



HAL
open science

Cardiac Specific Increase in Aldosterone Production Induces Coronary Dysfunction in Aldosterone Synthase-Transgenic Mice

Anne Garnier, Jennifer K Bendall, Sebastien Fuchs, Brigitte Escoubet, Francesca Rochais, Jacqueline Hoerter, Johnny Nehme, Marie-Lory Ambroisine, Noeleen de Angelis, Gilles Morineau, et al.

► **To cite this version:**

Anne Garnier, Jennifer K Bendall, Sebastien Fuchs, Brigitte Escoubet, Francesca Rochais, et al.. Cardiac Specific Increase in Aldosterone Production Induces Coronary Dysfunction in Aldosterone Synthase-Transgenic Mice. *Circulation*, 2004, 110 (13), pp.1819-1825. 10.1161/01.CIR.0000142858.44680.27 . hal-03161417

HAL Id: hal-03161417

<https://hal.science/hal-03161417>

Submitted on 15 Mar 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

CARDIAC SPECIFIC INCREASE IN ALDOSTERONE PRODUCTION INDUCES CORONARY DYSFUNCTION IN ALDOSTERONE SYNTHASE TRANSGENIC MICE

Short Title: Garnier A. Cardiac aldosterone and coronary dysfunction

Garnier A, PhD^{(1)*}, Bendall JK, PhD^{(1)*}, Fuchs S, MD, PhD⁽²⁾, Escoubet B, MD, PhD⁽³⁾, Rochais F⁽⁴⁾, Hoerter J, PhD⁽⁴⁾, Nehme J⁽¹⁾, Ambroisine ML⁽¹⁾, De Angelis N, PhD⁽⁵⁾, Morineau G, PhD⁽⁶⁾, d'Estienne P⁽¹⁾, Fischmeister R, PhD⁽⁴⁾, Heymes C, PhD⁽¹⁾, Pinet F, PhD⁽⁷⁾, Delcayre C, PhD⁽¹⁾.

* equivalent contributors.

1 : INSERM U572, Université Paris 7, Paris, France.

2 : INSERM U36, Collège de France, Paris, France.

3 : INSERM U426, CEFI IFR02, AP-HP, Faculté de Médecine Bichat, Paris, France.

4 : INSERM U446, Faculté de Pharmacie, Châtenay-Malabry, France.

5 : Instituto Mario Negri, Milano, Italy.

6 : Laboratoire d'hormonologie, Hopital St Louis, Paris, France

7 : INSERM U508, Institut Pasteur, Lille, France.

Word count: 4364.

Financial support: Pharmacia France, Inserm, CNRS, Fondation de France.

Correspondence to Dr Claude Delcayre, INSERM U572, Hôpital Lariboisière, 41 Boulevard de la Chapelle, 75475 Paris cedex 10, France.

Tel: +33-1 4463 1729.

Fax: +33-1 4874 2315.

E-mail: Claude.Delcayre@larib.inserm.fr

ABSTRACT

Background: Elevated circulating aldosterone level is associated with impaired cardiovascular function. While the mechanisms are not fully understood, aldosterone antagonists decrease total and cardiovascular mortality in heart failure and myocardial infarction. Aldosterone induces cardiac fibrosis in experimental models, and it is locally synthesized in rat heart. These observations suggest pathological effects of aldosterone in heart that remain unclear.

Methods and Results: Transgenic mice (TG) that overexpress the terminal enzyme of aldosterone biosynthesis, aldosterone-synthase (AS), in heart have been raised by gene targeting with the alpha-myosin heavy chain promoter. AS mRNA increased 100-fold and aldosterone concentration 1.7-fold in hearts of male TG mice relative to wild-types. No structural or myocardial alterations were evidenced as ventricle/body weight, AT1 and AT2 receptor binding and collagen content were unchanged in TG. No alteration in cardiac function was evidenced by echocardiography, isolated perfused heart or whole-cell patch clamp experiments. In contrast, coronary function was impaired since basal coronary flow was decreased in isolated perfused heart (-55% of baseline values), and vasodilatation to acetylcholine, bradykinin and sodium nitroprusside were decreased by 75%, 60% and 75%, respectively, in TG mice as compared to wild-types, showing that the defect was not related to NO production.

Conclusions: Increased cardiac aldosterone production in male mice induces a major coronary endothelium-independent dysfunction with no detectable alterations in cardiac structure and function. However coronary dysfunction may be harmful for coronary adaptation to increased flow demand.

CONDENSED ABSTRACT

Aldosterone is involved in rat heart remodeling. Transgenic mice overexpressing aldosterone-synthase in heart were created. In male transgenic mice, aldosterone concentration in heart increased 1.7-fold, without any change in heart weight, AT1 receptor binding, collagen content and cardiac function relative to wild-types. Baseline values of coronary flow studied on isolated perfused heart were decreased by 55%, as well as acetylcholine (by 75%), bradykinin (by 60%) and sodium nitroprusside

(by 75%) vasodilatory responses, indicating an endothelium-independent alteration. Despite without any consequences on cardiac function, this coronary alteration may be harmful in cardiac challenging conditions.

Key Words: Aldosterone, Cardiac hormones, Transgenic mice, Coronary dysfunction, Heart function.

The RALES study has shown that the mineralocorticoid-receptor (MR) antagonist, spironolactone, decreases mortality in patients with heart failure (HF) (1). Decreased mortality was also observed in patients with left ventricular (LV) dysfunction after myocardial infarction (MI) treated with the MR-specific antagonist eplerenone (2). In both studies, beneficial effects were obtained using doses of aldosterone antagonists that had no effect on blood pressure.

Aldosterone has deleterious effects on heart structure and function (review in 3). The MR is present in heart, supporting the possibility that aldosterone may have actions on this organ, independently of its renal and blood pressure effects. Experimentally, cardiac fibrosis is induced in aldosterone-salt-treated rats (4,5), but the mechanisms remains partly unclear (3). A pericoronary inflammatory phenotype is an early step of cardiac damage in aldosterone-salt-treated animals (6,7,8), suggesting that coronary arteries may be a target for aldosterone in heart.

Aldosterone is produced at low levels in rat heart (9), its production increasing following MI where it participates in MI-associated fibrosis (10,11). In humans, several steroidogenic genes are detected in the failing heart (12), and aldosterone-synthase (AS) mRNA is upregulated in ventricles of patients with HF (13) or with hypertrophic cardiomyopathy (14). Another study has linked the increase in cardiac aldosterone production to LV dysfunction (15). These observations strengthen the possibility that cardiac aldosterone may have harmful effects in the myocardium.

