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Pulmonary vascular dysfunction in end-stage cystic fibrosis: Role of NF-kB and endothelin-1.

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Abstract

Rationale: Pulmonary hypertension, rare in chronic respiratory diseases but strongly impacting on their prognosis, is partly underlied by factors other than hypoxemia.
Objectives: to assess the potential role of endothelin-1 (ET-1) and NF-kB vasoconstrictive pathways.
Methods: the effects of endothelin-1 receptors blockers (BQ 123 and 788) and of genistein were assessed on response to acetylcholine of pulmonary vascular rings from cystic fibrosis (CF) lung transplant recipients (n=23). NF-kB and ET-1 receptors expression were immunodetected in pulmonary arteries and quantitated using Western blotting. ET-1 vascular content was quantitated using ELISA.
Measurements and Main Results: 14/23 subjects exhibited a strongly impaired pulmonary vasodilation ($p<0.01$ vs 9/23 subjects with a normal response) associated with an activation of ET-1 receptors A and NF-kB pathways. Genistein restored vasodilation in subjects with an abnormal response.
Conclusions: pulmonary vascular dysfunction is frequent in end-stage CF, involving the NF-kB pathway and that of ET-1 through its ET-A receptors. These data leave a conceptuable place to ET-A blockers and to isoflavones in the management of the devastating vascular complication of chronic obstructive respiratory diseases such as CF.

Number of words: 183
Key words: NF-kB, endothelin-1, genistein, obstructive lung disease.

Abbreviations:

Ach: Acetylcholine

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

ET-1: Endothelin-1

ET-A: Endothelin-1 receptor A

ET-B: Endothelin-1 receptor B

PE: L-Phenylephrine Dichloride

PH: Pulmonary Hypertension

PAP: Pulmonary Arterial Pressure

SNP: Sodium Nitroprusside

PDTC: Pyrrolidine dithiocarbamate

INTRODUCTION

Pulmonary hypertension (PH) occurs in some patients with end-stage pulmonary diseases, bearing a particularly severe prognostic value (1, 2). Although hypoxemia is usually present in these patients and undoubtedly partly underlies the increase in pulmonary vascular resistance, other mechanisms are likely to be involved. They have been poorly elucidated so far maybe due to the fact that very few studies have focused on the pulmonary vascular tone and remodeling in humans affected by chronic lung diseases, in contrast to idiopathic PH (3-5). In order to get some insights into the mechanisms underlying the pulmonary vascular impact of chronic obstructive lung diseases, we set up a study in one of them: cystic fibrosis (CF). The reasons of this choice were triple. Firstly, CF is a widely distributed chronic obstructive lung disease in which PH and right-sided cardiac failure have long been described (6-9). Although PH seems to be relatively rare in this context, it is recognized as a very significant clinical issue with a heavy impact on the prognosis (10-12). Therapeutic recommendations on CF patients have not included so far specific issues on pulmonary vascular derangement but sporadic observations have been reported on the beneficial effect of vasodilative drugs in this context (13). Secondly, we thought that it would be of interest to have a pharmacological approach of the pulmonary vascular tone in this condition in addition to the morphological studies. Indeed, endothelial dysfunction is thought to precede the structural changes known as "vascular remodeling" which in turn leads to the increase in vascular resistance and ultimately to irreversible pulmonary hypertension. This occurs possibly through an imbalance between the production of vasoconstrictive and vasodilative factors. Generally defined as an impaired endothelial-dependent relaxation to acetylcholine (Ach) (14), this dysfunction has been reported before in one study evaluating a very small group of end-stage CF (15), with no specific particular hint to its mechanisms. Since then, if systemic endothelial dysfunction (16) or secretory endothelial dysfunction (17) have been studied in CF, no study, to our knowledge, has focused on pharmacological pulmonary endothelial dysfunction and on its pathogenesis in CF. NF-kB is constitutively expressed in CF as an upstream mediator or as a result of the intense inflammation which characterizes CF. This might lead to the transcription of vasoactive and proliferative mediators. Among these, ET-1 appears relevant in the context of CF. Increased serum levels of ET-1 have been reported in CF patients (18) as well as its airway overproduction (19). Produced by endothelial cells, this 21-amino acid peptide has very potent vasoconstrictive and mitogenic capacities via signalling through its receptors, ET-A and ET-B. ET-1 activity therefore

strongly contributes to both pulmonary remodeling and hypertension (20-22). As more recently hypothesized, NF-kB expression could also be driven through ET-1 itself (23)

We therefore designed this study to 1) confirm the endothelial dysfunction in pulmonary arteries from end-stage CF subjects 2) assess the role of two pathways critical to vascular homeostasis, NF-kB and endothelin-1 (ET-1) and 3) evaluate the effects of targeted drugs in restoring normal endothelial-dependent vasodilative response.

MATERIAL AND METHODS

Subjects

Explants from 23 subjects, aged 27 ± 4 years, undergoing lung transplantation for end-stage CF were studied. All subjects had undergone a preoperative echocardiography during which systolic pulmonary arterial pressure (PAP) was measured. In addition, PAP was measured by right heart catheterization in operative theatre just before lung transplantation. Pulmonary hypertension (PH) was defined as a mean PAP > 25 mmHg (20). No patient was receiving a vasodilative drug. All patients were informed of the aims of the study, according to the new French law (Bioethics law, August 2004), which was approved by our Institutional Review Board.

Tissue preparation

Immediately after excision, lung samples were placed in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (Sigma-Aldrich, UK) and transported without delay to our laboratory. Intralobar arteries were carefully dissected free of parenchyma and adhering connective tissue, then several rings (3- to 5-mm length x 1.5- to 2-mm inside diameter) from a single artery were prepared. Some of them were used immediately for pharmacological studies, while others were snap frozen and stored in liquid nitrogen for subsequent protein extraction. In parallel, samples of lung parenchyma were fixed in 10% neutral buffered formalin for 48h, embedded in paraffin, and kept in dry storage at room temperature for histological analyses.

