Available online at www.sciencedirect.com
SCIENCE DIRECT®
Growth Hormone & IGF Research xxx (2005) xxx–xxx
www.elsevier.com/locate/ghir

Growth hormone- and pressure overload-induced cardiac hypertrophy evoke different responses to ischemia-reperfusion and mechanical stretch

Hinrik Strömer [a], Emiliano A. Palmieri [b], Mark C.H. De Groot [a], Francesca Di Rella [b], Andrea Leupold [a], Michael Horn [a], Maria G. Monti [b], Raffaele Napoli [b], Angela Di Gianni [b], Jörgen Isgaard [c], Luigi Saccà [b], Stefan Neubauer [d], Antonio Cittadini [b,*]

[a] *Department of Medicine, Medizinische Universitätsklinik Würzburg, 97080 Würzburg, Germany*
[b] *Department of Internal Medicine and Cardiovascular Sciences, University Federico II, Via S. Pansini, 5, 80131 Naples, Italy*
[c] *Research Center for Endocrinology and Metabolism, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden*
[d] *University of Oxford Centre for Clinical Magnetic Resonance Research, Department of Cardiovascular Medicine, John Radcliffe Hospital, OX3 9DU Oxford, UK*

Received 28 January 2005; revised 5 September 2005; accepted 6 September 2005

Abstract

Objective. To compare the molecular, histological, and functional characteristics of growth hormone (GH)- and pressure overload-induced cardiac hypertrophy, and their responses to ischemia-reperfusion and mechanical stretch.
Design. Four groups of male Wistar rats were studied: aortic banding ($n = 24$, AB) or sham ($n = 24$, controls) for 10 weeks, and GH treatment ($n = 24$; 3.5 mg/kg/day, GH) or placebo ($n = 24$, controls) for 4 weeks. At 13 weeks, the rats were randomly subjected to: (i) assessment of basal left ventricular mRNA expression of sarcoplasmic reticulum calcium-ATPase (SERCA-2), phospholamban (PLB), and Na^+–Ca^{2+} exchanger (NCX) and collagen volume fraction (CVF) (*Protocol A*, 8 rats in each group); (ii) left ventricular no-flow ischemia with simultaneous evaluation of intracellular Ca^{2+} handling and ATP, phosphocreatine (PCr) and inorganic phosphate (Pi) content (*Protocol B*, 12 rats in each group); or (iii) left ventricular mechanical stretch for 40 min with assessment of tumor necrosis-α (TNF-α) mRNA (*Protocol C*, 4 rats in each group). *Protocol B* and *C* were carried out in a Langendorff apparatus.
Results. In *Protocol A*, no difference was found as to myocardial mRNA content of Ca^{2+} regulating proteins and CVF in GH animals vs controls. In contrast, in the AB group, myocardial mRNA expression of SERCA-2 and PLB was downregulated while that of NCX and CVF were increased vs. controls ($p < 0.05$). In *Protocol B*, recovery of left ventricular function was significantly decreased in AB vs GH groups and controls and this was associated with 1.6-fold increase in intracellular Ca^{2+} overload during reperfusion ($p < 0.05$). Baseline ATP content was similar in the four study groups, whereas PCr and Pi was lower in AB vs GH rats and controls. However, the time courses of high-energy phosphate metabolic changes did not differ during ischemia and reperfusion in the four study groups. In *Protocol C*, no detectable TNF-α mRNA level was found in the left ventricular myocardium of GH treated rats and controls at baseline, while a modest expression was noted in AB animals. Mechanical stretch resulted in de novo myocardial TNF-α mRNA expression in GH group and controls, which was dramatically increased in AB animals (≈5-fold above baseline, $p < 0.001$).

* Corresponding author. Tel.: +39 81 7464375; fax: +39 81 7463199.
E-mail address: antonio.cittadini@unina.it (A. Cittadini).

1096-6374/$ - see front matter
doi:10.1016/j.ghir.2005.09.002

Conclusions. The data show that cardiac hypertrophy activated by short-term GH treatment confers cardioprotection compared with pressure overload with regard to molecular and histological characteristics, and responses to ischemia-reperfusion and mechanical stretch.

Keywords: Ischemia-reperfusion; Calcium handling; Cytokines; Somatotropin; Hypertrophy

1. Introduction

Augmenting muscles mass is one of the means by which the heart faces a hemodynamic burden, in addition to the recruitment of neurohormonal mechanisms and the use of the Frank–Starling mechanism [1]. However, load-induced hypertrophy (*pathologic hypertrophy*) is characterized by interstitial fibrosis, molecular remodeling with reduced sarcoplasmic reticulum and increase of sarcolemmal content of Ca^{2+} regulating proteins, and progressive cell death [2]. Several studies have documented that post-ischemic recovery of mechanical function is impaired in load-induced hypertrophy [3–6]. However, little data is available with regard to the pathophysiological mechanisms of such impaired recovery, particularly its dependency on perturbations of intracellular Ca^{2+} handling and/or energy metabolism, nor myocardial TNF-α expression has been tested in response to acute mechanical stretch in hypertrophied hearts.

On the other hand, myocardial growth stimulated by the activation of the growth hormone/insulin-like growth factor I (GH/IGF-I) axis appears more "*physiologic*" than load-induced hypertrophy, at least in the short-term, insofar as it is associated with unchanged capillary density, no interstitial remodeling, normal or even augmented systolic and diastolic function, and reduced apoptotic rate [7,8]. Such observations have prompted several experimental investigations focused on GH/IGF-I activation in heart failure by genetic manipulation or pharmacological means [8–11].

However, no study has compared vis-à-vis the molecular and histological characteristics of GH- and load-induced hypertrophy. In addition, there is no information as to whether these two kinds of hypertrophy entail different responses to ischemic and mechanical injury. The current study was performed to clarify these issues using classical models of ischemia-reperfusion and mechanical stretch in vitro.

2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and experimental procedures were approved by the local Animal Care Committee. Wistar rats of male sex were used and the study design is shown in Fig. 1. Transverse banding of ascending aorta (AB) was performed placing a tantalum hemoclip in rats aged 3 weeks, as previously described [12]. Sham rats underwent the same surgical procedures, although the clip was not placed. Growth hormone treatment (3.5 mg/kg body weight rhGH per day via two subcutaneous injections) or placebo (normal saline) were randomly started in rats aged 9 weeks and continued for 4 weeks. Ex vivo studies were performed at 13 weeks of age. No death was observed during surgery and before the final experiments. Timing of AB and GH treatment, and even more GH dosage, were established on the basis of pilot experiments (unpublished data) showing that at 13 weeks of age, AB and GH-treated rats achieved similar Δ left ventricular growth responses (see below).

2.1. Assessment of cardiac growth in AB and GH-treated rats

Although the optimal method for comparing heart weights in the rat is unknown, normalizing left ventricular weight to tibial length relates cardiac size to the amount of lean body tissue and to cell size more than does normalization to body weight [13]. Accordingly, at the end of the final experiments, right hind legs of the rats were removed by disarticulating the femurs from the acetabulum at the hip. The tibias were dissected free of soft tissue and frozen at −20 °C. Four radiographic films (X-Omat XTL2, Eastman Kodak Co) of the tibias were then obtained, and the tibial length of each animal was assessed with a caliper from the radiograph.

2.2. Protocol A

A total of 32 hearts ($n = 8$ in each group) underwent basal molecular analysis and histochemistry. Immediately after rats were sacrificed, left ventricles were carefully separated form right ventricles and stored for subsequent analysis.