Until now, the effects of aldosterone on cardiac structure and function have been observed in experimental models based on increased plasma aldosterone plus salt. To better define the specific cardiac effects of aldosterone, we created a mouse model in which aldosterone was specifically increased in the heart, without changes in its plasma level. Phenotypic analysis of these mice showed that AS overexpression induced a major coronary dysfunction without changes in cardiac structure and function in transgenic male mice.

METHODS

Detailed methods are included in an on-line Data supplement to this manuscript.

Transgenic mice. Expression of the transgene containing the 1500 bp coding sequence of AS (Fig. 1a) was driven by the mouse alpha-MHC gene promoter (16). Two independent founder lines (8282 M and 8283 M) were established in FVB mice.

Collagen and AngII receptors assay. Quantification of collagen was determined as described (10). Binding analysis of AngII-receptors was performed as described (17). AT-receptor subtypes were identified by incubation with losartan or PD 123319.

RNA analysis. Extraction of heart total RNA and RT-PCR of AS and 11-beta-hydroxylase were performed as described (10). Total RNA yields were not different in WT and TG mice.

Western blot. Mitochondrial proteins were isolated from ventricles (18). Blots were incubated with an antibody against the rat AS that detected the mouse AS (kind gift of Dr Gomez-Sanchez). AS protein was detected using an ECL+ kit (Amersham, Paris, France).

Hormones. Aldosterone in cardiac homogenates was assayed by radioimmunoassay using a Immunotech kit (Beckman, Marseille, France), and corticosterone with an in-house radioimmunoassay.

Echocardiography. Echocardiography was performed as published (19).

Isolated heart perfusion. Contractile properties of hearts perfused in non-recirculating Langendorff mode at constant pressure (80 mm Hg) were recorded as published (20).

Coronary function. Coronary vascular responsiveness to vasoactive agents was measured in non-recirculating Langendorff mode at constant flow as published (21). Vasoactive drugs were administered by bolus injections just above the aortic cannula.

Electrophysiological analysis of calcium and potassium currents. Whole cell patch-clamp of ventricular myocytes was used to record the L-type Ca^{2+} current ($I_{\text{Ca,L}}$) as published (20), and the time-dependent and time-independent components of the potassium transient outward currents, respectively I_{to1} and I_{sus} as published (22).

Statistical analysis. Results were expressed as means \pm SEM. Mean values were compared by one-way ANOVA followed by a Fisher's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

AS overexpression.

Two independent mouse TG founder lines (8282 M and 8283 M) were established. Post-natal development and basic phenotype parameters were similar in both TG lines. Results presented here were obtained in male mice of line 8282 M.

Figure 1b shows the increase of about 100-fold of the AS mRNA in ventricles of TG mice compared to age-matched WT. In WT samples the 633 bp band contained 2 products: AS and 11-beta-hydroxylase. Their quantification, using *Ava*I that cuts AS and *Nhe*I that cuts 11-beta-hydroxylase (not shown), gave an AS/11-beta-hydroxylase ratio of 1/7. Figure 1b shows that the 633 bp band in TG samples was much more intense than in WTs, due to presence of transgenic rat AS. AS mRNA was also induced in lung but to a lower level than in heart, in agreement with the known alpha-MHC expression in this organ (16), and not detected in adrenal gland, kidney, skeletal muscle and brain (not shown). AS was detected by Western blot only in TG mice ventricles (Fig.1c). Aldosterone concentration in ventricles was increased by 1.7 fold in TG mice (Table 1). Aldosterone levels were identically increased in either left or right ventricles and left or right atria (not shown). Interestingly, there was neither a change in aldosterone concentration in adrenal glands or plasma, nor of corticosterone concentration in any samples.

Phenotype.

Table 1 shows that no anatomical changes were observed in TG mice compared with WT mice. Histologic examination of ventricles showed no alteration of ventricular tissue, and of coronary vessels including the perivascular area, in TG mice (not shown). Levels of ventricular AT1 and AT2 receptors and the collagen volume fraction were also unchanged in 3 month-old male TG mice (Fig. 2). Using real-time PCR, we found no difference in the levels of expression of mRNAs coding for the Endothelin Converting Enzyme, Pre-Pro-endothelin and the Endothelin-1 type A receptor in TG mice ventricles compared to WT ones (see Data supplement file).

Echocardiographic evaluation was performed in 15 and 27 week-old mice. The LV of TG mice was neither dilated nor hypertrophied, and systolic and diastolic echocardiographic parameters were similar in TG and WT mice (Table 1). At 36 weeks, LV function was also normal (not shown). Fig. 3a shows that AS overexpression did not alter L-type calcium current $I_{Ca,L}$ of isolated cardiac myocytes, both under basal conditions and under maximal β -stimulation (isoproterenol 10^{-7} M). Similarly, AS overexpression did not alter the characteristics of the time-dependent and -independent components of the transient outward potassium channel current, respectively I_{to1} (Fig. 3b) and I_{sus} (Fig. 3c). T-type calcium current was absent in ventricular myocytes from both WT and TG mice (see Data supplement).

Using the perfused isolated heart, LV function in unstimulated WT and TG hearts was found to be similar with a LV developed pressure of 73.8 ± 2.4 vs 78.5 ± 3.6 mmHg, as was the sensitivity to Ca_o measured in hearts paced at 650 bpm (Fig. 4), kinetics of contraction and relaxation (dP/dt max and dP/dt min normalized to LV developed pressure, time to peak tension and half relaxation time) (not shown). Interestingly, coronary flow (CF) was reduced by 55 % in TG hearts compared to WT mice (36.7 ± 3.5 mL/min/g vs 81.1 ± 14.7 mL/min/g, respectively; $p < 0.05$) at Ca_o 1.8 mM.

Coronary function.

Coronary function was studied using a constant flow isolated perfused heart set-up (21). Infusion of acetylcholine in the perfusion line induced a decrease in coronary resistance, expressed here in percent change of coronary perfusion pressure (CPP) relative to baseline values in WT mice (Fig. 5a). The vasodilatory response was observed at 10^{-6} M acetylcholine and was greater for higher acetylcholine concentrations (maximal decrease 28.8 ± 3.6 mm Hg at 10^{-4} M acetylcholine). In contrast, responses to acetylcholine were almost abolished in TG mice since 10^{-4} M acetylcholine did not induce a significant decrease in CPP (Fig. 5a). Similarly, coronary responses to bradykinin were decreased by 60% in TG compared to WT mice (Fig. 5b). The maximal response to sodium nitroprusside was also decreased by 75% in TG hearts, indicating that the defect was endothelium-independent (Fig. 5c).