Design of pharmacological experiments

Arterial rings were suspended on tissue hooks in 5 ml-organ baths containing Krebs-Henseleit solution (mM : 120 NaCl, 4,7 KCl, 2,5 $CaCl_2$, 1,2 $MgCl_2$, 15 $NaHCO_3$, 1,2 KH_2PO_4, 11 D-glucose and 10 Hepes, pH 7,4) at 37°C and bubbled with 95% O2 and 5% CO2. Each

preparation was connected to a force displacement transducer (Statham UF-1) and changes for isometric tension were recorded as previously described (24). An initial tension of 1g was applied to the rings, which were then left to equilibrate for 30 minutes until a stable resting tension (RT1) was obtained, with changes in fresh Krebs-Henseleit solution every 10 minutes (Fig 1). Ring viability was verified by adding KCl (40 mM) which induced a contraction. Viable rings were then washed 3 times until full relaxation (resting tension 2, RT2), and were left at rest for 20 minutes. They were then precontracted with L-phenylephrine dichloride 10^{-5}M (PE) to obtain a stable plateau of contraction. Serial dilutions of acetylcholine were then added to produce a cumulative dose response curve (10^{-10} to 10^{-4}M). Relaxation to Ach is expressed as a percentage of relaxation to PE induced tone. A contractile response to Ach is expressed as a negative value. Endothelium-independent relaxation was assessed by measuring the response to sodium nitroprusside 10^{-5}M (SNP) at the end of each experiment.

To assess the role of endothelium in pulmonary vasoactivity we compared response to Ach in presence and absence of endothelium, condition which was achieved by carefully removing the endothelium with a pipe cleaner (15).

For each patient, some rings were pre-treated with various drugs for 30 minutes after PE precontraction. To evaluate the NO-dependent vasorelaxation, rings were pre-treated with L-NAME (a non selective NOsynthases inhibitor).To evaluate the role of ET-1 in pulmonary vasoactivity, we assessed the effects of ET-A (BQ 123, 10^{-5} M) or ET-B (BQ 788, 10^{-5} M) ET-1 receptors antagonists on dose response curves to Ach in all samples (n=23).

To evaluate the effects of phytoestrogens genistein and daidzein (structural analog of genistein lacking inhibitory effects on tyrosine kinases), we performed dose response curves to these drugs in the presence and absence of L-NAME (n=4). We then evaluated the effects of genistein (10^{-6}M) and daidzein (10^{-6} M) on dose response curves to Ach. All experiments were performed in duplicate, with a variability between rings less than 10%.

Immunohistochemistry

Parenchymal serial sections (4-µm thick) were mounted on Superfrost Plus slides (Fischer Scientific, Fairlawn, NJ). Sections were deparaffinized in toluene, rehydrated through graded concentrations of ethanol, and heated for 40 min in citrate buffer pH 6 in a 97°C water bath. The slides were incubated with 20% normal swine serum for 30 min to block nonspecific antibody binding sites, and with hydrogen peroxide for 10 min to block endogenous peroxidase activity. The slides were then incubated for 1 h at room temperature with 1) goat

polyclonal antibodies raised against a peptide mapping near the N-terminus of ET-A receptor (ETAR (N-15) : sc-21193, 1:150 dilution) and ET-B receptor (ETBR (N21): sc-21199, 1:200 dilution) (Santa Cruz Biotechnology, Lexington, UK) of human origin and 2) a rabbit polyclonal antibody raised against amino acids 1-286 of NF-kB subunit p65 of human origin (NF-kB p65 (H-286) : sc-7151, 1:500 dilution) (Santa Cruz Biotechnology, Lexington, UK). Negative controls omitted the primary antibody. Immunostaining was performed with the use of the labeled streptavidin-biotin peroxidase system (K0679 Universal Dakocytomation LSAB+Kit; horseradish peroxidase). Hematoxylin was used as the counterstain.

Western blot analysis

ET-A and ET-B receptors and subunit p65 of NF-kB were assayed from homogenized extracts of frozen arterial rings. Total proteins were extracted with a lysis buffer (10mM Tris-HCl pH 7,4, 50 mM NaCl, 0,1% NP-40, and 20% antiprotease cocktail) and were measured with a bicinchoninic acid (BCA) protein assay kit (Pierce) on a microplate according to the manufacturer's instructions. Total proteins (30µg/lane) were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel, and then, transferred onto nitrocellulose membranes and immunodetected. Nonspecific binding was blocked with 10% milk powder in Tris-buffered saline for 1h at room temperature. The blots were incubated with the same antibodies as those used for immunohistochemistry against ET-A receptor (1:500 dilution), ET-B receptor (1:500 dilution) and NF-kB p65 (1:1000 dilution). Membranes were subsequently stripped and reprobed with β-actin to verify equal loading. The proteins were detected with an enhanced chemiluminescence (ECL) kit (GE Healthcare Europe) and Hyperfilm ECL high-performance chemiluminescence film (GE Healthcare Europe). The intensities of protein staining on the immunoreactive Western blot bands were analyzed with ImageJ software. The relative amounts of immunoreactive proteins were obtained by dividing the scanning unit values by the respective value of β-actin protein (primary anti β-actin mouse monoclonal antibody: AANO2, 1:1000 dilution; Cytoskeleton) and expressed in arbitrary units.

ELISA

The Biotrak ET-1 ELISA system (Amersham Pharmacia Biotech., Buckinghamshire, UK) was used to assess ET-1 levels in protein extracts from arterial rings. ET-1 in the samples tested was captured by microtiter plates precoated with anti-ET-1 antibody and detected by a peroxidase-labeled Fab' fragment of ET-1 antibody conjugate.

Expression of results and statistical analysis

Results are presented as mean ± SE. Comparisons between groups were made by an unpaired t-test. Sets of pharmacological data were compared using repeated-measures ANOVA followed by the Bonferroni post-hoc test. Comparisons between the relaxation observed in the presence or absence of various drugs were made using a Wilcoxon test. A p value < 0.05 was considered statistically significant.

