Cardiac extrinsic apoptotic pathway is silent in young but activated in elder mice overexpressing bovine GH: interplay with the intrinsic pathway

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Abstract

Apoptosis may occur through the mitochondrial (intrinsic) pathway and activation of death receptors (extrinsic pathway). Young acromegalic mice have reduced cardiac apoptosis whereas elder animals have increased cardiac apoptosis. Multiple intrinsic apoptotic pathways have been shown to be modulated by GH and other stimuli in the heart of acromegalic mice. However, the role of the extrinsic apoptotic pathways in acromegalic hearts is currently unknown. In young (3-month-old) acromegalic mice, expression of proteins of the extrinsic apoptotic pathway did not differ from that of wild-type animals, suggesting that this mechanism did not participate in the lower cardiac apoptosis levels observed at this age. On the contrary, the extrinsic pathway was active in elder (9-month-old) animals (as shown by increased expression of TRAIL, FADD, TRADD and increased activation of death inducing signaling complex) leading to increased levels of active caspase 8. It is worth noting that changes of some pro-apoptotic proteins were induced by GH, which seemed to have, in this context, pro-apoptotic effects. The extrinsic pathway influenced the intrinsic pathway by modulating t-Bid, the cellular levels of which were reduced in young and increased in elder animals. However, in young animals this effect was due to reduced levels of Bid regulated by the extrinsic pathway, whereas in elder animals the increased levels of t-Bid were due to the increased levels of active caspase 8. In conclusion, the extrinsic pathway participates in the cardiac pro-apoptotic phenotype of elder acromegalic animals either directly, enhancing caspase 8 levels or indirectly, increasing t-Bid levels and conveying death signals to the intrinsic pathway.

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Introduction

Acromegalic cardiomyopathy is characterized by biventricular hypertrophy and diastolic abnormalities and, in the late stage, by systolic dysfunction, eventually leading to cardiac failure (Colao et al. 2004, Giustina et al. 2008a, b, Melmed 2009).

Cardiomyocyte apoptosis is a low abundance process, identified in the diseased heart of human and rodents, the activation of which is considered sufficient to lead to heart failure (Movassagh & Roger 2008).

Moreover, cardiomyocyte apoptosis may directly cause dilated cardiomyopathy in animals. Mice expressing an FKBP-caspase 8 fusion protein, or constitutively activated Gαq, had increased incidence of cardiac apoptosis, leading to myocardial hypertrophy that progressed to dilatation and leading to higher mortality rate (Yussman et al. 2002, Wencker et al. 2003).

It has been reported that patients with advanced acromegalic cardiomyopathy have increased apoptosis (Frustaci et al. 1999). However, in animal models of acromegaly apoptosis degree was related to the duration of disease, being higher in elder and lower in young animals (Bogazzi et al. 2008). In fact, acromegalic mice aged 3 months had lower while 9-month-old animals had higher cardiomyocyte apoptosis than littermate controls (Bogazzi et al. 2008).

Mechanisms underlying changes of cardiac apoptosis in acromegalic mice involved regulation of the expression of several pro- and anti-apoptotic proteins of the intrinsic pathway, driven by GH-dependent or GH-independent signals (Bogazzi et al. 2008).

On the other hand, the extrinsic pathway has been involved in the regulation of either post-ischemic or stretch-induced cardiomyocyte apoptosis (Jeremias et al. 2000, Liao et al. 2005). In addition, death receptors and death ligands are constitutively expressed in cardiomyocytes (Chao et al. 2002) and their expression may increase following diastolic wall stress due to volume overload (Wollert et al. 2000) or to mechanical stretch (Liao et al. 2005).
These findings suggest a role of the extrinsic apoptotic pathway in cardiac pathology. However, the involvement of the extrinsic pathway in acromegalic cardiomyopathy is unknown.

We evaluated the expression and activation of pro- and anti-apoptotic proteins of the extrinsic pathway in the heart of young and elder mice overexpressing bovine GH (bGH), and its interplay with the intrinsic pathway.