DISCUSSION

This work shows that cardiac specific AS overexpression induces a slight increase in the aldosterone concentration in cardiac tissue and a major coronary vascular dysfunction in male mice, without altering cardiac structure and function.

In hearts of TG mice, we observed an important induction of AS mRNA (x 100), a moderate increase in AS protein (x4) and a slight increase (of 1.7 fold) in aldosterone concentration compared to WT mice. This suggests that AS synthesis is controlled by mechanisms working downstream from the transcription of AS gene. It is noteworthy that this modest increase reflects the change in aldosterone content of whole homogenized hearts, and is likely to underestimate the actual increase in aldosterone concentration within or in the close vicinity to cardiac myocytes, where the AS gene has been targeted. One major finding is the marked alteration of coronary vascular responsiveness to acetylcholine and bradykinin observed at 15 weeks. To date, it is unknown whether this coronary dysfunction was present at birth, or if it developed progressively after birth. However, the well-described alpha MHC expression pattern in heart suggests that the AS overexpression, and presumably the cardiac overproduction of aldosterone, began early in the life of the TG mouse. TG mice had a normal post-natal

development and no post-natal mortality was observed. These observations indicate that the coronary alteration is not life-threatening under physiological/basal conditions. On the other hand, this silent coronary dysfunction may have important consequences if cardiac work was suddenly to change. Moreover, these results may have an important pathophysiological significance. Several studies have demonstrated an increased production of cardiac aldosterone in pathological states. This was demonstrated for the first time in a rat model of MI (10) but the situation in human species is more confused. It is generally accepted that aldosterone production in the normal human heart is very low. However, several observations in patients with HF are in favour of a strongly increased production of aldosterone within the diseased heart (14,15, review in 23). Remarkably, our results demonstrate that a slight elevation of aldosterone synthesis is able to seriously impair coronary reserve. It is also possible that the long term cardiac impregnation with elevated aldosterone concentrations is a crucial factor. In this respect, it is worthy to note that long-lasting yet moderately increased aldosterone concentrations are typical features of human chronic cardiac diseases, where the neuro-hormonal stimulation may last for months (24).

Interestingly, the AS overexpression did not alter the cardiac phenotype. There was no cardiac hypertrophy nor cardiac fibrosis, likely related to the modest increase of aldosterone concentration in heart and to the absence of hypertension in this model. Cardiac structure and parameters of LV contraction and relaxation, evaluated both *in vivo* and *ex vivo*, were unaltered in TG mice whatever their age. Besides, previous studies have demonstrated an increased L-type Ca^{2+} current and a decreased transient outward K^{+} current after 1-2 days incubation of rat cardiomyocytes with high (100 nM) concentrations of aldosterone (25,26). These currents were not different in ventricular myocytes from TG and WT mice, suggesting either a dose dependent-effect or different mechanisms. At last, the absence of cardiac hypertrophy and fibrosis indicated no perturbing effect of the alpha MHC promoter.

This study showed that locally produced aldosterone alters coronary vascular function. It is established that aldosterone has effects on vascular reactivity but the mechanisms are not well understood (for

review, see 27). Most results have been obtained after chronic aldosterone blockade by spironolactone or eplerenone. In patients with heart failure spironolactone improved acetylcholine-mediated endothelium-dependent vasodilation and increased nitric oxide bioactivity (28). The causative role of oxidative stress in the aldosterone-mediated vascular alterations is strongly suggested by the protective action of either spironolactone or antioxidants in either coronary (7) or peripheral (29,30) vessels. As demonstrated by Bauersachs et al, the addition of spironolactone to ACE inhibition in rats with heart failure results in improvements in endothelial vasomotor dysfunction that can be attributed to the normalization of nitric oxide-mediated relaxation through the beneficial modulation of nitric oxide balance and superoxide anion formation (29). Similarly, in rabbits fed a proatherosclerotic diet, treatment with spironolactone normalized superoxide formation and improved endothelial function (31). The mechanism for the coronary dysfunction observed in TG mice is yet unknown. Aldosterone stimulates the production of endothelin-1 in heart (32). The expression of mRNAs coding the main components of the endothelin system were not changed in TG mice, suggesting that endothelin is likely not involved in the observed abnormality in coronary vasomotor reactivity. Since the effect of exogenous nitric oxide (nitroprusside) was also blunted in TG animals, it is possible that the alteration may involve vascular smooth muscle cells.

The suggested interactions between aldosterone-synthesizing myocytes and other cell types, requires further study. The present model of cardiac-specific aldosteronism has distinct features when compared with the well known model of systemic aldosteronism with added salt (4,5). In the latter, the high plasma aldosterone level and the still ambiguous effect of sodium chloride result in major damage to cardiac cells and fibrosis. In contrast, cardiac fibrosis did not develop in our TG model, likely because the increase in the cardiac concentration of aldosterone was less marked than in the aldo-salt model. On the other hand, this mouse model demonstrated a specific effect on coronary vascular function that has not, to date, been described by aldosterone-salt challenge. Therefore, our results indicate that coronary arteries may be a target for aldosterone. This finding extends previous observations obtained from the rat aldosterone-salt model, in which fibrinoid necrosis and the presence of inflammatory cells surrounding coronary arteries have been demonstrated (33). More recently,

several laboratories have shown a pericoronary inflammatory phenotype that appears early in the aldosterone-salt-treated rats (6-8,34). Preliminary results indicate that there was no inflammatory cells proliferation around coronary arteries of TG mice hearts, probably because of the slight increase of cardiac aldosterone. Again, the present mouse model where plasma aldosterone was not elevated, reveals aldosterone-induced alterations whose origin is cardiac specific.

The present study provides the first evidence that locally produced aldosterone alters coronary vascular function. Since the pathophysiological relevance of cardiac aldosterone synthesis has been questioned, it is important to underscore that this work demonstrates a novel deleterious effect of cardiac aldosterone. Moreover, it opens new perspectives by suggesting that at levels below those inducing cardiac fibrosis, locally produced aldosterone may have discrete effects that may facilitate the progression towards disease. At last, since it induces a coronary dysfunction at levels in the range of those reached in clinical situations, it may be hypothesized that the beneficial effects of anti-aldosterone treatment in cardiovascular pathologies such as congestive HF may be due, in part, to protection against aldosterone-induced alterations of coronary function. This point deserves further investigation.

Acknowledgements.

