

# Cardiac expression of adenine nucleotide translocase-1 in transgenic mice overexpressing bovine GH

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## Abstract

Heart hypertrophy is a common finding of acromegaly, a syndrome due to GH excess. Impairment of adenine nucleotide translocase-1 (*ANT-1*) gene, the main mitochondrial ADP/ATP exchanger, leads to cardiac hypertrophy. The aim of the study was to evaluate cardiac expression and the functional role of ANT-1 in 1- to 12-month-old transgenic mice overexpressing bovine GH (acromegalic mice, Acro) and littermate controls (wild-type mice, Wt). GH specificity of protein degree variation was assessed treating Acro with pegvisomant, a GH receptor competitor. Tissue levels of ANT-1, NF- $\kappa$ B, ATP, and lactic acid were evaluated by western blot, bioluminescence, and Fourier transform infrared spectroscopy respectively. The degree of ANT-1 expression was higher in 1-month-old Acro than in Wt ( $47 \pm 5\%$  OD vs  $33 \pm 4\%$  OD,  $P < 0.01$ ). On the contrary, ANT-1 expression was lower in 3- to 12-month-old Acro than in Wt ( $P < 0.03$ ). Changes in ANT-1 expression were associated with consistent changes of cellular ATP content,

increasing at 1 month ( $P < 0.05$ ) and reducing thereafter in Acro when compared with Wt ( $P < 0.04$ ). Treatment with pegvisomant abolished ANT-1 and ATP changes observed in 1- and 3-month-old Acro, thus supporting a GH-dependent mechanism. Reduced ATP generation in hypertrophied hearts of older Acro was associated with increased lactic acid levels suggesting that part of energy was due to glycolysis. Variations in ANT-1 expression were linked to GH through changes in NF- $\kappa$ B, the levels of which changed accordingly. In conclusion, 1-month-old acromegalic mice had increased ANT-1 expression and higher degree of ATP production. Long-standing disease was associated with a consistent reduction of ANT-1 and ATP tissue levels, which became GH-independent in older animals. This study demonstrated a direct effect of GH on key proteins involved in energy metabolism of acromegalic hearts.

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## Introduction

Cardiac hypertrophy is a common finding of systemic manifestations of growth hormone/insulin-like growth factor-I (GH/IGF-1) excess in acromegalic patients (Lie & Grossman 1980, Saccà *et al.* 1994, Melmed 2006). Acromegaly is often complicated by hypertension, diabetes, or dyslipidemia, which may contribute to cardiovascular disease; however, many observations support the concept of a peculiar acromegalic cardiomyopathy characterized by biventricular hypertrophy and diastolic dysfunction (Fazio *et al.* 1994, Ciulla *et al.* 1999, Colao *et al.* 2004).

Hypertrophic hearts use many sources of energy substratum to compensate for their increased energy requirements (Allard *et al.* 1994): oxidation of long-chain fatty acids could be reduced and glycolysis accelerated (el Alaoui-Talibi

*et al.* 1992, Allard *et al.* 1999, Wambolt *et al.* 1999). The latter has been proposed as a compensatory response to low fatty acid oxidation (Allard *et al.* 1994), although glycolysis may increase also in absence of reduced lipid oxidation (Schonekess *et al.* 1996). On the other hand, glucose oxidation may also be reduced, contributing to impaired pyruvate oxidation and enhancing its reduction to lactic acid. Overall, hypertrophic hearts may have reduced fatty acid oxidation, increased glycolysis, and possibly increased lactate (Stanley & Chandler 2002). A key step of energy metabolism, i.e. ATP production, is the substrate (ADP) availability. Mitochondrial ATP/ADP exchange largely depends on the function of adenine nucleotide translocase 1 (ANT-1), a homodimeric protein of the mitochondrial inner membrane, transporting ADP inside mitochondria and ATP outside (Klingenberg 1989, Fiore *et al.* 1998, Palmieri *et al.* 2005). ANT<sup>-/-</sup> mice