2.2.1. Basal myocardial mRNA expression of Ca^{2+} regulating proteins ($n = 4$ in each group)

Total RNA was prepared from left ventricular myocardium according to the method of Chomczynski et al., as previously described [14]. For sarcoplasmic

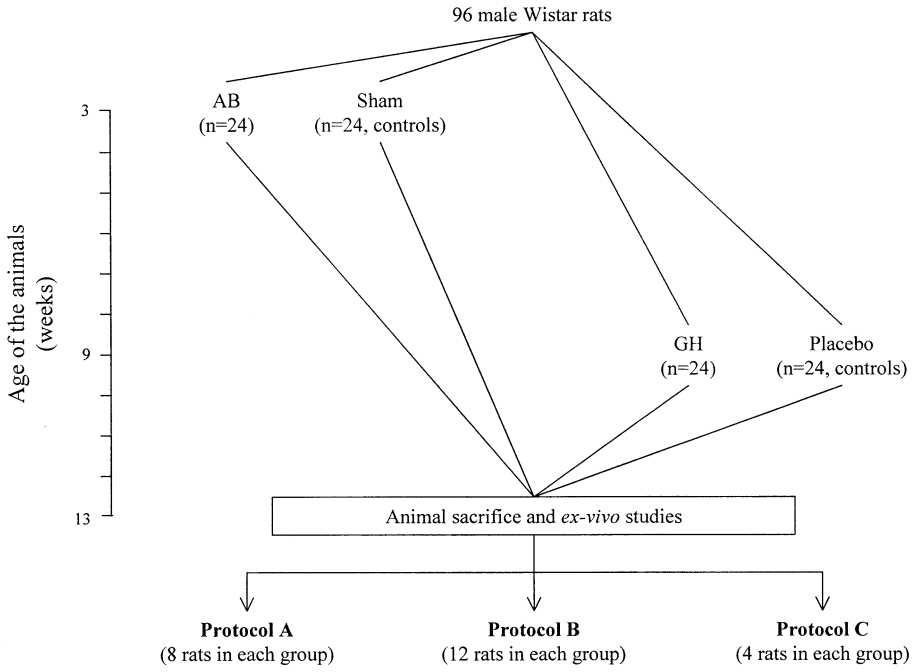

Fig. 1. Study design. AB = aortic banding; GH = growth hormone.

reticulum calcium-ATPase (SERCA-2), Na–Ca^{2+} exchanger (NCX) and phospholamban (PLB), the polymerase chain reaction products which were 523, 429 and 535 bp respectively, were cloned into a Bluescript vector. They were subsequently transformed into competent bacteria which where selected and sequenced using the Big Dye TM terminator cycle sequencing kit (ABI Prism, PE Applied Biosystems, Warrington, UK). RNAse protection assay (RPA II kit Ambion) and the solution hybridization RNAse protection assay were performed according to the manufacturer's instructions with 20 μg of total RNA, prepared as previously described [14].

2.2.2. Collagen volume fraction (n = 4 in each group)

The whole left ventricles were cut serially into 10–14 transverse sections (varying according to the heart's size) 1 mm thick each, from apex to base. Six μm thick transverse sections were subsequently stained with Picrosirius red. Slides were observed with a Nikon Microphot FXA light microscope equipped with a polarized set and analysed with Zeiss KS300 software. Collagen volume fraction (CVF) was expressed as the mean percentage of connective tissue areas divided by total tissue area in the same field [15].

2.3. Isolated whole heart preparation

A total of 64 rats ($n = 16$ in each group), underwent isolated whole heart preparation protocol (Fig. 1, Protocol B and C). Under deep anesthesia, hearts were rapidly excised and immersed in ice-cold Krebs–Henseleit buffer, weighted and mounted in a Langendorff apparatus at a constant temperature of 37 °C, as previously described [16–19]. Perfusion was set at a constant flow of 12 ml/min per gram of heart weight by means of a roller pump using phosphate-free, Krebs–Henseleit buffer (118 mmol/l NaCl, 4.7 mmol/l KCl, 1.75 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 0.5 mmol/l EDTA, 25 mmol/l NaHCO3, 11 mmol/l glucose), which was inline filtered (45 μm pore size) and bubbled with 95% O_2 and 5% CO_2 to yield a pH of 7.4; coronary perfusion pressure was measured with a Statham P23Db transducer (Gould Instruments, Oxnard, CA, USA) connected to the perfusion line. To calculate the coronary flow, the total effluent was collected intermittently in a calibrated cylinder over a time period of 1 min. Cardiac temperature was measured by a temperature probe inserted into the right ventricle and was kept constant within ±0.1 °C by regulating the temperature of the perfusate. Left ventricular mechanical performance was continuously measured with a water-filled latex balloon inserted into the left ventricle through an incision in the left atrial appendage, via the mitral valve, and secured by a ligature. The balloon was attached to a stiff plastic tube and connected to a Statham P23Db pressure transducer (Gould Instruments, Oxnard, CA, USA) for continuous measurement of isovolumic pressure. The hearts were paced at 5 Hz using monophasic square-wave pulses delivered from a Grass Stimulator (model S 88 S1U5) and two pericardial electrodes on the free wall of the right ventricle. Left

ventricular pressure and coronary perfusion pressure curves were digitized using a commercially available computer. In order to achieve comparable loading conditions (i.e., balloon volumes) in hearts of different sizes and geometry, the left ventricular parameter of interest was acquired with the intraventricular balloon inflated at 50% of the volume that resulted in the maximum left ventricular developed pressure (50% of Vol_{max}), as previously validated [18]. Left ventricular wall stress (σ) and relative wall thickness (h_r) were derived from left ventricular pressure measurements, intraventricular balloon volume, and weight of left ventricle as previously described [18].

2.4. Protocol B

A total of 48 hearts ($n = 12$ in each group), underwent ischemia-reperfusion protocol with assessment of intracellular Ca^{2+} handling and high-energy phosphate metabolism. After an equilibration period of 15 min at 50% of Vol_{max}, the perfusion line was clamped (no-flow ischemia) and hearts were subjected to global normothermic ischemia for 20 min followed by 30 min of reperfusion. Half of the hearts in each group were studied in the Ca^{2+}-setup, the other half in the ^{31}P-NMR setup.

2.4.1. Aequorin loading and quantification of intracellular Ca^{2+} ($n = 6$ in each group)

Intracellular Ca^{2+} $[Ca^{2+}]_i$ was measured in parallel experiments by aequorin normalized by fractional luminescence as described below [17,19]. After stabilization for 10 min at 25 °C, 3–5 μl of an aequorin-containing solution (1 μg/ml) were injected with a glass micropipette into the interstitium of the inferoapical region of the left ventricle. The heart was positioned in an organ bath with the aequorin-loaded area of the left ventricle directed towards the cathode of a photomultiplier (model 9635QA, Thorn-EMI, Gencom, Inc., Fairfeld, NJ, USA) and submerged in Krebs–Henseleit solution. Subsequently, the temperature was increased to 37 °C within 10 min and kept constant. Aequorin light signals were recorded on the 4-channel recorder (Graphtec, Seefeld 82229, Germany), digitized by a 12 bit analog-digital converting board at a sampling rate of 1 kHz (DAP 800/3, Microstar, Bellevue, WA, USA) and stored on the hard drive of a computer (PC 133 MHz). Normalization of aequorin light signals and analysis of calcium-overload in the first minute of reperfusion was performed as previously described [17,19]. Specifically, the following parameters were used: $[Ca^{2+}]_{isch}$ = end-ischemic $[Ca^{2+}]_i$, peak = initial peak of Ca^{2+} signal in the first minute of reperfusion, $Oscill_{sys}$ = maximum of the first 10 transients of Ca^{2+} oscillations in the first minute of reperfusion (absolute value), $I_{(0-60)}$ = time integral of aequorin light signal from the beginning until 60 s of reperfusion, normalized by L_{max} [19]. A representative tracing showing an original pressure and light signals and the first 30 s of reperfusion is depicted in Fig. 2.

