Changes by tacrolimus of the rat aortic proteome: Involvement of endothelin-1

José J. Zamorano-León, Antonio J. López-Farré, Maria Marques, Pablo Rodríguez, Javier Modrego, Antonio Segura, Carlos Macaya, Alberto Barrientos

Cardiovascular Research Unit of the Cardiology Department, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain

Nephrology Department, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain

Health Science Institute Talavera de la Reina, Toledo, Spain

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Abstract

The aim was to analyze the effect of tacrolimus on the aortic expression of proteins associated with the energetic metabolism and cytoskeleton and if it could be reverted by ET-1-receptor antagonist bosentan. Wistar Kyoto rats were divided into: control (n = 10), tacrolimus (n = 10, 0.5 mg/kg bw/day tacrolimus for 30 days) and tacrolimus + bosentan (n = 10, 0.5 mg/kg bw/day tacrolimus and 100 mg/kg bw/day bosentan for 30 days). Rat aortic segments were homogenized and submitted to 2-dimensional electrophoresis and mass spectrometry. Tacrolimus treatment did not modify neither systolic nor diastolic arterial pressure but increased ET-1 content, ET-A and ET-B-type receptor expression in aorta. Proteomic study revealed that tacrolimus treatment modified the expression of aortic proteins associated with the cytoskeleton as some isotypes of lamin A and β-tropomyosin; and energetic metabolism such as ATP synthase gamma chain, NADH dehydrogenase ubiquinone, acyl CoA dehydrogenase long
chain mitochondrial and phosphatidylinositol 3-kinase regulatory subunit gamma. Aortic expression of gp91-phox and MnSOD was also increased by tacrolimus. Bosentan co-administration with tacrolimus prevented also changes in ET-1 content and the expression of proteins associated with energetic metabolism. Bosentan did not affect the increased expression of gp91-phox related to tacrolimus although significantly enhanced aortic MnSOD expression. As conclusion, tacrolimus treatment increased ET-1 content in aortic wall and modified the expression of proteins associated with the cytoskeleton and energetic metabolism independently of changes on blood pressure. Bosentan reverted some effects induced by tacrolimus in the aorta and increased the antioxidant defense system.

Graphical abstract

Highlights

► Tacrolimus treatment did not modify blood arterial pressure. ► Tacrolimus treatment increased ET-1 content and aortic ET\textsubscript{A}-type receptor expression. ► Tacrolimus treatment altered the protein expression profile in aorta. ► Bosentan prevented changes in ET-1 content and energetic metabolism proteins.

Keywords
Aortic proteome; Endothelin-1; Rats; Tacrolimus

1. Introduction
Tacrolimus (FK506) is a potent immunosuppressive agent mainly used after allogenic organ transplant [1]. Incidence of negative effects in patients treated with tacrolimus seems to be lower than with other calcineurin inhibitors [2] and [3]. However, there is a great controversy about tacrolimus effects on vascular functionality. In this regard, different studies have reported that tacrolimus treatment preserved vasomotor function, decreased sensitivity to vasospasm and maintained normal vascular homeostasis [4] and [5]. Contrarily, tacrolimus treatment has been also associated with wide
spectrum of adverse effects, including vascular events [6]. In this regard, a number of “in vitro” and “in vivo” studies have reported that tacrolimus is strongly linked to increased systemic blood pressure and a decreased vascular functionality [7] and [8]. However, the molecular mechanisms by which tacrolimus may produce alterations in the vascular functionality remains poorly understood [7] and [9].

Endothelin-1 (ET-1) is a vasoconstrictor peptide involved in cyclosporine A-induced hypertension [10]. ET-1 has been also associated with the increased vascular resistance observed after tacrolimus administration. Moreover, in vitro studies have demonstrated that tacrolimus causes ET-1 release in cultured rat mesangial cells [11], increases pre-pro ET-1 mRNA expression in human umbilical vein endothelial cells [12] and increases urinary and plasma ET-1 levels in humans [13]. Bosentan, a dual ETA- and ETB-types receptor antagonist, used in humans to treat pulmonary arterial hypertension [14], attenuated renal vascular resistance and reversed noradrenaline-induced vasoconstriction by tacrolimus in rat kidneys [15] and [16]. Nevertheless, the molecular mechanisms by which ET-1 may be involved in the vascular effects of tacrolimus remain to be elucidated. In this regard, an important non-answered question is if vascular effects attributed to tacrolimus are related to tacrolimus by itself or it is secondary to the increased mean blood pressure induced by tacrolimus.

There are several molecular processes crucial for the vascular functionality. Between them, vascular energetic metabolism and vascular cytoskeleton have relevant importance for vascular functionality. Different works have postulated that cytoskeleton and mitochondrial ATP production in aortic smooth muscle and endothelium play an important role for vascular functionality and in the generation of vascular substances [17], [18] and [19]. In vitro studies have also suggested that FK506 binding protein (FKBP52) may be involved in the regulation of cytoskeleton functionality [20] and [21]. However, in our knowledge, it has not been yet explored, in an integrated form, whether tacrolimus by itself may influence the vascular expression of proteins associated with either energetic metabolism or cytoskeleton in the aortic vascular wall. Until now it has been difficult to monitor changes in the expression of several proteins at the same time in a single sample. However, new technologies such as proteomics may facilitate to determine changes in the level of expression of multiple proteins and protein isotypes in a sample. In this regard, by using two-dimensional electrophoresis (2-DE) in combination with mass spectrometry several proteins expressed in a single sample may be identified.

