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# Inadequate Myocardial Oxygen Supply/Demand in Experimental Pulmonary Hypertension

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## 1. Introduction

Right ventricular (RV) hypertrophy is an adaptive response to chronic pulmonary hypertension, but can progress to chronic or advanced RV heart failure. The treatment of pulmonary hypertension is improving but remains unsatisfactory (Humbert et al., 2010). A better understanding of the transition from hypertrophy to RV failure may lead to new insights in the treatment of pulmonary hypertension.

The increased workload of the right heart due to pulmonary hypertension increases oxygen consumption of right ventricular cardiomyocytes because the mitochondria have to operate at a higher rate. Hypertrophy can normalize right ventricular wall stress, thereby also normalizing the rate of oxygen consumption by mitochondria. However, hypertrophy may cause hypoxia in cardiomyocytes because intracellular diffusion distances for oxygen increase and capillary density decreases when the myocytes enlarge. Increased diffusion distances imply that the interstitial oxygen tension preventing core hypoxia in cardiomyocytes ( $PO_{2crit}$ ) increases, whereas reduced capillary density likely reduces interstitial  $PO_2$ . In addition to the increased power output of the right ventricular myocytes, the rate of oxygen consumption of the cells also increases because their mechanical efficiency decreases (Wong et al., 2010). These changes can lead to oxidative stress (Redout et al., 2007) and may lead to apoptosis of RV cardiomyocytes (Écarnot-Laubriet et al., 2002).

Whether or not hypoxia occurs in hypertrophied cardiomyocytes is a matter of debate because measurement of core  $PO_2$  in cardiomyocytes in vivo is technically impossible. Previous reports, in which myoglobin saturation was measured to estimate mean intracellular  $PO_2$  in the left heart, suggest that hypoxic cores in cardiomyocytes are absent (Bache et al., 1999; Kreuzer et al., 2001). However, Hill et al. (1989) demonstrated that supplemental oxygen reduces hypertrophy in experimental pulmonary hypertension.

We use calibrated histochemistry to obtain the physiological parameters required to evaluate oxygen demand and supply at the cellular level in hypertrophied right ventricular myocardium of pulmonary hypertensive rats (des Tombe et al., 2002). To investigate whether the cardiomyocytes adapt to hypoxia, we determined the expression of the hypoxia inducible transcription factor HIF 1 $\alpha$  (Wang & Semenza, 1995) by quantitative immunohistochemistry in cardiomyocyte nuclei.

## 2. Calculation of PO<sub>2</sub>'s

The mean extracellular oxygen tension required to drive oxygen from the interstitial space into cardiomyocytes preventing hypoxic cores (PO<sub>2crit</sub>) is calculated from a Hill-type diffusion model including myoglobin-facilitated diffusion (Murray, 1974):

$$PO_{2crit} = (VO_{2max} CSA - 4\pi D_{Mb} [MbO_2]_R) / 4\pi K_{O_2} \quad (1)$$

where VO<sub>2max</sub> is the maximum rate of oxygen consumption of the cardiomyocyte, CSA is the cross-sectional area of the cardiomyocyte, D<sub>Mb</sub> is the radial diffusion coefficient of myoglobin in the cardiomyocyte, [MbO<sub>2</sub>]<sub>R</sub> is the concentration of oxygenated myoglobin at the sarcolemma of the cardiomyocyte and K<sub>O<sub>2</sub></sub> is Krogh's diffusion coefficient for oxygen in heart muscle. [MbO<sub>2</sub>]<sub>R</sub> can be calculated from:

$$[MbO_2]_R = PO_{2crit} Mb_{tot} / (PO_{2crit} + P_{50}) \quad (2)$$

where Mb<sub>tot</sub> is the total myoglobin concentration in the cardiomyocyte and P<sub>50</sub> is the oxygen tension at which 50% of myoglobin is oxygenated. Substitution of equation 2 into equation 1 allows the calculation of PO<sub>2crit</sub>. CSA, VO<sub>2max</sub> and myoglobin concentration were determined as described below. The values for the other parameters were taken from the literature: D<sub>Mb</sub> = 2.0 10<sup>-5</sup> mm<sup>2</sup> s<sup>-1</sup> (Papadopoulos et al., 2001), K<sub>O<sub>2</sub></sub> = 1.5 nM mm<sup>2</sup> s<sup>-1</sup> hPa<sup>-1</sup> (van der Laarse et al., 2005), and P<sub>50</sub> = 8.7 hPa. The latter value was calculated from P<sub>50</sub> of rat myoglobin and the temperature dependency of P<sub>50</sub> (Gayeski & Honig, 1991; Enoki et al., 1995; Schenkman et al., 1997).

Assuming that oxygen supply is limiting VO<sub>2max</sub> by a negligible percentage in normal muscle (Spriet et al., 1986; Mootha et al., 1997), it is possible to calculate the permeability of the capillary endothelium and the interstitial space for oxygen using Fick's law, because in the steady state the flux of oxygen entering the cardiomyocytes equals the flux of oxygen crossing the capillary endothelium. This flux per unit myocyte length is:

$$CSA VO_{2max} = D_{cap} (PO_{2cap} - PO_{2crit}) \quad (3)$$

where D<sub>cap</sub> is the permeability of capillary endothelium and interstitial space (in nmol mm<sup>-1</sup> myocyte s<sup>-1</sup> hPa<sup>-1</sup>), and PO<sub>2cap</sub> is the average capillary oxygen tension. PO<sub>2cap</sub> - PO<sub>2crit</sub> is the minimum driving force required to prevent hypoxia in the cardiomyocytes. The average capillary PO<sub>2</sub> used in the calculation in normal myocardium is 66 hPa, which equals P<sub>50</sub> of rat blood (Schmidt-Nielsen & Larimar, 1958; Gray & Steadman, 1964) and approximates the mean of capillary arterial and venous PO<sub>2</sub>. We assume that D<sub>cap</sub> is proportional to the number of capillaries per cardiomyocyte (for discussion, see Bekedam, 2010). The permeability in normal myocardium was used to calculate PO<sub>2cap</sub> in hypertrophied myocardium, taking changes in CSA VO<sub>2max</sub>, capillaries per cell and PO<sub>2crit</sub> into account.