Materials and Methods

**Animals**

Transgenic mice overexpressing a coding sequence of bGH gene under the control of metallothionein promoter in the C57BL/6j × CBA genetic background have been described (Bohlooly-Y et al. 2001). C57BL/6j × CBA mice were purchased from Harlan Italy (Udine, Italy). Briefly, transgenic mice overexpressing bGH had: i) increased body weight compared with Wt animals; ii) increased serum IGF1 and GH levels; iii) increased organ mass in relation to body weight—in particular, heart weights, even when normalized to BW, were double those of Wt. Moreover, myocardial hypertrophy was a constant finding at histology (Bollano et al. 2000, Fu et al. 2000). Transgenic animals have been shown to have reduced global left ventricle function (on average, EF 58 vs 78% of controls), whereas diastolic function was usually normal (Bollano et al. 2000).

The identity of bGH transgenic mice (acromegalic animals) was confirmed by PCR analysis as previously reported (Bogazzi et al. 2008, 2009a). Thirty animals were studied. 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**

A group of acromegalic animals (see animals) were treated with pegvisomant (Pfizer, Rome, Italy), a specific antagonist of GH receptor (GHR; 0.1 mg/daily, s.c. for 15 days) as previously reported (Liao et al. 2006, Bogazzi et al. 2008, 2009b). To exclude any antagonist’s proper effect, Wt animals were treated with pegvisomant; in addition, Wt and Acro animals were also treated with vehicle. Treatment with pegvisomant (or vehicle) lasted 15 days owing to the rapid effect of pegvisomant and for a better comparison with our previous studies on cardiac apoptosis (Bogazzi et al. 2007, 2008, 2009b). Effectiveness of pegvisomant was evaluated by measuring serum IGF1 concentrations before and at the end of treatment.

**Assays**

Serum IGF1 concentrations were measured using a commercial kit (Diagnostic System Laboratories, Webster, TX, USA), as previously reported (Bogazzi et al. 2008, 2009b).

**Tissue samples**

Animals were killed under ether anesthesia by bleeding and cervical dislocation; ventricles were separated and then immediately frozen in liquid nitrogen until further examination.

**Apoptosis**

Apoptosis was evaluated by TUNEL assay (Roche Diagnostic) and by Annexin V (Santa Cruz Biotecology, Santa Cruz, CA, USA) in mice heart, as previously reported (Bogazzi et al. 2008).

**Tissue extracts**

Total tissue extracts were obtained by homogenizing left ventricles in lysis buffer, in keeping with previous reports (Bogazzi et al. 2008). Briefly, tissues were homogenized 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 inhibitors cocktail tablets (benzamidine, phenanthroline, aprotinin, leupeptin, pepstatin, and phenylme-thylsulphonyl fluoride); after incubation on ice for 30 min and subsequent centrifugation, supernatants were recovered and stored at −80°C. Protein concentration was measured by Bradford assay using the Bio–Rad reagent (Bio–Rad Laboratories).

**Antibodies**

The following antibodies were used: TNFα (N-19) goat IgG antibody, FAS-L (Q-20) rabbit IgG antibody, TRAIL (H-257) rabbit IgG antibody, TRADD (H-278) rabbit IgG antibody, FADD (H-181) rabbit IgG antibody, caspase 8 p20 (H-134) rabbit IgG antibody, procaspase 8 (T-16) goat IgG antibody, p53 (FL-393) rabbit IgG antibody polyclonal, NFκB (E-10) mouse monoclonal IgG antibody, FLIPS/L (H-202) rabbit IgG antibody, GHR (S-19) goat IgG antibody (all from Santa Cruz Biotecology) mouse monoclonal α-sarcomeric actin (clone 5CS) (Sigma–Aldrich), Bid and t-Bid rabbit IgG antibody (Abcam, Cambridge, UK).

**Western blotting**

Total (50 μg) myocardial protein extracts (and protein extracts from immunoprecipitation, as appropriate) were resolved on a 12% SDS–PAGE, transferred onto nitrocellulose membrane,
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FAD antibody (5 µg) was incubated with 1 mg of total tissue extracts overnight at 4 °C. The mixture was then incubated with 50 µl of a 1:1 suspension of n-protein A Sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) for 1 h at 4 °C with gentle rotation. The beads were pelleted and washed three times with lysis buffer. The immunocomplexes were dissociated by boiling the mixture in SDS-PAGE sample buffer and analyzed by western blot, using TNFα, TRAIL, FAS-L, and caspase 8 antibody, as appropriate.