2. Objective
The aim of the present study was to determine by proteomics whether a non-modifier blood pressure treatment with tacrolimus may change the level of expression of proteins associated with the cytoskeleton and with the energetic metabolism in the rat aorta. Moreover, the effect of bosentan coadministration with tacrolimus on the aortic expression of the above mentioned proteins was also tested.

These aims are based on the hypothesis that vascular energetic metabolism and vascular cytoskeleton have relevant importance for vascular functionality. Accordingly, it has been previously reported possible effects of tacrolimus on vascular function. Therefore, as hypothesis tacrolimus by itself, independently of changes in blood pressure, may modify the level of expression of proteins involved in either cytoskeleton or energetic metabolism in the vascular wall.
3. Materials and methods

3.1. Experimental groups design
Experiments were completed in 30 male Wistar Kyoto rats with age range between 12 and 16 weeks. Rats were maintained in temperature controlled room under 12-hour dark/light cycles and free access to food (standard laboratory chow) and water. Before the experiments, the animals were maintained overnight without food and with water ad libitum. Rats were divided into three groups: a) control (n = 10); b) tacrolimus (Fujisawa Pharmaceutical Co., Ltd., Osaka) (0.5 mg/kg bw/day for 30 days, n = 10) which was daily intramuscularly injected, and c) tacrolimus-administered rats which they were co-treated with bosentan (Hoffman-La Roche, Basel, Switzerland) (100 mg/kg bw/day in the food, n = 10). Tacrolimus was dissolved in isotonic saline and was injected in a different leg each day; moreover the injection site on leg was also changed to avoid muscle damages. Injection volumes were limited to 20–25 μl. The control group only received isotonic saline following the same schedule of tacrolimus-treated groups. The dose of tacrolimus used was carefully chosen based in a previous study that demonstrated that 0.5 mg/kg bw/day failed to modify blood pressure in Wistar–Kyoto rats [22]. The day 25th from the beginning of tacrolimus administration, rats were placed in individual metabolic cages. The day 28th urine was collected over 24 h and a blood sample (0.5 ml) was also taken from the tail to measure urine creatinine and plasma creatinine. Plasma and urine creatinine were measured by picric acid colorimetric assay (Raichem, San Diego, CA) using a Cobas–Mira machine (Roche). Creatinine clearance was calculated according to formula of DuBois and DuBois: CrCl = UCr * UVol / PCr * Tmin [ml/min].

Thirty days after tacrolimus administration, rats were anesthetized with ketamine/xylazine mixture (90 and 5 mg/kg i.p.) and, as reported [23], femoral artery catheterized for mean arterial pressure measurement using a blood pressure transducer (s/5 Anesthesia Monitor, Datex Ohmeda, Finland). Rats were then exsanguinated through the femoral catheter. Thoracic aorta was carefully removed under sterile conditions, washed in isotonic saline and carefully fragmented in similar pieces. These aortic fragments were quickly frozen in liquid nitrogen for proteomic and Western blot analyses and for measurement of pyruvate content. The experimental procedure was approved by the Institutional Animal Care and Use Committee.

3.2. Two dimensional electrophoresis (2-DE)
One aortic fragment from each rat (n = 30) was separately homogenized using an Ultra-Turrax T8 IKA-Werke in a buffer containing 8 mol/L urea, 2% CHAPS w/v, 40 mmol/L dithiothreitol, 0.2% Bio-Lyte™ ampholyte (Bio-Rad) and 0.01% w/v bromophenol blue. Protein concentration was estimated by bicinchoninic acid reagent (Pierce), using bovine serum albumin as standard [24]. Two hundred fifty micrograms of total protein contained in aorta were loaded on immobilized gradient IPG strips (pH 3–10) and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad Laboratories). The gels were actively rehydrated at 50 V for 60 h, then rapid and linear voltage ramping steps, limited by a maximum current of 50 μA per gel [25], [26] and [27]. In the second dimension, the proteins from the strips were resolved on 10% SDS-PAGE gels using a Protean II XL system (Bio-Rad). Afterwards, the gels were fixed and silver stained using a Silver Stain Plus Kit (Bio-Rad).
3.3. Image acquisition and analysis
The silver-stained gels were scanned in a UMAX POWERLOOK III Scanner operated by the software Magic Scan V 4.5. Intensity calibration was carried out using an intensity stepwedge prior to gel image capture to avoid signal saturation. Image analysis was carried out using Quantity One 4.2.3 (Bio-Rad). Spots areas used for densitometry were automatically detected and matched by image analysis software, and then only some spots areas were manually edited in order to avoid comparison mistakes among gels belongs to different experimental groups. Intensity calibration was carried out using an intensity stepwedge prior to gel image capture. Each spot intensity volume was automatically processed by background subtraction.

