Altered cardiac expression of peroxisome proliferator-activated receptor-isoforms in patients with hypertensive heart disease

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Abstract

Objective: To investigate whether cardiac expression of the nuclear peroxisome proliferator-activated receptor α (PPARα) is altered in patients with hypertensive heart disease (HHD).

Methods: We studied endomyocardial septal biopsies from 24 patients with essential hypertension divided into three groups: 6 without left ventricular hypertrophy (LVH) (HT group), 10 with LVH (LVH group), and 8 with LVH and heart failure (HF) (HF group). The expression of two PPARα isoforms (the native active and the truncated inhibitory) was analyzed by Western blot and reverse transcription polymerase chain reaction (RT–PCR), and two PPARα target genes were evaluated by RT–PCR. Histomorphological features were evaluated in a second myocardial sample from LVH and HF groups.

Results: Whereas the expression of native PPARα protein was lower (p < 0.05) in LVH and HF groups than in the HT group, truncated PPARα protein was overexpressed (p < 0.001) in the HF group as compared with LVH and HT groups. The mRNA expression of native and truncated PPARα was similar in the three groups of hypertensives. In addition, a progressive decrease (p for trend <0.05) in the two PPARα target genes mRNA expression was observed among HT, LVH and HF groups. The amount of truncated PPARα protein correlates directly with cardiomyocytes apoptosis and inversely with cardiomyocytes density in patients with HHD. In addition, the expression of truncated PPARα protein was directly correlated with left ventricular volumes, and inversely with ejection fraction in all hypertensives.

Conclusions: These findings suggest that post-transcriptional regulation of PPARα isoforms is altered in patients with HHD, namely in those developing HF. An excess of the truncated inhibitory isoform may be involved in hypertensive left ventricular failure and remodeling.

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1. Introduction

Previous studies have shown that myocardial fatty acid utilization is decreased in the failing heart of spontaneously hypertensive rats [1,2] and patients with essential hypertension [3]. It has also been shown that expression of genes encoding cardiac fatty acid oxidation enzymes is coordinately repressed in the myocardium of hypertensive rats and patients with heart failure (HF) [4]. This defect in lipid metabolism can be regarded as a result of alterations in
peroxisome proliferator-activated receptor α (PPARα) transcription factor activity. An abnormally low expression of several fatty acid transporters and mitochondrial fatty acid-metabolizing enzymes has been found in the heart of PPARα null mice [5]. Thus PPARα seems to be a critical regulator of myocardial energetics, through the activation of some genes encoding key limiting steps in the fatty acid utilization pathway [6]. Moreover, recent evidences suggest that PPARα may be an antiinflammatory and antifibrotic transcription factor [7–10]. In fact, the heart from PPARα-null mice shows progressive myocardial degeneration associated with contraction band necrosis, inflammatory infiltrates and diffuse fibrosis besides an abnormally low expression of several fatty acid transporters and mitochondrial fatty acid-metabolizing enzymes [5].

Two variants of the human PPARα transcription factor have been described. Tugwood et al. [11] and Palmer et al. [12] found a splice variant of PPARα mRNA lacking exon 6 in human liver. Later on, this variant was also reported in other human tissues including the heart [13]. Whereas the native PPARα mRNA is known to give rise to an active PPARα protein (53 kDa), the truncated PPARα mRNA gives rise to a form of PPARα protein (30 kDa) which lacks the ligand-binding domain [14]. In addition, recent findings suggest that, upon nuclear translocation, the truncated isoform of PPARα exerts repressive action on the native PPARα function, probably through competition for essential coactivators [14].

We have hypothesized that an imbalance between the native active and the truncated inhibitory isoforms of PPARα may occur in patients with hypertensive heart disease (HHD). This study was therefore designed to investigate the expression of PPARα isoforms and the obligate heterodimeric partner retinoid X receptor α (RXRα) in the myocardium of patients with HHD. In addition, the expression of PPARα target genes involved in fatty acid oxidation and histomorphological features of myocardial remodeling were also assessed in tissue samples from the same patients.

2. Methods

2.1. Patients

All subjects gave written informed consent to participate in the study, and the institutional review committee approved the protocol. The study was approved by the ethical committee of Donostia University Hospital and conformed to the principles of the Helsinki Declaration.

The hypertensive population consisted of 24 Caucasian patients with systolic blood pressure >139 and/or diastolic blood pressure of >89 mm Hg. All patients had appropriate clinical and laboratory evaluation to exclude secondary hypertension. Other cardiac diseases (e.g., coronary artery disease, aortic stenosis, hypertrophic cardiomyopathy) were excluded after complete medical examination, which included a diagnostic cardiac catheterization. Patients with diabetes mellitus or metabolic syndrome, as defined by the ATPIII criteria, were excluded from the study [15].

