

# Protein Profiling of Human Atrial and Ventricular Musculature: the Effect of Normoxaemia and Hypoxaemia in Congenital Heart Diseases

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## Summary

Samples of myocardial tissue were obtained during surgical intervention from children operated for different types of congenital heart disease (tetralogy of Fallot, ventricular and atrial septal defect). Sarcoplasmic, contractile and collagenous proteins were isolated by stepwise extraction from the both right ventricular and atrial musculature. It has been found that: a) the concentration of contractile proteins is significantly higher in the ventricles, b) the concentration of collagenous proteins is significantly higher in the atrium, c) the concentration of sarcoplasmic proteins was not different, d) in children with chronic hypoxia the above atrio-ventricular differences persisted. Moreover, the proportion of the soluble collagenous fraction in the atria was significantly increased.

## Key words

Protein profiling of cardiac muscle – Contractile proteins – Collagenous proteins – ATPase of atrial and ventricular myosin – Congenital heart disease – Tetralogy of Fallot – Ventricular septal defect – Atrial septal defect

## Introduction

The structure of ventricles and atria of the mammalian myocardium differs considerably. The ventricular myocytes are organized into layers lying in the same direction, while in the atrial musculature cells form bundles of varying sizes. Myocytes from ventricular musculature are large with closely interdigitating intercalated discs arranged in a stepwise manner, they have more abundantly articulated T-tubules and mitochondrial matrices without granules. On the other hand, atrial cells are more slender, simpler, with intramitochondrial inclusions in the mitochondria; they contain "specific atrial granules" (Sartore *et al.* 1981, Gorza *et al.* 1982, Sommer and Jennings 1992). Gross biochemical analysis of both cardiac compartments showed no differences in dry weight and water content or in glycogen concentration, but atria were found to have a higher lipid content (Arminger *et al.* 1984). Significant atrio-ventricular

differences were also observed at the level of contractile proteins; the structure and enzyme activity of myosin in the two compartments differs (Swynghedauw 1986, Syrový 1987, Yazaki *et al.* 1989). Surprisingly little attention has been paid to a comparison of collagen proteins; a number of studies have appeared which are concerned with ventricular collagenous structures (Weber 1989, Borg and Terracio 1990) but only isolated data are available on atrial collagen (Imataka *et al.* 1989).

Our previous study (Bass *et al.* 1988) showed that in the myocardium of children that had undergone surgery of congenital heart defects, ventricular musculature has a higher capacity of enzymes that catalyze the aerobic and anaerobic splitting of various substrates; the atrial muscle preferentially utilizes glucose. Chronic hypoxaemia, which accompanies congenital heart defects of the cyanotic type, lowered

the capacities of oxidative enzymes in both myocardial compartments but the changes were more conspicuous in the atria (Šamánek *et al.* 1989). In the present study main attention was paid to the protein composition of the myocardium in normoxaemic and hypoxaemic patients. The work aimed at determining whether: a) the protein profiling of atria and ventricles differs and b) whether chronic hypoxaemia affects the qualitative and quantitative composition of collagenous and noncollagenous structures.

## Material and Methods

The examination was performed on 51 samples of human cardiac tissue obtained during surgery of children with congenital heart disease (patients' age: 6 months to 16 years). The samples were taken from right atria (n=25) and right ventricles (n=8) obtained from patients with a normoxaemic defect, and also from right atria (n=9) and right ventricles (n=9) from patients with a hypoxaemic defect (Table 1). Preliminary observations did not reveal significant differences in individual protein fractions during the postnatal period studied. The samples from children of different ages were therefore pooled. The atrial tissue was excised before commencement of the cardiopulmonary bypass; the ventricular samples were taken 2 to 80 min after the heart beat had been arrested; no "in vitro aging" of collagenous and non-collagenous proteins was observed during that time. Samples were rapidly weighed and transferred into precooled homogenization test tubes with 200  $\mu$ l of 50 mmol.l<sup>-1</sup> sodium-potassium-phosphate buffer, pH 7.4, containing 10 mmol.l<sup>-1</sup> EDTA and 1% Triton X-100. Subsequently they were frozen to -50 °C and kept at this temperature until the next isolation stage. Later they were thawed, the volume made up to 20-fold original with a buffer, homogenized and centrifuged at 15 000 x g. This step was repeated once more and pooled supernatants (a 40-fold multiple of the original amount) were used for the determination of sarcoplasmic proteins. In subsequent steps, the pellet was resuspended and fractions of contractile and collagenous proteins were obtained in a stepwise manner by extracting contractile proteins into a supernatant with a phosphate buffer (100 mmol.l<sup>-1</sup>, pH 7.4, containing 1.1 mol.l<sup>-1</sup> KCl) (cf. Fig. 1); the pellet was shortly washed with 0.5 mol acetic acid then extracted with 0.5 mol.l<sup>-1</sup> CH<sub>3</sub>COOH-pepsin (pH 1.45); pepsin concentration was kept in the range 1:100 - 1:50. After 24 h at 4 °C the extracts were centrifuged. The supernatant contained the fraction of soluble collagenous proteins. The pellet was further resuspended in 1.1 mol.l<sup>-1</sup> NaOH and left for 45 min at 105 °C. This fraction contained insoluble collagenous