3.4. Mass spectrometry
After image analysis, spots whose intensities were significantly different among the experimental groups were identified by mass spectrometry. Spots from two-three different gels were manually excised from the gels using biopsy punches. The spots were washed twice with water, shrunk with 100% acetonitrile and dried in a Savant SpeedVac. The samples were then reduced with 10 mmol/L dithiothreitol in 25 mmol/L ammonium bicarbonate and subsequently alkylated with 55 mmol/L iodoacetamide in 25 mmol/L ammonium bicarbonate. They were digested with 12.5 ng/μl sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mmol/L ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatant was collected and, as reported [25], [26] and [27], 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of a 3 mg/ml of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature. All mass spectra were calibrated externally using a standard peptide mixture (Sigma). Peptides from the auto digestion of trypsin were used for the internal calibration. The analysis by MALDI-TOF mass spectrometry produced peptide mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. MS/MS sequencing analyses were carried out using the MALDI-tandem time-of-flight mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). Peptides with a signal to noise greater than 20 were considered. For protein identification Mascot database 1.9 (http://www.matrixscience.com) was used as algorithm to match the peptides obtained by mass spectrometry.

3.5. Western blot analysis
The protein expression of triosephosphate isomerase, ET-1, ETA-, ETB-type receptor, gp91-phox and Mn superoxide dismutase (MnSOD) were analyzed by Western blot. Western blot experiments for triosephosphate isomerase, ETB-type receptor, gp91-phox and MnSOD were performed in a subgroup of rats from each of the three experimental groups. Aortic fragments were separately homogenized, as above mentioned for 2-DE. Homogenized aortic segments were then solubilized in Laemmli buffer containing 2-mercaptoethanol. The obtained proteins were separated on denaturing SDS/15% (w/v) polyacrylamide gels. Equal amount of proteins (40 μg/lane), estimated by bicinchoninic acid reagent (Pierce) [24], was loaded. In order to assess that the same amount of proteins was loaded into the gel, a parallel gel with identical samples was run and stained with Coomasie Blue. Proteins were then blotted onto nitrocellulose
(Immobilion-P; Millipore) and the blot was blocked overnight at 4 °C with 5% (w/v) non fat dry milk in TBS-T mmol/L Tris/HCl (pH 5.2), 137 mmol/L NaCl and 0.1% Tween 20, as reported [28]. Nitrocellulose membrane was then incubated with a monoclonal antibodies against triosephosphate isomerase (1:500; sc-30145, Santa Cruz Biotechnology), ET-1 (1:1500; Alexis Biochemicals), ET-1-type A receptor (1:1500; ALX-210-507, Alexis Biochemicals), ET-1-type B receptor (1:500; Sc-21196, Santa Cruz Biotechnology), gp91-phox (1:500; Sc5827, Santa Cruz Biotechnology), and MnSOD (1:1000; Sc-30080, Santa Cruz Biotechnology). After that, nitrocellulose membranes were incubated with HRP (horseradish peroxidase)-conjugated anti-mouse IgG antibodies at a dilution of 1:2000 for the ETA-type receptor and triosephosphate isomerase detection, HRP-conjugated-anti-goat IgG antibody (1:2000) for the ETB-type-receptor and gp91-phox, HRP-conjugated-anti-rabbit IgG antibody (1:2000) for the MnSOD and with HRP-conjugated-anti-sheep IgG antibody (1:2500) for ET-1 detection. As reported, the proteins were detected by enhanced chemiluminescence's (ECL®, Amersham Biosciences) and evaluated by densitometry (Quantity One; Bio-Rad Laboratories). Pre-stained protein markers (Sigma) were used for molecular mass determinations.

3.6. Determination of the pyruvate content in rat aorta

Pyruvate content in the aorta from the same rats and experimental groups of the Western blot experiments was quantified using a colorimetric pyruvate assay kit (K609-100, BioVision Research products, USA). To quantify pyruvate content, 80 μg of each homogenized aorta sample was used and the pyruvate assay was performed following manufacturer's instructions.

3.7. Statistical analysis

Results are expressed as mean ± SEM. Kruskal–Wallis test was used for determining statistical differences in the means of the protein expression among the three experimental groups. A significance level of 0.05 was set for a type I error in all analyses. When Kruskal–Wallis test revealed a p < 0.05, Mann–Whitney's test was also used to identify differences between the specific experimental groups and p < 0.05 was also considered as significative. Kruskal–Wallis and Mann–Whitney's tests were used in the SPSS 12.0 program (Statoft Inc., Tulsa, Oklahoma, USA).

4. Results

4.1. Blood pressure and renal parameters

As Table 1 shows, 30 days after tacrolimus treatment there were no statistical differences in the body weight among the three groups of rats although tacrolimus treatment apparently tended to reduce body weight. Moreover, although both systolic and diastolic blood pressures tended to slightly increase 30 days after tacrolimus treatment, it did not reach statistical significance compared with those in control rats (Table 1). Bosentan coadministered with tacrolimus did not modify either systolic or diastolic blood pressures compared with both control and tacrolimus alone (Table 1).