## 3. Animals and histochemical methods

### 3.1 Preparations

All experiments were approved by the local Animal Ethics Committee. Male Wistar rats (n = 33) were used for this study. At 180 g body mass, 18 rats were injected s.c. with 40 mg monocrotaline (MCT) per kg body mass, to induce pulmonary hypertension (Okumura et al., 1992). Body mass was determined daily. The body mass change (in %/day) was

calculated as the mean of the last three days. Untreated, age-matched rats served as controls. Right ventricular systolic pressure of monocrotaline-injected rats in our laboratory increases after the injection from 25 (SEM 0.9) mmHg in control (CNTR) to 45 (SEM 4.6) mmHg after two weeks (MCT2) and to 64 (SEM 2.8) mmHg after 4 weeks (MCT4, Henkes et al., 2007). We did not measure pulmonary arterial pressure in the rats used in the present experiments because the measurement and the anaesthesia may interfere with HIF 1 $\alpha$  expression (YY Wong and WJ van der Laarse, unpublished results). After 2 or 4 weeks, 6 or 12 MCT rats and 6 or 9 control rats, respectively, were anaesthetized with halothane and the lungs and heart were excised. The heart was perfused with Tyrode solution (120 mM NaCl, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 2.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 27 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>, 10 mM glucose and 20 mM 2,3-butanedione monoxime, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>; pH 7.3-7.4 at 10°C) to slow down metabolism and remove the blood and a biopsy was taken from the right ventricular wall within 10 min after excision. The biopsy was frozen in liquid nitrogen (van der Laarse & Diegenbach, 1988). Sections, 5  $\mu$ m thick, were cut in a cryostat, air-dried for 30 min and incubated for succinate dehydrogenase (SDH) activity or stored at -80°C until use. The wet mass of the lungs and the dry mass after freeze-drying were determined.

### 3.2 Calibrated enzymehistochemistry

SDH activity was demonstrated as described (des Tombe et al., 2002). The incubation medium consisted of 37.5 mM sodium phosphate buffer, pH 7.6, 75 mM sodium succinate, 5 mM sodium azide, and 0.4 mM tetranitro blue tetrazolium. Sections were incubated at 37°C in the dark for 7 min. To calculate the extracellular oxygen tension required to prevent a hypoxic core in the cardiomyocyte (PO<sub>2crit</sub>), an estimate of VO<sub>2max</sub> (in nmol mm<sup>-3</sup> s<sup>-1</sup> = mM s<sup>-1</sup>) was calculated from the absorbance of the formazan precipitate measured in the section, based on the proportional relationship between VO<sub>2max</sub> and SDH activity under hyperoxic conditions *in vitro*: VO<sub>2max</sub> = 2.9 mM s<sup>-1</sup> per unit of absorbance at 660 nm (des Tombe et al., 2002; van der Laarse et al., 2005).

The sections for the determination of the myoglobin concentration and cytosolic cytochrome c were vapour-fixed with formaldehyde as described (van Beek-Harmsen et al., 2004, van Beek-Harmsen & van der Laarse, 2005). Myoglobin peroxidase activity was determined as described (Lee-de Groot et al., 1998), in a medium consisting of 59 ml 50 mM Tris(hydroxymethyl)aminomethane and 80 mM KCl, pH 8.0, 25 mg ortho-tolidine (Sigma T8533, St Louis, MI) dissolved in 2 ml 96% ethanol (at 50°C) and 1.43 ml 70% tertiary-butylhydroperoxide (Fluka Chemie 19995, Buchs, Switzerland). The myoglobin concentration was determined using gelatine sections with known concentrations of myoglobin. The concentration was calculated from the absorbance using an extinction coefficient of 363 mM<sup>-1</sup> cm<sup>-1</sup> (van Beek-Harmsen et al., 2004).

### 3.3 Immunohistochemistry of HIF 1 $\alpha$ , cytochrome c, and collagen type IV

Incubations were carried out at room temperature (22-25°C). Sections for HIF 1 $\alpha$  and collagen type IV were fixed in 4% formaldehyde in 150 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub> (PBS), pH 7.4, for 10 min. sections for cytochrome c were fixed as indicated above. Sections were incubated with 1:100 dilution of anti-HIF 1 $\alpha$  (H-206, Santa Cruz Biotechnology, Santa Cruz, CA), anti-collagen type IV (Rockland, Gilbertsville PA), or anti-cytochrome c (Santa Cruz) for 1h, followed by incubation with a secondary biotin-labelled

anti-rabbit antibody in a 1:100 dilution (Vector Laboratories, Burlingame, CA) for 30 min. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol for 30 min. Subsequently, following the manufacturers guidelines, the sections were incubated with Vectastain ABC reagent (Vector Laboratories). Each step was followed by rinsing in PBS containing 0.05% (v/v) Tween 20. Peroxidase activity was demonstrated in a solution containing 3 mg 3,3'-diaminobenzidine in 30  $\mu$ l dimethyl sulphoxide, 10 ml 0.05 M Tris(hydroxymethyl)aminomethane, 10 mM imidazole and 10 mM sodium azide, pH 7.6. Hydrogen peroxide was added before use (final concentration 0.003%).

### 3.4 Microdensitometry and morphometry

Staining intensities and morphometry were quantified using a DMRB microscope (Leica, Wetzlar, Germany) fitted interference filters at 660 nm for SDH activity (Pool et al., 1979) and 436 nm for myoglobin (Lee-de Groot et al., 1998), cytochrome c (van Beek-Harmsen et al., 2005), and HIF 1 $\alpha$ . Images were obtained using a 40x objective, and a monochrome Charge-Coupled Device camera (Sony XC 77CE, Towada, Japan), connected to a LG-3 frame grabber (Scion, Frederick, MD) in an Apple Macintosh computer. Images were analysed using NIH Image 1.61 (<http://rsb.info.nih.gov/nih-image/>). Grey values were transformed to absorbance values using calibrated grey filters. Morphometry was calibrated using a slide micrometer taking the pixel to aspect ratio into account.