proteins. The procedure (Fig. 1) yielded 3 basic fractions:

- sarcoplasmic proteins (containing predominantly enzyme systems for aerobic and anaerobic substrate utilization see Bass *et al.* (1988);
- contractile proteins (complex of proteins that transduce the chemical energy of ATP to mechanical contractile work);
- structural collagenous proteins (extracellular matrix - the fraction includes collagens, elastins, proteoglycans and glycoproteins) which can be divided into two fractions: 1) soluble collagenous proteins are constituted mainly by collagen I and III, and 2) insoluble collagenous proteins include collagen aggregates, elastins and other proteins of the extracellular matrix. This methodological approach has been described in detail elsewhere (Pelouch *et al.* 1980, 1984, 1992).

## ISOLATION OF PROTEIN FRACTIONS

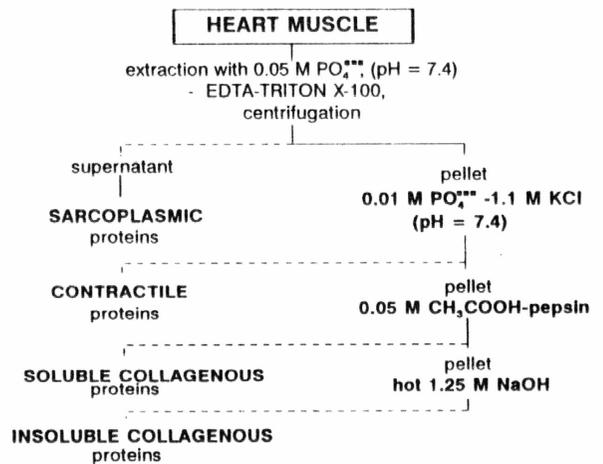


Fig. 1

Scheme of isolation of protein fractions from heart muscle.

Table 1

Subject of study

Diagnosis	n	Arterial oxygen Saturation % ± S.E.M.
Tetralogy of Fallot	17	61.5 ± 3.8
Ventricular septal defect	18	94.6 ± 1.3
Atrial septal defect	11	93.6 ± 0.9
Others	5	94.0 ± 3.0

Protein concentration in individual fractions was determined according to Lowry *et al.* (1951). Total protein was the sum of concentrations of noncollagenous and both fractions of collagenous proteins while the sum of sarcoplasmic and contractile proteins corresponds to total noncollagenous proteins. The results are expressed as  $\text{mg}\cdot\text{g}^{-1}$  sample wet weight. Concentration of hydroxyproline ( $\text{mg}\cdot\text{g}^{-1}$  sample wet weight) was determined in the pepsin-soluble and pepsin-insoluble fraction of collagenous proteins (Huszar 1980). Total hydroxyproline was obtained by pooling the concentrations of hydroxyproline in the fraction of soluble and insoluble collagenous structures. The ATPase activity of heart myosin was determined by measuring enzymatically released phosphate (Fiske and Subbarow 1925) after 10-min incubation of a sample of contractile proteins at 25 °C. The activity was expressed in  $\text{mol P}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$  (Pelouch *et al.* 1980, 1985, 1987, 1989).

#### Statistical evaluation

Experimental values are given as means  $\pm$  S.E.M. Differences were evaluated by Student's t-test and were considered significant for  $p < 0.05$  or less.