The authors thank Stephane Cailmail, Françoise Marotte, Philippe Mateo, Patricia Oliviero and Estelle Robidel for expert technical assistance, Dr Sylvie Salenave for hemodynamic measurements, Drs Jeffrey Robbins and Thierry Pedrazzini for gift of MHC promoter, Pr Celso Gomez-Sanchez for gift of anti-AS antibody, Dr Leigh Pascoe for gift of adrenoreductase, Prs Alessandro Capponi and Pierre Corvol, Drs Lydie Rappaport and Jane-Lise Samuel for helpful discussions. This work was supported by Pharmacia France (grant #92036-4), by Inserm and CNRS and Fondation de France. AG was recipient of a Pharmacia fellowship grant .

REFERENCES

1. Pitt B, Zannad F, Remme WJ, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. *N Engl J Med* 1999;341:709-717.
2. Pitt B, Remme W, Zannad F, et al. Eplerenone, a Selective aldosterone Blocker, in Patients with Left Ventricular Dysfunction after Myocardial Infarction. *N Engl J Med* 2003;348:1309-21.
3. Delcayre C, Swynghedauw B. Molecular mechanisms of myocardial remodeling. The role of aldosterone. *J Mol Cell Cardiol* 2002;34:1577-84.
4. Brilla C, Matsubara LS, Weber KT. Anti-aldosterone treatment and the prevention of myocardial fibrosis in primary and secondary hyperaldosteronism. *J Mol Cell Cardiol* 1993;125:563-575.
5. Robert V, Silvestre JS, Charlemagne D, et al. Biological determinants of aldosterone-induced cardiac fibrosis in rat. *Hypertension* 1995;26:971-978.
6. Rocha R, Rudolph AE, Friedrich GE, et al. Aldosterone induces a vascular inflammatory phenotype in the rat heart. *Am J Physiol Heart Circ Physiol* 2002;283:H1802-H1810.
7. Sun Y, Zhang J, Lu L, Chen SS, et al. Aldosterone-induced inflammation in the rat heart. *Am J Pathol.* 2002;161:1773-1781.
8. Gerling IC, Sun Y, Ahokas RA, Wodi LA, Bhattacharya SK, Warrington KJ, Postlethwaite AE, Weber KT. Aldosteronism: an immunostimulatory state precedes proinflammatory/fibrogenic cardiac phenotype. *Am J Physiol Heart Circ Physiol.* 2003;285:H813-21.
9. Silvestre JS, Robert V, Heymes C, et al. Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. *J Biol Chem* 1998;273:4883-4891.

10. Silvestre JS, Heymes C, Oubénaïssa A, et al. Activation of cardiac adosterone production in rat myocardial infarction: effect of angiotensin II blockade and role in cardiac fibrosis. *Circulation* 1999;99:2694-2701.
11. Casal AJ, Silvestre JS, Delcayre C, Capponi AM. Expression and modulation of the Steroidogenic Acute Regulatory (StAR) protein mRNA in rat cardiocytes and after myocardial infarction. *Endocrinology* 2003;144:1861-1868.
12. Young MJ, Clyne CD, Cole TJ, Funder JW. Cardiac steroidogenesis in the normal and failing heart. *J Clin Endocrinol Metab* 2001;86:5121-5126.
13. Yoshimura M, Nakamura S, Ito T, et al. Expression of aldosterone synthase gene in failing human heart: quantitative analysis using modified real-time polymerase chain reaction. *J Clin Endocrinol Metab* 2002;87:3936-40.
14. Tsybouleva N, ZhangL, Chen S, et al. Aldosterone, through novel signaling proteins, is a fundamental molecular bridge between the genetic defect and the cardiac phenotype of hypertrophic cardiomyopathy. *Circulation* 2004;109:1284-91.
15. Mizuno Y, Yoshimura M, Yasue H, et al. aldosterone production is activated in failing ventricle in humans. *Circulation* 2001;103:72-77.
16. Subramaniam A, Jones WK, Gulick J, et al. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem* 1991;266:24613-20.
17. Oliviero P, Chassagne C, Kolar F, et al. Effect of pressure overload on angiotensin receptor expression in the rat heart during early post-natal life. *J Mol Cell Cardiol* 2000;32:1631-1645.
18. Cherradi N, Brandenburger Y, Rossier MF, et al. Atrial natriuretic peptide inhibits calcium-induced steroidogenic acute regulatory protein gene transcription in adrenal glomerulosa cells. *Mol Endocrinol* 1998;12:962-72.
19. Beggah AT, Escoubet B, Puttini S, et al. Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. *Proc Natl Acad Sci USA* 2002; 99: 7160-7165.

20. Georget M, Mateo P, Vandecasteele G, et al. Augmentation of cardiac contractility with no change in L-type Ca²⁺ current in transgenic mice with a cardiac-directed expression of the human adenylyl cyclase type 8 (AC8). *FASEB J.* 2002;16:1636-8.
21. Bendall JK, Heymes C, Wright TJ, et al. Strain-dependent variation in vascular responses to nitric oxide in the isolated murine heart. *J Mol Cell Cardiol.* 2002;34:1325-33.
22. Tessier S, Karczewski P, Krause EG, et al. Regulation of the transient outward K⁺ current by Ca²⁺/calmodulin-dependent protein kinases II in human atrial myocytes. *Circulation Research* 1999;85:810-819.
23. White PC. Aldosterone: direct effects on and production by the heart. *J Clin Endocrinol Metab* 2003;88:2376-83.
24. Weber KT. Aldosterone in congestive heart failure. *N Engl J Med* 2001;345:1689-97.
25. Benitah JP, Vassort G. Aldosterone upregulates Ca(2+) current in adult rat cardiomyocytes. *Circ Res.* 1999;85:1139-45.
26. Benitah JP, Perrier E, Gomez AM, Vassort G. Effects of aldosterone on transient outward K⁺ current density in rat ventricular myocytes. *J Physiol* 2001;537:151-160.
27. Struthers AD, MacDonald TM. Review of aldosterone- and angiotensin II-induced target organ damage and prevention. *Cardiovascular Research.* 2004;61:663-670.
28. Farquharson CAJ, Struthers AD. Spironolactone increases nitric oxide bioactivity, improves endothelial vasodilator dysfunction, and suppresses vascular angiotensin I/angiotensin II conversion in patients with chronic heart failure. *Circulation* 2000;101:594– 7.
29. Bauersachs J, Heck M, Fraccarollo D, et al. Addition of spironolactone to angiotensin-converting enzyme inhibition in heart failure improves endothelial vasomotor dysfunction: role of vascular superoxide anion formation and endothelial nitric oxide synthase expression. *J Am Coll Cardiol.* 2002;39:351-8.