RESULTS

Endothelial function was evaluated by the response to Ach of pulmonary arterial rings isolated from 23 CF subjects, and dysfunction was defined as an impaired response such as a lack of relaxation or a contraction (25). As in systemic vessels, the pulmonary vascular response to Ach in CF is endothelium- and NO-dependent, as demonstrated by the abrogation of the vasodilative response in the absence of endothelium or in the presence of L-NAME (data not shown).

Pulmonary vasorelaxation

In CF as a whole, this response appeared quite heterogeneous (Fig 2, panel A). However, a closer analysis allowed to distinguish two subgroups among CF subjects: one characterized by the absence of relaxation or even a contraction in the presence of Ach (subjects with endothelial dysfunction, group ED+, n = 14) and one (subjects with no endothelial dysfunction, group ED-, n = 9) exhibiting a strong relaxant response (-23±3% vs 53±8% in groups ED+ and ED-, respectively; $p<0.005$) (Fig 2, panel B). Figure 2 panel C shows representative traces of isometric measurements in group ED+. Besides their marked differences in vascular response to Ach, the two groups were comparable in terms of resting tensions and phenylephrine-induced vasoconstriction (data not shown). Despite its contraction to Ach, group ED+ showed a relaxing response to SNP which was comparable to that of group ED- (115±6% vs 118±3%, NS, respectively), indicating that the endothelium-independent relaxation was conserved in ED+ patients (Fig 3).

As shown in Table 1, clinical and spirometric characteristics were comparable in groups ED+ and ED-. All CF patients were receiving oxygen support during preoperative periods and there was no difference between groups ED+ and ED- regarding their $P0_2$. In contrast, of note is the fact that 10 of 14 subjects in group ED+ (71%) had a preoperative PH vs none in group

ED- ($p < 0.001$, chi-square test). The presence of an impaired vascular response appeared independent of the type of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) mutations. The majority of subjects (14/23) were homozygotous for delta F508 mutations, the remaining nine being heterozygotous for delta F508 or for other mutations. These genotypes appeared similarly distributed between groups ED+ and ED- (Table 1).

To get an insight into the mechanisms underlying the reduced vasodilative response in group ED+, we evaluated the contribution of proximal (NF-kB) and distal mediators (ET-1) classically involved in endothelial dysfunction and pulmonary hypertension.

NF-kB expression.

The subunit p65 of NF-kB was immunolocalized in the pulmonary vessels of both groups of CF subjects (ED+, n=8 and ED-, n=8), with a clear staining of endothelial and smooth muscle cells (Fig 4, panel A). Interestingly, the quantitation by Western blot of their vascular expression exhibited a markedly higher expression of p65 in group ED+ (n=3) vs group ED- (n=3) ($p<0.05$) (Fig 4, panel B).

Endothelin-1 pathway

Vascular reactivity

We performed the dose response curves to Ach in the presence and absence of specific blockers of ET-1 receptors, BQ 123 (anti-ET-A) and BQ 788 (anti-ET-B) in all patients (n=23). The latter did not display any relaxing effect in group ED+ nor an enhanced vasodilative effect in group ED- (Fig 5, panel A). In contrast, BQ 123 exhibited a marked although not complete vasodilative effect in group ED+ (26±12 *vs* -22.9±3.5% in the presence and absence of BQ 123, respectively; $p < 0.01$, Fig.5 panel B), and a moderate but not significant enhancement of the vasodilative response in group ED- (71±13 and 53±7% in the presence and absence of BQ 123, respectively, NS) (Fig 5, panel B).

ET-1 concentration

The protein content of arterial rings in ET-1 was measured in ED+ (n=8) and ED- (n=8) subjects. ET-1 was significantly higher in group ED+ compared to group ED- (10.8±5.2 vs 2.2±1.9 pg/mg protein, respectively; $p < 0.01$) (Fig 6, panel A)

ET-A and ET-B expression

ET-1 receptors were localized in arterial rings using immunohistochemistry ED+ (n=8) and ED- (n=8) subjects. ET-A was mainly detected on vascular smooth muscle cells (Fig 6, panel B) whereas ET-B was detected in both smooth muscle and endothelial cells (Fig 6, panel C).

No difference was observed between the groups as to ET-1 receptor location. In both groups, ET-A appeared more intensely stained than ET-B. Moreover, the Western blotting analysis of both receptors in pulmonary arterial rings from ED+ (n=4) and ED- (n=4) subjects revealed a markedly higher ET-A expression in group ED+ compared to group ED-, ($p < 0.05$) (Fig 6, panel D), whereas that of ET-B appeared comparable in both CF groups (Fig 6, panel E) .

Effects of genistein

We firstly performed dose response curves to genistein and we observed a strong vasodilative effect of this drug with an EC50 at 10^{-6}M. This response was largely, although not fully, abrogated by L-NAME (Fig. 7). This concentration (10^{-6}M) was used thereafter in experiments evaluating the effects of the drug on the impaired relaxant response to Ach (ED+, n=8). Genistein was able to restore a largely vasodilative response to Ach (Fig 8). Daidzein exhibited a vasodilative effect with the same EC50 than genistein. Furthermore, and similarly to the latter, it exhibited clear vasodilative effects in subjects with an impaired response to Ach (ED+ group, n= 8) (Fig 8).

DISCUSSION

We have confirmed and largely extended some of our preliminary results showing that pulmonary vasoreactivity is severely impaired in a consistent fraction of subjects with end-stage CF. In an attempt to get insights into the mechanisms underlying this dysfunction, we have evaluated some critical mediators involved in the control of vascular tone, i.e. NF-kB and ET-1. We show here that CF-associated pulmonary endothelial dysfunction is related to classical mechanisms of vascular dysfunction and/or pulmonary hypertension, namely a high NF-kB expression and an activated ET-1 pathway.