develop mitochondrial myopathy and hypertrophic cardiomyopathy (Graham *et al.* 1997). Similar findings have been reported in patients with spontaneous mutations in *ANT-1* gene. In addition to hypertrophy and cardiomyopathy, increased serum lactic acid levels are common findings in patients with *ANT-1* mutations (Palmieri *et al.* 2005). Moreover, lower *ANT-1* transport capacity has been reported in patients with dilated cardiomyopathy, contributing to disturbed energy metabolism (Dorner *et al.* 2006). However, it is unknown whether variations of *ANT-1* degree might occur in hypertrophic hearts of animal models of acromegaly.

The aim of the study was to evaluate the effect of chronic GH excess on *ANT-1* expression in transgenic mice overexpressing bovine GH.

## Materials and Methods

### Animals

Transgenic mice overexpressing a coding sequence of bovine GH gene under the control of metallothionein (MT) promoter in the C57Bl/6J×CBA genetic background were a generous gift from Dr M Bohlooly-y (University of Goteborg, Sweden) and have been described elsewhere (Bohlooly *et al.* 2001). C57Bl/6J×CBA mice were purchased from Harlan Italy (Udine, Italy). The identity of transgenic mice was confirmed by PCR analysis of DNA from tail biopsy specimens using PCR primers located in the MT promoter and in the *bGH* gene as reported (Fu *et al.* 2000). Only male animals of the following age: 1-, 3-, 6-, 9- and 12-month-old were used for the experiments. The study groups included wild-type animals (Wt), acromegalic mice (Acro), and acromegalic mice treated with pegvisomant (Acro-Peg). Each group consisted of five animals of each age; thus, 75 animals were included in the study. The environment of the animal rooms was controlled with a 12 h light:12 h darkness cycle, a relative humidity of 45–55% and temperature of 20 °C. Animals had free access to tap water and standard pellet chow.

All procedures on animals followed the recommendations reported in The UFAW handbook on the care and management of laboratory animals (Universities Federation for Animal Welfare at the Old School, Brewhouse Hill, UK). The study was approved by the local board for animal experimentation at the University of Pisa.

### Treatment

Some acromegalic animals (see animals) were treated with pegvisomant (Pfizer, Rome, Italy), a specific antagonist of GH receptor (0.1 mg/daily, subcutaneously for 15 days). The effectiveness of pegvisomant was evaluated by measuring serum IGF-1 concentrations at the baseline (before starting therapy) and at the end of the treatment.

### Assays

Serum IGF-1 concentrations were measured using a commercial kit (Diagnostic System Laboratories, Webster, TE, USA). Sensitivity was 21 ng/ml; intra- and interassay variations were 12 and 9% respectively.

### Tissue samples

Body and heart weights of Acro and Wt mice were determined after killing (bleeding and cervical dislocation under ether anesthesia). Organs were immediately frozen in liquid nitrogen until further examination.

### Histology

The hearts were fixed in 10% formalin, embedded in paraffin, and then subjected to light-microscopic examinations. Serial 4 µm tissue sections were deparaffinized and stained with hematoxylin and eosin. Fiber diameter was determined by calculating the mean of the shortest and longest diameters as reported (Lund & Tomanek 1978).

### Tissue extracts

Tissue extracts were obtained by homogenizing hearts with lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, and protease inhibitor). After incubation on ice for 30 min and subsequent centrifugation, supernatants were stored at –80 °C. Protein concentration was measured by Bradford assay using the Bio-Rad reagent (Bio-Rad Laboratories).