2.4.2. ^{31}P-NMR Spectroscopy ($n = 6$ in each group)

High-energy phosphates were measured by ^{31}P nuclear magnetic resonance (^{31}P-NMR) on a 7 T Bruker AM system, as previously described [16]. Data were averaged over 5 min time intervals with 4 K data points, 152 scans, TR = 1.93 s. Peak amplitudes were corrected for partial saturation (8% for P_i, 12% for PCr) by comparing to fully relaxed spectra. The concentration of the gamma ATP in control hearts was set to 10.8 mM [16] and normalized to the heart weight to obtain the relation between spectrometer intensities and absolute concentrations [20]. Concentrations of all other compounds were computed using this factor, taking into account the heart weight of each individual heart. Intracellular pH (pH_i) was measured by comparing the chemical shift, δ, between inorganic phosphate (Pi) and phosphocreatine (PCr) to values obtained from a standard curve.

2.5. Protocol C

A total of 16 hearts ($n = 4$ in each group), underwent mechanical stretch protocol with assessment of stretch-induced myocardial tumor necrosis-α (TNF-α) mRNA expression. After 15–30 min at 25 °C, the temperature was gradually increased to 37 °C and kept constant. After 15 min stabilization period, the balloon was further inflated to achieve 50% (control unstretched myocardium) or 140% of Vol_{max} (severe stretch), corresponding in controls hearts to a diastolic pressure of 5–10 and 35–40 mmHg, respectively [21]. This was done to compare hearts of different size at similar preload levels, insofar as it is well known that load-induced hypertrophy is accompanied by elevated left ventricular chamber stiffness, and therefore choosing the same value of left ventricular diastolic pressure in controls and AB would have resulted in a significant understretching of AB hearts [18]. At 10 min intervals during experiments, a sample of the coronary venous effluent was collected in a calibrated cylinder over a period of 1 min for measuring the lactate production (Lactate Reagent; Sigma Chemical Co.). After 40 min, respectively, the balloon was rapidly deflated to a volume just enough to obtain a pressure signal. After 5 min stabilization, perfusion was terminated and left ventricles, carefully separated from right ventricles, were quickly frozen in liquid nitrogen. Total RNA was extracted from homogenized left ventricular myocardium and Northern analysis was performed as previously described in details [21].

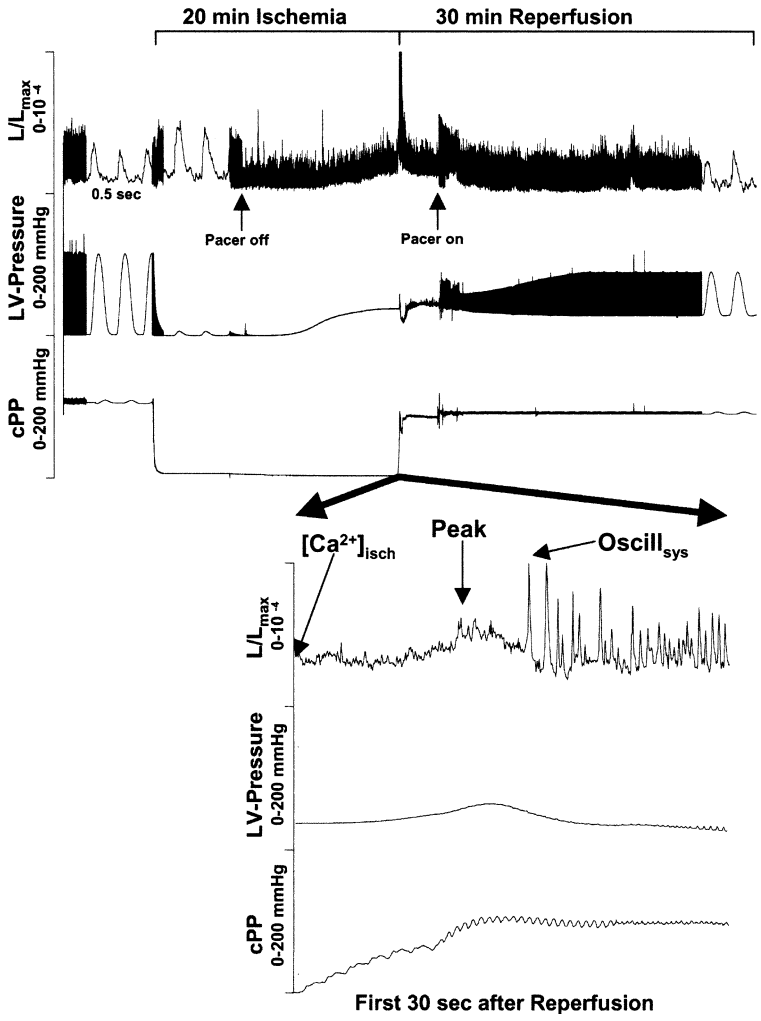

Fig. 2. Original tracing (upper panel) from a control heart showing coronary perfusion pressure (CPP), left ventricular pressure (LVP) and aequorin light signal normalized to L_{max} (L/L_{max}), where control is representative of sham and placebo rats. The lower panel depicts the first 30 s of reperfusion at an enlarged time scale. Note that reperfusion peaks consists of a sequence of Ca^{2+} transients, a phenomenon known as "Ca^{2+}-oscillations".

2.6. Statistical analysis

All data are presented as mean ± SEM. For statistical comparison among the various groups of hearts the multiple comparison factorial-one factor ANOVA was used. Significant differences were detected using the Neuman–Keuls test. A p values of <0.05 were considered significant.

3. Results

3.1. Characteristics of cardiac growth

AB and GH rats had a similar left ventricular concentric hypertrophy, as shown by a comparable values of both left ventricular to tibial length ratio and thickness to radius ratio, indicating similar myocardial growth responses in AB and GH groups (Table 1). Molecular analysis of left ventricular myocardial mRNA expression of SERCA-2, PLB and NCX revealed significant changes in the AB group, consistent with pathologic cardiac remodeling, while in the GH group message levels of the above calcium regulating proteins were unaffected (Table 1). In addition, left ventricular myocardial CVF was significantly increased in AB, while in the GH-treated animals it was unchanged (Fig. 3). Myocyte area was slightly but not significantly increased in AB and GH rats (not shown).