Table 1. Blood arterial pressure and renal parameters in control, tacrolimus-treated and tacrolimus + Bosentan-treated Wistar rats.
|                          | Control (n = 10) | Tacrolimus (n = 10) | Tacrolimus + bosentan (n = 10) | p Value  
(Kruskal–Wallis) |
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>335 ± 14</td>
<td>263 ± 42</td>
<td>293 ± 33</td>
<td>0.202</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>117.29 ± 4.09</td>
<td>121.50 ± 2.03</td>
<td>118.95 ± 2.13</td>
<td>0.444</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>109.67 ± 2.38</td>
<td>114.79 ± 1.15</td>
<td>114.60 ± 0.98</td>
<td>0.147</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.48 ± 0.02</td>
<td>0.60 ± 0.03</td>
<td>□ 0.57 ± 0.03□</td>
<td>0.026</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>1.55 ± 0.11</td>
<td>1.25 ± 0.05 □</td>
<td>1.06 ± 0.07□</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Data are represented in densitometric arbitrary units as mean ± SEM; Kruskal–Wallis test was used to compare for comparison among the three groups. When Kruskal–Wallis test revealed a p < 0.05, Mann–Whitney's test was also used to identify differences between each specific experimental group with the others. p < 0.05 with respect to control rats.

Full-size table

Thirty days after tacrolimus treatment, tacrolimus plasma levels were 9.8 ± 0.6 ng/ml. There were no differences in tacrolimus plasma levels by bosentan coadministered with tacrolimus (9.7 ± 0.4 ng/ml).

After tacrolimus and tacrolimus + bosentan treatment, a slightly although statistically significant increase of plasma creatinine levels was found with respect to control (Table 1). Creatinine clearance was also slightly reduced after tacrolimus treatment, that it was not modified by bosentan (Table 1).

### 4.2. ET-1, ETA- and ETB type receptor expression

Aortic protein content of ET-1 was significantly enhanced in aortic segments from tacrolimus-treated compared with those obtained from control rats (Fig. 1). Bosentan prevented the increased content of ET-1 induced by tacrolimus (Fig. 1). Protein expression of both ETA- and ETB-type receptors in the aortic wall from tacrolimus treated rats were also increased with respect to control group (Fig. 1). Bosentan coadministration with tacrolimus tended to reduce aortic ETA-type receptor expression, although it did not reach statistical significance with respect to tacrolimus and to control (Fig. 1). Bosentan coadministration with tacrolimus did not modify ETB-type receptor expression in the aortic segments that remained increased with respect to control (Fig. 1).
Fig. 1. Representative Western blot showing the effect of tacrolimus (0.5 mg/kg bodyweight/day for 30 days) and tacrolimus + bosentan (0.5 mg/kg bodyweight/day tacrolimus + 100 mg/kg bw/day bosentan for 30 days) on the aortic content of endothelin-1 (ET-1) and ETA-, ETB-type receptors. At the bottom is shown the densitometric values of all Western blots. The number of samples used in each group was as follows: control group (n = 10), tacrolimus group (n = 10), and tacrolimus + bosentan group (n = 10). Results are represented as mean ± SEM. *p < 0.05 with respect to control. #p < 0.05 with respect to tacrolimus alone.

4.3. Proteomic study
The present study was focussed in possible modifications by tacrolimus on the expression of vascular proteins associated with cytoskeleton and with energetic metabolism. Therefore, only proteins associated with such processes were analyzed in the aortic proteomes (Fig. 2). The spots observed in the aortic proteomic maps were densitometrically analyzed and identified by comparison with those found in human saphenous vein published by McGregor et al. [29] and in bovine aorta published by Modrego et al. [24]. The entire of the here identified spots were at least expressed in 60% of the 2-DE gels within each experimental condition. The identity of only spots that reached statistical differences between the experimental groups was further confirmed by mass spectrometry.
Fig. 2. Representative bidimensional gel electrophoresis (2DE) of rat aortic proteins developed using a pH range between 3 and 10. The numbers represent the isotype within each specific protein. View thumbnail images

4.4. Cytoskeleton-related proteins
Alpha and β-actins were identified in the rat aortic proteome (Fig. 2). Only the expression level of β-actin was reduced in tacrolimus-treated rats, although Kruskal–Wallis test did not reach statistical significance between the three experimental groups ($p = 0.086$). This tendency to decrease β-actin expression by tacrolimus was partially prevented by bosentan (Table 2).