Cytochrome c release in cardiomyocytes or in the interstitial space was categorized as 0: no release, 1: some release in one or two areas of the section, 2: maximum release in more than two areas of the section, 3: maximum release detectable in the entire section.

HIF 1 $\alpha$  was measured by using the threshold option in NIH Image to measure the absorbance and size of the stained nuclei. The total staining of a nucleus was calculated as the product of absorbance and size. The mean absorbance of positive nuclei was multiplied by the fraction of positive nuclei to obtain the mean HIF 1 $\alpha$  expression in the heart.

The number of capillaries per cardiomyocyte was determined after incubation with anti-collagen type IV (Madsen & Holmskov, 1995) in two areas of a section where cardiomyocytes were cut perpendicularly to their longitudinal axis.

The sarcomere length was determined using a 100x phase contrast objective in areas of the section where the cardiomyocytes were cut along their longitudinal axis. This value was used to normalise the cross-sectional area to a sarcomere length of 2  $\mu$ m, assuming that the volume of cardiomyocytes does not change when the cells contract. The volume of cytoplasm per nucleus was determined in sections fixed for 10 min in 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and stained with hematoxylin and eosin (Loud & Anversa, 1984; des Tombe et al., 2002). The number of cardiomyocyte nuclei in the right ventricular free wall was determined from the volume of the wall (calculated as wet mass/1.04). The volume of interstitial space was subtracted, and the volume occupied by cardiomyocytes was divided by the volume of cytoplasm per nucleus to obtain the number of cardiomyocyte nuclei.

### 3.5 Statistics

Values are given as mean (standard deviation, SD). Students t-test with equal or unequal variances was used to determine differences between groups. P<0.05 was considered significant.

## 4. Results

### 4.1 Lung and body mass

Fig. 1 shows lung masses and the change in body mass of control and MCT-injected rats. The wet mass of the lung was slightly but significantly increased after two weeks, as was the change in body mass. The wet/dry mass of the lung in MCT2 was higher than control 5.64 (SD 0.42) and 5.22 (SD 0.34), respectively, indicating oedema in MCT2. The wet/dry mass of MCT4 rats was intermediate between control and MCT2 (5.33, S.D. 0.31; not different from control or MCT2). After 4 weeks, both wet and dry lung mass were significantly higher than control and MCT2, and the body mass decreased by 2% per day on average. However, the change in body mass in MCT4 rats was rather variable, as indicated by the large standard deviation (Fig. 1C); the range was +3.0 to -8.5 % per day.

The changes in lung and body mass are indicative of the severity of pulmonary hypertension (Mouchaers et al., 2007; Handoko et al, 2009).

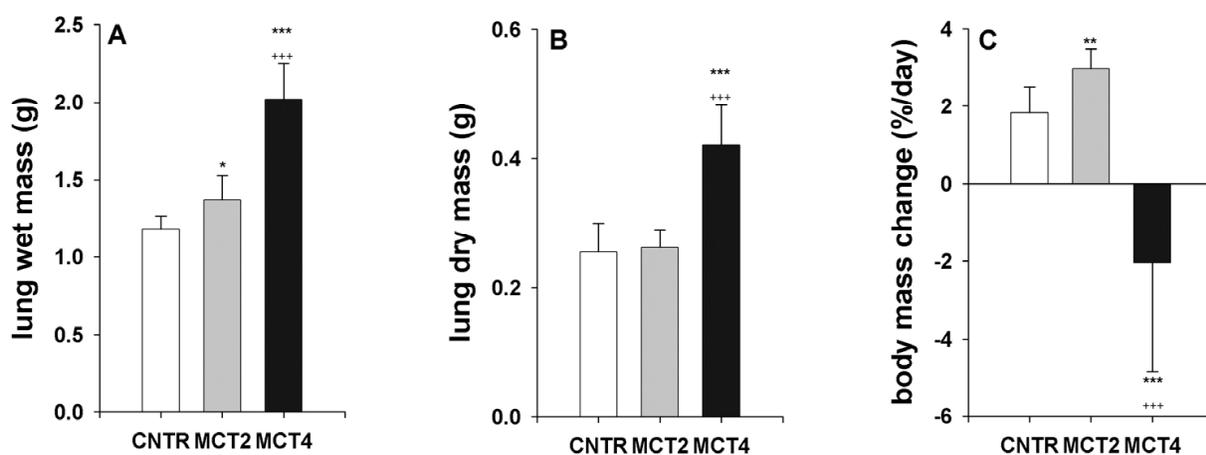


Fig. 1. A: Lung wet mass, B: lung dry mass and C: body mass change of control and monocrotaline-injected rats after 2 (MCT2) and 4 (MCT4) weeks. The body mass change is the mean value over the last three days. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ : difference between MCT and control. \*\*\*  $P < 0.001$ : difference between MCT2 and MCT4. Controls at 2 and 4 weeks are similar and were pooled.

### 4.2 Right myocardial histology and histochemistry

The histology is shown in Fig 2. Hematoxylin and eosin staining clearly shows hypertrophy of cardiomyocytes of the MCT4 rat, and also shows considerable heterogeneity of the cross-sectional area of cardiomyocytes in the hypertrophic heart and many interstitial cells, which are leucocytes (Handoko et al. 2009).

SDH activity was distributed fairly evenly in controls whereas it clusters in hypertrophic heart. Myoglobin shows a similar distribution as SDH activity in control and hypertrophic hearts. This staining was lost when sections were preincubated in saline before fixation, indicating that the peroxidase activity is not structurally bound (result not shown).