3.2. Mechanical performance, calcium handling and high-energy phosphate metabolism

Data on baseline left ventricular mechanical performance are shown in Table 2. Developed pressure was significantly increased in the hypertrophied compared with

Table 1
Somatic growth, morphometric histology and myocardial mRNA expression of Ca^{2+} regulating genes

	BW (g)	TL (mm)	LVW (mg)	LVW/BW (mg/g)	LVW/TL (mg/mm)	h_r	SERCA-2 (pg/μg RNA)	PLB (pg/μg RNA)	NCX (pg/μg RNA)	CVF (%)
N	48	48	48	48	48	32	8	8	8	8
Sham	376 ± 18	39.4 ± 0.5	979 ± 56	2.51 ± 0.08	24.3 ± 0.9	0.99 ± 0.05	4.31 ± 0.05	1.38 ± 0.11	0.36 ± 0.03	3.8 ± 0.1
AB	361 ± 14	38.9 ± 0.3	1336 ± 94*	3.70 ± 0.11*	34.3 ± 1.8*	1.23 ± 0.03*	3.21 ± 0.17*	0.43 ± 0.17*	0.51 ± 0.02*	7.7 ± 0.3*
N	48	48	48	48	48	32	8	8	8	8
Placebo	383 ± 13	38.8 ± 0.4	967 ± 69	2.60 ± 0.05	24.9 ± 1.4	1.07 ± 0.03	4.37 ± 0.02	1.31 ± 0.17	0.39 ± 0.01	3.6 ± 0.3
GH	441 ± 16*,**	40.8 ± 0.5*,**	1298 ± 78*	2.94 ± 0.07*,**	32.3 ± 1.5*	1.18 ± 0.02*	4.26 ± 0.05**	1.25 ± 0.18**	0.38 ± 0.03**	3.7 ± 0.1**

Data are mean ± SEM; *N* = number of animal considered for each variable; AB = aortic banding; GH = growth hormone; BW = body weight; TL = tibial length; LVW = left ventricular weight, expressed as anatomical wet weight; h_r = relative wall thickness; SERCA-2 = sarcoplasmic reticulum calcium-ATPase; PLB = phospholamban; NCX = Na^+–Ca^{2+} exchanger; CVF = collagene volume fraction.

* $p < 0.05$ vs. sham and placebo.

** $p < 0.05$ vs. AB.

control hearts. However, developed wall stress, an index which normalizes pressure to left ventricular weight and geometry, was not significantly different among the three groups, indicating similar left ventricular intrinsic contractility. While in GH treated rats there was no evidence of diastolic impairment, in pressure-overloaded animals end-diastolic pressure, time to 90% relaxation, and Tau at 50% of V_{max} were significantly higher compared with controls. The perfusion pressure in the GH and control group was 80 ± 2 (mean ± SE) and 81 ± 4 mmHg, respectively, while in AB group it was 98 ± 5 mmHg. During baseline perfusion, the Ca^{2+} transients were not significantly different among the three groups; however, there was a tendency to a prolonged Ca^{2+} transient in the AB group, which did not come out statistically significant (Fig. 4). After the onset of global ischemia, hearts stopped beating within 2 min. During ischemia, contracture started after approximately 10 min in all groups and end-diastolic pressure showed the well-described overshoot upon reperfusion (Fig. 5). Recovery of developed and end-diastolic pressures were both markedly altered in the AB group, compared with GH and control hearts (Fig. 5). Heart rate was similar in all groups during the ischemia protocol.

Quantitative high energy phosphate data from the whole experimental protocol are depicted in Table 2 and Fig. 5. Under baseline perfusion, PCr content was lower in the AB group and Pi was increased compared with the control group, whereas ATP was similar. GH treated rats exhibited no differences regarding ATP, PCr or Pi as compared with controls, but had lower PCr and Pi content than AB rats. No differences in intracellular pH were evident during baseline perfusion among the three study groups. PCr fell rapidly below 20% of its baseline value within 5 min and was undetectable after 15 min of ischemia. Pi started to increase several fold to values as high as 25 mM at the end of ischemia. ATP decreased to about 2 mM after 15 min of ischemia, however, the time course was slower than that of the PCr decrease. The time courses of metabolic changes did not differ during ischemia among the three study groups. Intracellular pH fell to 6.0 at the end of ischemia and recovered during reperfusion within 5 min to control values in all groups. Post-ischemic recovery of PCr as percentage of the pre-ischemic value was similar (about 70%) in all groups, although absolute PCr content was reduced in the AB group (Fig. 5). ATP recovered to the same extent in control, AB, and GH groups; (40 ± 5, 39 ± 3, and 34 ± 6%, respectively) and Pi decreased, but remained elevated to about 350% of pre-ischemic values. Maximal intracellular Ca^{2+} overload occurring in the first minute of reperfusion as defined by $Oscill_{sys}$ showed a 1.6-fold increase in the AB hearts (Fig. 6). [Ca^{2+}] at the end of ischemia, peak [Ca^{2+}] or integrated light emission during the first minute of reperfusion showed no differences between groups.

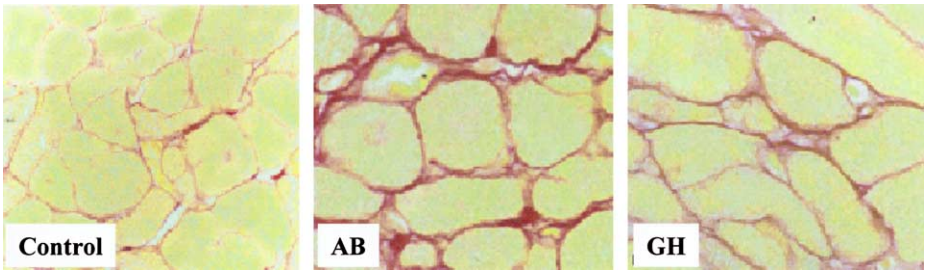

Fig. 3. Left ventricular collagen deposition stained with picrosirius red in control, aortic banding (AB) and growth hormone (GH) group, where control is representative of sham and placebo rats. Note the marked increase of the collagen framework surrounding cardiomyocytes in the group subjected to AB.

Table 2
Left ventricular mechanical performance and high energy phosphate metabolism during baseline perfusion

	dP (mmHg)	dσ (kdynes/cm^2)	ed-P (mmHg)	$T_{90}R$ (ms)	Tau (ms)	ATP (mmol/L)	PCr (mmol/L)	Pi (mmol/L)	pH_i
N	12	12	12	12	12	12	12	12	12
Sham	86 ± 5	29 ± 3	8 ± 1	77 ± 5	39 ± 5	10.6 ± 0.2	14.4 ± 0.6	2.7 ± 0.3	7.1 ± 0.01
AB	117 ± 2*	28 ± 2	13 ± 2*	94 ± 4*	52 ± 2*	10.3 ± 0.6	11.9 ± 0.9*	4.8 ± 0.7*	7.1 ± 0.01
N	12	12	12	12	12	12	12	12	12
Placebo	89 ± 2	27 ± 1	6 ± 2	72 ± 3	41 ± 2	10.1 ± 0.7	14.9 ± 0.2	2.4 ± 0.5	7.1 ± 0.02
GH	110 ± 3*	31 ± 1	6 ± 1**	71 ± 3**	39 ± 3**	11.1 ± 0.5	15.3 ± 0.6**	2.6 ± 0.4**	7.1 ± 0.01

Data are mean ± SEM; *N* = number of animal considered for each variable; AB = aortic banding; GH = growth hormone; dP = developed pressure; dσ = developed wall stress; ed-P = end-diastolic pressure; $T_{90}R$ = time to 90% relaxation; PCr = phosphocreatine; Pi = inorganic phosphate.
* $p < 0.05$ vs. sham and placebo.
** $p < 0.05$ vs. AB.

3.3. Stretching protocol

Under basal conditions, no myocardial TNF-α mRNA expression was found in control and GH left ventricles, whereas detectable TNF-α message levels were noted in myocardium from pressure-overloaded animals (Fig. 7). No change in basal myocardial TNF-α mRNA expression was noted during perfusion with maintenance of the uninflated balloon (≈5 mmHg left ventricular end-diastolic pressure). On the contrary, myocardial stretch at 140% of V_{max}, corresponding to a diastolic pressure of 40 mmHg in controls and GH animals, and to 63 mmHg in the AB group, was associated with de novo myocardial TNF-α expression in both controls and GH hearts, with a further and remarkable increase in TNF-α transcript levels in hypertrophied hearts subjected to AB (≈5-fold vs. baseline; $p < 0.001$, Fig. 7). No lactate production was found in the coronary venous effluent during or after 40 min of stretch (data not shown).