The fact that CF is associated with a strong impact on pulmonary arterial pressure and right ventricle function had long been demonstrated (6-8), bearing a strongly negative prognostic value (10, 12). Their exact frequency is not known. Our study was not designed to accurately assess the prevalence of pulmonary arterial disorders in CF, but we come up, to the best of our knowledge, with the largest series of CF addressing this question. In our group of 23 end-stage CF subjects, pulmonary endothelial dysfunction was present in almost two thirds (14/23, group ED+). Two observations should be noted in this group. Firstly, 10 of these 14 subjects displayed an echocardiographic pulmonary hypertension, subsequently confirmed by

right heart catheterization, whereas none was noted in group ED- (chi square test $p< 0.001$), underlining the strong relationship between pulmonary endothelial dysfunction and hypertension. Secondly, the pharmacological demonstration of an endothelial dysfunction was the sole marker of the vascular impact of CF in the remaining four subjects in group ED+, who displayed normal echocardiographic parameters. Their condition therefore remained clinically undetectable. These subjects might however be at risk for the subsequent development of pulmonary hypertension considering the general agreement according to which endothelial dysfunction might pave the way for vascular remodeling, increase in vascular resistance and irreversible pulmonary hypertension.

To get an insight into the mechanisms of the vascular dysfunction defining the group ED+, we focused on two main pathways classically involved in the control of vascular tone, i.e. NF-kB and ET-1. We observed that both of them were upregulated. NF-kB is well known to be activated in CF airway epithelial cells and to participate in their dysregulated inflammation (26-28). We extended these findings by demonstrating for the first time to our knowledge a clear overexpression of NF-kB in the pulmonary vascular structures of CF subjects who exhibited an impaired response to Ach. The reason why NF-kB is upregulated in vessels can only be speculative. CF is characterized by an intense pulmonary inflammation, and NF-kB upregulation is among its key markers. It is a major inflammatory transcription factor (29) partly responsible for the high levels of IL-8, among other inflammatory mediators (30, 31). In addition to the latter, NF-kB might also lead to the transcription of vasoactive and proliferative mediators, such as ET-1 for instance. Alternatively we can hypothesize that NF-kB overexpression might be driven by ET-1 pathway upregulation. Zhao et al. have indeed demonstrated strong connections between these two pathways in cardiomyocytes with ET-1 deficient mice tissues having diminished NF-kB expression (23). Whether NF-kB is upregulated by a paracrine increased release of ET-1 or whether ET-1 overproduction results from its increased transcription by an activated NF-kB pathway cannot be differentiated by our data. In any case, NF-kB upregulation is known to impact on endothelial function. Inhibition of this pathway by PDTC is able to restore of a normal vasodilative response in an ovine model of pulmonary dysfunction (32).

ET-1 is a potent and long-lasting vasoconstrictor, strongly involved in vascular remodeling by a direct mitogenic effect on smooth muscle cell and by inhibition of their apoptosis. The critical role of ET-1 in PH has been largely demonstrated (20-22), underlying the now widely used therapeutic strategies in idiopathic PH based on ET-1 receptor blocking either dually (33, 34) or specifically directed to ET-A (35). Very few data other than increased

ET-1 levels in serum (18) and in sputum (19) of CF patients exist as to a potentially pathogenic role of ET-1 in CF. To the best of our knowledge, this is the first report on the involvement of ET-1 in CF-associated pulmonary endothelial dysfunction. Our data argue for a largely predominant effect of this vasoconstrictive pathway in these subjects, involving more specifically the ET-A receptor subtype. Indeed, its selective blocking by BQ-123 partly restored this response, whereas protein analysis revealed a marked expression of ET-A and of ET-1 predominantly in the group with endothelial dysfunction.

Importantly, we also show in this study that genistein is able to overcome the negative effect of ET-1 on the pulmonary vasodilative tone in ED+ subjects. This isoflavone has various effects potentially involved in its vasodilative properties. First of all, the main hypothesis is that the dilative effect of both isoflavone drugs in our model is mediated through their activation of eNOS and the production of NO. This has been documented as one of their classical effects. Furthermore, the strong inhibition by L-NAME of the vasodilative response to Ach in the presence of genistein strongly argues for this mechanism. Alternative hypothesis could be that genistein is a strong CFTR potentiator (36). The latter has recently been demonstrated to be markedly involved in vasodilation (37-39). In addition, genistein could act through its wide enhancing effects on the transcription of vasodilative proteins, some of them involving different tyrosine kinase activities. Daidzein is devoid of any potentiating effect on CFTR and of any tyrosine kinase activity. The fact that daidzein exhibits vasodilative effects comparable to those of genistein rules out the two above cited mechanisms. Finally, the fact that genistein is a strong inhibitor of activated NF-kB and acts as a potent antiinflammatory agent (30, 40, 41) should also be considered. Indeed, PDTC, another NF-kB inhibitor, has shown potent dilative effects in an ovine pulmonary model of vascular dysfunction (32).

In conclusion, we report here the first demonstration of a very frequent vascular dysfunction affecting lung vessels in end-stage CF. This dysfunction associates an endothelial upregulation of two vasoconstrictive pathways: NF-kB and ET-1. If our data are confirmed, this might leave a conceptual place for isoflavones in the therapeutic armenterium aiming at controling the potentially devastating impact of CF on pulmonary artery and ultimately right ventricle. ET-1 blockers, largely used in other causes of pulmonary hypertension but rarely in CF might also be considered based on our experimental data.

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Table 1: Comparison of general characteristics and lung function measurements of CF patients.

	ED-	**ED+**	***p***
n	n=9	n=14	
Age, yr	27±3	27±4	NS
Male:female ratio	6:3	8:6	NS
CFTR Mutation			
Delta F508/F508	5	9	
Delta F508/1066C	1	2	
Delta F508/E60X	1	1	
1677 DES TA/D 614G		1	
G 542 X heteroz	2	1	
Spirometry			
FEV_1, % predicted	28±2	31±2	NS
FVC, % predicted	44±3	48±2	NS
FEV_1/FVC,%	65±4	66±5	NS
Pulmonary Arterial Pressure			
PAPs, mmHg	27±2	44±3*	<0.001
PH	n=0/9	n=10/14**	<0.001

Values are means ± SE. FEV_1, forced expiratory volume in 1s; FVC, forced vital capacity; PAPs, Systolic Pulmonary Arterial Pressure .

