EXPERIMENTAL STUDY

Effect of amiodarone and dronedarone administration in rats on thyroid hormone-dependent gene expression in different cardiac components

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Abstract

Objective: In view of their different actions on thyroid hormone receptor (TR) isoforms we set out to investigate whether amiodarone (AM) and dronedarone (Dron) have different and/or component-specific effects on cardiac gene expression.

Design: Rats were treated with AM or Dron and the expression of TR α 1, TR α 2, TR β 1 and several tri-iodothyronine (T₃)-regulated genes was studied in different parts of the heart, namely the right atrium (RA), left ventricular wall (LVW) and apex.

Methods: Rats were treated for 14 days with 100 mg/kg body weight AM or Dron. The expression of TR α 1, TR α 2, TR β 1 and T₃-regulated genes was studied using real-time PCR and non-radioactive *in situ* hybridisation.

Results: AM and Dron affected TR expression in the RA similarly by decreasing TR α 1 and β 1 expression by about 50%. In the LVW, AM and Dron decreased TR β 1 and, interestingly, AM increased TR α 1. In the apex, AM also increased TR α 2. The changes seen in T₃-dependent gene expression are reminiscent of foetal reprogramming.

Conclusion: Taken together, our results indicate that AM and Dron have similar effects on the expression of TR isoforms in the RA, which could partly contribute to their ability to decrease heart rate. On the other hand, the more profound effect of AM appears on TR- and T₃-dependent gene expression in the left ventricle suggests foetal reprogramming.

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Introduction

Thyroid hormone (TH) has profound effects on the heart via the different TH receptor isoforms, for example as a chronotrope. Specialised cells generate the rhythm and guide the impulse throughout the heart. The conduction system can be divided into the sino-atrial node (SAN), the atrioventricular node (AVN) and the peripheral ventricular conduction system (PVCS). From our recent studies, it has become apparent that the TH receptor (TR) isoforms α 1, α 2 and β 1 are expressed in a component-specific manner in the heart (2). In the mouse, it has been found that TR α 1 is important in setting heart rhythm and regulating the expression of genes important for cardiac function such as α -myosin heavy chain (α MHC; up-regulated by TH), β MHC (down-regulation reciprocal to α MHC), sarcoplasmic reticulum Ca-ATPase2a (SERCA2a; up-regulated by TH) and guanine nucleotide regulatory proteins (like HCN4; up-regulated by TH; (3, 4)). Furthermore, TR β 1 appears to have a function in mediating the tri-iodothyronine (T₃)-induced increase in the heart rate in the mouse (5).

The situation is slightly different in the rat where it has been suggested that TR β 1 also plays a role in the expression of SERCA2a and β MHC (6, 7).

We speculated whether the component-specific expression of TRs had any relation to certain TH antagonistic effects of the class III anti-arrhythmic drugs amiodarone (AM) and dronedarone (Dron). AM has been suggested to induce a localised hypothyroid condition in the heart (8), which is partly due to its TR inhibiting action (9, 10). Similarly Dron also appears to mimic hypothyroidism at the level of the heart (6, 11). Furthermore, it has been shown that a high dose of AM intensifies cardiac remodelling (12). Remodelling after cardiac failure by itself has been suggested to bring about a hypothyroid cardiac phenotype with accompanying changes in TR expression as well as changes in the expression of genes like SERCA (13). In the present study, we look at a combination of both phenomena on the expression of the TR isoforms α 1, α 2 and β 1, and the T₃-dependent genes α and β MHC, SERCA2a, guanine nucleotide regulatory protein (HCN4) and atrial natriuretic factor (ANF) in the different components of the rat heart.

Materials and methods

In vivo studies

Male Wistar rats, divided into three groups (eight rats per group), were housed under normal conditions with free access to standard lab chow and tap water. The rats received either water (controls), an aqueous suspension of Dron 100 mg/kg body weight or AM 100 mg/kg body weight by gastric tubes daily for 2 weeks as described previously (11). Rats were anaesthetised and killed by i.p. infusion of 0.5 mg/kg medetomidine (Dormitor 1 mg/ml) and 0.75 mg/kg ketamine (Ketalar 10 mg/ml). Blood was collected by cardiac puncture and plasma was stored at -20°C . The hearts were removed and washed in PBS. Five hearts of each group were dissected under a stereomicroscope into right atria, part of the left ventricle wall and cardiac apex. These components were immediately frozen and stored in liquid nitrogen for isolation of mRNA. Three hearts were washed in PBS and processed for *in situ* hybridisation. The animal experiments were approved by the animal welfare committee of the Academic Medical Centre, University of Amsterdam.

