

EXPERIMENTAL STUDY

Desethylamiodarone antagonizes the effect of thyroid hormone at the molecular level

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Abstract

Objective: To evaluate the molecular mechanisms of the inhibitory effects of amiodarone and its active metabolite, desethylamiodarone (DEA) on thyroid hormone action.

Materials and methods: The reporter construct ME-TRE-TK-CAT or TSH β -TRE-TK-CAT, containing the nucleotide sequence of the thyroid hormone response element (TRE) of either malic enzyme (ME) or TSH β genes, thymidine kinase (TK) and chloramphenicol acetyltransferase (CAT) was transiently transfected with RSV-TR β into NIH3T3 cells. Gel mobility shift assay (EMSA) was performed using labelled synthetic oligonucleotides containing the ME-TRE and *in vitro* translated thyroid hormone receptor (TR) β .

Results: Addition of 1 μ mol/l T₄ or T₃ to the culture medium increased the basal level of ME-TRE-TK-CAT by 4.5- and 12.5-fold respectively. Amiodarone or DEA (1 μ mol/l) increased CAT activity by 1.4- and 3.4-fold respectively. Combination of DEA with T₄ or T₃ increased CAT activity by 9.4- and 18.9-fold respectively. These data suggested that DEA, but not amiodarone, had a synergistic effect with thyroid hormone on ME-TRE, rather than the postulated inhibitory action; we supposed that this was due to overexpression of the transfected TR into the cells. When the amount of RSV-TR β was reduced until it was present in a limited amount, allowing competition between thyroid hormone and the drug, addition of 1 μ mol/l DEA decreased the T₃-dependent expression of the reporter gene by 50%. The inhibitory effect of DEA was partially due to a reduced binding of TR to ME-TRE, as assessed by EMSA. DEA activated the TR-dependent down-regulation by the negative TSH-TRE, although at low level (35% of the down-regulation produced by T₃), whereas amiodarone was ineffective. Addition of 1 μ mol/l DEA to T₃-containing medium reduced the T₃-TR-mediated down-regulation of TSH-TRE to 55%.

Conclusions: Our results demonstrate that DEA, but not amiodarone, exerts a direct, although weak, effect on genes that are regulated by thyroid hormone. High concentrations of DEA antagonize the action of T₃ at the molecular level, interacting with TR and reducing its binding to TREs. This effect may contribute to the hypothyroid-like effect observed in peripheral tissues of patients receiving amiodarone treatment.

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Introduction

Amiodarone is an iodine-rich benzofuranic derivative frequently used for the treatment of refractory cardiac arrhythmias. During chronic treatment, amiodarone and its main metabolite, desethylamiodarone (DEA), reach high concentrations in several tissues, including the thyroid gland (1). The massive increase in available iodine and the high intrathyroidal concentration of amiodarone and DEA are responsible for changes in thyroid function observed in a substantial proportion of patients during chronic treatment with amiodarone. Amiodarone-induced thyroid dysfunction occurs in

16–49% of patients (2–5) soon after initiation of treatment or even long after withdrawal of the drug.

Amiodarone treatment can cause both hypothyroidism and thyrotoxicosis; the latter can be due to excess iodine or to a thyroid-destructive process (6–10).

At the cellular level, in addition to the inhibition of type I and type II iodothyronine 5'-deiodinase (11, 12), amiodarone can also act as a competitive antagonist of tri-iodothyronine (T₃) in cultured rat pituitary cells (13), and induce a hypothyroid-like condition. Similar to what is observed in hypothyroidism, amiodarone decreases the number of catecholamine receptors (14, 15), and reduces the effect of T₃ on β -adrenergic

receptors (16). Amiodarone is also likely to antagonize the effects of thyroid hormone by a competitively binding to thyroid hormone receptor (TR) both *in vivo* and *in vitro* (17–19). Furthermore, the drug inhibits the thyroid hormone-mediated increase in growth hormone mRNA levels in cell cultures (13). Hudig *et al.* (20) reported that, in amiodarone-treated animals, the drug-induced hypercholesterolaemia was due to a reduction of low-density lipoprotein (LDL) receptor mRNA levels, thus suggesting that the drug induced a hypothyroid-like condition in the liver. However, the molecular mechanisms underlying these competitive effects of amiodarone on thyroid hormone-responsive genes are not completely understood. In addition, although DEA is considered to be the active metabolite of amiodarone, it is not clear whether the effects observed at the molecular level are due to DEA alone.

