conditions (8). Pipettes with a 1- to 3-MΩ resistance were routinely used and connected to the headstage of a RK400 amplifier (Biologic; Grenoble, France) with a 100-MΩ feedback resistor. Cells were placed in a plastic petri dish containing the appropriate extracellular medium (see below); the cell from which the recording was being made was continuously superfused with solutions flowing by gravity from a set of five capillaries located near the cell and allowing rapid changes (within 5 s) of the extracellular medium. The superfusion flow rate was 20–40 μl/s.

Membrane capacitance was systematically measured and calculated by analyzing the capacitive surge produced by a small voltage step as described previously (4).

The series resistance ($R_s$) ranged from 4 to 5 MΩ when filled with the internal solution (see below). Membrane capacitance and $R_s$ were not compensated. Because of the presence of $R_s$, the membrane potential ($V_m$) deviates from the command potential ($V_c$) according to the following equation: $V_m = V_c[1 - (R_s/R_c + R_m)]$, where $R_s/R_c + R_m$ is the error factor. From the slope of the $I_{Vc}$-voltage relationships, we determined $R_s + R_m$ which gives a good estimate of the lowest $R_m$ value. The maximal error factor was estimated to be $0.24 ± 0.03$ and $0.23 ± 0.03$ ($n = 19$ in each group; $P = 0.81$) during the flow of $I_{Vc}$ in the control day 0 group and in the regression day 0 group, respectively (see RESULTS for a more detailed description of these groups). Note that the difference between the error factors of the two groups is not significant and thus should not affect the comparison between them. Action potentials were elicited by a 5-ms depolarizing current pulse at a rate of 0.2 Hz and sampled at 1 kHz. Voltage-clamp protocols are described in RESULTS. Current traces were sampled at 5 kHz and uncorrected for the
leak. Current or voltage commands and simultaneous signal recordings used pCLAMP software (Axon Instruments).

For action potential recording, the internal solution in the patch electrode contained (in mM) 7 NaCl, 110 potassium aspartate, 30 KCl, 2 MgCl₂, 10 glucose, 0.2 EGTA/KOH, and 5 HEPES/KOH (pH = 7.2), and the external solution contained (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 10 HEPES/NaOH (pH = 7.4).

For the \( I_{\text{Ca}} \) recording, the internal solution in the patch electrode contained (in mM) 130 potassium aspartate, 5 KCl, 5 MgCl₂, 10 glucose, 3 K₂-ATP, 5 Na₂-CP, 0.4 Na₂-GTP, 5 EGTA/KOH, and 10 HEPES/KOH (pH = 7.2), and the external solution contained (in mM) 135 choline-Cl, 1.1 MgCl₂, 2.5 CaCl₂, 0.5 CdCl₂, 10 glucose, 0.01 atropine sulfate, and 10 HEPES/KOH (pH = 7.4).

For the \( I_{\text{Na}}/I_{\text{Ca}} \) recording, the internal solution in the patch electrode contained (in mM) 7 NaCl, 20 CsCl, 110 cesium aspartate, 1.1 MgCl₂, 0.2 EGTA/CsOH, and 5 HEPES/CsOH (pH = 7.2), and the external solution contained (in mM) 140 NaCl or LiCl, 5 CsCl, 2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES/NaOH or LiOH (pH = 7.4).

Osmolarity was between 300 and 320 mosM for every solution.

**Statistical Analysis**

All values are expressed as means ± SE; \( n \) = number of experiments. The statistical significance of differences between groups was determined by a nonparametric Mann-Whitney test. A difference was assumed to be statistically significant when \( P < 0.05 \). Selection of representative records was based on close similarity of their APD or the amplitude of the current density to mean values.

**RESULTS**

**Morphometric Changes**

Figure 2 shows the evolution of the RV mass of control and altitude-exposed rats (Reg rats) at 0, 20, and 40 days after the end of hypoxic exposure. To isolate the regression phenomenon, differences (expressed in percentages) can be considered between exposed and control rats. At \( \text{day 0} \), this difference for the RV weight-to-heart weight ratio (Fig. 2A) was 40% and only 18% and 7% at 20 and 40 days of regression, respectively. Figure 2B shows the representative change in macroscopic morphology of the heart from a control day 0 rat (a) and from Reg day 0 (b), Reg day 20 (c), and Reg day 40 rats (d). The thickness of the RV free wall in Reg day 0 and Reg day 20 rats was markedly increased, whereas the LV and septal wall thicknesses were unaffected. The difference for the RV wall thickness (Fig. 2C) between exposed and control rats was 62.1%, 57.8%, and 12% at 0, 20, and 40 days of regression, respectively.