30. Viridis A, Neves MF, Amiri F, et al. Spironolactone improves angiotensin-induced vascular changes and oxidative stress. *Hypertension* 2002 ;40:504-10.
31. Rajagopalan S, Duquaine D, King S, et al. Mineralocorticoid receptor antagonism in experimental atherosclerosis. *Circulation* 2002;105: 2212–6.
32. Lariviere R, Deng LY, Day R, et al. Increased endothelin-1 gene expression in the endothelium of coronary arteries and endocardium in the DOCA-salt hypertensive rat. *J Mol Cell Cardiol.* 1995;27:2123–2131.
33. Robert V, NV Thiem, SL Cheav, et al. Increased cardiac collagens (I) and (III) mRNAs in aldosterone-salt hypertension. *Hypertension* 1994; 24: 30-36.
34. Young MJ, Moussa L, Dilley R, Funder JW. Early inflammatory responses in experimental cardiac hypertrophy and fibrosis: effects of 11 beta-hydroxysteroid dehydrogenase inactivation. *Endocrinology.* 2003;144:1121-5.

FIGURE LEGENDS

Figure 1. **Cardiac overexpression of AS gene.** **a)** Transgenic construct of the α -MHC promoter driving AS expression. **b)** AS mRNA expression in mouse ventricles of TG and WT mice. **c)** Western blot of AS in adrenal glands and ventricles of TG and WT mice. Abbreviations WT: wild-type; TG: transgenic; Adr: adrenal gland; V: ventricles.

Figure 2. **AngII receptors and collagen quantification in 15 wk-old male mouse ventricles.** **a)** Specific binding for AngII receptors was calculated as total minus non-specific binding. n = 6-10. **b)** Sirius red collagen staining of ventricular tissue. Bar = 1 mm. **c)** Total collagen volume fraction. n = 10-12. Abbreviations as in Fig. 1.

Figure 3. **Patch-clamp analysis of calcium and potassium currents in 27 wk-old isolated ventricular myocytes.** Current density to voltage relationship in 4 month-old mice **a)** Slow calcium I_{CaL} under basal conditions (WT: n=8, TG: n=10) and following stimulation by isoproterenol (ISO, 0.1mmol/L) (n= 6). **b)** Potassium current I_{to1} (n= 10). **c)** Sustained potassium current I_{sus} (WT: n=8, TG: n=10). All activation and inactivation curves were similar in WT and TG (not shown).

Figure 4. **Cardiac contractile function of 15 wk-old male mice.** Hearts were perfused in Langendorff mode at 80 mm Hg. Left ventricular developed pressure (LVDevP) response changes of Ca^{2+} (n = 7). Abbreviations as in Fig. 1.

Figure 5. **Coronary function of 15 wk-old WT and TG male mice.** Hearts were perfused in Langendorff mode at constant flow without an intraventricular balloon. The changes in CPP in response to acetylcholine (**a**), bradykinin (**b**), and sodium nitroprusside (**c**). n = 6. Abbreviations as in Fig. 1; CPP: coronary perfusion pressure; SNP: sodium nitroprusside.

Table 1. Phenotype of male Wild Type (WT) and Transgenic (TG) mice.

	WT		TG	
Age	15 wks	27 wks	15 wks	27 wks
Anatomy	(n=9)		(n=9)	
Systolic pressure, mm Hg	151±13		152±15	
Diastolic pressure, mm Hg	110±31		109±37	
Body weight, g	25.5±0.5		26.2±0.5	
Ventricles weight, mg	101.1±3.1		109.4±3.6	
VW/BW, mg/g	3.97±0.11		4.17±0.9	
Adrenal glands weight, mg	2.47±0.11		2.36±0.06	
Adrenal glands W/BW	0.10±0.01		0.09±0.00	
Ventricles	(n=4)		(n=4)	
Capillaries density, n/mm ²	39.1±3.2		41.5±3.0	
Myocytes density, n/mm ²	23.4±3.3		21.5±1.3	
Capillaries/myocytes ratio	1.6±0.19		1.9±0.14	

Echocardiography		(n=11)	(n=6)	(n=11)	(n=6)
Heart rate, bpm		545±17	530±15	518±15	516±13
LVEDD/BW		0.16±0.00	0.16±0.01	0.17±0.00	0.18±0.01
EF, %		76±3	86±3	78±3	82±3
VcFc, circ/s		2.42±0.15	2.98±0.2	2.53±0.18	2.73±0.14
IVRT, ms		12.4±0.5	15.0±0.91	13.6±0.67	18.0±1.6
E/Ea		23.9±1.53	19.8±1.31	26±1.78	24.1±1.16
Hormones (nM/L)		(n=6)		(n=6)	
Aldosterone	Plasma	0.40±0.09		0.60±0.22	
	Adrenals	128±38		158±11	
	Ventricles	9.8±1.6		16.8±2.1*	
Corticosterone	Plasma	422±90		551±320	
	Adrenals	12698±3799		12917±2380	
	Ventricles	111±22		99±12	

*p<0.02. Abbreviations: VW: Ventricles Weight; BW: Body Weight; AGW: Adrenal Glands Weight; EDD: End Diastolic Diameter; EF: Ejection Fraction; VcFc: Velocity of circumferential Fibers shortening corrected for HR; IVRT: IsoVolumic Relaxation Time; E: maximal velocity of E wave of transmitral blood flow; Ea: maximal velocity of E wave of the mitral annulus tissue Doppler.

DATA SUPPLEMENT TO MANUSCRIPT

METHODS

Transgenic mice. Experimental procedures were conducted in accordance with institutional guidelines and those formulated by the European community for use of experimental animals (L358-86/609/EEC). The 1500 bp coding sequence of AS gene (NCBI accession number #D14092) was amplified by RT-PCR from rat adrenal gland RNA. AS expression was driven by the mouse alpha-MHC gene promoter (1). The transgene (Fig. 1a), composed of a 1.5 kb rat AS cDNA linked to the 5.8 kb mouse alpha-MHC gene promoter and containing 1.2 kb of the beta-globin intronic sequence (16), was microinjected into fertilized eggs of FVB mice. Two independent founder lines (8282 M and 8283 M) were established.

Blood pressure was measured with a Millar probe (EMKA, Paris, France). Mice were anesthetized by I.P. injection of sodium pentobarbital (60 mg/kg) and blood was collected for hormone assays. Hearts were frozen in liquid nitrogen and stored at -80°C until use.

Collagen and AngII receptors assay. Quantification of collagen was determined on equatorial heart 7 µm sections stained with picosirius red F3BA as described (2). Binding analysis of AngII-receptors was performed on 20 µm equatorial sections of rat ventricles using ¹²⁵I-Sar1-Ile8-AngII, as described (3). AT-receptor subtypes were identified by incubation with losartan or PD 123319.