Immunoprecipitation

and stained with Ponceau red to verify the amount of proteins per lane. Transferred proteins were incubated overnight at 4 °C in 50% Tris-buffered saline (200 mm Tris–HCl (pH 7-6) and 1·4 m NaCl) and 50% TTBS (Tris-buffered saline with 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, an IgG HRP-conjugated secondary antibody was added for 1 h at RT; positive proteins were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Membranes were incubated at 70 °C for 10 min in stripping buffer (5 mm Tris–HCl (pH 6·8), 2% SDS, 67·5 ml ultrapure water, 0·8% β-mercaptoethanol) and reprobed for α-sarcomeric actin for loading normalization.

Films were scanned on densitometry (Bio-Rad Life Science) and band intensity was evaluated using Interfocus software (Interfocus GmbH, Sonnenblumenring, Mering, Germany). Each sample value was normalized for loading errors (dividing by intensity of α-sarcomeric actin); data were expressed as arbitrary units (A.U.) that represent the ratio between the intensity of the band of interest and the intensity of the band corresponding to the control protein. The resulting values within the same mouse group but from different blots were then combined.

Immunoprecipitation

Figure 1 Cardiomyocyte apoptosis. Cardiomyocyte apoptosis was determined by TUNEL (panel A) and Annexin V (panel B) according to the procedure described in the Materials and Methods section. Wt, wild type; Acro, acromegalic; Acropeg, acromegalic animals treated with a GH-receptor antagonist; Wtpeg, wild-type animals treated with a GH-receptor antagonist; Wtvehicle, wild-type animals treated with vehicle; Acrovehicle, acromegalic animals treated with vehicle. Data are expressed as mean ± S.D.*P < 0·05. Full colour version of this figure available via http://dx.doi.org/10.1330/JOE-10-0402.

Figure 2 Death ligands, adaptors, and caspase 8 expression in cardiac tissue from 3-month-old animals. Representative western blot of cellular extract of left ventricles obtained from 3-month-old transgenic mice (Acro), controls (Wt), or Acro treated with a GH-receptor antagonist (Acropeg). The expression of death ligands, adaptors, and caspase 8 was normalized for α-sarcomeric actin (α-actin). Data are expressed as arbitrary units (A.U.), which represent the ratio between the intensity of the band corresponding to the control protein and represent the mean ± S.D. of measurements obtained in five animal of each group. All differences between groups were not statistically significant.
Results were expressed as mean ± S.D. ANOVA was used to evaluate differences in the prevalence of cardiomyocyte apoptosis, degree of expression of pro- and anti-apoptotic proteins and caspases among groups of animals. A P value < 0.05 was considered statistically significant.

Results

As expected, and in line with previous data (Bogazzi et al. 2008) young acromegalic mice had a lower but elder animals had a higher degree of apoptosis than their littermate controls (Fig. 1). Experiments were carried out in animals aged 3 and 9 months to exploit expression of GHR. GHR expression did not differ in Acro and Wt animals aged 3 months and in those aged 9 months.

The extrinsic pathway in 3-month-old acromegalic mice

Expression of death ligands, TNFα, TRAIL, and FAS-L did not differ significantly between acromegalic and wild-type mice at this age. In keeping with this observation, a 15-day course with a GHR inhibitor was without effect (Fig. 2). Likewise, expression of adaptor proteins, FADD and TRADD, was unchanged in acromegalic, wild type and acromegalic mice treated with a GHR antagonist. The unchanged expression of procaspase 8 or caspase 8 (Fig. 2) further supports that the extrinsic pathway was not altered. Taken together, these results suggested that the extrinsic pathway through formation of death inducing signaling complex (DISC) was not involved in the regulation of cardiac apoptosis in acromegalic mice aged 3 months.

However, expression of FLIP (the anti-apoptotic FADD-like interleukin β converting enzyme-like inhibitory protein) was increased in a GH-dependent manner in 3-month-old acromegalic mice (Fig. 3), whereas that of the proapoptotic Bcl-2 family member Bid was reduced. Expression of both proteins was similar to that of wild-type mice after treatment with a GHR antagonist. On the contrary, the level of expression of t-Bid was not affected by a GHR antagonist.

Figure 3 Expression of FLIP, Bid, NfκB, and p53 in 3-month-old animals. Expression of FLIP, Bid, NfκB, and p53 was determined in cellular extract from left ventricles as described in the legend to Fig. 2. Data are expressed as A.U., which represent the ratio between the intensity of the band corresponding to the control protein and represent the mean ± s.d. of measurements obtained in five animal of each group. **P < 0.001; ***P < 0.0001; §P < 0.0004; NS, not significant.