Figure 3 shows the evolution of some RV myocyte parameters. The difference for the myocyte size (Fig. 3A) between exposed and control rats was 10.6%, 5.9%, and 3.2% at 0, 20, and 40 days of regression, respectively. For the myocyte capacitance (Fig. 3B), this difference was 50.8%, 23.6%, and 13.9% at 0, 20, and 40 days of regression, respectively, whereas for myocyte width (Fig. 3C), it was 35.4%, 27.2%, and 3.9% at 0, 20, and 40 days of regression, respectively.

Figure 4C shows the evolution of pericellular fibrosis (see MATERIALS AND METHODS). The difference between exposed and control rats was 25.6%, 17.4%, and 0.6% at 0, 20, and 40 days of regression, respectively.

It is noteworthy that for all the morphometric parameters measured, the differences between exposed
 Action Potential Changes

Typical action potential recordings in control and in Reg RV myocytes are illustrated in Fig. 5, A–C. There were no significant differences in resting membrane potential or action potential amplitude among the three groups or between the individual groups nor in the APD of control myocytes between the three groups except between control APD at 25% repolarization (APD25) at day 0 and at day 40 (Fig. 5D). This latter result is qualitatively similar to those reported previ-ously in the rat atrium and attributed to an age-dependent prolongation (3). The marked difference in APD between control and Reg rats at day 0 was no longer recorded 20 days after the end of hypoxic expo-

Fig. 3. Evolution of some RV myocyte parameters of control and altitude-exposed rats at different time intervals after the end of hypoxic exposure. A: evolution of RV myocyte perimeter; n = number of measurements in each condition made from 6 hearts at day 0 and 2 hearts at days 20 and 40, respectively. See MATERIALS AND METHODS for details. B: evolution of RV myocyte capacitance; n = number of cells in each condition isolated from 9 to 12 rats. C: evolution of RV myocyte width; n = number of cells in each condition isolated from 3 rats. Bar values are means ± SE. *P < 0.05 compared with control.

Fig. 4. Evolution of heart collagenic content of altitude-exposed rats at different time intervals after the end of hypoxic exposure. A: collagenic material evidenced in the extracellular space by the Gordon Sweet silver staining method. Bar = 100 μm. B: enlargement of 3 cardiomyocytes in A to see the outlining produced by silver impregnation. M, myocyte; CP, capillary. Bar = 20 μm. C: evolution of pericellular fibrosis (expressed as a percentage of control). The correction factors were applied when necessary. See MATERIALS AND METHODS for details. Bar values are means ± SE; n = number of measurements in each condition made from 6 hearts at day 0 and 2 hearts at days 20 and 40, respectively. *P < 0.05 compared with control.
sure (Fig. 5D). Indeed, this difference measured at 90% of repolarization was 219.2%, 8.5%, and 9.8% at 0, 20, and 40 days of regression, respectively.

\( I_{to1} \) and \( I_{Na/Ca} \) Changes

Figure 6, A–C, shows representative \( I_{to1} \) recordings elicited by a step depolarization to +30 mV from a holding potential of −80 mV in control and Reg RV myocytes. \( I_{to1} \) was evaluated as the difference between peak current and the current at the end of the 600-ms pulse. As previously shown (4), significant differences in mean \( I_{to1} \) densities between control and Reg myocytes were observed only at day 0 between +10 and +60 mV (Fig. 6D). For instance, at +30 mV, the difference was −43.9%, −22.2%, and −5.8% at 0, 20, and 40 days of regression, respectively. Neither the inactivation time constant nor the time to peak for \( I_{to1} \) were different among the three groups or between the individual groups (results not shown). There was also no change in the recovery from inactivation for \( I_{to1} \) between control and Reg day 0 groups when assessed by a low stimulation frequency (0.05 Hz, results not shown).