Plasma assays

Total thyroxine (T_4) and T_3 were measured by an in-house RIA (14), using rat null plasma as diluent. TSH was determined by Delfia fluoroimmunoassay (Perkin–Elmer Wallac GmbH, Freiburg, Germany). Plasma total cholesterol was measured using a fully enzymatic dye method (Modular P analyzer, Roche Molecular Biochemicals). All samples were measured in one run. Data are expressed as mean $\pm$ s.d.

mRNA analyses by real-time PCR

mRNA from the right atrium (RA), apex and left ventricular wall (LVW) was obtained with the MagNa Pure LC RNA Isolation Kit II (Tissue; Roche) using the MagNa Pure LC Instrument (Roche), after which cDNA was made using the first-strand cDNA synthesis kit (containing avian myeloblastosis virus (AMV) reverse transcriptase) on which real-time PCR was performed using the LightCycler (Roche). Primers were purchased from Biolegio BV (Malden, The Netherlands). $\text{TR}\alpha 1$ and $\text{TR}\alpha 2$ were quantified as described previously (15). For

$\text{TR}\beta 1$, primer sequences were as follows: forward, 5'-CACCTGGATCCTGACGATGT-3' and reverse, 5'-ACAGGTGATGCAGCGATAGT-3'. SERCA2a was detected using the following primers: forward, 5'-ATGGACGA-GACGCTCAAGTT-3'; reverse, 5'-GAAGCGGTTACTC-CAGTATTGC-3'; ANF using forward, 5'-AACACA-GATCTGATGGATTTCAAG-3'; reverse, 5'-CGCTTCATC-GGTCTGCTC-3'; α MHC using forward, 5'-CTGCAGTCA-GCCCTGGAG-3'; reverse, 5'-GGAGGATCTTGCCCTCCT-3'; β MHC using forward, 5'-CATCAAGGAGCTCACC-TACCA-3'; reverse, 5'-TCCTGCAGTCGCACTAGGTT-3'; and finally HCN4 using forward, 5'-ATCCAGTCGCTG-GACTCCT-3'; reverse, 5'-TGTACTGCTCCACCTGCTTG-3'.

Non-radioactive *in situ* hybridisation for T_3 -regulated genes

The hearts were processed for *in situ* hybridisation using a fixation protocol and digoxigenin (DIG)-labelled, single-strand RNA probes as described previously (16). All probes have also been described previously: SERCA2a (17), α MHC and β MHC (18), HCN4 (19) and ANF (20). Images of sections were taken using a digital camera Leica DFC 320 RZ 0793, coupled with a Zeiss Axiophot microscope, equipped with differential interference contrast optics.

Statistical analysis

Differences between groups in plasma TH parameters and body weights were evaluated using ANOVA followed by a *t*-test when the ANOVA appeared significant to identify individual changes. Differences between groups in mRNA levels were evaluated using the Mann–Whitney test.

Results

Plasma parameters and body weight

To confirm the efficacy of the drug treatment, we checked several plasma parameters (Table 1). As expected, AM-treated animals had higher T_4 , TSH and total cholesterol and lower T_3 plasma concentrations than either Dron-treated animals or controls (11). Body weight

Table 1 Plasma parameters and body weight (BW).