Table 2. Aortic expression of proteins associated with cytoskeleton.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control (N = 10)</th>
<th>Tacrolimus (N = 10)</th>
<th>Tacrolimus + bosentan (N = 10)</th>
<th>(Kruskal–Wallis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular cytoskeleton</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>α-Actin</td>
<td>3165.99 ± 806.89</td>
<td>3419.72 ± 739.73</td>
<td>4603.49 ± 664.24</td>
<td>0.282</td>
</tr>
<tr>
<td>β-Actin</td>
<td>249.40 ± 48.83</td>
<td>97.50 ± 15.55</td>
<td>168.48 ± 39.73</td>
<td>0.086</td>
</tr>
<tr>
<td>β-Tropomyosin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Control (N = 10)</td>
<td>Tacrolimus (N = 10)</td>
<td>Tacrolimus + bosentan (N = 10)</td>
<td>p Value (Kruskal–Wallis)</td>
</tr>
<tr>
<td>--------------</td>
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<td>-------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Isotype 1</td>
<td>39.31 ± 15.53</td>
<td>97.62 ± 33.20</td>
<td>98.33 ± 24.88 □</td>
<td>0.049</td>
</tr>
<tr>
<td>Isotype 2</td>
<td>92.80 ± 25.07</td>
<td>31.80 ± 9.58</td>
<td>47.93 ± 7.35</td>
<td>0.093</td>
</tr>
<tr>
<td>Isotype 3</td>
<td>302.98 ± 65.34</td>
<td>298.96 ± 59.86</td>
<td>310.07 ± 72.24 □</td>
<td>0.976</td>
</tr>
<tr>
<td>Vimentin</td>
<td>59.39 ± 13.03</td>
<td>45.06 ± 10.72</td>
<td>65.91 ± 16.52</td>
<td>0.498</td>
</tr>
<tr>
<td>Lamin A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotype 1</td>
<td>260.16 ± 56.54</td>
<td>85.57 ± 27.34 □</td>
<td>164.60 ± 35.11 □</td>
<td>0.036</td>
</tr>
<tr>
<td>Isotype 2</td>
<td>203.26 ± 42.73</td>
<td>49.62 ± 11.76 □</td>
<td>67.91 ± 14.31 □</td>
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<td>Isotype 3</td>
<td>39.84 ± 11.97</td>
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<td>21.90 ± 9.89</td>
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<tr>
<td>Annexin-A2</td>
<td>185.43 ± 21.16</td>
<td>134.02 ± 29.91</td>
<td>186.83 ± 39.16</td>
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<tr>
<td>Annexin-A5</td>
<td>244.15 ± 102.66</td>
<td>211.44 ± 108.97</td>
<td>220.96 ± 54.60</td>
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Data are represented in densitometric arbitrary units as mean ± SEM; Kruskal–Wallis test was used to compare for the three experimental groups. When Kruskal–Wallis test revealed a $p < 0.05$, Mann–Whitney's test was also used to identify differences between each specific experimental group with the others.$p < 0.05$ with respect to control rats.$#p < 0.05$ with respect to tacrolimus alone.

Full-size table

Three different tropomyosin isotypes were identified in the rat aortic proteome (Fig. 2 and Fig. 3A). The expression level of tropomyosin isotype 1 was significantly increased in the aortic segments from tacrolimus-treated rats with respect to control (Table 2). This increased expression of tropomyosin isotype 1 was not prevented by bosentan co-administered with tacrolimus (Table 2). Tacrolimus treatment tended to reduce the level of expression of tropomyosin isotype 2, although it did not reach statistical significance (Kruskal–Wallis $p = 0.093$) (Table 2).
Fig. 3. (A) Representative MS/MS spectrum of beta-tropomyosin isotype 2 and lamin A isotype 1, (B) ATP synthase gamma chain and NADH dehydrogenase ubiquinone isotype 1, and (C) Acyl CoA dehydrogenase mitochondrial precursor isotype 2 and phosphatidylinositol 3-kinase regulatory subunit gamma. On the right is shown the matched peptides and the sequence coverage.

Vimentin was also identified in the rat aortic proteome although its expression was not modified by neither tacrolimus nor tacrolimus + bosentan treatment (Table 2). Three different lamin A isotypes were identified (Fig. 2 and Fig. 3A). The protein expression of lamin A isotypes 1 and 2 was significantly decreased in the aortic segments from tacrolimus-treated rats compared with those from control (Table 2). Bosentan prevented the decreased expression of lamin A isotype 1 induced by tacrolimus, but bosentan has no effect on the decreased expression of lamin isotype 2 observed after tacrolimus treatment (Table 2). Annexins A2 and A5 were also identified in the rat aortic proteome and they were not different among the three groups of rats (Table 2).

### 4.5. Energetic metabolism-related proteins

The following proteins associated with energetic metabolism were identified in the aorta: ATP synthase gamma chain, four isotypes of NADH dehydrogenase ubiquinone, three isotypes of acyl CoA dehydrogenase long chain mitochondrial precursor, phosphoglycerate mutase and phosphatidylinositol 3-kinase regulatory subunit gamma (Fig. 2 and Fig. 3B and C).

Protein expression of ATP synthase gamma chain, enzyme involved in mitochondrial oxidative phosphorylation, was significantly reduced in aortic segments from tacrolimus-treated rats compared with control which it was prevented by bosentan (Table 3). Four NADH dehydrogenase ubiquinone isotypes enzymes involved in mitochondrial respiratory chain, were identified. The protein expression of NADH dehydrogenase ubiquinone isotype 2 was found reduced in aortic segments from tacrolimus-treated rats with respect to that in control and it was not prevented by bosentan co-administered with tacrolimus (Table 3). In tacrolimus-treated rats NADH dehydrogenase ubiquinone isotype 4 also tended to be reduced although it did not reach statistical significance (Table 3).