## Results

### A. Atrio-ventricular differences in protein composition

Protein concentrations in individual fractions isolated from atria and ventricles from the normoxaemic patients are given in Table 2. The concentration of total atrial protein in the normoxaemic group significantly exceeds the ventricular one; this atrio-ventricular difference reflects a higher concentration of collagenous proteins. The concentration of contractile proteins is higher in the ventricles while the concentration of sarcoplasmic proteins in the two compartments is about equal. No significant differences were found between the enzyme activity of atrial and ventricular myosin ( $5.57 \pm 0.78$  for atrial,  $5.49 \pm 0.75$  for ventricular musculature).

The higher concentration of collagenous proteins in the atria of normoxaemic patients corresponds to a higher concentration of hydroxyproline (Table 3). The qualitative composition of collagenous proteins in the two cardiac compartments also differs: ventricles contain a significantly (by some 12 %) higher amount of soluble collagenous proteins (expressed as hydroxyproline concentration) whereas atria exhibit a higher concentration (by about 85 %) of insoluble collagenous fraction (Table 3).

### B. Effect of hypoxaemia on protein composition of atria and ventricles

The atrio-ventricular difference in total protein concentration observed in normoxaemic samples, however, disappeared in hypoxaemic patients: protein concentration in hypoxaemic atria was significantly lower than in normoxaemic patients (Table 2). On the other hand, hypoxaemia had no effect on the total protein concentration in ventricular musculature. The atrio-ventricular differences in the concentration of contractile and collagenous proteins were also about the same in the two groups. The concentration of collagenous (total extracellular proteins) and sarcoplasmic proteins in hypoxaemic atrial musculature (Table 2) was, however, significantly lower (Table 2) The concentration of sarcoplasmic proteins in hypoxaemic atrial musculature was, however, significantly lower as compared with the normoxaemic group.

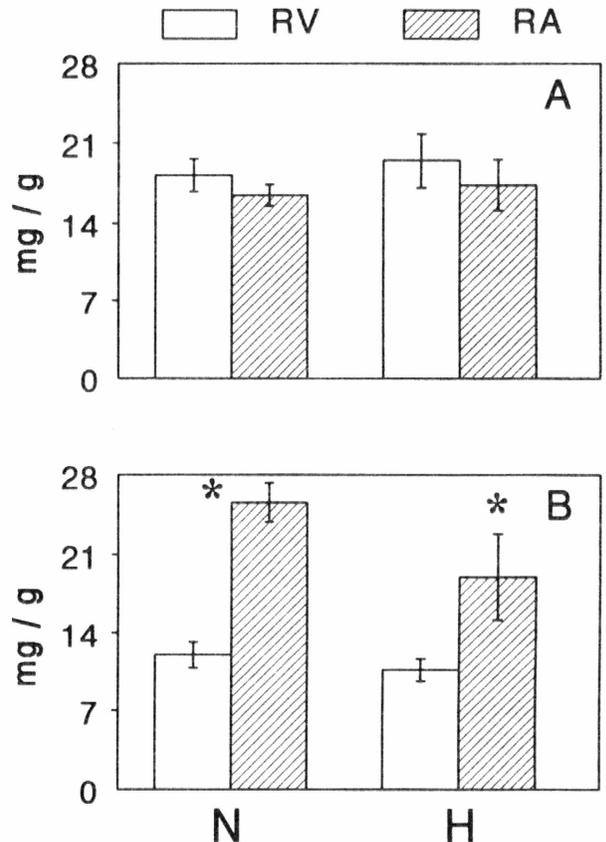


Fig. 2

Protein profiling of collagenous fraction. Concentration of pepsin-soluble (upper part-A) and pepsin-insoluble (lower part-B) collagenous proteins in the human right ventricle (RV-white bars) and right atrium (RA-black bars) in normoxaemic (N) and hypoxaemic (H) patients. Values are means  $\pm$  S.E.M. (expressed in  $\text{mg}$  per  $\text{g}$  of wet weight). Significance: \*  $p < 0.01$  vs RV

**Table 2**

Protein profiling of the human right ventricle (RV) and right atrium (RA) in patients with normoxaemic and hypoxaemic congenital heart disease

Proteins (mg.g <sup>-1</sup> wet weight)	Normoxaemia		Hypoxaemia	
	RV n=8	RA n=25	RV n=9	RA n=9
Total	99.91 ±4.06	110.44* ±2.10	97.85 ±3.61	96.17 <sup>a</sup> ±2.17
Sarcoplasmic	42.26 ±1.76	44.10 ±1.01	41.47 ±2.11	39.64 <sup>a</sup> ±3.21
Contractile	27.78 ±1.92	23.41* ±0.80	26.80 ±2.41	22.11* ±1.30
Collagenous	30.19 ±1.99	42.01* ±1.76	30.14 ±2.30	36.28* ±2.50

n - number of experiments. Values are means ± S.E.M.