### Western blotting

Proteins (50 µg) were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane and stained with red Ponceau to verify the amount of proteins per lane. Transferred proteins were incubated overnight at 4 °C in 50% TBS (200 mM Tris-HCl (pH 7.6) and 1.4 M NaCl) and 50% TTBS (TBS, 0.05% Tween-20), containing 5% nonfat dry milk, and subsequently incubated with the appropriate primary antibody for 1 h at RT. After TTBS washing, a chicken anti-goat IgG horseradish peroxidase-conjugated secondary antibody was added for 1 h at RT; positive proteins were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The intensity of bands of interest was quantified by densitometry (Interfocus GmbH, Sonnenblumenring, Mering, Germany).  $\alpha$ -Sarcomeric actin was used as internal control. Degree of *ANT-1* or NF- $\kappa$ B was expressed as percentage of optical density (OD) measured by densitometry and corrected by OD of  $\alpha$ -sarcomeric actin.

### Antibodies

Goat polyclonal anti-*ANT* (s.c.: 9299) and mouse monoclonal anti-NF- $\kappa$ B p50 (s.c.: 8414) were obtained

from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse monoclonal anti- $\alpha$ -sarcomeric actin was obtained from Sigma–Aldrich. Chicken anti-goat IgG horseradish peroxidase-conjugated secondary antibody were obtained from Bio-Rad and used for revealing ANT. Donkey anti-mouse IgG horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz and used for revealing NF- $\kappa$ B or  $\alpha$ -sarcomeric actin.

#### Fourier transform infrared spectrometry (FTIR)

Four micrometer tissue slices were used for evaluating the presence of lactic acid in heart samples. The FTIR analysis was carried out as reported (D'Alessio *et al.* 2005). An autoimage microscope connected with a Perkin–Elmer 2000 spectrophotometer was employed, and measurements were carried out using 15 $\times$  Reffachromat lenses in the 4000–600/cm region with a 2/cm resolution with 100 scans signal on average. Lactic acid (Sigma–Aldrich) was used as internal standard control.

#### ATP assay

ATP concentration in tissue samples was evaluated using a bioluminescence assay (Calbiochem, Milan, Italy) following the manufacturer's instructions. The assay is based on the firefly luciferase-catalyzed oxidation of D-luciferin in the presence of ATP and oxygen, whereby the amount of ATP is quantified by the amount of light produced. Relative degree of ATP at each age was expressed as ATP Acro/ATP weight ratio.

#### Statistical analysis

Results were expressed as mean  $\pm$  S.D.; comparison of parameters among the study groups was performed by Kruskal–Wallis analysis; comparison of parameters between two study groups was performed by the Mann–Whitney *U*-test. The correlation between ANT-1 expression and serum IGF-I levels was evaluated by Spearman correlation.

## Results

As expected, mean body weight of Acro was greater than that of Wt (Table 1); heart weight of Acro was greater than that of Wt even when corrected by body weight, and increased during the life span (Table 1). Cardiac hypertrophy was revealed in 6- to 12-month-old Acro, at histology (data not shown), confirming previous data (Bollano *et al.* 2000, Fu *et al.* 2000). Mean serum IGF-I concentrations were 291  $\pm$  45 ng/ml in Wt, 683  $\pm$  74 ng/ml in Acro, and 391  $\pm$  93 ng/ml in AcroPeg ( $P < 0.04$ ).

ANT-1 degree of expression was higher in 1-month-old Acro (47  $\pm$  5% OD) than in 1-month-old Wt (33  $\pm$  4% OD,  $P < 0.01$ ; Fig. 1). Thereafter, in 3- to 12-month-old Acro, the degree of ANT-1 expression was lower than that of littermate control (Fig. 1;  $P < 0.03$ ). Reduced expression of ANT-1 was more evident in older Acro (9-month-old (63  $\pm$  4% OD vs 38  $\pm$  5% OD) and 12-month-old (62  $\pm$  5% OD vs 34  $\pm$  6% OD)); these changes were due to increased expression of ANT-1 in Wt and reduced degree in Acro. Changes in cardiac ANT-1 expression in Acro were due to GH excess as clearly shown by treatment with pegvisomant, a selective GH receptor competitor. In 1- to 3-month-old Acro, pegvisomant restored ANT-1 expression to levels superimposable to those found in Wt (Fig. 1). However, in 6- to 12-month-old Acro, pegvisomant treatment did not restore the ANT-1 expression observed in Wt, suggesting that in hypertrophied hearts of older acromegalic mice ANT-1 down regulation is no longer GH dependent.