	Control	Dron	AM	ANOVA (<i>P</i>)
TSH (ng/ml)	2.11 $\pm$ 0.80	1.45 $\pm$ 0.75	6.70 $\pm$ 3.39*	<0.0001
T_4 (nmol/l)	72 $\pm$ 10	68 $\pm$ 14	150 $\pm$ 21*	<0.0001
T_3 (nmol/l)	1.21 $\pm$ 0.13	1.09 $\pm$ 0.19	0.78 $\pm$ 0.11*	<0.0001
Total cholesterol (mmol/l)	1.94 $\pm$ 0.26	1.95 $\pm$ 0.25	2.89 $\pm$ 0.53*	<0.0001
Baseline BW (g)	287 $\pm$ 9	295 $\pm$ 9	288 $\pm$ 7	N.S.
Δ BW (g)	64 $\pm$ 13	58 $\pm$ 7	41 $\pm$ 11*	<0.0001

Values are represented as mean $\pm$ s.d. **P* < 0.01 versus controls.

gain during the experiment in the AM-treated animals was lower compared with the Dron-treated group and controls.

Cardiac component-specific TR gene expression

We studied the mRNA expression of TR isoforms $\alpha 1$, $\alpha 2$ and $\beta 1$ in three parts of the heart, which represent different cardiac components, namely the RA (including the SAN), the LVW (mainly working myocardium) and the apex (contains a large proportion of PVCS). Dron caused a decreased expression of TR $\alpha 1$ in the RA but had no effect in the LVW or apex. TR $\alpha 2$ expression was not influenced by Dron. TR $\beta 1$ expression in RA, LVW and apex was decreased by Dron (Fig. 1). AM lowered TR $\alpha 1$

expression in the RA, but interestingly increased it in the LVW. In the apex, no change was seen in TR $\alpha 1$, but TR $\alpha 2$ increased as a result of AM (Fig. 1). TR $\beta 1$ expression was decreased by AM in all the three components (Fig. 1).

TR-regulated cardiac genes

The same parts of the rat heart were also used to quantify mRNA expression by PCR of a number of T₃-dependent genes after AM or Dron treatment, in parallel with visualisation using *in situ* hybridisation. From the latter experiments, it appears that in the control animals, SERCA2a and α MHC are expressed throughout the heart. ANF is expressed in the atria, apex and PVCS

Figure 1 Expression of the TR isoforms $\alpha 1$, $\alpha 2$ and $\beta 1$ in different cardiac components. The mRNA values of control, AM- and Dron-treated rats are corrected for GAPDH and presented as mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus controls.

(Figs 3 and 4). Although α MHC is expressed throughout the heart, β MHC is found only in the PVCs. HCN4 showed positive staining only in the SAN.

Dron treatment caused a decrease in α MHC expression in the cardiac apex and shows a trend towards decreased ANF expression in the LVW (Fig. 2). None of the other genes studied were affected by Dron treatment. This is reflected in the *in situ* hybridisation results as shown in Fig. 3. SERCA2a expression in the RA showed a trend towards a decrease due to AM treatment ($P < 0.1$). AM increased ANF expression in

the apex (Figs 3 and 4). Furthermore, it caused a reciprocal regulation of α MHC and β MHC in this component; α MHC showed a trend towards a decrease in the RA ($P < 0.1$) and decreased in the apex, whereas β MHC increased in both the components. No difference between the effect of AM and Dron on α MHC expression in the apex was observed. The same observations can be made looking at the *in situ* hybridisation data (Fig. 3). We did not observe any significant differences in the expression of HCN4 in the RA after AM or Dron treatment (mRNA data not shown; Fig. 4).

Figure 2 Expression of thyroid hormone-regulated genes in components of the rat heart. α MHC, β MHC, SERCA2a, HCN4 and ANF were measured in mRNA derived from different cardiac components of control, AM- and Dron-treated rats. Values are corrected for GAPDH and presented as mean $\pm$ s.d. # $P < 0.1$, * $P < 0.05$, ** $P < 0.02$ and *** $P < 0.01$ versus controls.

Figure 3 Cardiac mRNA expression of thyroid hormone-dependent genes. Overview of the left ventricle of control rats (left), Dron-treated (middle) and AM-treated (right) rats, stained with a sequence-specific, antisense probes, against ANF (first row), α MHC (second row), β MHC (third row) and SERCA2a (fourth row). LWV, left ventricular wall; A, apex. All images are oriented in the same direction.