* : Mann Whitney U test

** : chi-square test

FIGURE LEGENDS

Figure 1: Representative traces of isometric tension recording in ED- patients

Figure 1

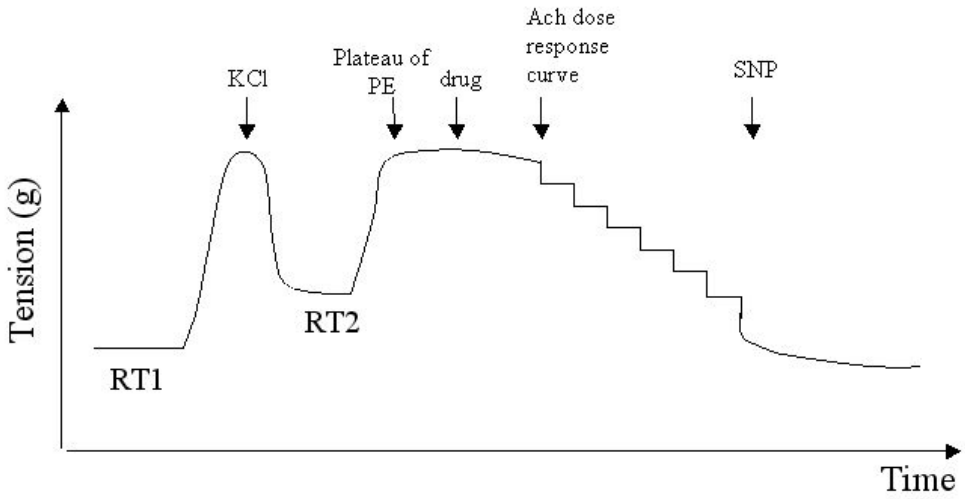


Figure 2: Cumulative dose response curves to Ach in pulmonary arterial rings from CF subjects. CF subjects (n=23) as a whole (panel A). CF subjects broken down in two groups according to the presence (ED+, n=14) or absence (ED-, n=9) of pulmonary endothelial dysfunction (panel B). Data points are means ± SE.*$p<0.005$. Representative traces of isometric tension recording in ED+ patients (panel C).

Figure 2

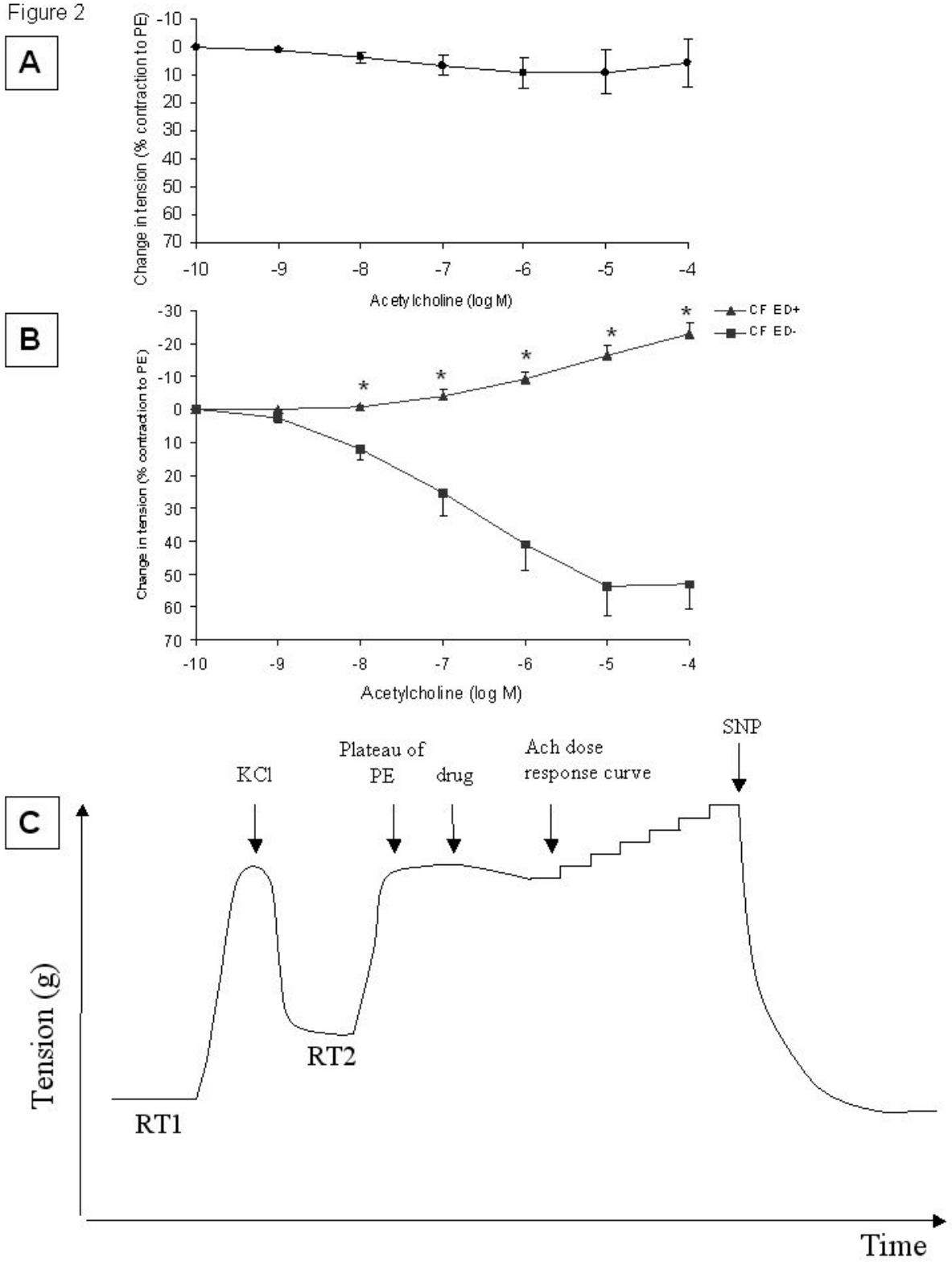


Figure 3: Endothelial vs non endothelial vasoactivity in ED+ and ED- patients. Whereas Ach produced endothelium-dependent vasorelaxation in ED- and vasoconstriction in ED+ patients

(here represented at Ach concentration of 10^{-4}M), SNP (10^{-5}M) produced a similar endothelium-independent vasorelaxation in both groups.