\* p < 0.01 vs RV in corresponding group; <sup>a</sup> p < 0.01 vs RA in normoxaemic group

**Table 3**

Concentration of hydroxyproline in the human right ventricle (RV) and right atrium (RA) in patients with normoxaemic and hypoxaemic congenital heart disease

Hydroxyproline (mg.g <sup>-1</sup> wet weight)	Normoxaemia		Hypoxaemia	
	RV n=8	RA n=25	RV n=9	RA n=9
Total	1.65 ±0.22	3.53* ±0.39	1.64 ±0.09	3.45* ±0.35
In soluble - collagenous fraction	0.42 ±0.02	0.42 ±0.03	0.44 ±0.06	0.76* <sup>a</sup> ±0.12
In insoluble collagenous fraction	1.23 ±0.20	3.11* ±0.20	1.20 ±0.12	2.63* ±0.36

For legends see Table 2. Values are means ± S.E.M.

\* p < 0.01 vs RV in corresponding group, <sup>a</sup> p < 0.01 vs RA in normoxaemic group

More detailed analysis of the collagenous fraction has shown that the amount of soluble collagenous proteins in atrial and ventricular myocardium did not differ (Fig. 2A). On the other hand, the concentration of insoluble collagenous proteins (Fig. 2B) is approximately 100 % higher in atrial musculature, in both the normoxaemic and hypoxaemic groups.

Hypoxaemia had no effect on the total hydroxyproline concentration or on its concentrations

in soluble fraction of collagenous proteins of ventricular musculature (Table 3). However, hypoxaemia significantly increased hydroxyproline concentration in the soluble fraction of atrial collagenous proteins, and thereby also the relative proportion of the two types of collagenous proteins. By contrast, the relative proportion of individual collagenous fractions in ventricles remained unaffected (Table 3).

## Discussion

The results imply that the proportion of individual protein fractions is significantly different in the right ventricular and atrial musculature of the human myocardium. The concentration of contractile proteins is significantly higher in ventricles whereas collagenous proteins are more abundant in atria. Quantitative atrio-ventricular differences stem from the different structure, function and protein turnover rate (for details see Swynghedauw 1986). Although the Ca-ATPase activity of atrial myosin in most animal species is higher than the ventricular one (Dechesne *et al.* 1985, Syrový 1987) in the case of human cardiac myosin this value was found to be higher only when the measurement of the ATPase activity of myosin was performed at temperatures above 25 °C (Hoffman *et al.* 1987a,b). This is apparently the reason why no differences in the enzyme activity of myosin were found in our experimental setup; the primary change is likely to be in the quantitative and qualitative profile of light myosin chains (Takeda *et al.* 1985, Hirzel *et al.* 1985).

An important atrio-ventricular difference was observed in the fraction of collagenous proteins (a mixture of different collagens, elastins and glycoproteins) which form an extensive network of myocardial connective tissue; these proteins are synthesized by fibroblasts, extruded molecular forms are transformed to fibrillar ones in the extracellular space (Henney *et al.* 1982, Weber *et al.* 1987, 1989, 1990, Laurent 1987, Kozlovskij *et al.* 1987, Kawahara *et al.* 1990). There is a relative wealth of literary data on the quantitative and qualitative properties of the ventricular collagenous structure (Laurent *et al.* 1987, Weber *et al.* 1989, 1990, Kawahara *et al.* 1990), while considerably less is known about the composition of extracellular proteins in the atria of different animal species. The atrial musculature has always been found to have a higher content of hydroxyproline (Caspari *et al.* 1976, Pelouch *et al.* 1988, Imataka *et al.* 1989). In the present study we describe not only a higher concentration of collagen in the atria but also a different composition of atrial collagens; they contain more pepsin-insoluble collagenous proteins (the fraction comprises primarily collagen aggregates and elastins). Since this fraction contains no newly synthesized collagenous proteins (these occur in the fraction of soluble collagenous proteins) we may assume that the higher concentration of collagen in atrial musculature, along with different qualitative parameters, originated probably from a different activation of fibroblasts. It should be noted that two types of growth factors were detected in the myocardium; the concentration of the basic fibroblast growth factor in different animal species was always higher in the atria than in the ventricles (Kardami and Fandrich 1989).