Whereas 18 patients exhibited HHD, 6 patients did not (HT group). HHD was defined by the presence of left ventricular hypertrophy (LVH) in the echocardiogram (see below) in the absence of a cause other than arterial hypertension. Whereas 10 patients with HHD did not present medical history or current manifestations of HF (LVH group), 8 patients with HHD had a previous diagnosis of HF (HF group). The diagnosis of HF was made on a clinical basis, by the presence of, at least, 1 major and 2 minor Framingham criteria [16]. After echocardiographic evaluation (see below) the patients were classified into two groups: 4 patients exhibiting an ejection fraction (EF)<0.40 (i.e. patients with systolic HF) and 4 patients presenting an EF ≥ 0.40 (i.e. patients with diastolic HF). Furthermore, hemodynamic evidence of myocardial failure was obtained in each patient by measuring elevated left ventricular end-diastolic pressure and pulmonary capillary wedge pressure (>12 mm Hg in both cases).

2.2. Assessment of left ventricular dimensions, mass and function

2D echocardiographic imaging, targeted M-mode recordings and Doppler ultrasound measurements were obtained in each patient. Left ventricular end-diastolic and end-systolic volumes (LVEDV and LVESV, respectively) were calculated according to Doughty et al [17]. Left ventricular mass index (LVMI) was calculated as previously described [18]. The presence of LVH was established when LVMI was >111 g/ m² for men and >106 g/m² in women [19]. The following pulsed Doppler measurements were obtained: maximum early transmitral velocity in diastole; maximum late transmitral velocity in diastole; the deceleration time of the early mitral filling wave; and isovolumic relaxation time. Left ventricular EF was calculated according to Quinones et al. [20].

2.3. Sample acquisition

Transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum with a bioptome, Cordis 960 mm (7F), under fluoroscopic guidance after angiographic examination, as previously reported [18]. Two biopsies were taken in each intervention. The biopsy procedure was well tolerated in all cases and no complications were recorded. The first sample was divided into two smaller pieces and frozen separately in liquid nitrogen following different processes for protein or mRNA obtention. Protein was extracted in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% Chaps, 1% dithiothreitol), and mRNA was initially isolated with TRIZol (Invitrogen) and subsequently purified using QIAGEN’s RNasy Total RNA
Isolation kit. The second myocardial sample was immediately fixed in 4% buffered formalin, embedded in paraffin, and serially sectioned in 4-μm-thick sections for the histomorphological studies.

Human liver tissue, kindly provided by Dr. José Ignacio Riezu-Boj, was used as a positive control in analyzing the expression of the two PPARα isoforms. The hepatic sample was processed in the same way as the cardiac samples.

2.4. mRNA studies

To identify the presence of the splice variant of PPARα mRNA, reverse transcription polymerase chain reaction (RT–PCR) followed by sequentiation was performed. Reverse transcription was performed with 200 ng of total RNA and two primers flanking exon 6 were used to amplify human cDNA: (i) 5′-GCCAGTATTGTCGATTTCACAAGT–3′ corresponding to sequences 674–697 of the human PPARα cDNA, located in exon 5; (ii) 5′-TCCTTGTTCTGGATGCTTG–3′ corresponding to sequences 996–1016 of the human PPARα cDNA, located in exon 7. PCR products were separated by electrophoresis, showing two different fragments, one of 343 bp and other about 140 bp (Fig. 1). The smaller fragment was excised from the gel and cloned into a pCR 2.1 vector for sequencing. The DNA sequence of this fragment was determined using the dideoxy chain-termination method [21]. This fragment contained the published sequence of exon 5 followed by exon 7, lacking exon 6 [22] (Fig. 1).

To corroborate the presence of the truncated PPARα mRNA, a specific TaqMan fluorescent probe that recognizes the last nucleotides of exon 5 and the first nucleotides of exon 7 of PPARα cDNA was designed. The fluorescent signal of this probe can only be detected when exon 5 is followed by exon 7, lacking exon 6 [22] (Fig. 1).

Fig. 1. Left panel: Fragments of 343 bp and of 140 bp obtained by amplification of cDNA with the primers flanking exon 6 and electrophoresis. Right panel: Nucleotide sequence of the 140 bp fragment, with the corresponding exons.
bands of 53 kDa and 30 kDa using the amino-terminal antibody and only one band of 53 kDa with the carboxy-terminal antibody.