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<th>Tacrolimus + bosentan (N = 10)</th>
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<tr>
<td>ATP synthase gamma chain</td>
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<td>NADH dehydrogenase ubiquinone</td>
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<td>Isotype 1</td>
<td>210.05 ± 39.2</td>
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<tr>
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<td>Tacrolimus + bosentan (N = 10)</td>
<td>p Value</td>
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</tr>
<tr>
<td>Isotype 2</td>
<td></td>
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<tr>
<td></td>
<td>7</td>
<td>7</td>
<td></td>
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<tr>
<td></td>
<td>309.40 ± 29.7</td>
<td>188.72 ± 30.71</td>
<td>181.90 ± 37.26 □</td>
<td>0.031</td>
</tr>
<tr>
<td>Isotype 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>175.51 ± 38.68</td>
<td>200.10 ± 12.60</td>
<td>288.01 ± 55.21</td>
<td>0.222</td>
</tr>
<tr>
<td>Isotype 4</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>82.64 ± 17.26</td>
<td>50.89 ± 11.05</td>
<td>93.64 ± 16.28</td>
<td>0.140</td>
</tr>
<tr>
<td>Acyl CoA dehydrogenase long chain mitochondrial precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isotype 1</td>
<td>121.69 ± 50.99</td>
<td>43.33 ± 14.92</td>
<td>99.61 ± 24.94</td>
<td>0.082</td>
</tr>
<tr>
<td>Isotype 2</td>
<td>195.82 ± 57.6</td>
<td>47.38 ± 18.25</td>
<td>77.67 ± 19.36</td>
<td>0.012</td>
</tr>
<tr>
<td>Isotype 3</td>
<td>47.50 ± 34.70</td>
<td>Undetectable</td>
<td>58.89 ± 27.83 #</td>
<td>0.049</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>69.58 ± 21.12</td>
<td>103.46 ± 70.00</td>
<td>94.12 ± 40.14</td>
<td>0.484</td>
</tr>
<tr>
<td>Phosphatidylinositol 3-kinase regulatory subunit gamma</td>
<td>46.22 ± 13.56</td>
<td>9.53 ± 3.82</td>
<td>19.69 ± 8.52</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Data are represented in densitometric arbitrary units as mean ± SEM; Kruskal–Wallis test was used to compare the three experimental groups. When Kruskal–Wallis test revealed a p < 0.05 value, Mann–Whitney's test was also used to identify differences between each specific experimental group with the others. p < 0.05 with respect to control rats. #p < 0.05 with respect to tacrolimus alone.

Full-size table

Kruskal–Wallis analysis showed that acyl CoA dehydrogenase mitochondrial precursor isotypes 2 and 3 reached statistical differences between the three experimental groups (Table 3). However, the protein expression of the three identified acyl CoA dehydrogenase mitochondrial precursor isotypes tended to be reduced in the aortic segments from tacrolimus-treated with respect to those from control (Table 3). In the tacrolimus group, protein expression level of acyl CoA dehydrogenase mitochondrial precursor isotype 3 was under the detection limit (1 ng) of silver staining (Table 3). The reduction in the protein expression of acyl CoA dehydrogenase mitochondrial precursor isotype 3 induced by tacrolimus was prevented by bosentan (Table 3).

The protein expression of phosphoglycerate mutase, enzyme that catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate in glycolysis, was not different among the three experimental groups (Table 3). However, phosphatidylinositol 3-kinase regulatory subunit gamma, enzyme involved in the PI3K/Akt signaling pathway that induces glucose uptake and stimulates glycolytic metabolism [30], [31] and [32], was significantly decreased in rats treated with tacrolimus compared to control. Bosentan
coadministration with tacrolimus did not prevent the effect of tacrolimus on phosphatidylinositol 3-kinase regulatory subunit gamma expression (Table 3). Pyruvate content in the aortic segments, the end product of the glycolytic pathway, was also analyzed. In tacrolimus-treated rats, aortic pyruvate content was enhanced with respect to that observed in control and bosentan + tacrolimus-treated rats (Fig. 4, panel A). Moreover, in aortic segments from bosentan + tacrolimus-treated rats, triosephosphate isomerase, a key-step glycolytic protein, was found enhanced with respect to both control and tacrolimus-treated rats (Fig. 4, panel B).

Fig. 4. Panel A shows aortic pyruvate content (mmol/L) in control (n = 6), tacrolimus-treated (n = 6) and bosentan + tacrolimus-treated (n = 5) rats. Panel B shows a representative Western blot of the effect of tacrolimus and bosentan + tacrolimus on the aorta expression of triosephosphate isomerase (TPI). At the bottom is shown a bar graph with the densitometric analysis of all the Western blots, in arbitrary units. The number of samples used in each group was as follows: control group (n = 6), tacrolimus group (n = 6) and tacrolimus + bosentan group (n = 6). Results are mean ± SEM. *p < 0.05 with respect to control. #p < 0.05 with respect to tacrolimus alone.

The protein expression of gp91-phox and MnSOD was also tested in the aortic segments. Gp91-phox and MnSOD aortic expression was significantly higher in tacrolimus-treated rats compared with those from control rats (Fig. 5). Bosentan coadministration with tacrolimus did not modify the increased aortic expression of gp91-phox observed with tacrolimus alone (Fig. 5). However, MnSOD expression found in the tacrolimus + bosentan group was significantly enhanced compared with those observed in the vascular wall from tacrolimus and control rats (Fig. 5).
Fig. 5. Representative Western blot showing the effect of tacrolimus (0.5 mg/kg bodyweight/day for 30 days) and tacrolimus + bosentan (0.5 mg/kg bodyweight/day tacrolimus + 100 mg/kg bw/day bosentan for 30 days) on the aortic content of gp91-phox and MnSOD. At bottom is shown gp91-phox and MnSOD densitometric values of all Western blots within each experimental group. The number of samples used in each group was as follows: control group (n = 6), tacrolimus group (n = 6), and tacrolimus + bosentan group (n = 6). Results are represented as mean ± SEM. *p < 0.05 with respect to control. #p < 0.05 with respect to tacrolimus alone.