Discussion

Here, we study the expression of TH-dependent genes and TRs in relation to certain TH antagonistic effects of the class III anti-arrhythmic drugs AM and Dron. In an earlier paper, we characterised our animal model (11), and the changes in TH parameters seen in this study are essentially the same as in our former one. Furthermore, we showed in our earlier study that both AM and Dron decreased the heart rate and significantly increased the QTc interval (11). We now analyse the mRNA levels, by both *in situ* hybridisation and quantitative PCR, of a

number of well-known T₃-dependent genes as well as the levels of the TR isoforms α 1, α 2 and β 1 in the three different components of the rat heart, namely the RA, the LWV and the apex. Since we did not find any major discrepancies between the mRNA and protein levels of the TR isoforms in an earlier paper (21) using hyper- and hypothyroid mice, we concentrate on the mRNA levels of the TR isoforms in the present study.

In the present study, we observe that TR β 1 is down-regulated by AM and Dron in all the heart components tested. This is probably due to a direct effect of the inhibition by these compounds of TR α 1 (11). Indeed,

Figure 4 Cardiac mRNA expression of thyroid hormone-dependent genes in the right atrium and SAN. Upper row – overview of the RA and superior vena cava (SVC), stained with an ANF-specific, antisense probe, reveals ANF expression throughout RA and SCV (positive staining is in blue). The SAN is indicated by an arrow. Middle row – higher magnification shows similar sizes of the SAN between control animals (left), Dron-treated (middle) and AM-treated animals (right). The SAN is indicated by an arrow. Bottom row – HCN4 is expressed in the SAN of the animals of all groups. The SAN is indicated by an arrow.

Sadow *et al.* (22) suggested that TR α 1 also has a major role in cardiac regulation of both itself and TR β 1. We found a decreased expression of TR α 1 in the RA, which contains the pacemaking components SAN and AVN due to both Dron and AM, which in case of AM has been shown before (23). The similarity of the effects of AM and Dron on TR isoform expression matches their ability to inhibit TR α 1, the isoform determining cardiac rhythm (3, 4). The down-regulation of TR α 1 itself could be a result of more complicated mechanisms involving TR α 1, TR β 1 and/or other (component-specific) factors (22). The latter suggestion is supported by our observation that TR α 1 is regulated in a component- and compound-specific manner. For instance, the effect of AM on the expression of TR α 1 in the LVW differs from that of Dron. In this component, TR α 1 is up-regulated by AM, which may signify a compensatory mechanism for the inhibition of T₃ action due to the drug. Interestingly, the effects of AM on heart rate and contractility resemble those of the euthyroid TR β 1 knock-in mouse (24) and correlates with AM's ability to inhibit both TR α 1 and TR β 1.

The ability of AM and Dron to differentially inhibit TR α 1 and TR β 1 is reflected in their effects on TR-regulated cardiac gene expression. We could not find changes in the expression of HCN4 in the RA despite the bradycardia in the AM-treated animals (8). The bradycardia could, however, be explained by the non-genomic effects of AM on β -adrenoceptor density (25).

As expected, AM showed a trend to suppress the expression of the TR α 1-dependent SERCA2a. In addition, α MHC shows a trend of being down-regulated in the RA and it is decreased in the apex by AM but not in the LVW, correlating with the up-regulation of TR α 1 in that compartment. Dron caused down-regulation of the TR α 1-dependent α MHC in the apex, which tallies with earlier reports showing that Dron changes α MHC expression but not β MHC and SERCA2a (6). The differences between Dron and AM could be due to AM's additional inhibition of TR β 1.

An interesting finding is that AM, but not Dron, causes an up-regulation of β MHC and ANF. Since AM antagonises both TR α 1 and TR β 1, our results support the earlier hypothesis that TR β 1 has an important role for β MHC regulation (7). Furthermore, ANF was up-regulated specifically in the apex, subendocardial and pericoronary cardiomyocytes, which form the PVCS, thus suggesting that TR β 1 may have a specific role there. The induction of β MHC and ANF as a result of AM treatment is reminiscent of the induction of these genes after heart failure and could indicate foetal reprogramming, as has been suggested by Kinugawa *et al.* (26).

Taken together, our results indicate that AM and Dron have similar effects on the expression of TR isoforms in the RA, which could partly contribute to their ability to decrease heart rate. On the other hand, the more profound effect of AM appears on TR- and

T₃-dependent gene expression in the left ventricle suggests foetal reprogramming.

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