Statistical analysis

Results were expressed as mean ± S.D. ANOVA was used to evaluate differences in the prevalence of cardiomyocyte apoptosis, degree of expression of pro- and anti-apoptotic proteins and caspases among groups of animals. A P value < 0.05 was considered statistically significant.

Figure 4 Death ligands, adaptors, and caspase 8 expression in cardiac tissue from 9-month-old animals. Representative western blot of cellular extract of left ventricles obtained from 9-month-old transgenic mice (Acro), controls (Wt), or Acro treated with a GH-receptor antagonist (Acropeg). The expression of death ligands, adaptors, and caspase 8 was normalized for α-sarcomeric actin (α-actin). Data are expressed as A.U., which represent the ratio between the intensity of the band corresponding to the control protein and represent the mean ± s.d. of measurements obtained in five animal of each group. **P < 0.001; ***P < 0.0001; §P < 0.0004; NS, not significant.
Among death ligands, only the expression of TRAIL was higher in acromegalic mice than in controls, whereas that of TNF was not affected by treatment with a GHR antagonist (Acropeg). Also, levels of caspase 8 and pro- and anti-apoptotic proteins FLIP, Bid, and t-Bid were decreased and increased respectively in acromegalic mice. These changes were not influenced by a GHR antagonist.

Interplay between the intrinsic and extrinsic pathway in young and elder acromegalic animals

The intrinsic pathway influenced the extrinsic pathway changing NFkB, the expression of which was higher in acromegalic animals aged 3 months and lower in those aged 9 months (Figs 3 and 6). NFkB may be responsible for variations of FLIP expression as previously reported (Kanetaka et al. 2008). In addition, we evaluated the expression of p53, which is a regulator of Bid expression. As shown in Figs 3 and 6, p53 expression decreased in young acromegalic mice and increased in elder animals, in keeping with corresponding variations of Bid expression.

On the other hand, the extrinsic pathway may participate in the regulation of the intrinsic apoptotic pathway changing the expression of the activated t-Bid (Figs 3 and 6).

In fact, increased t-Bid expression may activate the mitochondrial pathway of the apoptotic process. A proposed mechanism of the extrinsic pathway and its interplay with the intrinsic pathway is shown in Fig. 7.

Discussion

Cardiac apoptosis has been linked to acute lesions (myocardial infarction and ischemia/reperfusion) or chronic disease of the heart (ischemic and dilated cardiomyopathies) (Kang & Izumo 2000, Niessner et al. 2009). In fact, increased apoptosis in hypertrophic cardiomyopathy may be harmful, leading to dilation and finally to systolic failure (Das et al. 2010). Acromegaly is often complicated by acromegalic cardiomyopathy featured by biventricular hypertrophy and diastolic dysfunction, which may progress to systolic failure (Colao et al. 2004, Giustina et al. 2008a,b, Melmed 2009).

Figure 5 Formation of DISC. Cellular extracts obtained from 9-month-old transgenic mice (Acro), controls (Wt), or Acro treated with a GH-receptor antagonist (Acropeg) were firstly immunoprecipitated with a FADD antibody and resolved on 12% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with TNFα, TRAIL, FAS-L, caspase 8 antibody, as appropriate, to verify the formation of protein–protein interaction leading to formation of activated extrinsic pathway (DISC, death inducing signaling complex). ***P<0.0001; NS, not significant.

Figure 6 Connection between intrinsic and extrinsic pathway in elder animals. Expression of FLIP, Bid, NFkB, and p53 was determined in cellular extract from left ventricles as described in the legend to Fig. 4. The expression of death ligands, adaptors, and caspase 8 was normalized for α-sarcomeric actin (α-actin). Data are expressed as arbitrary units (A.U.), which represent the ratio between the intensity of the band corresponding to the control protein and represent the mean ± s.d. of measurements obtained in five animals of each group. ***P<0.0001; NS, not significant.
We have previously reported that cardiomyocyte apoptosis is reduced in young and enhanced in elder acromegalic mice (Bogazzi et al. 2008). While blockage of GH–IGF action using a GHR antagonist abolished the anti-apoptotic effect of GH in young mice, this action was ineffective in elder animals. However, GH still had anti-apoptotic action in acromegalic mice aged 9 months, as demonstrated by induction of anti-apoptotic proteins of the intrinsic pathway at that age (reduced proapoptotic Bax and increased anti-apoptotic AKT levels Bogazzi et al. (2008)). However, the anti-apoptotic GH action on the intrinsic pathway was overridden by GH-independent stimuli in elder animals, which led to increased apoptosis degree (Bogazzi et al. 2008).