This study was undertaken to investigate the interaction of amiodarone and DEA with T₃ and TR at the molecular level. For this purpose, we used either a malic enzyme (ME) or a thyroid-stimulating hormone (TSH) thyroid hormone response element (TRE)–thymidine kinase (TK)–chloramphenicol acetyltransferase (CAT) reporter cotransfected into NIH3T3 cells with RSV-TR β in the absence or presence of thyroid hormone, amiodarone or DEA, alone or in combination. We herein demonstrate that DEA, but not amiodarone, is a weak thyroid hormone agonist that antagonizes T₃ action.

Materials and methods

Plasmids

The RSV-TR β expression plasmid, containing the cDNA sequence of rat TR β , inserted into the HindIII/HpaI restriction sites of RSV, has been described previously (21). The reporter plasmids ME-TRE-TK-CAT and TSH-TRE-TK-CAT containing the nucleotide sequence of the ME-TRE or TSH β -TRE have also been described in detail (22). The plasmid PCH110 containing the β -galactosidase coding region was purchased from Pharmacia (Uppsala, Sweden) and used as an internal control to account for variations in transfection assays.

Cell culture and transfection assay

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as described previously (21). Transfection assays were performed by the calcium phosphate precipitation methods as detailed previously (22). Briefly, 200 000 cells were seeded into a 35-mm dish 24 h before transfection in DMEM containing 10% FBS deprived of thyroid hormone by treatment with cationic exchange resin (AG-1-X8; Bio-Rad, Richmond, CA, USA) and activated charcoal. A typical experiment

consisted of 4 μ g reporter plasmid, 1.5 μ g RSV-TR β and 0.4 μ g pCH110, cotransfected into each 35-mm dish. In a dose–response experiment, RSV-TR β -containing plasmid in amounts from 0.1 to 1.5 μ g were put in each 35-mm dish and the smallest amount allowing a T₃-specific response (0.25 μ g) was chosen for competition experiments. Hormones or analogues were added at concentrations ranging from 1 pmol/l to 100 μ mol/l, as appropriate. The CAT activity was determined 48 h later by a phase extraction method and normalized by measuring the β -galactosidase activity (21). Results represent the mean \pm s.d. of at least five different experiments, each carried out in duplicate.

Gel mobility shift assay (EMSA)

Expression vector pSP72 containing cDNA of the rat TR β was used for *in vitro* transcription–translation using a reticulocyte lysate system. The rat ME-TRE has been described elsewhere (21). For the binding reaction, 2.5 μ l *in vitro* translated TR β or control was used. After preincubation for 15 min on ice in a total volume of 20 μ l (20 mmol/l HEPES, pH 7.9, 0.5 mmol/l dithiothreitol, 5 mmol/l MgCl₂, 0.5 mmol/l EDTA, 100 mmol/l KCl, 10% glycerol, 5 μ g polyA-dT), 3 ng (15 000 c.p.m.) of probe was added and the incubation continued for 45 min. DNA–protein complexes were resolved on a 5% linear non-denaturing

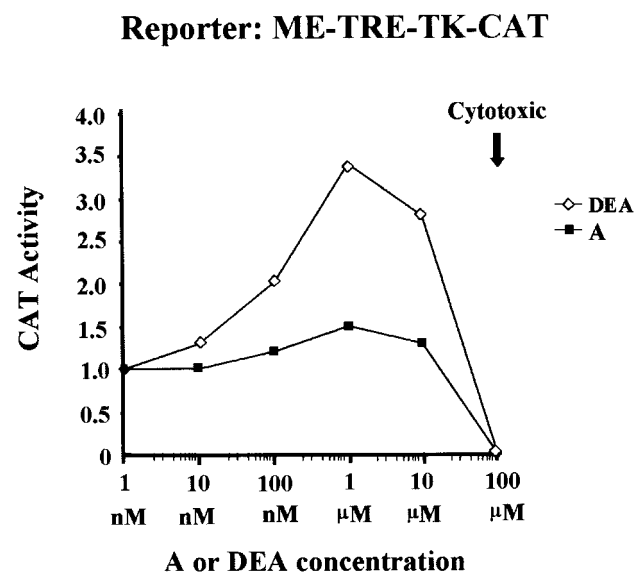


Figure 1 Dose response of ME-TRE-TK-CAT to amiodarone (A) or DEA. NIH3T3 cells transfected with ME-TRE-TK-CAT and TR β were exposed to different concentrations of amiodarone or DEA, as indicated. Responses were expressed as fold of induction of the reporter gene in the presence and absence of amiodarone or DEA. Concentrations of amiodarone or DEA greater than 10 μ mol/l were cytotoxic for the cells. Results represent the average of four different experiments, performed in duplicate.

polyacrylamide gel at room temperature in $0.5 \times$ TBE (45 mmol/l Tris, 45 mmol/l boric acid, 1 mmol/l EDTA). Gel was dried and exposed at -70°C .