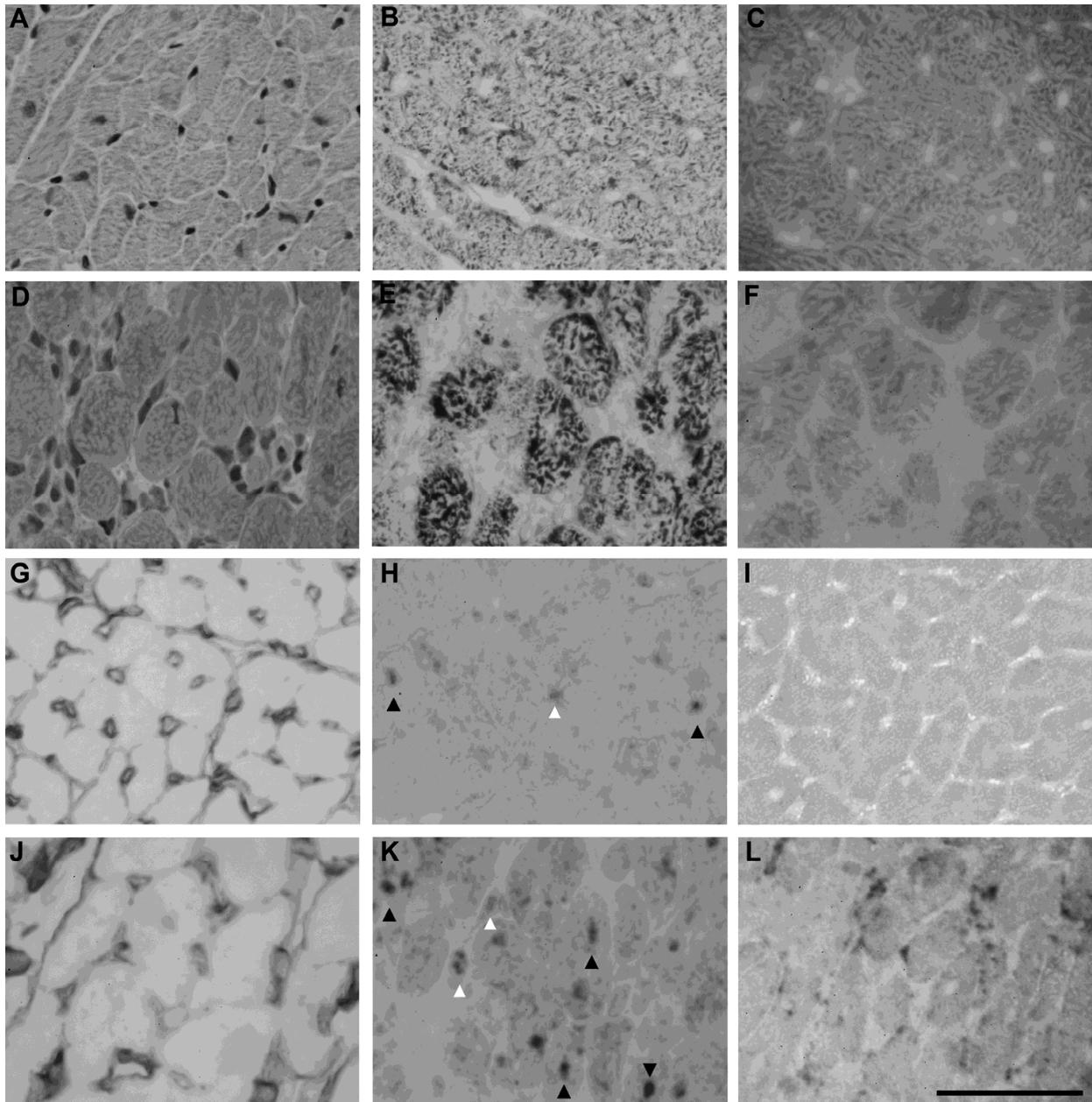


Fig. 2. Sections of a control and of a MCT-injected rat after 4 weeks. A,D: hematoxylin and eosin; B,E: SDH activity; C,F: Myoglobin concentration, G,J: Collagen type IV (capillaries); H,K: HIF 1 $\alpha$  expression; black arrow heads point at cardiomyocyte nuclei, white arrow heads point at nuclei in interstitial cells; I,L: cytochrome c. Top and third row: control; second and bottom row: MCT4. Bar 50  $\mu$ m.

The sections incubated for collagen type IV show a decrease of capillary density expressed per unit of area in the hypertrophic heart, indicating that maximum oxygen supply per unit mass of the hypertrophic myocardium was reduced, and intracellular diffusion distances were increased. HIF 1 $\alpha$  staining was very weak in control cardiomyocyte nuclei, whereas intensive nuclear staining as well as expression in interstitial cells was present in MCT4 rats.

Cytochrome c release was absent in controls. In MCT rats, cytochrome c was detected in a variable fraction of RV cardiomyocytes and could also be present in the interstitial space.

### 4.3 Oxygen demand and supply

The oxygen demand of the right heart can increase threefold due to the increase in pulmonary artery pressure (Handoko et al., 2009) and by a similar factor due to a decrease of mechanical efficiency (Wong et al., 2010). Fig. 3 shows the parameters relevant to oxygen demand and supply. Four weeks after monocrotaline the mean cross-sectional area of cardiomyocytes was significantly increased, whereas the mean SDH activity was similar to control. The maximum rate of oxygen uptake per unit of cardiomyocyte length ( $VO_{2max}CSA$ , in  $\mu\text{mol mm}^{-1} \text{s}^{-1}$ ) was increased, whereas the myoglobin concentration was slightly decreased in MCT4. These changes increased  $PO_{2crit}$ . The number of capillaries per cardiomyocyte in MCT4 is slightly higher compared to MCT2, but not different from control.