While the intrinsic apoptotic pathway may be modulated directly by GH through activation of p44/42, p38, and p38 pathway, the extrinsic pathway requires the formation of the DISC complex after binding of a death ligand to its cognate receptor (Movassagh & Roger 2008).

In this study, we showed that the extrinsic pathway is silent in young acromegalic animals as clearly demonstrated by unchanged levels of expression of proteins forming the DISC as well as of procaspase and caspase 8. However, silencing signals coming from the intrinsic pathway conveyed on the extrinsic pathway through p53-induced upregulation of the anti-apoptotic FLIP and NFκB-associated downregulation of Bid. Thus, the extrinsic apoptotic pathway was silenced in acromegalic mice aged 3 months (Fig. 7).

On the contrary, the extrinsic apoptotic pathway was activated in elder acromegalic mice, contributing, along with the intrinsic pathway, to the pro-apoptotic phenotype of 9-month-old acromegalic animals. Activation of the extrinsic pathway may increase apoptosis both directly, enhancing the expression of active caspase 8, triggering the apoptotic cellular death, and indirectly, increasing proportion of t-Bid. This may activate the intrinsic pathway, through mitochondrial damaging (Fig. 7). Although we showed an interplay between intrinsic and extrinsic apoptotic pathways, the relative contribution of each pathway to cardiac apoptosis in this murine model of acromegaly cannot be evaluated.

While activation of the intrinsic pathway in older animals is GH-independent (Bogazzi et al. 2008), the increased activity of the extrinsic pathway, seems to be, at least partially, dependent of GH excess. In fact, 9-month-old acromegalic mice had increased expression of pro-apoptotic adaptor proteins FADD and TRADD, which reverted after blocking
GH/IGFI action with a GHR antagonist. Thus, GH excess in elder animals seems to have a pro-apoptotic effect. However, it is worth noting that DISC formation and caspase 8 activation were no longer reverted by blocking GHR, suggesting that other stimuli intervene in activation of the extrinsic pathway at that age.

The increased expression of the proapoptotic TRAIL is consistent with a lower level of expression of NFκB, supporting a previous report suggesting that GH excess contributes to modulation of TRAIL expression through NFκB reduction (Steenbergen et al. 2002). Changes in the expression or sensitivity of either death ligands or death receptors may be a key step in regulating apoptosis and its effects. In fact, a beneficial effect of GH on heart remodeling in patients with idiopathic dilated cardiomyopathy through reduction in soluble death ligand TNFα and FAS-L (Parissis et al. 2005) has been reported. Moreover, it has been shown that autocrine IGFI reduced apoptosis in human adipocytes reducing sensitivity of death receptor (Fischer-Posovszky 2004). It is tempting to speculate that in the increased formation of DISC in 9-month-old acromegalic mice converge GH-dependent (increased FADD and TRADD) and GH-independent (increased TRAIL) stimuli; it is likely that, although the expression of some pro-apoptotic proteins was no longer reverted by a GHR antagonist, they were a consequence of chronic GH excess because they are evident in acromegalic mice but not in wild-type animals.

In conclusion, our data demonstrated the activation of the extrinsic pathway of cardiac apoptosis in elder acromegalic animals, which are characterized by ventricular hypertrophy (Bohlooly-Y et al. 2001) and abnormalities of energetic (Bohlooly-Y et al. 2001, Bogazzi et al. 2007) and lipid (Bogazzi et al. 2009b) metabolism. Interestingly, those changes may, at least partially, revert in young Acro animals during pegvisomant therapy, supporting the concept that changes in energetic metabolism and apoptosis degree might have different impacts on the cardiac function of young and elder animals. Moreover, it is expected that pegvisomant therapy may reduce ventricular hypertrophy improving cardiac function, as occurs in acromegalic patients (Pivonello et al. 2007), although direct proof is currently unavailable in our murine model.

The demonstration of the involvement of the extrinsic pathway in the cardiac apoptosis of elder acromegalic mice further enhances the level of complexity in the regulation of programmed cell death in conditions of GH excess. Both GH-dependent and GH-independent activation of both intrinsic and extrinsic pathways were involved in a final event, namely cardiomyocyte apoptosis. Although the relative contribution of each pathway to apoptosis degree remains to be elucidated, the interplay between the two pathways was clearly evident.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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