4. Discussion

The main findings of the current study are: (i) *pathologic* left ventricular hypertrophy induced by pressure overload is characterized by increased collagen volume fraction, diastolic dysfunction, significant changes in the myocardial mRNA expression of key Ca^{2+} regulating proteins, and greater vulnerability to ischemia-reperfusion injury and mechanical stretch compared with normal hearts; (ii) by contrast, GH-induced myocardial growth appears to be substantially different in nature insofar as is not associated with fibrosis and changes in Ca^{2+} regulating proteins, and diastolic function is preserved. Importantly, the susceptibility to ischemia-reperfusion and mechanical stretch in vitro is not significantly altered by GH-induced myocardial growth. The data support the concept that this kind of growth is not detrimental but rather confers cardioprotection against ischemic and mechanical injury compared with pressure-overload hypertrophy.

4.1. Current study

The current study provides a potential explanation for the altered post-ischemic recovery of left ventricular function in load induced hypertrophy. Indeed, direct measurement of intracellular Ca^{2+} showed a marked increase of Ca^{2+} overload in AB hearts upon reperfusion. $Oscill_{sys}$ reflects the amount of Ca^{2+} released by the overloaded sarcoplasmic reticulum on top of the cytosolic Ca^{2+}-overload, and are likely generated by rapid Ca^{2+} release and re-uptake by the sarcoplasmic reticulum since inhibitors of the sarcoplasmic reticulum abolishes them [19]. Ca^{2+} oscillations in turn are known

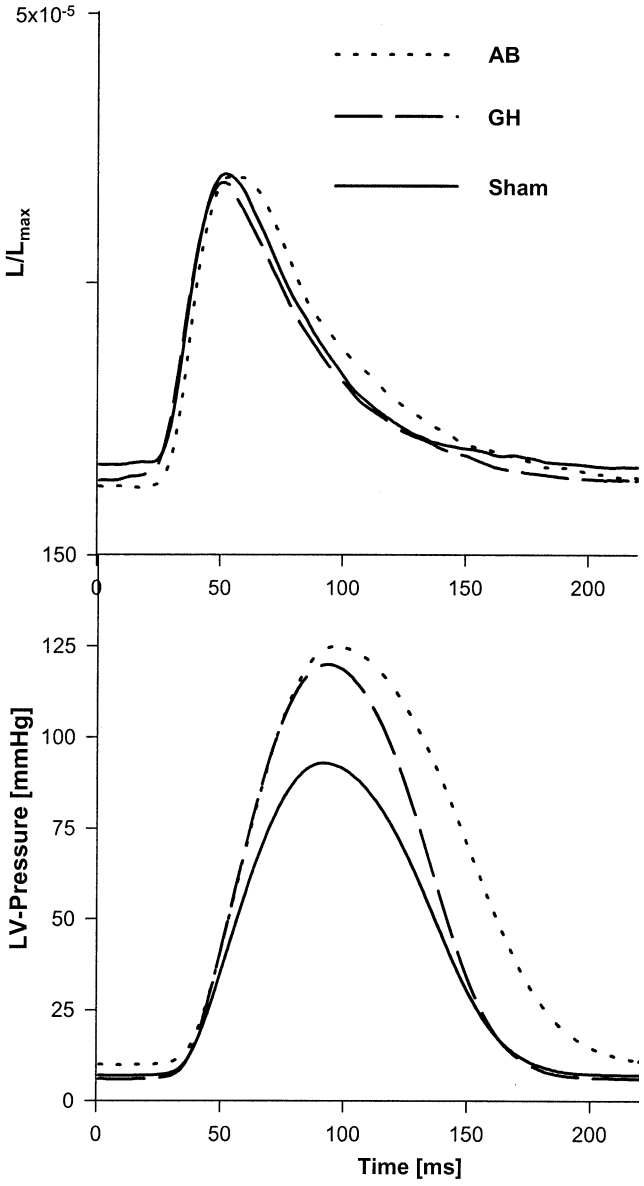

Fig. 4. Representative signal averaged Ca^{2+} transients (upper panel) and corresponding mechanical contractions (lower panel) of sham, growth hormone treated (GH) and hearts with hypertrophy due to aortic banding (AB), where sham is also representative of placebo rats. Pressure development is similarly increased in both hypertrophy groups compared to sham. Diastolic function is impaired in AB but not in GH rats. The Ca^{2+} transients were not significantly different, however there was a tendency of a Ca^{2+} transient prolongation in the AB group.

to induce reperfusion arrhythmias, particularly ventricular fibrillation and myocardial dysfunction.

Impaired myocardial high energy phosphate metabolism was also explored as an alternative potential culprit of the increased post-ischemic left ventricular dysfunction. However, this mechanism appears unlikely, given the similar time-course and percent recovery of PCr, ATP, and Pi content among the three study groups, despite baseline differences. The negative impact of load-induced hypertrophy on the myocardium is further supported by the stretching experiments. Myocardial TNF-α mRNA was already detectable under baseline conditions in the AB group, at variance with GH-treated and control animals. Moreover, its upregulation in response to mechanical stretch was much more marked than that occurring in the other study groups. This finding bears relevant implications in view of the important role that TNF-α appears to play in the pathogenesis of heart failure. Indeed TNF-α stimulates myocyte apoptosis, decreases contractility, and disrupts the extracellular matrix via the activation of metalloproteases [22,23].

Contrary to the situation with pressure overload hypertrophy, the current study supports the notion that GH-induced myocardial growth displays unique physiologic features. Indeed, not only was the extracellular matrix integrity maintained in GH treated rats and no changes were observed in candidate Ca^{2+} regulating genes, but also the susceptibility to ischemia-reperfusion injury and mechanical stretch was similar to normal hearts.

4.2. Comparison with previous studies

Left ventricular hypertrophy induced by chronic hemodynamic overload is commonly accompanied by fibrosis, decreased capillary density, and decreased energy reserve for use under stress situations [2,20]. In addition, hypertrophic hearts exhibit an impaired functional recovery from ischemia-reperfusion injury [3–6]. Allard et al. have suggested the possibility that Ca^{2+} overload may be augmented during reperfusion, although no direct measure of intracellular Ca^{2+} was provided in that study [3]. As to myocardial metabolism, our findings of decreased PCr during baseline perfusion are in agreement with those reported by Tian et al., who found the energy reserve to be decreased during baseline perfusion in hearts with pressure overload hypertrophy [20]. No data concerning high energy phosphate metabolism after global ischemia was reported in that study. On the contrary, despite significant hypertrophy, GH treated hearts displayed indices of high energy phosphate metabolism not different from control hearts. Such favorable energetic profile was described originally by Mercadier's group, who proposed an increased number of active cross-bridges despite a reduced cross-bridge cycling velocity, typical of V3 isomyosin, in hearts subjected to chronic GH hypersecretion [24].