RNA analysis. Extraction of heart total RNA and RT-PCR of AS and 11-beta-hydroxylase were performed as described (3). Total RNA yields were not different in WT and TG mice. AS and 11-beta-hydroxylase were co-amplified to give a 633 bp PCR product, and subsequently differentiated using *Ava*I and *Nhe*I restriction enzymes. Presence of an additional *Ava*I restriction site in rat AS sequence enabled quantification independently of endogenous mouse AS. PCR products were separated on 5% PAGE and signals analyzed using a Fujifilm LAS-1000 phosphoimager (Fuji, Paris, France).

Realtime PCR. Gene sequences were obtained from Genbank database. Primers were designed using Primer express 1.5 Software (ABI Prism, Paris, France). Primers sequence were : Endothelin-1 type a receptor (Eta) (accession number GeneBank NM_010332):5'AAACCTCCCAAgtTCTCTCCCAT3' (forward) and 5'GAAATGACATGCGCGGTATG3' (reverse); Pre-proendothelin (PPET) (accession number GeneBankAB081657): 5'CTggACATCATCTgggTCAACA3'(forward) and 5'TCTTgTCTTTTTggTgAgCgC3' (reverse); Endothelin converting enzyme (ECE) (accession number GeneBankNM_199307): 5'TgAACTTCggTggCATTggT3'(forward) and 5'CACgCTgTAgTTgCTgTATTgCT3'(reverse). Primers for GAPDH were purchased from the Taqman rodent GAPDH control reagents kit (Applied Biosystems, Paris, France). PCR reactions were run using a Perkin Elmer GeneAmp 5700 Sequence Detection System (Applied Biosystems, Paris, France). mRNA expression levels were given as Ct (threshold cycle number). Gene expressions were normalized using the GAPDH gene.

Western blot. Mitochondrial proteins were isolated from ventricles (4) and proteins assayed (5). Blots were incubated with an antibody against the rat AS that detected the mouse AS (kind gift of Dr Gomez-Sanchez) (dilution 1:10,000) for 2 hours at RT. AS protein was detected using an ECL+ kit (Amersham, Paris, France) and revealed using the Fuji phosphoimager.

Hormones. Hearts were homogenised in 1 mL methanol with an added minute amount of tritium-labelled tracer. Cardiac homogenates were centrifuged 15 min at 15,000 rpm, the supernatant dried under vacuum and redissolved in the kit buffer. Aldosterone was assayed by radioimmunoassay using a Immunotech kit (Beckman, Marseille, France), and corticosterone with an in-house radioimmunoassay.

Echocardiography. Echocardiography was performed as published (6).

Isolated heart perfusion. Hearts were perfused in non-recirculating Langendorff mode at constant pressure (80 mm Hg) as published (7). After 15 min of equilibration hearts were paced at 650

beats/min. Contractile properties were recorded using an intraventricular balloon. Sensitivity to external calcium (Ca_o) was studied by changing Ca_o stepwise from 0.5 to 2.5 mM.

Coronary function. Coronary vascular responsiveness to vasoactive agents was measured as described (8). Briefly, hearts were perfused in non-recirculating Langendorff mode at constant flow and the change in coronary perfusion pressure measured as an index of coronary vascular tone. During 20-min equilibration, coronary flow (CF) was adjusted to achieve a coronary perfusion pressure (CPP) of 60 ± 5 mmHg. CPP was measured via a pressure transducer and data recorded using Chart software. Vasoactive drugs were administered by bolus injections just above the aortic cannula.

Electrophysiological analysis of calcium and potassium currents. Ventricular myocytes were isolated from hearts by perfusion with Ca^{2+} -free Tyrode's solution containing liberase blendzyme III (Roche, Paris, France). Whole cell patch-clamp was used to record the L-type Ca^{2+} current ($I_{Ca,L}$), and the time-dependent and time-independent components of the potassium transient outward currents, respectively I_{to1} and I_{SUS} . $I_{Ca,L}$ was measured in the presence of external barium (0.5 mM), 4-aminopyridine (2 mM) and tetrodotoxin (25 μ M, from Latoxan, France) using a routine protocol consisting in an initial short prepulse at -50 mV (50 msec duration) followed by a test pulse to 0mV (400 msec duration) elicited every 8 sec from a holding potential of -80 mV. Although tetrodotoxin was used in the external solution to block the fast Na^+ current (I_{Na}), the short prepulse was aimed at inactivating any residual I_{Na} which might otherwise contaminate the measure of $I_{Ca,L}$ during the test pulse. It was also used at examining the presence of a T-type Ca^{2+} current ($I_{Ca,T}$). I_{to1} and I_{SUS} were measured by depolarising the cells during 400 ms in presence of tetrodotoxin and cadmium (0.5 mM) (9).

Statistical analysis. Results were expressed as means \pm SEM. Mean values were compared by one-way ANOVA followed by a Fisher's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Endothelin-1. Using real time PCR, the expression of mRNAs coding the Endothelin converting enzyme (ECE), Pre-proendothelin (PPET) and the Endothelin type a receptor (Eta) have been assayed. Gene expressions were normalized using the GAPDH gene. There was no statistical difference in the levels of expression of these genes in TG mice ventricles compared to WT ones (Table 1). The ratios of expression (TG/WT) were 0.99 for ECE, 1.04 for PPET and 1.03 for Eta mRNAs (n=5).

T-type Ca²⁺ channels currents. According to the electrophysiological characteristics of I_{Ca,T}, that current should activate at voltages <-50 mV, so that some I_{Ca,T} should have been measured during the prepulse in our recording conditions. However, no current at all (neither unblocked I_{Na} nor I_{Ca,T}) was detected during this prepulse, neither under basal conditions (WT: n=8, TG: n=10), nor during beta-adrenergic stimulation (n=6). These results indicate that T-type calcium current was absent in ventricular myocytes from both WT and TG mice.

References

1. Subramaniam A, Jones WK, Gulick J, et al. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem* 1991;266:24613-20.
2. Silvestre JS, Heymes C, Oubénaïssa A, et al. Activation of cardiac adosterone production in rat myocardial infarction: effect of angiotensin II blockade and role in cardiac fibrosis. *Circulation* 1999;99:2694-2701.
3. Oliviero P, Chassagne C, Kolar F, et al. Effect of pressure overload on angiotensin receptor expression in the rat heart during early post-natal life. *J Mol Cell Cardiol* 2000;32:1631-1645.