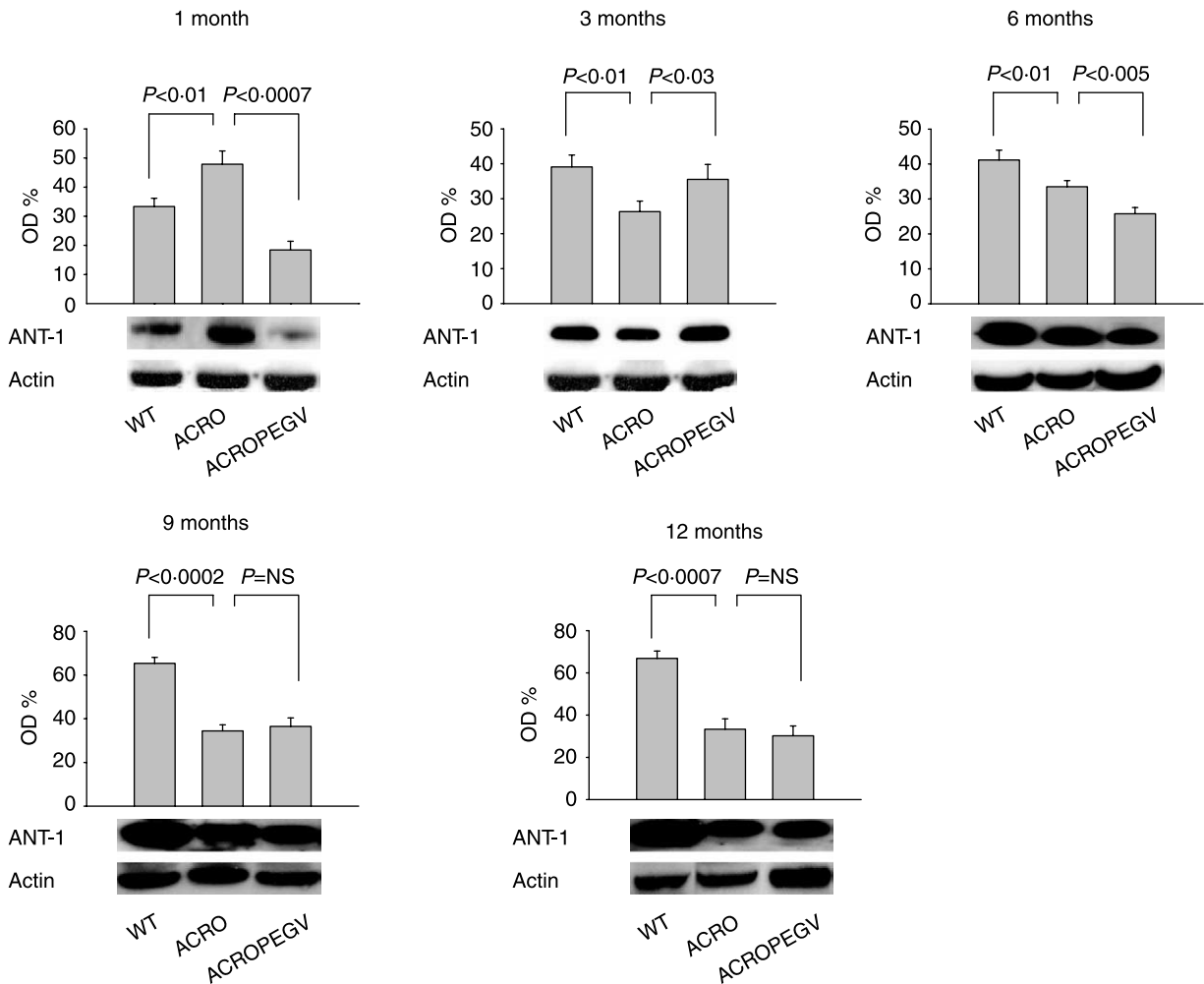
Cardiac content of ATP was measured by a bioluminescence method. ATP degree increased in 1-month-old Acro when compared with Wt (ATP Acro/ATP Wt ratio, 1.85  $\pm$  0.35), and this effect was abolished by pegvisomant treatment (Fig. 2). Cardiac ATP levels in 3- to 12-month-old Acro were lower than those of Wt ( $P < 0.04$ ), being ATP Acro/ATP Wt ratio 0.5–0.6; in addition, ATP levels did not change after treatment with pegvisomant. Overall, cardiac levels of ATP in Acro were consistent with those of ANT-1, leading support to the functional role of ANT-1, in the energy metabolism of hypertrophic hearts.