The expression of RXRα was assessed by using a specific rabbit polyclonal antibody (Santa Cruz Biotechnologies) at a dilution of 1:1000 and peroxidase-conjugated anti-rabbit IgG (Amersham) antibody at a dilution of 1:5000.

Both PPARα isoforms and RXRα protein expression was visualized with the ECL-Plus chemiluminescence system (Amersham) and autoradiograms analyzed using an automatic densitometer (Quantity One, Bio-Rad). The blots were reprobed with a monoclonal β-actin antibody (Sigma) as a control for loading. Data are expressed as arbitrary densitometric units (ADU) relative to β-actin expression.

2.6. Histomorphologic study

We analyzed some myocardial histomorphological alterations involved in the transition from LVH to HF (e.g., apoptosis, inflammation and fibrosis) in 7 patients from the LVH group and 8 from the HF group.

Apoptosis was assayed by the terminal deoxynucleotidyl transferase (TdT) reaction. DNA end-labeling (TUNEL) methodology was performed as previously described [24]. The discrimination between cardiomyocytes and non-cardiomyocytes was performed according to cytological characteristics of the different cell types [25]. Apoptotic indexes were calculated as the ratio between the number of TUNEL positive nuclei and the total number of nuclei for each type of cardiac cell evaluated. The determination of the total number of cardiomyocytes and non-cardiomyocytes was performed on a serial section from the one employed for the TUNEL technique, stained with Masson’s trichrome. Cardiomyocyte and non-cardiomyocyte densities were also analyzed. To assess myocardial inflammation, COX-2 protein expression was analyzed by immunohistochemistry using a specific goat polyclonal antibody (Santa Cruz Biotechnologies) at a dilution of 1:100 and biotinilated anti-goat IgG (Amersham) antibody at a dilution of 1:200. The staining of collagen fibers was performed as previously reported [18]. The fraction of myocardial volume occupied by fibrillar collagen (CVF) and COX-2 immunostained area were determined by quantitative morphometry with an automated image analysis system (AnalySYS, Soft Imaging System GmbH, Hamme). The histomorphologic studies were performed by two pathologists blinded to the other characteristics of the patients under study.

2.7. Statistical analysis

To analyze the differences among the three groups of patients, a 1-way ANOVA followed by Scheffé’s test was performed once normality had been proven (Shapiro–Wilks test); otherwise, the non-parametric Kruskal–Wallis test followed by a Mann–Whitney U test (adjusting the α-level by Bonferroni’s inequality) was used. To assess any tendency in the parameters measured as HHD develops and progresses, the linear test for trend was used. Differences between patients with LVH and patients with HF were tested by a Student’s t test for unpaired data once normality was demonstrated; otherwise, a non-parametric test (Mann–Whitney U test) was used. The correlation between continuously distributed variables was tested by univariate regression analysis. Values are expressed as mean±S.E.M. A value of p<0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of hypertensive patients

The clinical characteristics of the three groups of patients are presented in Table 1. Two patients in the HT group and five patients of the LVH group were receiving antihypertensive medication as monotherapy. No significant differ-
ences in blood pressure were found between treated and untreated patients in HT and LVH groups (data not shown). Most patients in the HF group were treated with the combination of a loop diuretic, a beta-blocker and either an angiotensin converting enzyme inhibitor or an angiotensin II type 1 receptor antagonist. The values of LVMI were higher in LVH and HF groups than in the HT group. The EF was lower in the HF group than in LVH and HT groups. The left ventricle volumes were higher in the HF group than in LVH and HT groups.

3.2. Expression of PPARα isoforms and RXRα

Compared with the HT group (1.16 ± 0.24 arbitrary densitometric units, ADU), the expression of native PPARα protein was decreased (p < 0.05) in the LVH group (0.52 ± 0.10 ADU) and the HF group (0.58 ± 0.06 ADU) (Fig. 2). No difference was found in native PPARα protein expression between LVH and HF groups. The expression of truncated PPARα protein was higher (p < 0.001) in the HF group (2.95 ± 0.32 ADU) than in the LVH group (0.89 ± 0.16 ADU) and the HT group (0.17 ± 0.09 ADU) (Fig. 2). Although the expression of truncated PPARα protein did tend to be higher in the LVH group than in the HT group, the differences did not reach statistical significance. As a consequence, a progressive decrease (p for trend < 0.01) was observed in the native PPARα:truncated PPARα ratio among the three groups of patients (HT: 4.17 ± 1.85 ADU; LVH: 0.78 ± 0.21 ADU; HF: 0.20 ± 0.01 ADU). No significant differences were observed in the expression of truncated PPARα mRNA among the three groups of patients (HT: 0.62 ± 0.09 AU; LVH: 0.43 ± 0.03; HF: 0.62 ± 0.09 AU).