We have shown that hypoxaemia significantly modulates the protein composition of the human heart muscle. Total protein concentration in the atrial musculature of hypoxaemic patients is, however, substantially lower than in patients with a normoxaemic heart disease. This decrease results from a lowered concentration of both noncollagenous and collagenous proteins. All congenital heart defects have, however, a hypertrophic myocardium as a consequence; in hypoxaemic defects this is occasioned by the increased pressure load (Morgan and Baker 1991), in normoxaemic diseases the volume load is the prime factor. Experimental hypertrophy is known to modify substantially the quantitative properties of contractile and collagenous proteins (Pelouch *et al.* 1980, 1987, Hoffman *et al.* 1987a,b, Morgan and Baker 1991, Nakanishi *et al.* 1987, Nostray *et al.* 1983, Dalla-Libero *et al.* 1983, Brooks *et al.* 1987). Examination of protein profiling did not provide an unambiguous answer to the question of why the total protein content (expressed as  $\text{mg}\cdot\text{g}^{-1}$  of wet weight) is lower in the hypoxaemic atrium. We assume this to be the consequence of a number of factors, e.g. changes in tissue hydration, metabolic adaptation of the myocardium, or a change of substrates in the hypoxaemic myocardium as documented by measurements of the activities of different enzymes of energy metabolism (Bass *et al.* 1988, Šamánek *et al.* 1989a,b). However, hypoxaemia did not change the atrio-ventricular difference in the concentration of contractile and collagenous proteins.

The finding that hypoxaemia did not significantly increase the concentration of collagenous proteins in the ventricular myocardium is seemingly surprising. It is namely known that intracellular oxygen tension may directly modify the rates of division of cardiac cells: a decrease in oxygen concentration increases cell division of both myocytes and fibrocytes (Hollenberger *et al.* 1976, 1977, Sen and Bumpus 1979). After birth, the mitotic activity of cardiac muscle cells declines rapidly, but the mitotic activity of fibrocytes persists throughout life. Chronic hypoxia in newborn rats (12-15 % oxygen) brought about a significant increase in the hydroxyproline content after 3 weeks; an absolute increase in the collagen content was not proportional to the growth of myocardial weight, therefore, the collagen concentration measured *via* hydroxyproline was actually lower (Hollenberger *et al.* 1976). An increase in collagen concentration in the myocardium of newborn animals (Pelouch *et al.* 1987) was observed only in the case of prolonged (5 weeks) and relatively intensive (8 % oxygen) hypoxia. The observation that neither the total concentration of collagenous proteins nor the concentration of hydroxyproline increased in hypoxaemic patients, despite the myocardial hypertrophy, may be due to the fact that the growth of the collagen stroma is proportional to the growth of contractile proteins. Furthermore, the analyzed tissue was collected during

surgery performed at the earliest possible date after diagnosis of the cardiac defect. A higher accumulation of collagen or cytoskeletal proteins (e.g. desmin, vimentin, vinculin-(Schapper *et al.* 1991) is thus likely to have been prevented.

The elevated concentration of hydroxyproline in the fraction of soluble collagenous proteins (predominantly collagen I and III) in the atria of hypoxaemic patients attests to a faster collagen synthesis. At present, little is known about collagen synthesis even in the normal myocardium (Caufield 1983, Factor 1990, Eghbali 1988). However, the

collagen growth appears to be a function of different stimuli, e.g. age, species, rapidity at which cardiac overload or hypoxia occurs (Weber 1987, 1990, Sen and Bumpus 1979, Schapper *et al.* 1991, Eghbali *et al.* 1988), the myocyte growth is primarily governed by ventricular work (Weber 1989, 1992) induced by the changes of haemodynamic parameters in congenital heart diseases. Lower arterial oxygen saturation probably affects the synthesis of collagen preferentially in atrial compartments. The reason for this is still far from clear.

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