Early upregulation of the TNF-α activity has been consistently documented within the stressed myocardium after persistent mechanical stimulation in end-stage human heart failure and after experimental load-induced hypertrophy, and in myocardial infarction [22,23,25]. In particular, myocardial generation of TNF-α following mechanical stress was recently characterized by our group in the same model system, showing that it

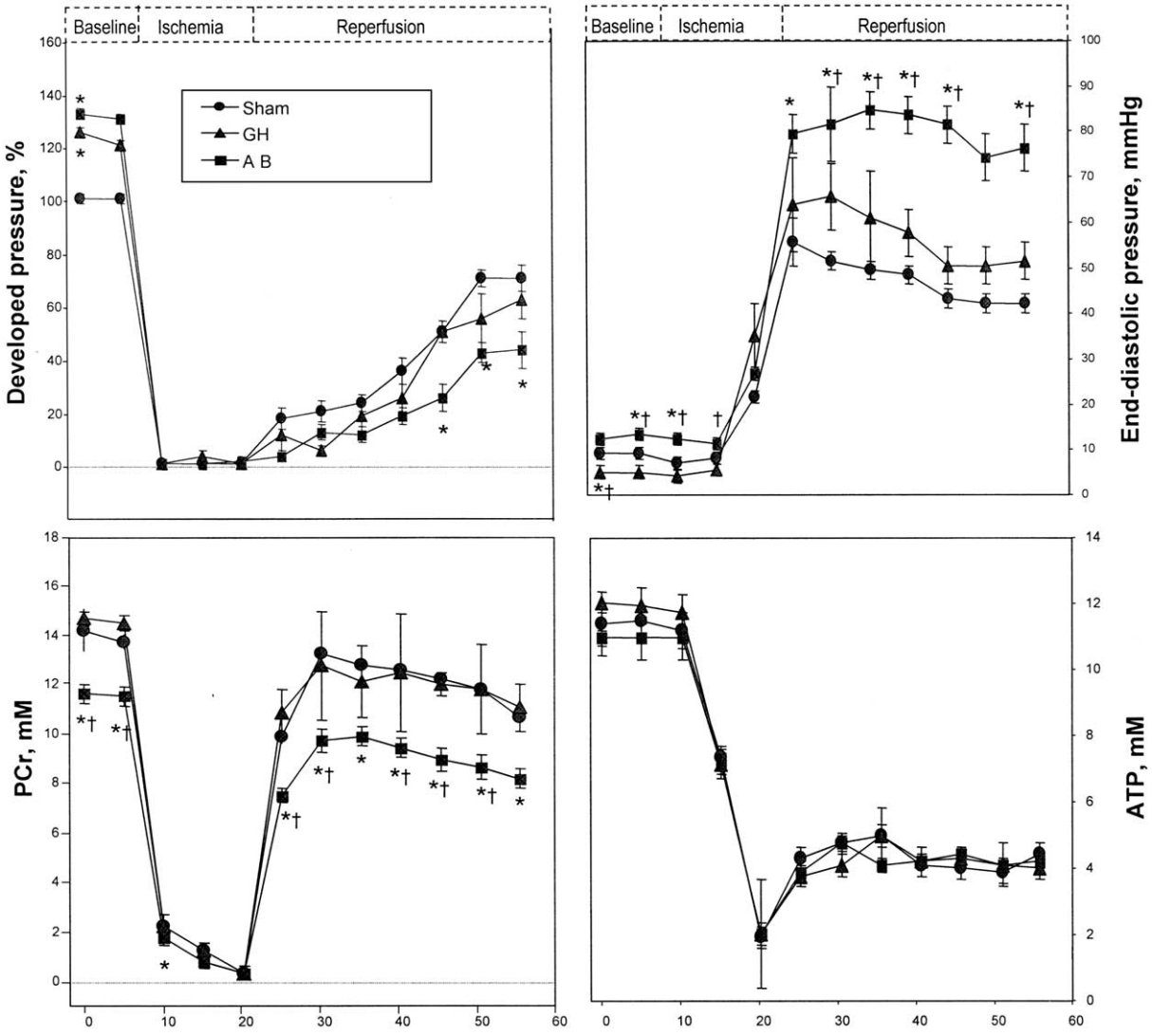

Fig. 5. Mechanical function and high energy phosphate metabolism in the sham, aortic banding (AB) and growth hormone (GH) group, where sham is also representative of placebo rats. Note the decreased post-ischemic recovery of left ventricular function and the higher end-diastolic pressure in the aortic banding (AB) group (upper panels). PCr content was decreased at baseline and reperfusion in the banded animals, but percent recovery is similar in all groups (lower left panel). ATP baseline differences were not evident upon reperfusion (lower right panel). * $p < 0.05$ vs. sham; † $p < 0.05$ vs. GH.

is not constitutively expressed in the myocardium under normal circumstances [21]. Conversely, graded mechanical stretch resulted in a progressive increase in TNF-α mRNA and protein levels. No data were available as to TNF-α in an already hypertrophic myocardium. Interestingly, the fact that myocardial TNF-α mRNA expression was remarkably upregulated in response to severe mechanical stretch suggests that load-induced myocardial hypertrophy has an increased susceptibility to hemodynamic challenges, which may promote and sustain the transition towards the heart failure phenotype.

In the short-term, GH-induced left ventricular hypertrophy is characterized by unchanged capillary density, absence of interstitial fibrosis, and normal diastolic function, a picture supported by both animal and human studies [7,26,27]. By contrast, in the long-term GH hypersecretion is associated with the typical stigmata of acromegalic cardiomyopathy, i.e. marked left ventricular enlargement, impaired systolic and diastolic function, and interstitial fibrosis [28]. Taken together, our findings lend further support to the hypothesis that, in the short-term, GH/IGF-1 axis activation induces a unique type of myocardial response that shares many features with physiological growth.

Our data are in agreement and expand on those reported by Ross Jr.'s group, showing that exogenous GH/IGF-1 in mice produce cardiac hypertrophy and a positive inotropic effect without causing significant changes in expression of fetal and other selected myocardial genes [29].

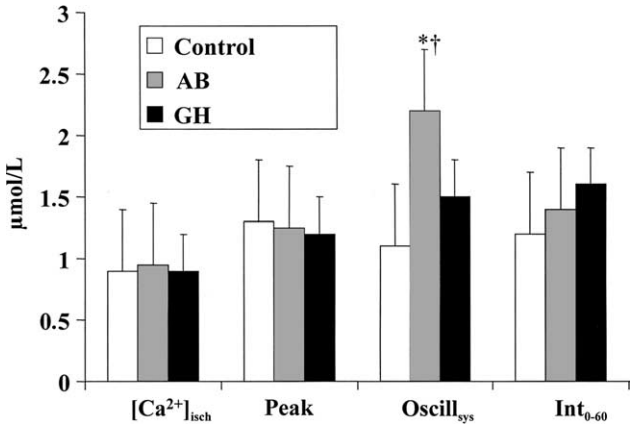

Fig. 6. Indices of Ca^{2+} overload (see Section 2 for details) in the first minute of reperfusion in control, aortic banding (AB) and growth hormone (GH) group, where control is representative of sham and placebo rats. The first 10 systolic Ca^{2+} oscillations were increased 1.6-fold in the aortic banding (AB) group vs. controls. * $p < 0.05$ vs. controls; † $p < 0.05$ vs. GH.