Tissue levels of lactic acid, evaluated by FTIR, were detected only in 9- and 12-month-old Acro (Fig. 3). In Fig. 3,

**Table 1** Body and heart weight of the animals included in the study. Data are shown as mean  $\pm$  S.D.

	Age (months)				
	1	3	6	9	12
Body weight (g)					
Wt	18 $\pm$ 5	27 $\pm$ 3	32 $\pm$ 6	35 $\pm$ 4	42 $\pm$ 5
Acro	25 $\pm$ 6	46 $\pm$ 3	52 $\pm$ 4	55 $\pm$ 3	60 $\pm$ 2
Heart weight (g)					
Wt	0.15 $\pm$ 0.03	0.21 $\pm$ 0.01	0.23 $\pm$ 0.02	0.25 $\pm$ 0.03	0.28 $\pm$ 0.06
Acro	0.24 $\pm$ 0.04	0.33 $\pm$ 0.02	0.34 $\pm$ 0.05	0.36 $\pm$ 0.04	0.38 $\pm$ 0.05

Differences between Acro and Wt at any age were statistically significant ( $P < 0.03$ ) as assessed by Kruskal–Wallis analysis; Wt, wild-type mice; Acro, transgenic mice overexpressing bovine growth hormone.



**Figure 1** Expression of ANT-1 in the heart of normal or acromegalic mice. ANT-1 expression was higher in 1-month-old acromegalic (Acro) mice than in littermate controls (Wt). Older Acro mice had lower ANT-1 degree. Pegvisomant (PEG) treatment abolished GH-dependent variations of ANT-1 in young Acro mice. Results represent mean  $\pm$  s.d. obtained in five animals for each group. Actin:  $\alpha$ -sarcomeric actin.

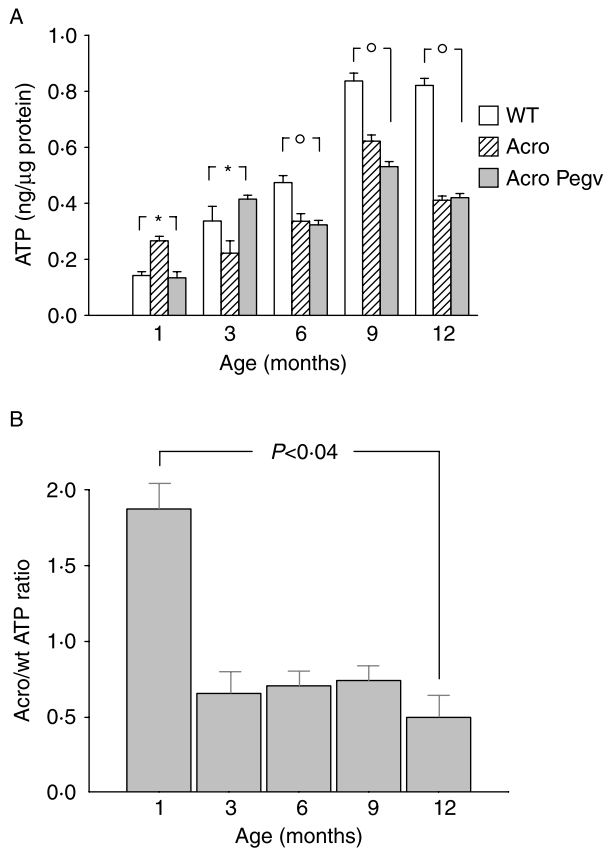
the spectrum of lactic acid with that of samples in the 1800–1600/cm region corresponding to the C=O stretching of lactic acid is compared. A small but significant peak, corresponding to lactic acid, was detected in cardiac tissue samples of 9- and 12-month-old Acro.