Results

Responsiveness of the ME-TRE-TK-CAT reporter gene to graded amounts of amiodarone or DEA was tested in transient transfection assays in NIH3T3 cells. DEA or amiodarone were added to the culture medium at concentration ranging from 1 nmol/l to 100 $\mu\text{mol/l}$. As shown in Fig. 1, the addition of 1 $\mu\text{mol/l}$ DEA increased the expression of the reporter gene, as assessed by the level of CAT activity, by 3.4-fold. CAT activity doubled with a 100 nmol/l concentration of DEA; at variance with this, amiodarone caused only a slight increase over the basal level of CAT activity at 1 $\mu\text{mol/l}$ concentration (1.4-fold). In keeping with previous reports (23), concentrations of amiodarone or DEA greater than 10 $\mu\text{mol/l}$ were cytotoxic for NIH3T3 cells, therefore we used 1 $\mu\text{mol/l}$ DEA or amiodarone in the next experiments.

Figure 2 illustrates the effect of 1 $\mu\text{mol/l}$ T_3 , T_4 , amiodarone or DEA, alone or in combinations of two, on the expression of ME-TRE-TK-CAT reporter plasmid cotransfected with a standard amount (1.5 $\mu\text{g/dish}$) of TR-containing plasmid (see Materials and methods). T_3 increased the CAT activity by 12.5-fold, T_4 by 4.5-fold, DEA by 3.4-fold and amiodarone by 1.4-fold, whereas iodide was completely ineffective. Combination of amiodarone with T_3 or T_4 gave a 14-fold and a sixfold induction of CAT activity. Addition of DEA to T_3 - or T_4 -containing medium increased CAT activity 18.9- and 9.4-fold respectively. These results suggested that DEA, but not amiodarone, was a TR agonist. However, these data were somewhat surprising, because the expected DEA- or amiodarone-induced inhibition of the thyroid hormone-dependent activity of the ME-TRE-TK-CAT reporter gene was not observed. We decided to titrate the amount of RSV-TR β plasmid, with the aim of obtaining a smaller amount of TR protein in the transfected cells (see Materials and methods). Cells were transfected with the RSV-TR β -containing plasmid in amounts ranging from 0.1 to 1.5 μg per dish, and the smallest amount still giving a T_3 -specific response (2.5-fold of induction of CAT activity) was used for competition experiments (0.25 μg). Addition of 1 $\mu\text{mol/l}$ T_3 to the cells transfected with a reduced amount of RSV-TR β (0.25 $\mu\text{g/dish}$) increased the CAT activity by 12-fold; 1 nmol/l and 1 pmol/l T_3 gave a 7.5- and fourfold increase in reporter gene activity respectively (Fig. 3). DEA (1 $\mu\text{mol/l}$) was ineffective when added to the 1 $\mu\text{mol/l}$ T_3 -containing medium, but it reduced the thyroid hormone-dependent activity of the reporter plasmid by 50% when in combination with 1 nmol/l or 1 pmol/l T_3 (Fig. 3). Amiodarone in these experimental conditions was ineffective.

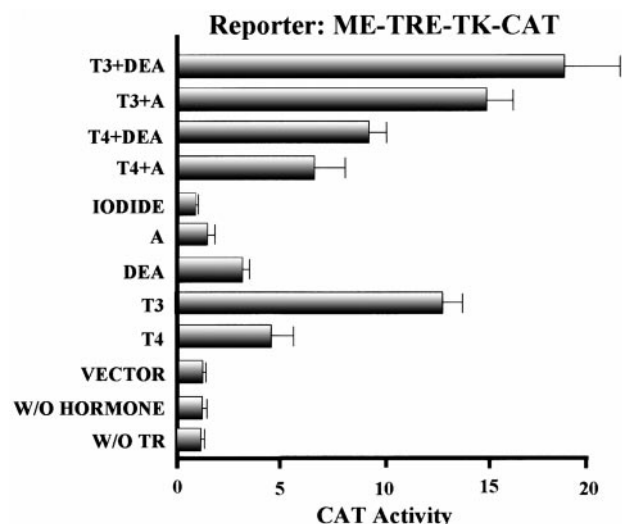


Figure 2 Thyroid hormone-dependent activity of ME-TRE-TK-CAT. Transient transfections were performed as described in Fig. 1 and in Materials and methods. A β -galactosidase-containing plasmid was included as an internal control. The cells were cultured in the absence or presence of 1 $\mu\text{mol/l}$ thyroid hormone or analogue. Results were expressed as a ratio of CAT activity in the presence and absence of the ligand and represent the mean \pm S.D. of at least four different experiments, performed in duplicate. W/O, without.