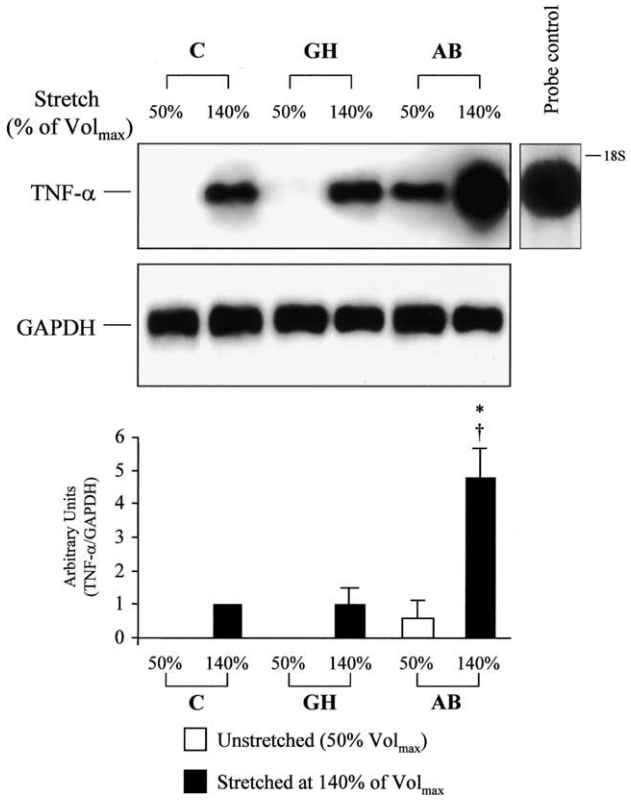

Fig. 7. Representative Northern blot and densitometric analysis of autoradiographic bands, expressed as mean ± SEM, for TNF-α expression in unstretched (50% of Vol_{max}) and severely stretched (140% of Vol_{max}) myocardium from controls ($n = 8$), growth hormone (GH, $n = 4$) and aortic banding (AB, $n = 4$) rats, where control is representative of sham and placebo rats. Twenty-five micrograms of total RNA was loaded for each lane. Probe control lane refers to endotoxin-stimulated (10 µg/mL for 8 h) rat macrophage cells. Fold-induction was referred to an arbitrary number, defined as 1, assigned to the level of expression estimated in severely stretched myocardium from control rats. Note the mechanical stretch-induced *de novo* myocardial TNF-α mRNA expression in GH group and controls, which was dramatically increased in AB animals. * $p < 0.001$ vs. the corresponding stretched groups; † $p < 0.001$ vs. the corresponding unstretched myocardium.

4.3. Pathophysiological and clinical implications

The current study provides two mechanisms potentially responsible for the poor outcome of *pathologic* left ventricular hypertrophy, namely intracellular Ca^{2+} overload during ischemia and enhanced stretch-induced myocardial TNF-α expression. Not only is this particular type of myocardial growth characterized by increased fibrosis, reduced capillary density, re-expression of the embryonic gene program, but it is also particularly susceptible to frequently occurring challenges. In the clinical scenario, repetitive episodes of ischemia may often occur in hypertrophied hearts, as well as sudden left ventricular mechanical stretch due to hypertensive bursts, or a combination thereof. Such events, by increasing intracellular Ca^{2+} overload and TNF-α myocardial production, may induce lethal ventricular arrhythmias and accelerate the transition to heart failure.

It is controversial whether reactivation of cardiac growth may be beneficial in heart failure. Recent studies using molecular techniques have shown that inhibiting myocardial growth by genetic manipulation, such as blocking *Gαq* or RAS signaling prevents cardiac dysfunction despite increased wall stress [30,31]. Conversely, stimulation of cardiac growth, particularly when oriented toward concentric remodeling, by activation of ERK, telomerase reverse transcriptase, or inhibition of nitric oxide synthesis appears to protect from cardiac decompensation in similar models of pressure overload [32]. The current data point to the existence of peculiar types of "*physiologic*" myocardial growth that may be beneficial in the setting of cardiac remodeling given the striking differences with pressure overload hypertrophy. In this regard, GH-induced myocardial growth also stimulates survival pathways, as recently suggested by two studies in the rat model of post-infarction heart failure [15,33]. The complex intracellular signaling involved in these such salutary actions is under active investigations and possibly involves *Akt*, a specific substrate of IGF-I positioned downstream of PI3-kinase [34]. In fact, relevant to the current study, *Akt*-induced hypertrophy is associated with a positive inotropic effect without re-expression of the fetal gene program and p44/42 MAPK activation *in vivo*, at variance with stress-induced *pathologic* hypertrophy [34]. In addition, more recently, McMullen et al. have shown that transgenic mice overexpressing IGF-1 receptor in the heart displayed compensated cardiac hypertrophy, characterized by increased myocyte size with no evidence of histopathology and enhanced systolic function over time,

which was associated with a significant activation of the PI3-kinase/*Akt*-p70^{S6K1} pathway [35].

Acknowledgements

The MRUI software package was kindly provided by A. van den Boogaart, Katholieke Universiteit Leuven. It is currently funded by the EC project TMR/Networks ERB-FMRX CT970160 (http://azur.univ-lyon1.fr/TMR/tmr.html). Human recombinant growth hormone was kindly supplied by Pharmacia & Upjohn GmbH, Erlangen Germany.