3.3. Expression of PPARα target genes

As an approach to evaluate whether the observed differences in the native PPARα:truncated PPARα ratio were accompanied by changes in PPARα transcriptional activity, mRNA expression of the PPARα target genes CPT-I and LCHAD was assessed. A progressive decrease (p for trend < 0.05) in CPT-I mRNA and LCHAD mRNA was observed among HT, LVH and HF groups (Fig. 3).

3.4. Assessment of myocardial apoptosis, inflammation and fibrosis

Data on the measured histomorphological parameters are presented in Table 2. The HF group showed increased (p < 0.01) cardiomyocyte apoptotic index compared with the LVH group. The non-cardiomyocyte apoptotic index did tend to be higher in the HF group than in the LVH group but the difference was not statistically significant. Whereas
cardiomyocyte density was lower \((p < 0.01)\) in the HF group than in the LVH group, no differences were observed in non-cardiomyocyte density between HF and LVH groups. COX-2 expression was higher \((p < 0.05)\) in the HF group than in the LVH group. CVF was increased \((p < 0.05)\) in the HF group compared with the LVH group.

3.5. Correlational analysis

Truncated PPAR\(\alpha\) protein was inversely correlated with EF \((r = -0.652, p < 0.01)\) (Fig. 4) and directly correlated with LVEDV \((r = 0.538, p < 0.01)\) and LVESV \((r = 0.579, p < 0.01)\) in all patients (Fig. 5). Truncated PPAR\(\alpha\) protein was directly correlated with the cardiomyocyte apoptotic index \((r = 0.572, p < 0.05)\) and inversely correlated with the cardiomyocyte density \((r = -0.695, p < 0.01)\) in patients with HHD (Fig. 6). No significant correlations were found between the native PPAR\(\alpha\) protein and the different parameters tested in this study. CPT-I mRNA expression was directly correlated with EF \((r = 0.640, p < 0.05)\).

4. Discussion

The main findings of this study can be summarized as follows: (1) the human heart expresses both the native active and the truncated inhibitory isoforms of PPAR\(\alpha\) protein, (2) whereas the expression of native PPAR\(\alpha\) protein is diminished in patients with HHD, the truncated PPAR\(\alpha\) protein is overexpressed only in those patients with HHD who develop HF, (3) the expression of the PPAR\(\alpha\) target genes decreases in parallel with the development and progression of HHD, and (4) the truncated PPAR\(\alpha\) protein is associated with the structural, geometric and functional deterioration of the hypertensive left ventricle.

A variant human PPAR\(\alpha\) mRNA species has been identified, which is generated by a post-transcriptional mechanism involving alternative splicing resulting in the

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<td>Myocardial apoptosis, inflammation and fibrosis</td>
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<tr>
<td>Parameter</td>
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<tr>
<td>CM apoptotic index (positive nuclei/10(^6) nuclei)</td>
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<tr>
<td>Non-CM apoptotic index (positive nuclei/10(^6) nuclei)</td>
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<td>CM density (nuclei/mm(^2))</td>
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<td>COX-2 (%)</td>
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LVH, means left ventricular hypertrophy; HF, heart failure; CM, cardiomyocyte; COX-2, cyclo-oxygenase 2; CVF, collagen volume fraction.

Values are expressed as mean ± S.E.M.
\(^a\) \(p < 0.01\), compared with LVH group.
\(^b\) \(p < 0.05\), compared with LVH group.
skipping of exon 6 \cite{11,12}. This splice variant of PPARα mRNA has been identified in a number of human tissues, including the heart \cite{13}. In this study, we detect the PPARα mRNA variant in all of the individuals examined, which suggests that exon skipping is associated with the processing of the human PPARα pre-mRNA, and that it does not reflect a rare allele. Exon skipping has been observed for transcripts of other genes and often leads to circular RNAs formed by the excised exons \cite{26}. However, neither the mechanisms leading to the excision of the exon and the formation of the circular RNA nor the factors that contribute to this process have been clearly defined.

Data presented in the current study demonstrate that the regulation of the two cardiac isoforms of PPARα protein is altered in a different way in patients with HHD. In fact, whereas repression of the native PPARα isoform is associated with the development of LVH, overexpression of the truncated PPARα isoform seems to be linked to the transition from LVH to HF. Therefore, although a diminished expression of native PPARα has been reported previously in the hypertrophied left ventricle of animals submitted to cardiac pressure overload \cite{27,28} and patients with HF \cite{29}, this is the first demonstration of alterations of truncated PPARα in the hypertensive heart. In addition, the origin of these alterations seems to be post-transcriptional since native and truncated mRNA PPARα expression was not modified along the three groups of hypertensives tested. However, further studies are necessary to investigate potential post-transcriptional mechanisms (e.g., changes in mRNA stability) influencing the two isoforms of PPARα in the hypertensive myocardium.