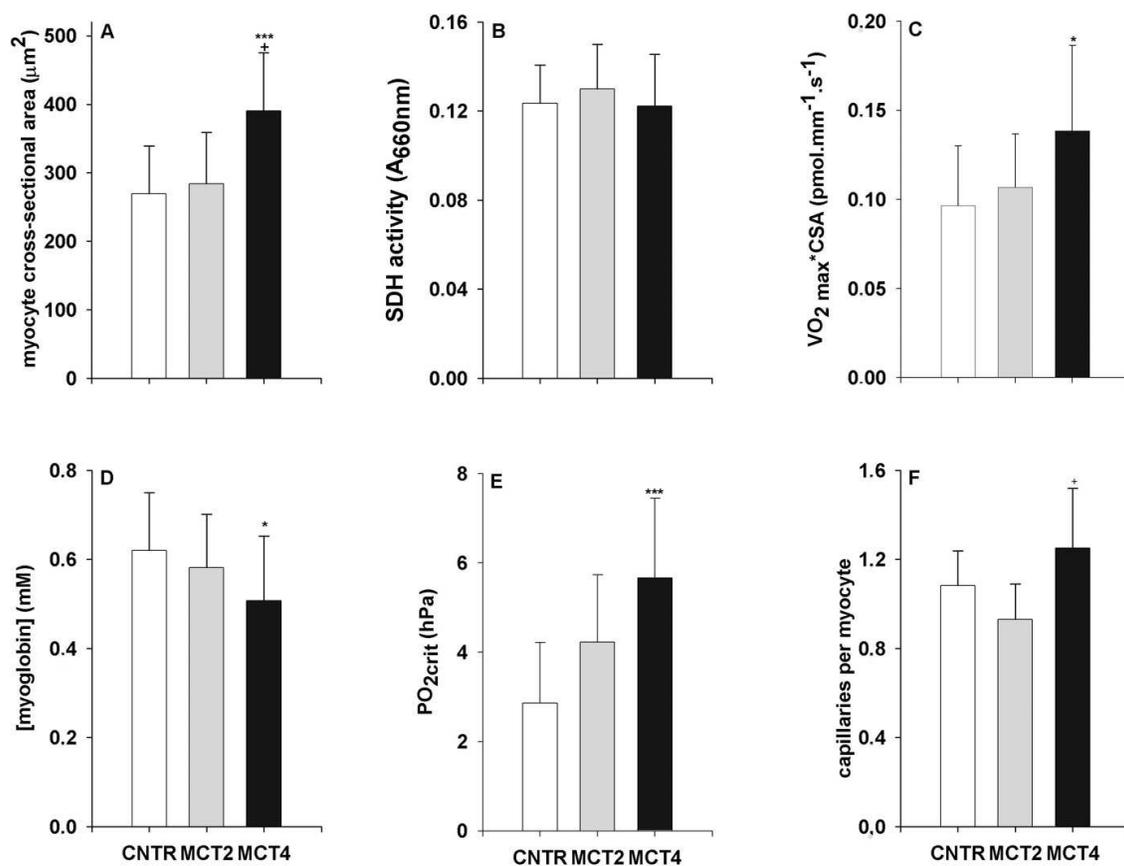


Fig. 3. Right ventricular cardiomyocyte characteristics of control rats (CNTR) and monocrotaline-injected rats after 2 (MCT2) and 4 weeks (MCT4). A: cardiomyocyte cross-sectional area (CSA); B SDH activity given as absorbance value at 660 nm; C: oxygen uptake per mm cardiomyocyte, calculated from data in A and B; D: myoglobin concentration; E:  $PO_{2crit}$  (see text); F: the number of capillaries per cardiomyocyte. \*  $P < 0.05$ , \*\*  $P < 0.001$ : difference between MCT and control; +  $P < 0.05$ , \*\*\*  $P < 0.001$ : difference between MCT2 and MCT4.