Typical \( I_{Na/Ca} \) recordings obtained in control and Reg RV myocytes using the following protocol are illustrated in Fig. 7, A–C. From a holding potential of −80 mV, a depolarization to −50 mV was applied for 20 ms to inactivate the TTX-sensitive transient sodium current. This was followed by a depolarization to +10 mV
for 30 ms to activate the L-type calcium current. This protocol was applied at 0.1 Hz until the L-type calcium current and the slow tail inward current recorded on repolarization to −80 mV reached a steady state (7). The sodium control solution surrounding the myocyte was then replaced in ~5 s with a lithium solution. The lithium-sensitive slow tail currents shown in Fig. 7, A–C (which can be attributed to the Na/Ca exchange mechanism), were obtained by subtraction of the current in the presence of sodium from that after 30 s in the presence of lithium. Figure 7D shows mean current density of the lithium-sensitive tail current measured 20 ms after the onset of the repolarization to −80 mV. As previously shown (7), significant differences in mean lithium-sensitive current densities between control and Reg myocytes were observed only at day 0. At day 0, this difference was 42.5%, and only 6% and 0% at 20 and 40 days of regression, respectively. The amount of charges transported by the exchanger at −80 mV was determined by the integral of the tail current between 9 and 130 ms after the start of repolarization. The charges moved were as follows: day 0 group, 0.019 ± 0.004 (n = 10) and 0.038 ± 0.002 pC/pF (n = 9, P < 0.05); day 20 group, 0.023 ± 0.004 (n = 9) and 0.019 ± 0.004 pC/pF (n = 8, P > 0.05); and day 40 group, 0.025 ± 0.001 (n = 10) and 0.021 ± 0.003 pC/pF (n = 12, P > 0.05) in the control and Reg groups, respectively.

**DISCUSSION**

The present work clearly demonstrates normalization of morphological and electrophysiological changes induced by permanent high-altitude exposure (4,500 m, 20 days) in the adult rat heart after removal of the animals from the hypoxic atmosphere for a sufficiently long period of time. Nevertheless, regression of morphological abnormalities appears to be slower than the regression of electrophysiological changes. As evidenced by both the RV weight-to-heart weight ratio and by the RV free wall thickness measurement (Fig. 2), the RVH induced by permanent simulated high-altitude exposure (4, 5, 7) was still significant at 20 days but no more at 40 days after exposure. The only data regarding regression of altitude-induced cardiac hypertrophy in rats come from intermittent high-altitude exposure (4–8 h/day, 5 or 6 days/week, stepwise up to 7,000 m, 24 exposures). Reported results vary from 2 to 4 wk (14, 22). Regression of heart weight to control values in our model is longer than these latter ones. The reason for these differences in the rate of reversibility is not clear. However, a possible explanation for some of this variability may be the differences in the inciting stimulus (intermittent vs. permanent exposure, duration of the exposure, degree of hypoxia). Regardless of the cause(s) of the variability, the return of heart weight to control values in our model was not a result of changes in water content, because no alteration was found in the ratio of the dry weight to wet weight (data not shown). Some of the hypertrophy reported in this study appears to originate from increased collagen (Fig. 4). Collagen is a very important determinant of myocardial stiffness (type I and III being the major constituents of the extracellular matrix network). Its accumulation in hypertrophied hearts is expected to increase stiffness and affect cardiac function. Indeed, the total collagenic mass was increased by ~26% in the hypertrophied RV but also by ~13% (result not shown) in the LV, which is not hypertrophied in this model (5). The increase in collagen content in both ventricles when only the RV has been
stressed is not specific to the permanent high-altitude hypoxia exposure model and has been reported in the model of RVH induced by chronic constriction of the main pulmonary artery in the cat (1) or by intermittent high-altitude hypoxia exposure in the rat (17). In this latter model, the increase in collagen content was 153% and 228% of control values in the RV and LV, respectively. The discrepancy between the two models may result from the difference in the simulated altitude used (intermittent altitude model, 7,000 m vs. permanent altitude model, 4,500 m) and thus to the higher deleterious degree of hypoxia in the intermittent altitude model. In fact, depending of the experimental model of hypertrophy production, either the LV or RV is principally concerned with a notable enlargement and fibrosis, but the numerous available data in the field proved that the ventricular myocardium was slightly affected in a global manner by a development of fibrous septa, perivascular fibrosis, and intercardiomyocyte collagenic thickening (16, 24). It has been shown that intermittent altitude exposures to 7,000 m can induce focal necrosis in both the RV and LV wall at a time when RVH, but not LVH, was present (27). We have never seen focal necrosis in the myocardium from rats exposed permanently to 4,500 m. These previous and present results corroborate the hypothesis that factors governing connective tissue proliferation may be independent from those governing the cardiomyocyte hypertrophy (12). The regression of collagen content toward the control value in the RV (Fig. 4) appears to parallel the recovery of some RV myocyte parameters (perimeter, capacitance, and width; Fig. 3). Nevertheless, it must be noticed that in the LV a persistent fibrosis was observed even 40 days after the end of altitude exposure (result not shown). Persistent fibrosis, particularly in the LV (17), has already been observed after regression of cardiac hypertrophy and could be due to a slower turnover of fibrillar collagen compared with myofibrillar proteins (6).