Figure 3

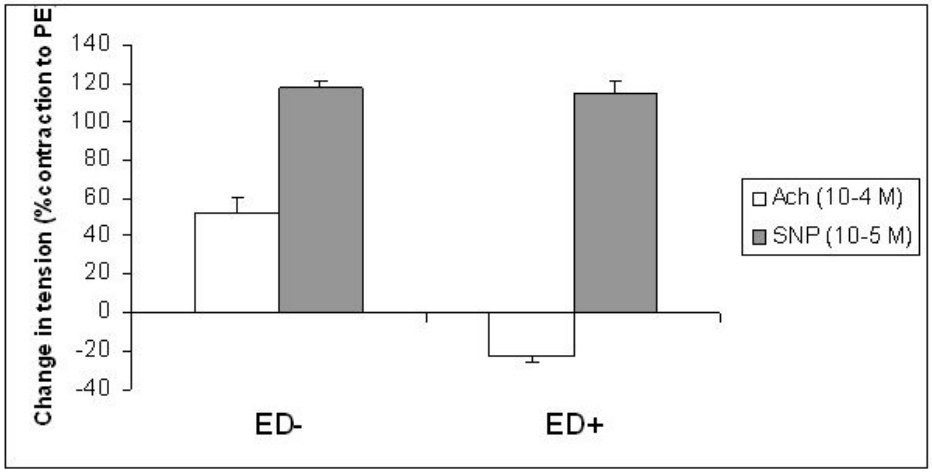
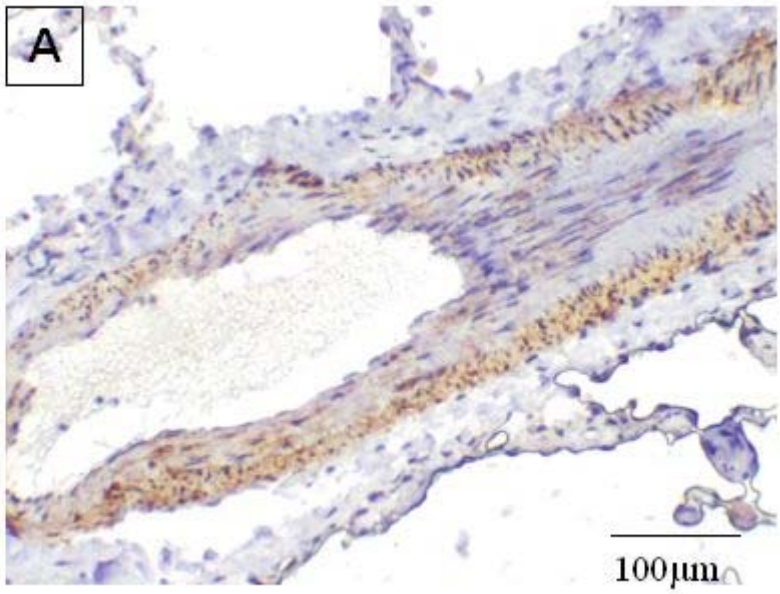


Figure 4: Immunolocalization and quantitation of p65. p65 was detected using immunohistochemistry (panel A) on smooth muscle and pulmonary endothelial cells (Hematoxylin staining) of ED+ (n=8) and ED- (n=8) patients. Western blotting technique was performed in 6 patients (ED+, n=3 and ED-, n=3). p65 expression was higher in ED+ than in ED- subjects (panel B) ($p<0.05$). Data are normalized for β-actin, and points represent means ±SE.

Figure 4



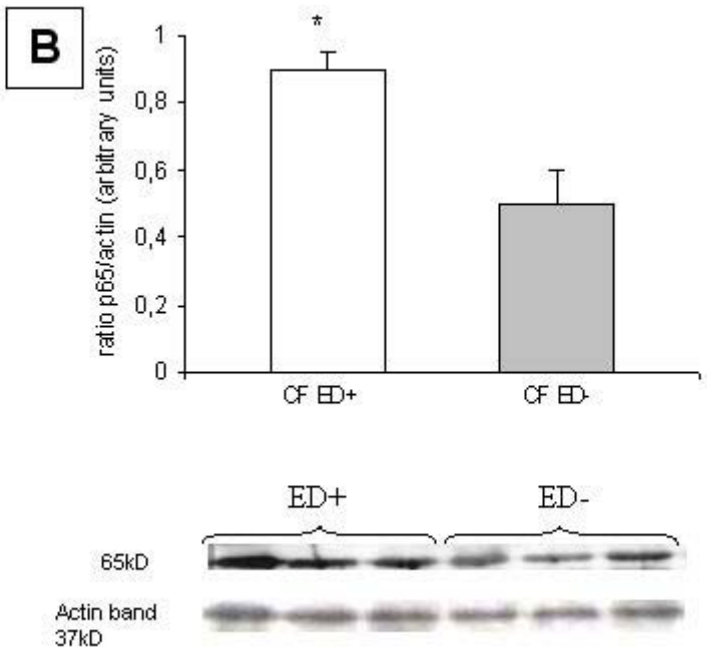


Figure 5: Effect of ET-B receptor antagonist, BQ 788 (panel A) and ET-A receptor antagonist, BQ 123 (panel B) on cumulative concentration response curves to Ach in group ED- (n=9) and in group ED+ (n=14). Data points are means ±SE. *p<0.01.