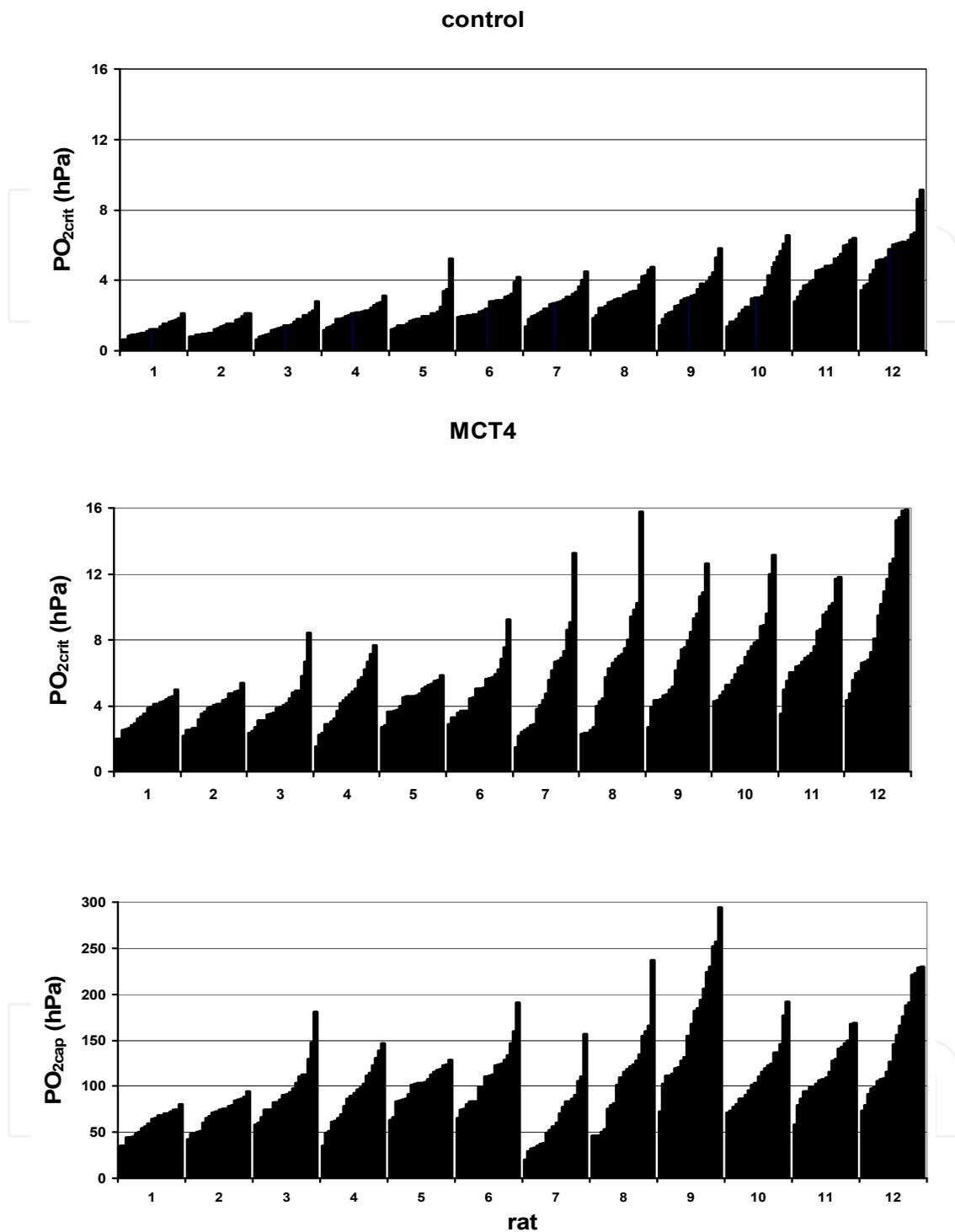


Fig. 4.  $PO_{2crit}$  of individual cardiomyocytes of 12 control rats (upper panel), and 12 MCT4 rats (middle panel), and required capillary  $PO_2$  in the MCT 4 rats (lower panel). 12 control rats were randomly selected and sorted by ascending mean  $PO_{2crit}$ .  $PO_{2crit}$  of 20 cardiomyocytes in each rat are shown, also sorted by ascending  $PO_{2crit}$ .  $PO_{2crit}$  was calculated using equations 1 and 2. This value was used to calculate the mean capillary  $PO_2$  required to prevent hypoxia in MCT4 cardiomyocytes using  $CSA \cdot VO_{2max} / PO_{2crit}$  from the middle panel, and the number of capillaries per cardiomyocyte (see text).

Fig. 4 shows the distributions of  $PO_{2crit}$  of individual cardiomyocytes. Individual rats differed with respect to  $PO_{2crit}$ , with mean  $PO_{2crit}$  ranging from 1.2 to 5.7 hPa; the overall mean was 2.8 hPa in control. In MCT4 rats, mean  $PO_{2crit}$  increased to 5.7 hPa mainly due to an increase in myocyte cross sectional area and slightly due to a decrease of the myoglobin concentration (Fig 3AD). However, the lowest  $PO_{2crit}$  values found in MCT4 rats were within the normal range observed in controls. The hypertrophic process increased the range of  $PO_{2crit}$  in MCT4 rats considerably, from 2 to 16 hPa. The control rats were used to calculate capillary permeability using Fick's law (equation 3). The permeability for oxygen normalised by the number of capillaries per myocyte (Fig. 3F) equalled 1.31 (SEM 0.17)  $\text{fmol mm}^{-1}\text{cap s}^{-1} \text{hPa}^{-1}$ . This value was used to calculate mean capillary  $PO_2$  in MCT4 rats. It can be inferred from the lower panel of Fig. 4 that a substantial fraction of the cardiomyocytes becomes hypoxic when the mitochondria are maximally activated and mean capillary  $PO_2$  equals 66 hPa. The highest required capillary  $PO_2$ 's found in MCT4 rats are well above normal arterial  $PO_2$  (about 133 hPa), indicating that the risk of oxygen supply limitation is substantial in MCT4 rats, especially during exercise.

#### 4.4 Cytosolic cytochrome c and the number of cardiomyocyte nuclei

It can be expected that the cardiomyocytes try to adapt to hypoxia. Indeed, Fig. 5 shows that HIF 1 $\alpha$  in cardiomyocyte nuclei was significantly increased in MCT4. Cytosolic cytochrome c was detected in cardiomyocytes of one out of six MCT2 rats and in nine out of twelve MCT4 rats. Interstitial cytochrome c was detected in two MCT2 rats and in eight MCT4 rats, indicating that HIF 1 $\alpha$  expression cannot prevent mitochondrial dysfunction. Release of cytochrome c can lead to apoptosis of cardiomyocytes, but surprisingly the numbers of cardiomyocyte nuclei in the right ventricular free wall of control and experimental groups were similar.

#### 4.5 Correlation analyses

Correlation analyses were carried out on the mean values of the parameters measured in MCT-injected animals, 2 and 4 weeks pooled ( $n = 18$ ). As shown in Fig 4B, the correlation of HIF 1 $\alpha$  expression and  $PO_{2crit}$  is significant ( $r = 0.64$ ,  $P=0.004$ ) but the explained variance of HIF 1 $\alpha$  expression only 41% ( $r^2 = 0.41$ ). The change of body mass - a decrease of body mass is indicative of the severity of heart failure in MCT injected rats - correlated with volume of cytoplasm per cardiomyocyte nucleus ( $r = -0.48$ ,  $P = 0.046$ ), lung wet mass ( $r = -0.74$ ,  $P < 0.001$ ) and lung dry mass ( $r = -0.79$ ,  $P < 0.001$ ).

HIF 1 $\alpha$  expression correlated strongly with the coefficient of variation of spatially integrated SDH activity (or  $VO_{2max}CSA$ , Fig 3C:  $r = 0.80$ ,  $P < 0.001$ ), indicating an increase of HIF 1 $\alpha$  expression with an increase of the variability of the maximum oxygen consumption per myocyte. There were no correlations between cytosolic cytochrome c or the presence of cytochrome c in the interstitial space and  $PO_{2crit}$  or HIF 1 $\alpha$  expression.

## 5. Discussion

### 5.1 Hypoxic cores in hypertrophied cardiomyocytes

The results demonstrate heterogeneous responses of most myocardial parameters to MCT-induced pulmonary hypertension, not only between rats within experimental groups, but

also between individual cardiomyocytes in a heart. The heterogeneity indicates that individual cardiomyocytes react differently to MCT-induced increased workload. Furthermore, the heterogeneity is strongly related to HIF 1 $\alpha$  expression.  $PO_{2crit}$  increases in most MCT rats, and correlates with the expression of HIF 1 $\alpha$  in nuclei of cardiomyocytes. The upper value of  $PO_{2crit}$  is similar to coronary sinus  $PO_2$  during maximum exercise, 20 hPa (swine: Merkus et al., 2003; dog: Tune et al., 2004). End capillary venous  $PO_2$  in the RV myocardium of MCT rats is not known. It can be lower than 20 hPa, because myocardial blood flow is heterogeneous (Zuurbier et al., 1999), arterial  $PO_2$  in awake, freely moving MCT rats is reduced (to  $88 \pm 7$  hPa (Hill et al., 1989), and because interstitial  $PO_2$  must be lower than capillary  $PO_2$  to extract oxygen. The present results provide an explanation for reduced survival induced by training in progressive pulmonary hypertensive rats (Handoko et al., 2009), because repeated increases of the workload of the right ventricle can lead to hypoxia-reoxygenation injury.