Variations of cardiac ANT-1 expression were associated with changes of NF- $\kappa$ B tissue levels (Fig. 4). NF- $\kappa$ B has been shown to regulate ANT-1 activity (Nebigil *et al.* 2003), and is part of cellular transduction of GH signaling (Jeay *et al.* 2002). Thus, we evaluate expression of NF- $\kappa$ B in cardiac tissue extracts of 1- and 12-month-old animals. One-month-old Acro have higher degree of NF- $\kappa$ B than Wt ( $P < 0.05$ ); NF- $\kappa$ B expression was GH dependent, reducing after pegvisomant treatment. Twelve-month-old Acro had reduced cardiac NF- $\kappa$ B degree, which was unaffected by the GH receptor antagonist ( $P < 0.04$ ).

Expression of ANT-1 and NF- $\kappa$ B was not related to serum IGF-1 concentrations ( $r = 0.1$ ,  $P = \text{NS}$ ).

## Discussion

Cardiac hypertrophy and interstitial fibrosis are common features of acromegaly (Melmed 2006). Chronic GH excess markedly affects cardiovascular system, leading to systemic hypertension, cardiac hypertrophy, arrhythmias, abnormal diastolic filling, and finally to systolic dysfunction (Saccà *et al.* 1994). The main effect of GH/IGF-1 excess in animals (Rubin *et al.* 1990, Donohue *et al.* 1994, Cittadini *et al.* 1996) is left ventricular hypertrophy due to increased myocyte diameter with moderate (Fu *et al.* 2000) or without (Cittadini *et al.* 1996) increase interstitial tissue. Short-term GH/IGF-1 administration to animals or healthy subjects had tropic effects on myocardium enhancing systolic function without increasing hemodynamic load (Cittadini *et al.* 1996). This occurred also in healthy subjects treated with high doses of GH, confirming a high cardiac output state associated with