4. Cherradi N, Brandenburger Y, Rossier MF, et al. Atrial natriuretic peptide inhibits calcium-induced steroidogenic acute regulatory protein gene transcription in adrenal glomerulosa cells. *Mol Endocrinol* 1998;12:962-72.
5. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.
6. Beggah AT, Escoubet B, Puttini S, et al. Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. *Proc Natl Acad Sci USA* 2002; 99: 7160-7165.
7. Georget M, Mateo P, Vandecasteele G, et al. Augmentation of cardiac contractility with no change in L-type Ca²⁺ current in transgenic mice with a cardiac-directed expression of the human adenylyl cyclase type 8 (AC8). *FASEB J.* 2002;16:1636-8.
8. Bendall JK, Heymes C, Wright TJ, et al. Strain-dependent variation in vascular responses to nitric oxide in the isolated murine heart. *J Mol Cell Cardiol.* 2002;34:1325-33.
9. Tessier S, Karczewski P, Krause EG, et al. Regulation of the transient outward K⁺ current by Ca²⁺/calmodulin-dependent protein kinases II in human atrial myocytes. *Circulation Research* 1999;85:810-819.

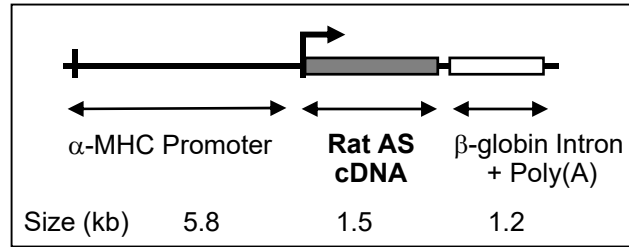
Table 1. RT-PCR analysis of of mRNAs coding the Endothelin converting enzyme (ECE), Pre-proendothelin (PPET) and the Endothelin type a receptor (Eta).

	WT	TG	TG/WT ratio
ECE/GAPDH (Ct)	1.34±0.10	1.33±0.09	0.99
PPET/GAPDH (Ct)	1.45±0.12	1.52±0.13	1.04
Eta/GAPDH (Ct)	1.39±0.12	1.43±0.10	1.03

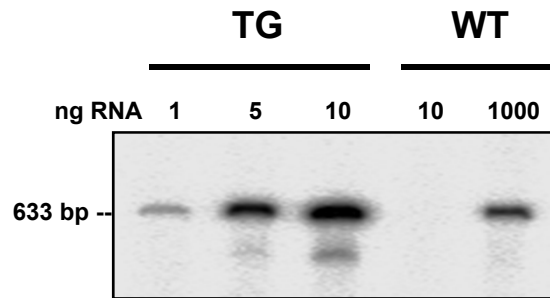
mRNA expression levels were given as Ct (threshold cycle number) (n = 5). Gene expressions were normalized using the GAPDH gene. WT: Wild Type; TG: Transgenic.

Fig 1

a)



b)



c)

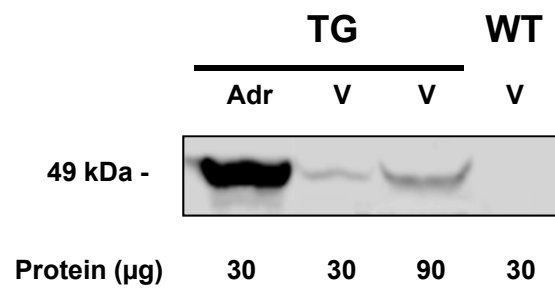


Fig 2

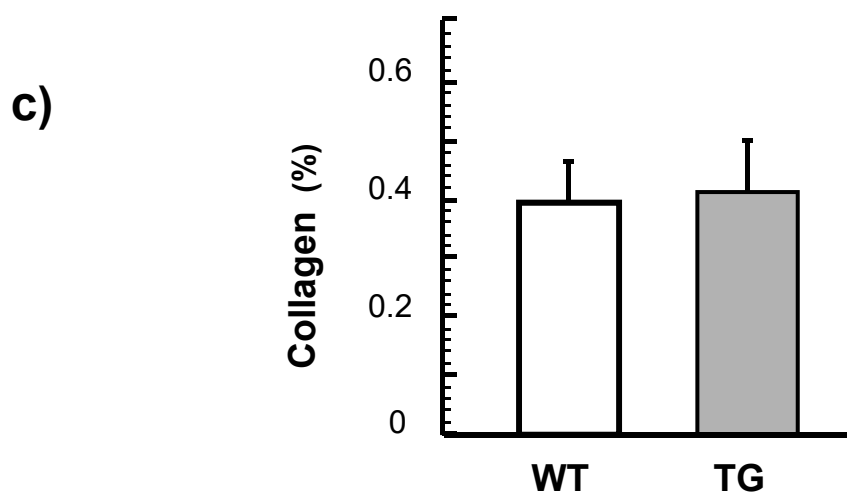
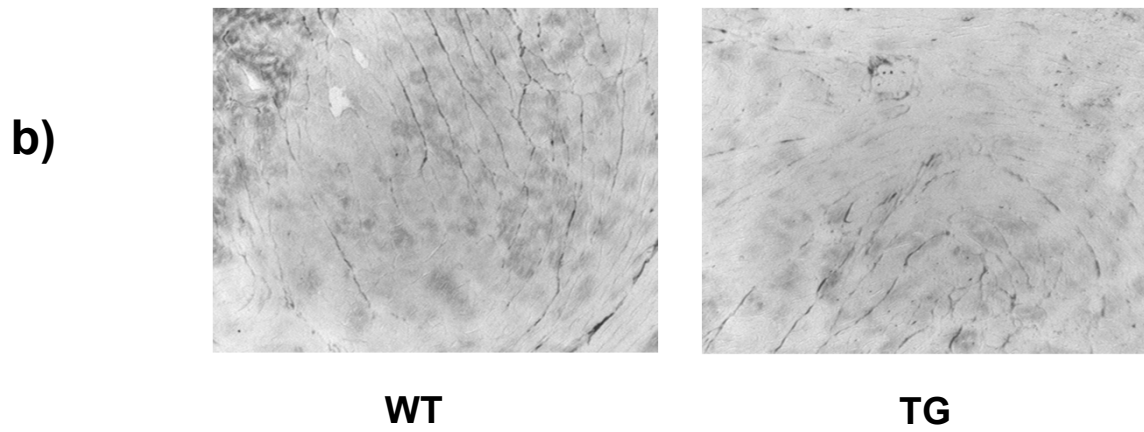
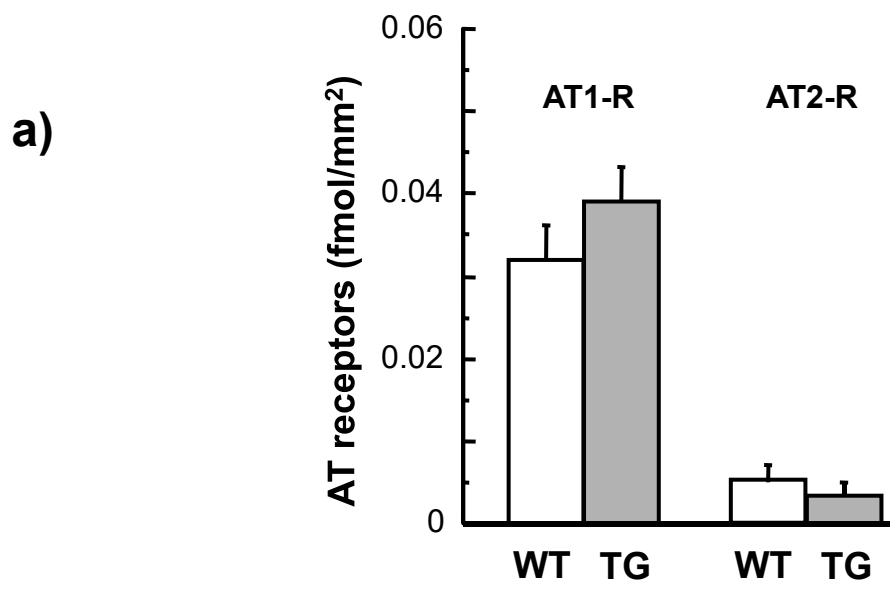