5. Discussion
The present work shows that tacrolimus treatment, in a dosage that did not change blood pressure, modified the expression of proteins associated with vascular cytoskeleton and vascular energetic metabolism further to increase the content of ET-1 as well as the level of expression of ETA- and ETB-type receptors in the aortic wall. Bosentan, a dual ETA and ETB receptor antagonist, co-administered with tacrolimus prevented the changes in the protein expression of some of the proteins that were modified in the aortic wall after tacrolimus treatment. It was accompanied of reduction of the aortic content of ET-1 but did not modify the increased expression of ETA- and ETB-type receptors observed with tacrolimus alone. Moreover, tacrolimus increased the protein expression of gp91-phox while the coadministration with bosentan promoted the expression of MnSOD suggesting that bosentan may favor an antioxidative state in the vascular wall.

5.1. Effects of tacrolimus on cytoskeleton-related proteins in the aortic vascular wall
The first observation of the present work was that tacrolimus reduced in the aorta the expression of cytoskeleton-related proteins such as one β-tropomyosin isotype and two lamin A isotypes. In addition, β-actin expression tended to be also decreased by tacrolimus although, it did not reach statistical significance. Although alterations of cytoskeleton architecture have been associated with vascular dysfunctionality in our knowledge, no previous reports have demonstrated direct effect of tacrolimus on
vascular cytoskeleton-related proteins. In this regard, it is well known that drugs disturbing cytoskeleton, and particularly intermediate filaments such as lamin A, may preferentially affect vascular dilatation [33] and [34]. Accordingly, reduction of endothelial-dependent vasodilatation has been demonstrated after tacrolimus treatment [35] and [9], which it may be associated with the here reported findings about that tacrolimus decreased the expression of cytoskeleton-related proteins in the vascular wall. Reduction in the expression of cytoskeleton proteins in the vascular wall may also reduce its vascular contractile capability [36]. Therefore, and taken together, reduction in both vascular relaxing ability and contractility may promote vascular stiffness which it has been previously reported after calcineurin immunosuppression in kidney transplant patients [37].

One finding of the present work was that ET-1 content in the aortic segments was significantly increased after tacrolimus treatment. It was accompanied of upexpression of both ETA and ETB type receptors in the aortic vascular wall by tacrolimus. In this regard, Takeda et al., also reported that tacrolimus exerted its negative effects on the vascular system through activation of ETA-type receptors [22].

In the present work, the observed changes induced by tacrolimus treatment on either ET-1 content and the upexpression of ETA- and ETB-type receptors in the aortic wall could be attributed to tacrolimus by itself better than to hemodynamic changes since neither systolic blood pressure nor diastolic blood pressure was modified after tacrolimus treatment. In this regard, the fact that tacrolimus administration enhanced the protein expression of both ETA- and ETB-type receptors, which they have extensively reported as receptors with opposite effects on vascular functionality, may occur as counterbalance response probably trying to attenuate the vasoconstrictor effect of tacrolimus related to ETA-type receptor. A previous work from Tepperman et al. [4] also showed that tacrolimus increased the expression of ETB receptors without changes on ETA-type receptors in aorta segments from Lewis rats, although these authors used shorter time of treatment with tacrolimus than the here followed.

5.2. Effect of tacrolimus treatment on energetic metabolism-related proteins in the aortic wall

With respect to proteins involved in energetic metabolism, 30 days after tacrolimus treatment, aortic expression of ATP synthase gamma chain was significantly decreased. Moreover, the expression of NADH dehydrogenase ubiquinone isotype 2 was reduced in the aortic segments from tacrolimus-treated rats compared with those from control. In addition, the expression of two acyl CoA dehydrogenase long-chain mitochondrial precursor isotypes, one of the main enzyme involved in fatty acid long-chain β-oxidation [38], was also reduced in the aortic segments from tacrolimus-treated rats. Taken together, tacrolimus treatment seems to decrease oxidative metabolism in the aortic vascular wall. Accordingly, in vitro studies have also suggested that tacrolimus reduced ATP production in different tissues decreasing oxidative phosphorylation [39] and [40]. Reduction of energetic metabolism in the vascular wall has been associated with cell injury and with decreased vascular functionality including an impaired endothelium-dependent relaxation [41] and [42].

Tacrolimus reduced the protein level of both ATP synthase gamma chain and NADH dehydrogenase ubiquinone which may suggest impaired mitochondrial metabolism. Mitochondrial dysfunction may result in an increased production of oxygen reactive species (ROS), which may diminish nitric oxide bioavailability [43]. Indeed, pharmacological inhibition of mitochondrial energy metabolism impairs endothelial-dependent vascular relaxation and may increase the production of reactive oxygen species [44]. Alteration of mitochondrial-related energetic metabolism seems also to
contribute to endothelial dysfunction in aging [45]. In the present study, it was observed that after tacrolimus treatment protein expression of the catalytic subunit of NADPH oxidase, gp91-phox, was found increased in the aortic segments. It suggests a putative increase of ROS in the aorta from tacrolimus-treated rats. In this regard, previous studies have demonstrated an increased production of ROS in tacrolimus-incubated bovine endothelial and glial cells [46] and [47].