Figure 5

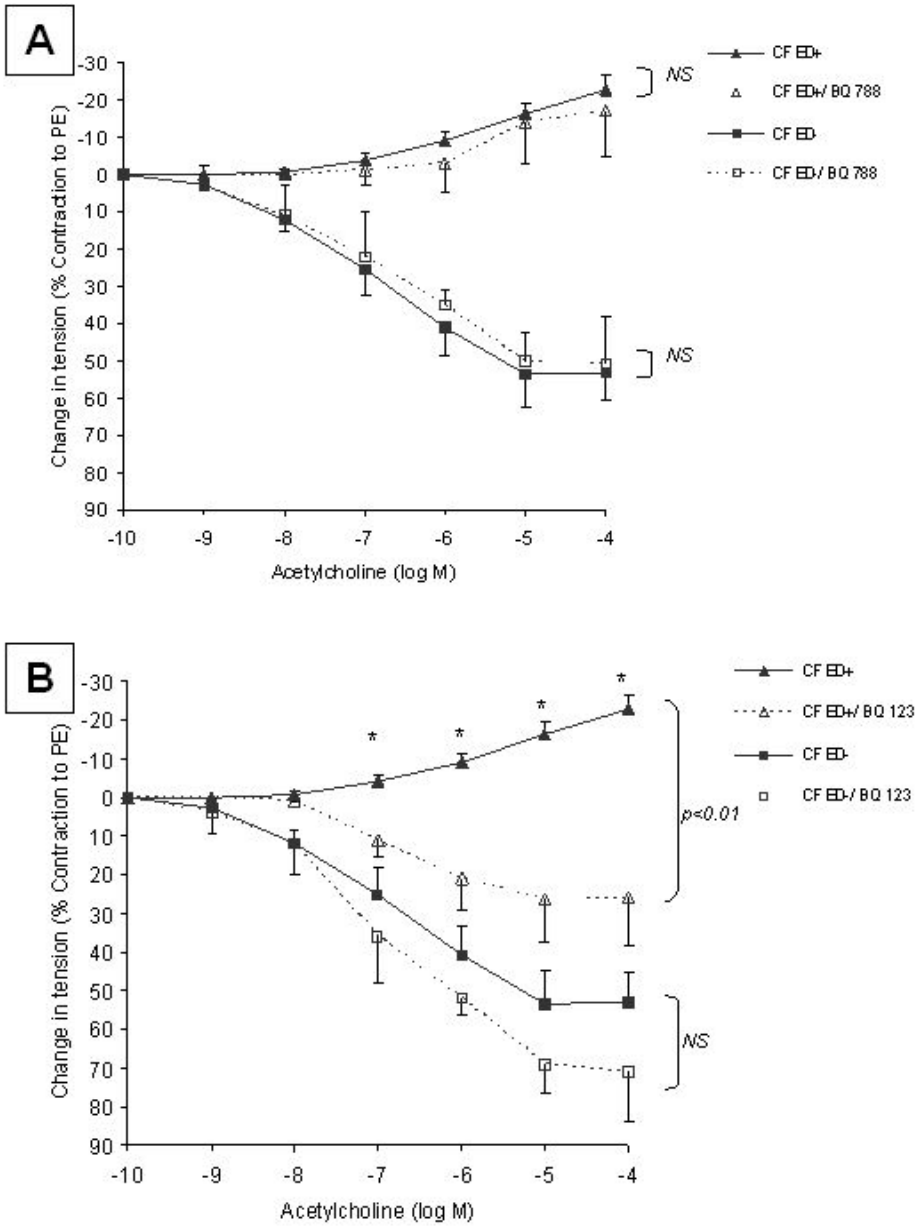


Figure 6: ET-1 quantitation, and ET-A / ET-B immunolocalization and quantification. ELISA quantitation of ET-1 was higher in pulmonary arteries from group ED+ (n=8) than from group ED- (n=8) (panel A) *p<0.01. ET-A (panel B) and ET-B (panel C) receptors were detected using immunohistochemistry in 16 patients (ED+, n=8 and ED-, n=8) in smooth muscle and pulmonary endothelial cells (arrows) and quantitated using Western blotting technique in 8 patients (ED+, n=4 and ED-, n=4) (panels D and E). Data are normalized for β-actin, and represent mean ±SE. *p<0.05.