Fig 3

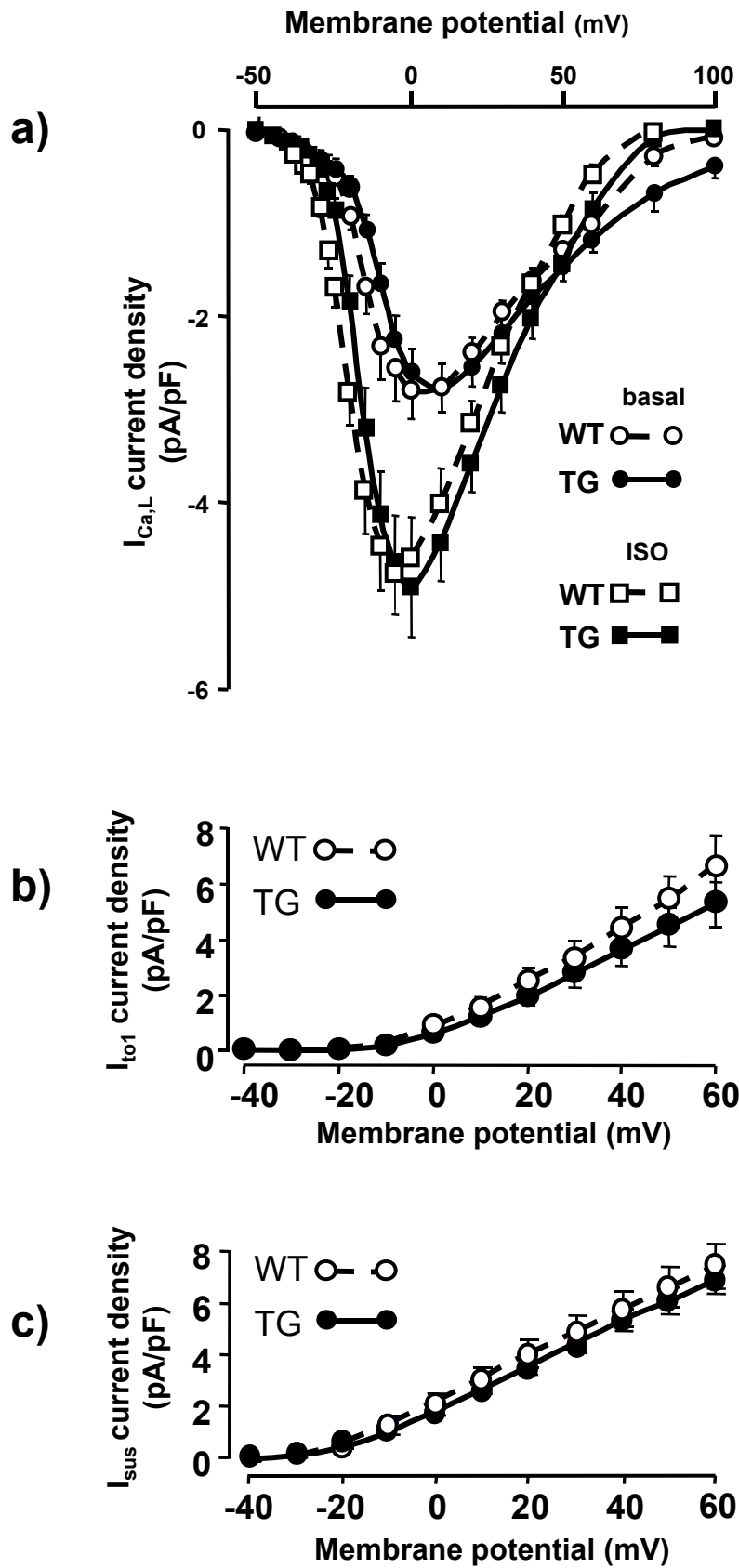


Fig 4

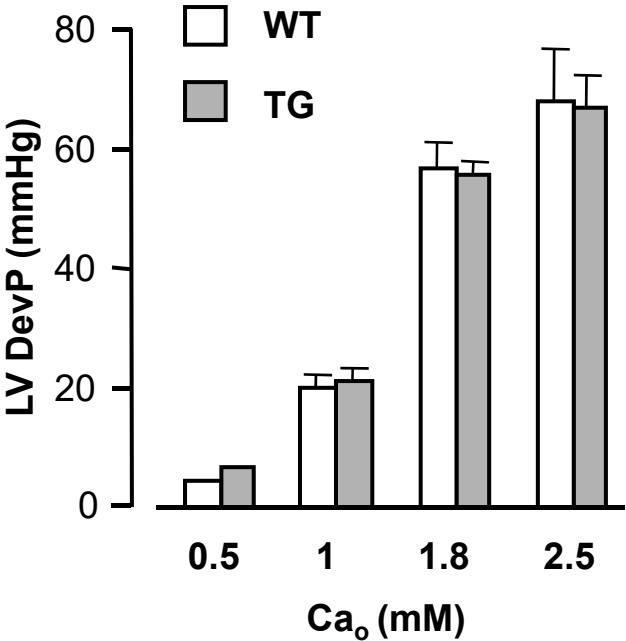


Fig 5