The most consistent electrical abnormality that has been described in association with cardiac hypertrophy is prolongation of the APD (9). In rat hearts, the ventricular action potential recorded at room temperature in either cardiac tissue (13) or isolated cardiomyocytes (4, 10) displays two distinct plateau phases: an early “high” plateau at positive potentials followed by the onset and slow decay of a “low” plateau, which occurs at potentials negative to −40 mV. We have previously shown, with chronic high-altitude hypoxia-induced RVH in the rat, that the decrease in \( I_{\text{to1}} \) and the increase in \( I_{\text{Na/Ca}} \) densities account for the lengthening of the high plateau and low plateau of the action potential, respectively (4, 7). Concerning L-type calcium current density, we reported a small but significant reduction for potential positive to +10 mV (4), a change that goes in the wrong direction to contribute to action potential, and consequently we did not study this current in our work. Our results show normalization of APD 20 days after the end of altitude exposure (Fig. 5) at a time where ventricular myocyte hypertrophy was still significant (see above). Moreover, our results show that in control myocytes the APD had a tendency to increase from day 0 to day 40 even if this increase was significant only between APD25 at day 0 and day 40. Such a result, qualitatively similar to those reported in the rat atrium and attributed to an age-dependent prolongation (3), could possibly be explained by a decrease in the density of \( I_{\text{to1}} \) during this period (see below). Previous studies used surgical (2, 18) or pharmacological methods (19, 20, 28) to produce regression of hypertrophy and reported a concomitant normalization of ventricular APD. It was then hypothesized that any intervention that produces regression of hypertrophy restores ventricular electrophysiology, that is to say that the regression of hypertrophy is responsible for the normalization of electrophysiological parameters (19). Data obtained from the chronic high-altitude hypoxia model are in conflict with this hypothesis. Furthermore, normalization of APD was associated with normalization of \( I_{\text{to1}} \) density and \( I_{\text{Na/Ca}} \) density (Figs. 6 and 7). Recently, molecular and biochemical techniques have been applied to address the mechanism underlying the reduced potassium current density in cardiac hypertrophy (for a review, see Ref. 26). This study shows that compensated hypertrophy after myocardial infarction in the rat is associated with a decrease in the transcription of Kv4.2 and Kv4.3, the probable molecular correlates of \( I_{\text{to1}} \) in the adult rat, whereas Kv1.4, the fetal and neonatal rat potassium channel gene, appears to be reexpressed. Further studies are needed to see whether the Kv4.2/Kv4.3 to Kv1.4 isofrom switch is a general feature during the cardiac hypertrophy phase whatever the experimental model used and whether the reverse one takes place during the regression phase. Because of the fact that \( I_{\text{to1}} \) is one of the major currents involved in the control of the duration of the rat ventricular action potential, it is tempting to involve it in the prolongation of action potential seen in the control rat (see above) even if we...
report no significant differences between the $I_{\text{to1}}$ densities measured in control myocytes at 0, 20, and 40 days (Fig. 6D). Figure 8 shows the evolution of the $I_{\text{to1}}$ density measured at +50 mV (open squares) as a function of age in RV control myocytes. We can see that the $I_{\text{to1}}$ density increased from 21 days postparturition to 2 mo, reached a maximum, and then decreased until 18 mo. These results are qualitatively similar to those reported previously on neonatal rat ventricular myocytes (21a) and in LV myocytes isolated from 3- and 18-mo-old normotensive rats (3a). The mean densities of $I_{\text{to1}}$ measured at +50 mV (solid circles) in control myocytes at days 0, 20, and 40 of Fig. 6D are reported in Fig. 8. We can see that these points fit very well with the curve and thus give substance to a possible role of $I_{\text{to1}}$ in the age-dependent prolongation of action potentials described in control myocytes.

We have previously suggested that the increase in the $I_{\text{Na/Ca}}$ density in hypertrophied ventricular myocytes by chronic high-altitude exposure could be linked to a modification of intracellular calcium homeostasis (7). The normalization of the $I_{\text{Na/Ca}}$ density 20 days after the end of altitude exposure suggests that the underlying mechanism(s) is plastic and corroborates the previous results of Kolar and Ostadal (14), which showed a normalization of RV contractility within 35 days after the termination of the hypoxic stimulus.

In conclusion, we have shown that reversal of electrophysiological changes observed at the end of a permanent high-altitude exposure (4,500 m, 20 days) seems to take place before the reversal of morphological changes. This differential normalization in chronic hypobaric high-altitude hypoxia is in contrast with the concomitant normalization recorded in animal models of LVH (2, 18–20).

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