5.3. Effects of bosentan coadministration with tacrolimus on cytoskeleton and energetic metabolism-related proteins

In cytoskeleton-related proteins, only lamin A isotype 1 was modified by bosentan coadministration with tacrolimus compared with tacrolimus alone. As previously mentioned, lamin A may be involved in modification of endothelial dependent vasodilation by tacrolimus although in our knowledge it has not been the role of the lamin A isotype 1 in such effect.

In the aortic segments more changes were observed by bosentan on energetic metabolism-related proteins. In this regard, Western blot experiments showed that aortic segments from bosentan + tacrolimus treated rats the expression level of triosephosphate isomerase increased with respect to that found in both control and tacrolimus-treated rats. It was accompanied with prevention of the decreased expression of ATP synthase gamma chain observed by tacrolimus alone. Moreover, bosentan co-administered with tacrolimus reduced the aortic pyruvate content, end-product of glycolytic pathway, which had been found increased in aortic segments from tacrolimus-treated rats. The increased vascular pyruvate content in tacrolimus-treated rats may be associated with a reduced pyruvate catabolism and it may be in accordance with the decreased expression of proteins involved in oxidative phosphorylation, as ATP synthase gamma chain, by tacrolimus.

It is important to note that the increased expression of triosephosphate isomerase in bosentan + tacrolimus-treated rats may occur as defensive response against a possible reduction of vascular glucose uptake induced by tacrolimus, which it has been associated with reduction of insulin secretion and insulin receptor expression better than to a direct effect of tacrolimus on glucose oxidative [48]. In this regard, with respect to control, tacrolimus-treated rats showed a reduced aortic expression of phosphatidylinositol 3-kinase regulatory subunit gamma, enzyme that induces glucose uptake and stimulates glycolytic metabolism [30], [31] and [32]. Accordingly, Radu RG et al. have demonstrated a direct effect of tacrolimus on the glycolytic pathway of pancreatic islets showing that tacrolimus reduced glucokinase activity which it is involved in glycolytic velocity [49].

Bosentan coadministration with tacrolimus did not change the increased expression of gp91-phox observed with tacrolimus alone which it may suggest that the increased expression of gp91-phox related to tacrolimus may be through an ET-1-unrelated mechanism. In this regard, a number of agonists for endothelial NADPH oxidase have been identified, including angiotensin II, growth factors (thrombin and VEGF) and cytokines (tumor necrosis factor α), etc. [50], [51] and [52]. Interestingly, some of these compounds have been also reported increased after tacrolimus treatment [53] and [54].

An interesting finding was the fact that coadministration of bosentan with tacrolimus markedly increased MnSOD expression in the vascular wall. This result may suggest that ET-1 may act as antioxidant repressor in the vascular wall. Indeed, this possible repressor role for ET-1 on the antioxidant system has been also suggested after myocardial ischemia. In this regard, Gupta et al., demonstrated antioxidant effects of bosentan restoring the cellular antioxidant defense mechanism in the ischemic myocardium [55]. In addition, as above mentioned, the fact that bosentan prevented
both oxidative metabolism and favored the antioxidant machinery, may promote better vascular functionality under tacrolimus treatment. In this regard, it is well known that reactive oxygen species, and particularly superoxide anion, breakdown nitric oxide following vascular dysfunctionality [56].

5.4. Considerations and study limitations
In the present work, the changes induced by tacrolimus on the expression of cytoskeleton and energetic metabolism-related proteins seem to be non-dependent of significant changes in neither systolic nor diastolic blood pressure; however, we cannot discard at all that slight blood pressure modifications may have some influence on the effect of tacrolimus in the vascular wall.

The study has several limitations. One of them was that since bosentan is a dual ET-receptor antagonist, it did not allow to delimit the role for each ET-receptor subtype in our findings. In this regard, the increased protein expression of ETA and ETB-type receptors observed after tacrolimus treatment was not modified by bosentan. Another limitation of the present study design is that it is not possible to delimit within the aortic wall where occurred the changes in the protein expression induced by tacrolimus (intima, media and adventitia). Further specific studies are needed to elucidate both study limitations.

6. Conclusion
Tacrolimus treatment increased the aortic content of ET-1 and ETA-, ETB-type receptors in addition to modify the aortic expression of specific proteins associated with cytoskeleton and with the energetic metabolism. Bosentan co-administered with tacrolimus prevented the changes in the expression induced by tacrolimus on some of such proteins mainly those associated with energetic metabolism. In addition, bosentan administration seems to favor the antioxidant system in the vascular wall. These results confer at least a partial role for ET-1 on the effects of tacrolimus on the aortic wall.

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Corresponding author at: Nephrology Department, Hospital Clínico San Carlos,
C/Profesor Martín Lagos s/n, Madrid 28040, Spain. Tel.: + 34 913303882; fax: + 34 913303492.
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