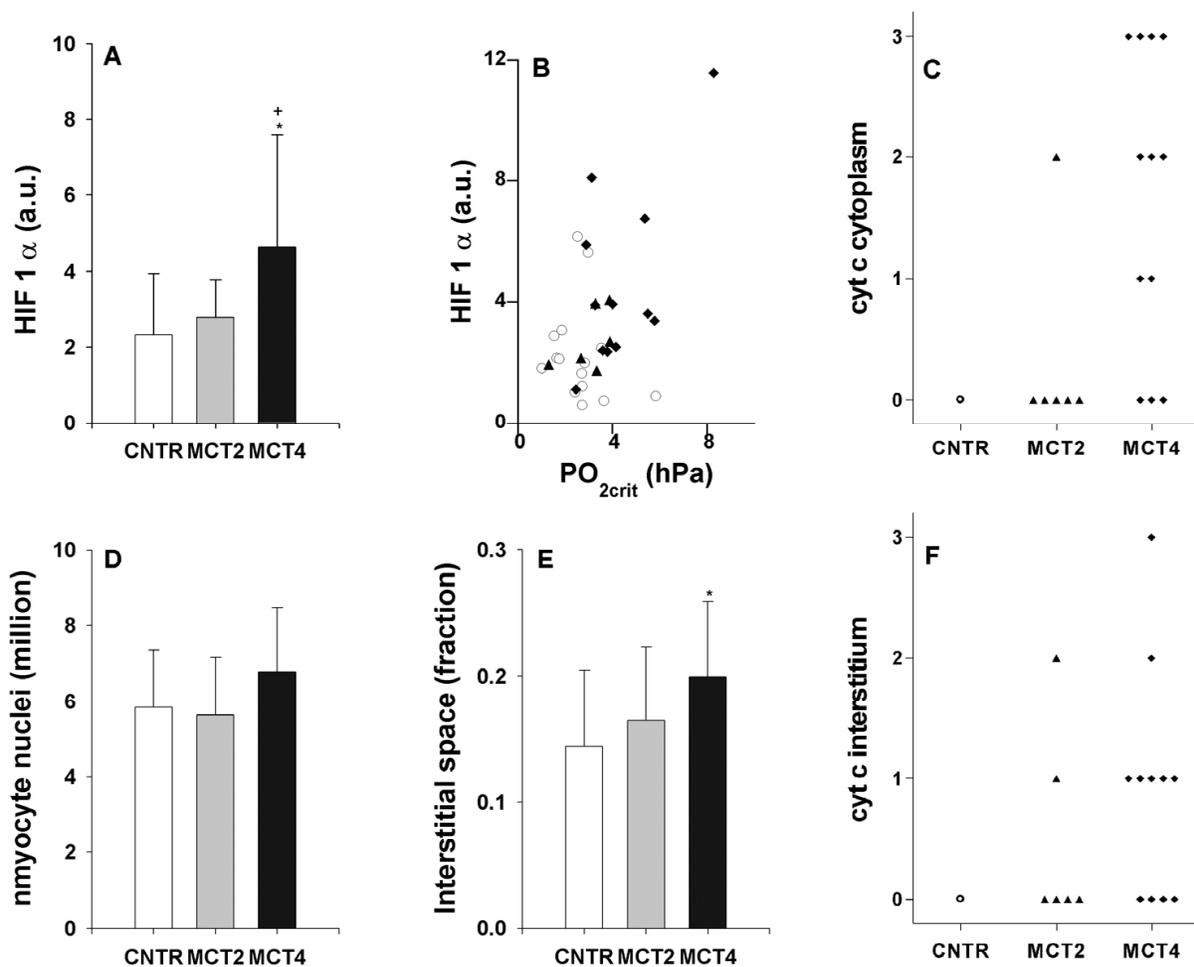


Fig. 5. A: Expression of HIF 1 $\alpha$  in arbitrary units (a.u.) in nuclei of right ventricular cardiomyocytes of control (CNTR) and monocrotaline-injected rats after 2 (MCT2) and 4 (MCT4) weeks. B: HIF 1 $\alpha$  expression in individual rats related to  $PO_{2crit}$ .  $\circ$ : control,  $\blacktriangle$ : MCT2;  $\blacklozenge$  MCT4, C: cytochrome c detected in cardiomyocyte cytoplasm; all 15 controls are negative; D: the number of cardiomyocyte nuclei in the right ventricular free wall; E: interstitial space; F: presence of cytochrome c in interstitium. \*  $P < 0.05$ : difference from control, +  $P < 0.05$ : different from MCT2.

## 5.2 Oxygen consumption and cytochrome c release

The effect of cytochrome c release on the rate of oxygen consumption and  $PO_{2crit}$  is not known. We previously found (van der Laarse et al., 2005) that the maximum rate of oxygen consumption of myocardial trabecula dissected from the right ventricular wall of MCT4 rats is not different from control, whereas the rate of oxygen consumption at rest is much higher than control. It is a possibility that the high rate of oxygen consumption of quiescent hypertrophied trabeculae is due to cytochrome c release because cytosolic cytochrome c can oxidize cytosolic NADH (La Piana et al., 2005). This will reduce the efficiency of metabolic recovery, i.e. lower ATP/O<sub>2</sub>. It is unlikely therefore, that the release of cytochrome c would cause a decrease of  $VO_{2max}$  and  $PO_{2crit}$  in failing myocardium. It is a possibility that mitochondrial damage is the reason for the reduced mechanical efficiency in observed in papillary muscles (Wong et al., 2010).

Bache et al. (1999) concluded that changes in energy rich phosphate concentrations that occur during high workload of hypertrophic hearts were not due to limited myocardial oxygenation using <sup>1</sup>H nuclear magnetic resonance to monitor myoglobin desaturation *in situ* in the anaesthetised dog. In these studies, however, coronary sinus  $PO_2$  was about 40 hPa. Because this  $PO_2$  is twice the value measured in exercising animals (see above), it is not surprising that myoglobin remained saturated with oxygen in these experiments. This argument may also hold for a similar study in normal rat myocardium by Kreutzer et al. (2001), in which coronary sinus oxygen content was 2.8 mM during dopamine infusion (34% of control, corresponding to  $PO_2 = 44$  hPa; Gray et al., 1964).