References

[1] B.H. Lorell, B.A. Carabello, Left ventricular hypertrophy: pathogenesis, detection, and prognosis, Circulation 102 (2000) 470–479.
[2] B. Swynghedauw, Molecular mechanisms of myocardial remodelling, Physiol. Rev. 79 (1999) 215–262.
[3] M.F. Allard, J.D. Flint, J.C. English, S.L. Henning, M.C. Salamanca, C.T. Kamimura, D.R. English, Calcium overload during reperfusion is accelerated in isolated hypertrophied rat hearts, J. Mol. Cell Cardiol. 26 (1994) 1551–1563.
[4] P.T. Buser, J. Wikman-Coffelt, S.T. Wu, N. Derugin, W.W. Parmley, C.B. Higgins, Postischemic recovery of mechanical performance and energy metabolism in the presence of left ventricular hypertrophy: A ^{31}P-MRS study, Circ. Res. 66 (1990) 735–746.
[5] D.J. Hearse, D.A. Stewart, D.G. Green, Myocardial susceptibility to ischemic damage: a comparative study of disease models in the rat, Eur. J. Cardiol. 7 (1978) 437–450.
[6] J.D. Sink, G.L. Pellom, W.D. Currie, R.C. Hill, C.O. Olsen, R.N. Jones, A.S. Wechsler, Response of hypertrophied myocardium to ischemia: correlation with biochemical and physiological parameters, J. Thorac. Cardiovasc. Surg. 81 (1981) 865–872.
[7] A. Cittadini, H. Strömer, S.E. Katz, R. Clark, A.C. Moses, J.P. Morgan, P.S. Douglas, Differential cardiac effects of growth hormone and insulin-like growth factor-1 in the rat. A combined in vivo and in vitro evaluation, Circulation 93 (1996) 800–809.
[8] Q. Li, B. Li, X. Wang, A. Leri, K.P. Jana, Y. Liu, J. Kajstura, R. Baserga, P. Anversa, Overexpression of insulin, and cardiac hypertrophy, J. Clin. Invest. 100 (1997) 1991–1999.
[9] A. Cittadini, J.D. Grossman, R. Napoli, S.E. Katz, H. Strömer, R.J. Smith, R. Clark, J.P. Morgan, P.S. Douglas, Growth hormone attenuates early left ventricular remodeling and improves cardiac function in rats with large myocardial infarction, J. Am. Coll. Cardiol. 29 (1997) 1109–1116.
[10] R.L. Duerr, S. Huang, H.R. Miraliakbar, R. Clark, K.R. Chien, J. Ross Jr, Insulin-like growth factor-1 enhances ventricular hypertrophy and function during the onset of experimental cardiac failure, J. Clin. Invest. 95 (1995) 619–627.
[11] W.L. Lee, J.W. Chen, C.T. Ting, T. Ishiwata, S.J. Lin, M. Korc, P.H. Wang, Insulin-like growth factor I improves cardiovascular function and suppresses apoptosis of cardiomyocytes in dilated cardiomyopathy, Endocrinology 140 (1999) 4831–4840.
[12] A.M. Feldman, E.O. Weinberg, P.E. Ray, B.H. Lorell, Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding, Circ. Res. 73 (1993) 184–192.
[13] R.C. Yin, H.A. Spurgeon, K. Rakusan, M.L. Weisfeldt, E.G. Lakatta, Use of tibial length to quantify cardiac hypertrophy: application in the aging rat, Am. J. Physiol. 243 (1982) H941–H947.
[14] J. Isgaard, H. Wahlander, M.A. Adams, P. Friberg, Increased expression of growth hormone receptor mRNA and insulin-like growth factor-I mRNA in volume-overloaded hearts, Hypertension 23 (1994) 884–888.
[15] A. Cittadini, J. Isgaard, M.G. Monti, C. Casaburi, A. Di Gianni, R. Serpico, G. Iaccarino, L. Saccà, Growth hormone prolongs survival in experimental heart failure, J. Am. Coll. Cardiol. 41 (2003) 2154–2163.
[16] S. Neubauer, M. Horn, A. Naumann, R. Tian, K. Hu, M. Laser, J. Friedrich, P. Gaudron, K. Schnackerz, J.S. Ingwall, Impairment of energy metabolism in the residual myocardium of rat hearts with chronic myocardial infarction, J. Clin. Invest. 95 (1995) 1092–1100.
[17] H. Strömer, A. Cittadini, P.S. Douglas, J.P. Morgan, Exogenously administered growth hormone and insulin-like growth factor-I alter intracellular Ca^{2+} handling and enhance cardiac performance: In vitro evaluation in the isolated isovolumetric buffer-perfused rat heart, Circ. Res. 79 (1996) 227–236.
[18] H. Strömer, A. Cittadini, G. Szymanska, C.S. Apstein, J.P. Morgan, Validation of different methods to compare isovolumic cardiac function in isolated hearts of varying sizes, Am. J. Physiol. 272 (1997) H501–H510.
[19] H. Strömer, M.C. de Groot, M. Horn, C. Faul, A. Leupold, J.P. Morgan, W. Scholz, S. Neubauer, Na^+/H^+ exchange inhibition improves postischemic recovery due to attenuation of Ca^{2+} overload and prolonged acidosis on reperfusion, Circulation 101 (2000) 2749–2755.
[20] R. Tian, L. Nascimben, J.S. Ingwall, B.H. Lorell, Failure to maintain a low ADP concentration impairs systolic dysfunction in hypertrophied rat hearts, Circulation 97 (1997) 1313–1319.
[21] E.A. Palmieri, G. Benincasa, F. Di Rella, C. Casaburi, M.G. Monti, G. De Simone, L. Chiariotti, L. Palombini, C.B. Bruni, L. Saccà, A. Cittadini, Differential expression of TNF-α, IL-6, and IGF-1 by graded mechanical stress in normal rat myocardium, Am. J. Physiol. 282 (2002) H926–H934.
[22] S.R. Kapadia, H. Oral, J. Lee, M. Nakano, G.E. Taffet, D.L. Mann, Hemodynamic regulation of tumor necrosis factor-α gene and protein expression in adult feline myocardium, Circ. Res. 81 (1997) 187–195.
[23] D.L. Mann, Recent insights into the role of tumor necrosis factor in the failing heart, Heart Fail. Rev. 6 (2001) 71–80.
[24] E. Mayoux, R. Ventura Clapier, J. Timsit, F. Behar-Cohen, C. Hoffman, J.J. Mercadier, Mechanical properties of rat cardiac skinned fibres are altered by chronic growth hormone hypersecretion, Circ. Res. 72 (1993) 57–64.
[25] G. Torre-Amione, S.J. Stetson, K.A. Youker, J.B. Durand, B. Radovancevic, R.M. Delgado, O.H. Frazier, M.L. Entman, G.P. Noon, Decreased expression of tumor necrosis factor-α in failing human myocardium after mechanical circulatory support. A potential mechanism for cardiac recovery, Circulation 100 (1999) 1189–1193.
[26] A. Cittadini, A. Berggren, S. Longobardi, C. Ehrnborg, R. Napoli, T. Rosen, S. Fazio, K. Caidahl, B.A. Bengtsson, L. Saccà, Supraphysiological doses of growth hormone induce rapid changes in cardiac morphology and function, J. Clin. Endocrinol. Metab. 87 (2002) 1654–1659.
[27] S. Fazio, A. Cittadini, B. Biondi, E.A. Palmieri, G. Riccio, F. Bone, U. Oliviero, L. Saccà, Cardiovascular effects of short-term growth hormone hypersecretion, J. Clin. Endocrinol. Metab. 85 (2000) 179–182.
[28] L. Saccà, A. Cittadini, S. Fazio, Growth hormone and the heart, Endocr. Rev. 15 (1994) 555–573.
[29] N. Tanaka, T. Ryoke, M. Hongo, L. Mao, H.A. Rockman, R.G. Clark, J. Ross Jr., Effects of growth hormone and IGF-I on

cardiac hypertrophy and gene expression in mice, Am. J. Physiol. 275 (1998) H393–H399.

[30] G. Esposito, A. Rapacciuolo, S.V. Naga Prasad, H. Takaoka, S.A. Thomas, W.J. Koch, H.A. Rockman, Genetic alterations that inhibit in vivo pressure overload hypetrophy prevent cardiac dysfunction despite increased wall stress, Circulation 105 (2002) 85–92.

[31] C. Indolfi, E. Di Lorenzo, C. Perrino, A.M. Stingone, A. Curcio, D. Torella, A. Cittadini, L. Cardone, C. Coppola, L. Cavuto, O. Arcucci, L. Saccà, E.V. Avvedimento, M. Chiariello, Hydroxymethylglutaryl coenzyme A reductase inhibitor simvastatin prevents cardiac hypertrophy induced by pressure overload and inhibits p21ras activation, Circulation 106 (2002) 2118–2124.

[32] M. Sano, M.D. Schneider, Still stressed out but doing fine. Normalization of wall stress is superfluous to maintaining cardiac function in chronic pressure overload, Circulation 105 (2002) 8–10.

[33] H. Jin, R. Yang, H. Lu, A.K. Ogasawara, W. Li, A. Ryan, F. Peale, N.F. Paoni, Effects of early treatment with growth hormone on infarct size, survival, and cardiac gene expression after acute myocardial infarction, Growth Horm. IGF Res. 12 (2002) 208–215.

[34] G. Condorelli, A. Drusco, G. Stassi, A. Bellacosa, R. Roncarati, G. Iaccarino, M.A. Russo, Y. Gu, N. Dalton, C. Chung, M.V. Latronico, C. Napoli, J. Sadoshima, C.M. Croce, J. Ross Jr., Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice, Proc. Natl. Acad. Sci. USA 99 (2002) 12333–12338.

[35] J.R. McMullen, T. Shioi, W.Y. Huang, L. Zhang, O. Tarnavski, E. Bisping, M. Schinke, S. Kong, M.C. Sherwood, J. Brown, L. Riggi, P.M. Kang, S. Izumo, The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3-kinase (p110alhpa) pathway, J. Biol. Chem. 279 (2004) 4782–4793.