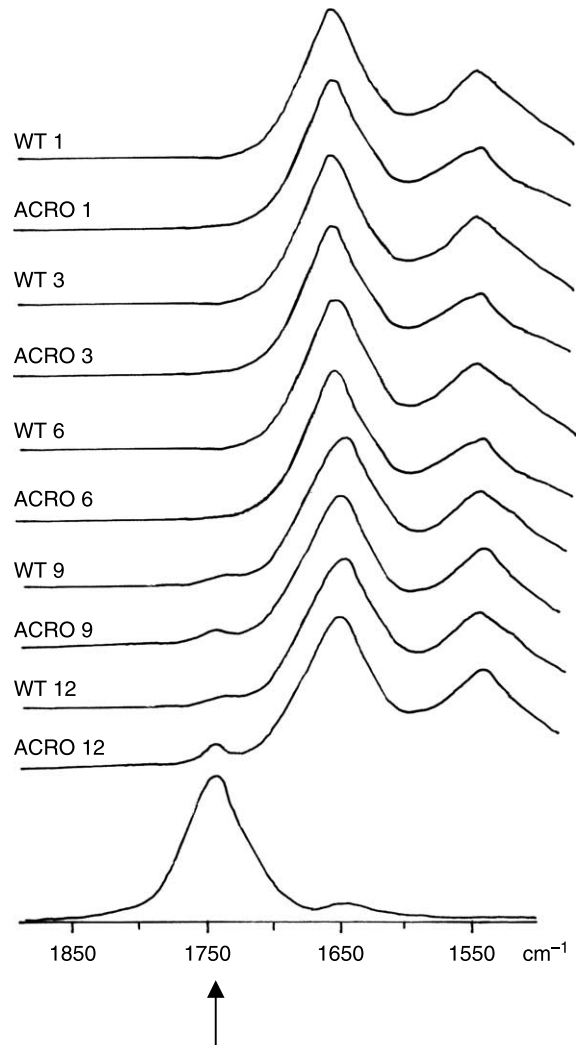


**Figure 2** ATP content in tissue extracts of acromegalic mice. (A) Tissue ATP levels were measured by bioluminescence assay as reported in Material and Methods. (B) ATP Acro/ATP Wt ratio in animals of different age. \* $P < 0.003$  Acro versus Wt or AcroPeg;  $^{\circ}P < 0.05$  Wt versus Acro or AcroPeg. Acro, acromegalic mice; Wt, wild-type animals; AcroPeg, acromegalic mice treated with pegvisomant. Results represent mean  $\pm$  s.d. obtained in five animals for each group.

concentric left ventricular remodeling (Cittadini *et al.* 2002). In addition to lower values of systolic wall stress and decreased systolic vascular resistance, increased myocardial contractility has been suggested for explaining the enhanced cardiac performance during short-term GH excess (Saccà *et al.* 1994, Cittadini *et al.* 2002).

The energy metabolism of normal heart relies on fatty acid and oxidative phosphorylation, generating adequate amounts of ATP, the efficiency of the latter reaction, largely depending on ADP availability. Mitochondrial ADP/ATP exchange is mediated mainly by ANT-1. Patients carrying mutations in the *ANT-1* gene or knockout (*ANT*<sup>-/-</sup>) animals develop myopathy and cardiomyopathy (Palmieri *et al.* 2005). These alterations are due, at least in part, to ADP transport alterations, affecting oxidative metabolism.

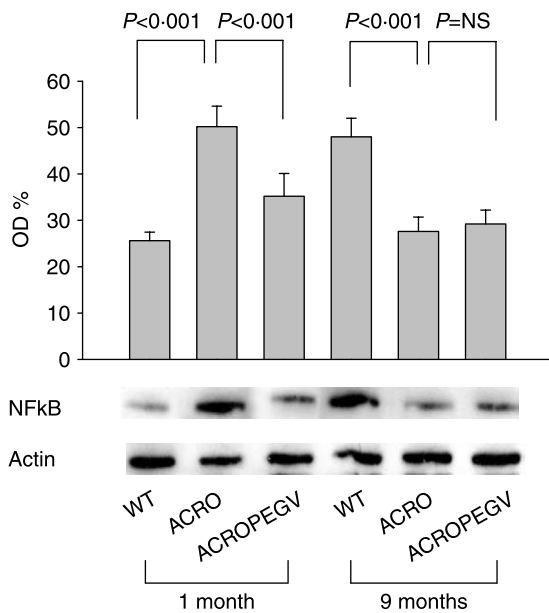
Our data clearly showed that ANT-1 expression was GH dependent and that GH excess increased the degree of expression of this transporter in the short-term. The increased



**Figure 3** Lactic acid assay in heart tissue. Presence of lactic acid in heart tissue extracts from acromegalic mice (Acro) or control animals (Wt) was assessed by FTIR as described in Materials and Methods. Arrow indicates lactic acid.