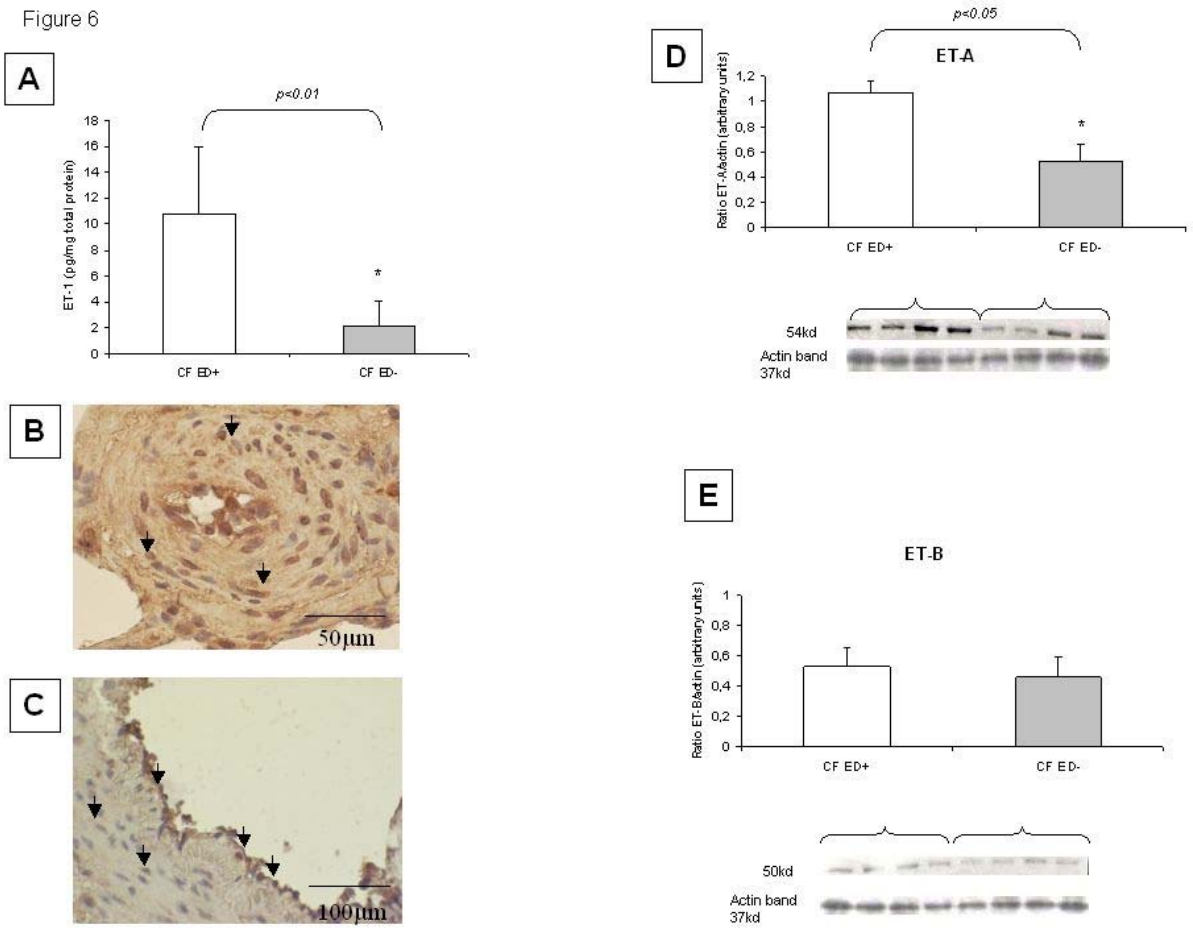


Figure 7: Cumulative dose response curve to genistein in the absence or presence of L-Name in ED+ patients (n=4). *p<0.05.

Figure 7

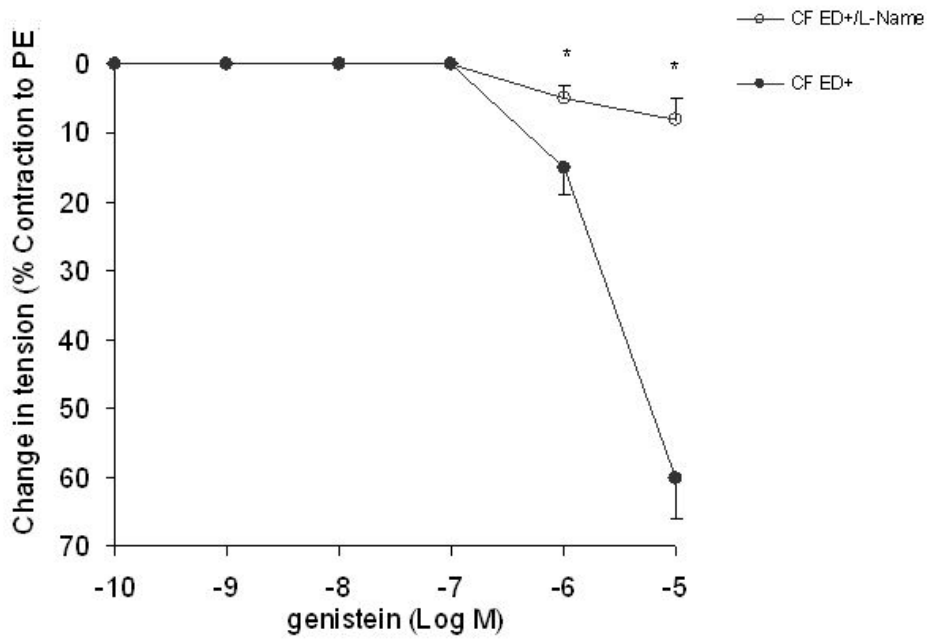
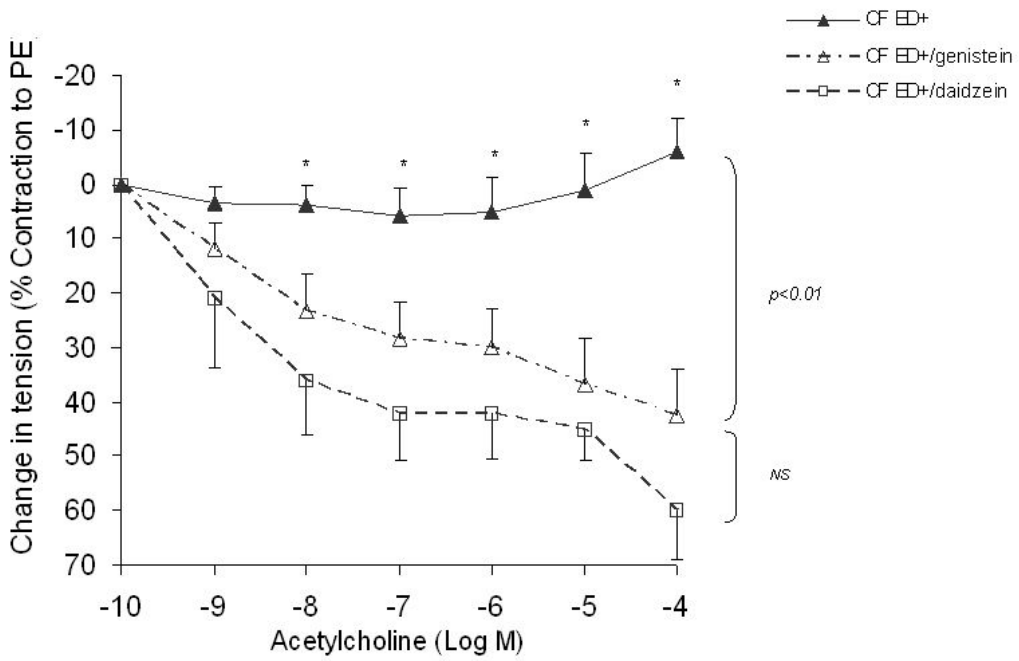


Figure 8: Effect of isoflavones genistein and daidzein on cumulative concentration response curves to Ach in 8 patients of group ED+. Data points are means ±SE. *p<0.01.

Figure 8



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