The release of cytochrome c from the mitochondria is a first sign of failure of cardiomyocytes to adapt to overload. It could already be observed two weeks after the monocrotaline injection when hypertrophy started to develop. We did not identify a single factor or a combination of factors that could fully explain the release, possibly because relevant factors were excluded in the analyses. For instance, arterial  $PO_2$  was not taken into account and interstitial cytochrome c may have been removed from the heart by lymph or blood (Radhakrishnan et al., 2007).

## 5.3 Cytochrome c release and the number of cardiomyocyte nuclei

The amount of cytochrome c released in different cardiomyocytes in one heart can vary from no to total release (van Beek-Harmsen & van der Laarse, 2005). Cytochrome c release did not induce apoptosis judging from the number of cardiomyocyte nuclei in the right ventricular free wall. This is surprising because we previously found a 40% decrease of the number of cardiomyocyte nuclei (des Tombe et al., 2002). Ecarnot-Laubriet et al. (2002) found apoptotic nuclei in the right ventricular myocardium of MCT-injected Wistar rats after 3 weeks but used a 1.5 times higher dose, 60 mg MCT/kg body mass. The reason for not finding a reduction of the number of cardiomyocyte nuclei in the present study could be that the occurrence of apoptosis in MCT-injected rats is dose-dependent and critically depends on type of rat strain, and food and housing conditions. This requires further study. We conclude that in the present experiments apoptosis is not the cause of the transition from hypertrophy to heart failure.

## 5.4 Myoglobin

The myoglobin concentration in hypertrophied cardiomyocytes decreased. It may be that the capacity to synthesize myoglobin (e.g. the increased cytoplasm/nucleus ratio or the

availability of iron (Rohbach et al., 2007) is the limiting factor, or that degradation of myoglobin in hypertrophied cardiomyocytes is increased, e.g. due to  $H_2O_2$  production. We previously found higher myoglobin concentrations in hyperthyroid rats compared to MCT-injected rats (Lee-de Groot et al., 1998).

Transcription of the myoglobin gene is thyroid hormone (Gianocco et al., 2004) and vascular endothelial growth factor (VEGF) dependent (van Weel et al., 2004). Myocardial overload can lead to a hypothyroid state due to induction of deiodinase (Simonides et al., 2008), while VEGF expression is regulated by HIF 1 $\alpha$ . (Forsythe et al., 1996). It has been shown that HIF 1 $\alpha$  in pressure overloaded mouse heart can be inhibited by p53 (Sano et al., 2007). Whether or not p53 plays a role in the monocrotaline-induced pulmonary hypertension model remains to be investigated. Myoglobin mRNA is reduced in dog and bovine dilated myocardium (O'Brien et al., 1995; Weil et al., 1997), but is increased in pressure overloaded mouse heart (Lindsey et al., 2007). These results indicate that myoglobin expression may critically depend on different types of interacting factors. Increasing the myoglobin concentration in hypertrophied cardiomyocytes is an important therapeutic target because it can lower  $PO_{2crit}$  and thereby increase oxygen extraction from capillaries.

## 5.5 Limitations

The values of  $PO_{2crit}$  and  $PO_{2cap}$  presented above are underestimates because the model calculation of  $PO_{2crit}$  assumes zero-order kinetics. Rumsey et al. (1990) showed that the Michaelis constant for oxygen of isolated rat heart mitochondria is 0.45 hPa at rest. However, any deviation from zero-order kinetics increases  $PO_{2crit}$ , impairing oxygen extraction from capillaries. The Michaelis constant may also increase due to inhibition of complex IV by nitric oxide (Cooper & Brown, 2008). This could increase intracellular  $PO_2$  at the expense of reduced ADP phosphorylation and cardiac output. The calculation of capillary and interstitial permeability is based on assuming a reasonable mean capillary  $PO_2$  but this permeability has not been verified. The calculation of capillary  $PO_2$  in MCT4 rats is based on the assumption that capillary permeability in control and pulmonary hypertensive rats is the same. This is a simplification because capillary circumference may increase, thereby increasing the endothelial diffusion area, whereas the permeability can decrease because the capillary basement membrane thickens. These changes require further study and direct measurements of capillary permeability for oxygen are required. The translation of the present results to clinical applications will require the demonstration of similar changes in pulmonary hypertensive patients. Preliminary results indicate that this is the case (van Beek-Harmsen et al., 2007; Ruiter et al., 2011).

## 5.6 Conclusions

We conclude that hypertrophying cardiomyocytes in MCT-injected pulmonary hypertensive rats adapt to hypoxia. However, the changes observed in the hypertrophied myocardium of MCT rats will lead to hypoxic cores in cardiomyocytes with high  $VO_{2max}CSA$  or  $PO_{2crit}$  when the mitochondria are maximally activated. The adaptation cannot prevent cytochrome c release from mitochondria. The results indicate that an increase of the number of capillaries per cardiomyocyte is required to normalize the oxygen supply/demand ratio in the hypertrophied RV myocardium and that an increase of the myoglobin concentration is required to normalize  $PO_{2crit}$ . When  $PO_{2crit}$  cannot be normalized, interstitial  $PO_2$  has to

increase to prevent core hypoxia in hypertrophied cardiomyocytes, or intramyocyte capillaries (Kobayashi et al., 1999) have to be induced.

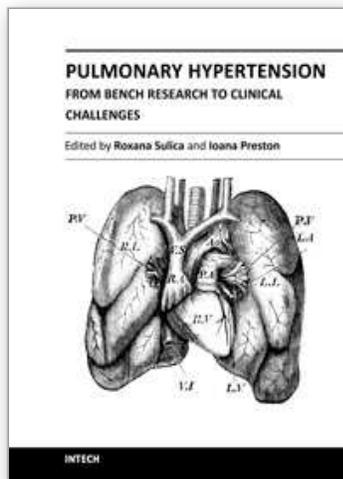
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## **Pulmonary Hypertension - From Bench Research to Clinical Challenges**

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