ATP levels found in heart tissue of 1-month-old Acro lend support to the concept of adaptive increased energy production upon a direct GH action. GH specificity of this coordinate variation of ANT-1 expression and ATP production was demonstrated by its reversal by pegvisomant treatment, a specific GH receptor antagonist. ATP changes were consistent with those of ANT-1, indicating the functional role of this transporter in the heart of acromegalic animals. It is worth noting that hypertrophic hearts of older Acro not only had a lower degree of ANT-1 expression than Wt, but also those variations that were not dependent on GH, as shown by lack of effect by pegvisomant. Although moderate, reduction of ANT-1 may have a role in energetic imbalance of Acro hypertrophic heart, having increased





**Figure 4** Expression of NF- $\kappa$ B. Degree of expression of NF- $\kappa$ B was evaluated in cardiac extracts from: acromegalic mice (Acro), wild-type animals (Wt), and acromegalic mice treated with pegvisomant (AcroPeg). Results represent mean  $\pm$  s.d. obtained in five animals for each group. Actin,  $\alpha$ -sarcomeric actin.

energy expenditure (Wambolt *et al.* 1999, Stanley & Chandler 2002). The figure drawn by our data is undoubtedly far from that occurring in *ANT-1*<sup>-/-</sup> animals, lacking *ANT-1* activity; however, detection of lactic acid in older Acro suggests that glycolytic pathway begins to be involved to compensate the increasing energy demands secondary to hypertrophy and reduced *ANT-1* expression.

Mechanisms underlying regulation of *ANT-1* are poorly understood. In astrocytes, *ANT-1* expression was regulated by a cooperative interaction of SMAD- and Sp1-binding elements located in the promoter region of *ANT-1* gene (Law *et al.* 2004). In a cardiomyocyte cell line, serotonin reduced *ANT-1* degree through activation of PI3K/Akt induction of NF- $\kappa$ B (Nebigil *et al.* 2003). On the other hand, NF- $\kappa$ B is a mediator of GH action at the molecular level, enhancing cell survival through activation of AKT pathway (Jeay *et al.* 2002).

Usually, activation of NF- $\kappa$ B is associated with inhibition of apoptosis (Beg & Baltimore 1996) while reduced NF- $\kappa$ B activity leads to enhanced programmed cell death (Wu *et al.* 1996). However, several reports emphasize that the function of NF- $\kappa$ B can be proapoptotic or antiapoptotic depending on cell type, degree of NF- $\kappa$ B activation and type of the apoptotic signals (Liu & Lin 2007). This notion fits well with our data in 1-month-old Acro in which NF- $\kappa$ B degree is increased suggesting reduced apoptosis; the contrary might be expected in 12-month-old Acro (Fig. 4).

On the other hand, NF- $\kappa$ B activation is necessary for development of cardiac hypertrophy after aortic banding

(Li *et al.* 2004) and its inhibition could favor hypertrophy regression (Gupta *et al.* 2005). In 12-month-old Acro with LV hypertrophy, NF- $\kappa$ B levels are not increased without variations during pegvisomant administration suggesting that other mechanisms are likely involved in cardiac hypertrophy of acromegalic mice.

Our data suggest an association among GH, *ANT-1*, and NF- $\kappa$ B: variations of NF- $\kappa$ B expression are consistent with an induction of *ANT-1* in 1-month-old Acro mice and with an inhibitory effect in older Acro mice. Variations of NF- $\kappa$ B levels were consistent with changes of *ANT-1* and reversed by pegvisomant treatment in youngest Acro. The fact that the reduced NF- $\kappa$ B protein degree in older Acro lost GH sensitivity suggests that other mechanisms likely converge on *ANT-1* regulation in older hypertrophic hearts.

In conclusion, our data suggest that GH excess increase expression of cardiac *ANT-1*, which leads to higher tissue content of ATP, in young animals. Chronic long-standing GH excess is associated with cardiac hypertrophy, reduced *ANT-1* degree, and less efficient energy metabolism involving glycolytic pathway. If these changes might be considered compensatory to cardiac hypertrophy remains to be elucidated. It is tempting to speculate that excessive GH exposure may induce heart hypertrophy which may become self-maintaining in the long-run. Adaptive energy metabolism seems to compensate at the beginning of GH excess and becoming not sufficient later-on. However, from our data, we cannot state that variations of *ANT-1* and energy production were associated with changes of cardiac function, albeit the latter have been reported to occur in 9-month-old mice (Bollano *et al.* 2000).

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