Nevertheless, overexpression of the truncated PPARα may also be a consequence of the loss of cardiac function. In fact, generation by alternative splicing of isoforms with repressive activity on the native isoform has been reported for other nuclear receptors (i.e., the glucocorticoid receptor and the thyroid hormone receptor) \cite{30,31} as a general mechanism to diversify and to adapt receptor action on pathophysiological conditions. Clearly, further studies are necessary to elucidate the significance and relevance of truncated PPARα protein in the hypertensive heart.

As suggested by in vitro studies \cite{14}, the association of diminished active PPARα isoform with enhanced inhibitory PPARα isoform may compromise PPARα-dependent...
transcriptional activity in the failing heart. In fact, we observed that a progressive decrease in native PPARα: truncated PPARα ratio was associated with a progressive decrease in the expression of the PPARα target genes CPT-I and LCHAD involved in fatty acid transport and oxidation. A continuous decrease in fatty acid oxidation has been found in hypertensives with LVH with and without HF compared with control subjects [3]. Since ATP availability and function of the heart are closely linked, it is reasonable to assume that deactivation of PPARα leading to diminished fatty acid oxidation and reduced production of ATP might be involved in the compromise of cardiac function in HF. In support of this possibility, we observed a direct association between CPT-I expression and ejection fraction. Thus it is tempting to speculate that progressive impairment of PPARα transactivation activity accompanies the development of HHD and may contribute to its progression to HF.

Alternatively, PPARα alterations could be involved in the transition from LVH to HF through the facilitation of some structural features characteristic of myocardial remodelling. In fact, a number of experimental evidences suggest that depressed myocardial PPARα expression and/or activity could be linked with the development of inflammation and fibrosis [7–10]. In addition, it has been shown that PPARα-null mice develop massive cardiomyocyte accumulation of fatty acid intermediates [5]. Some of these compounds, e.g., ceramide, can induce reactive oxygen species accumulation, inducible nitric oxide synthase expression and cardiomyocyte apoptosis [32]. Our findings showing associations of depressed PPARα activity with increased inflammation, fibrosis and cardiomyocytes apoptosis in the failing hypertensive heart could provide support to this view. Furthermore, the associations here reported of truncated PPARα with cardiomyocyte apoptosis and density on the one hand, and left ventricular dimensions and systolic performance on the other hand, raise the possibility that this molecule might contribute directly to the development of HF in hypertensive patients. In fact, it has been proposed that increased cardiomyocyte apoptosis present in the failing hypertensive heart could contribute to the impairment of systolic function due to the loss of contractile mass, and to the dilatation of the left ventricular chamber through side-to-side slippage of cardiomyocytes [33,34].

4.1. Limitations of the study

This was a study involving a small number of patients. Furthermore, no studies in normotensive subjects were performed. However, because of the nature of the goals under investigation this design is appropriate. In addition, it must be recognized that therapy with different types of drugs in the three groups of patients may have confounded the findings and their interpretation. For instance, angiotensin converting enzyme inhibitors have been reported to interfere with PPARα expression in the vascular wall [35] although no data are available yet on their effects on cardiac PPARα. Nevertheless, one must consider that, since such therapies are standard for hypertension and HF, it would have been unreasonable to withdraw them for the purposes of this research. Moreover, it is important to note that no patient was under treatment with drugs that modify PPARα expression and/or activity (i.e., thiazolidinediones, fibrates and statins). We performed biopsies of the right side of the interventricular septum to assess the molecular and cellular effects of left ventricular pressure loading. However, as shown by others [36], lesions present in the septum in postmortem tissue from hypertensive human hearts are representative of lesions existing in the free wall. Finally, we are aware that the real time RT–PCR method here used is not sensitive enough to detect small mRNA differences. However, the small amount of mRNA available in the samples forced us to select this method for mRNA expression measurement.

In summary, we report for the first time that HHD is associated with an excess of the truncated inhibitory PPARα isoform related to the native active isoform, which is accompanied by a diminished expression of a PPARα target enzymes involved in fatty acid utilization. Interestingly, these alterations evolve in parallel with both the development myocardial structural remodelling and the progression from compensated LVH to HF. In addition, results here presented suggest that the truncated PPARα isoform may be directly involved in the pathophysiology of the hypertensive left ventricle. Albeit preliminary and descriptive in nature, the data here presented set the stage for further studies intended to test the above hypothesis.

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