We next evaluated whether the reduction in T_3 activity was due to the inhibition of TR binding to the ME-TRE, using an EMSA. Addition of DEA in the concentration range 1 nmol/l–1 $\mu\text{mol/l}$ slightly reduced TR binding to ME-TRE. The same range of concentrations of amiodarone were less effective than

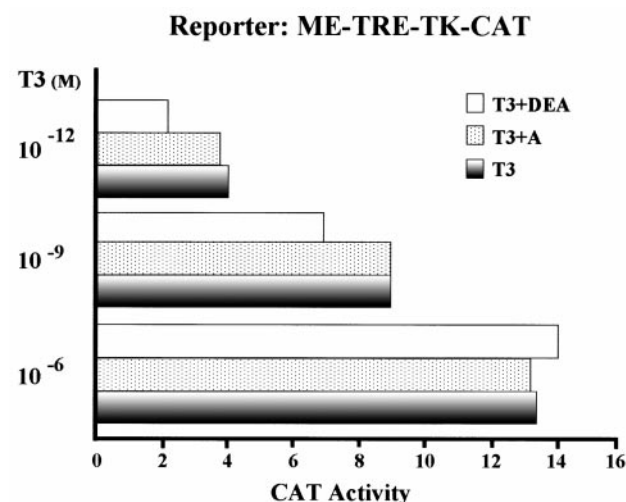


Figure 3 Effect of DEA excess on thyroid hormone-dependent activity of ME-TRE. Transfection conditions were as described in Fig. 2, except that TR β -containing expression plasmid was reduced to 0.25 $\mu\text{g/dish}$. CAT activity was expressed as in Fig. 2. Results represent the mean \pm S.D. of at least four different experiments, performed in duplicate.

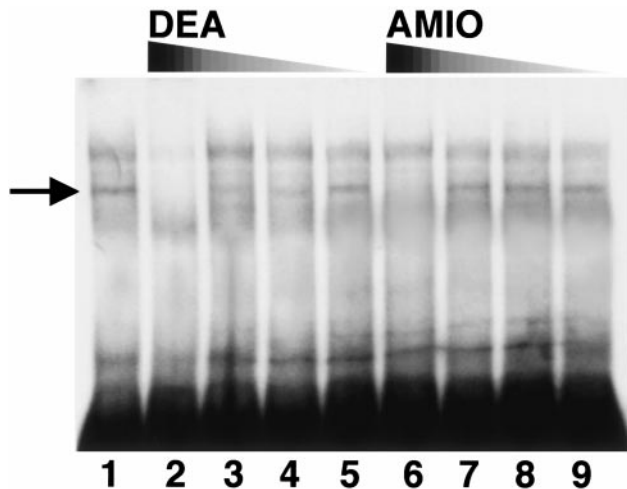


Figure 4 Effect of amiodarone (AMIO) and DEA on TR binding to ME-TRE. EMSA was performed using a synthetic oligonucleotide containing the ME-TRE sequence as a ^{32}P -labelled probe. TR β was translated *in vitro* using a reticulocyte lysate system. Complexes were resolved on a 5% non-denaturing polyacrylamide gel. Arrow indicates the specific complex. Amiodarone or DEA were used in concentrations of at 1 mmol/l (lanes 2, 6), 1 $\mu\text{mol/l}$ (lanes 3, 7), 1 nmol/l (lanes 4, 8) or 1 pmol/l (lanes 5, 9).

DEA. Higher concentrations (1 mmol/l) of DEA or amiodarone abolished TR binding (Fig. 4). However, the drugs were cytotoxic when used at this concentration.

We then evaluated whether the TSH-TRE, a negative TRE arranged as a direct repeat without spacing, could be activated by DEA or amiodarone. Addition of 1 $\mu\text{mol/l}$ DEA to NIH3T3 cells transfected with a limited amount of TR-expressing plasmid (0.25 μg) resulted in 35% reduction in CAT expression from the reporter gene compared with the basal level of CAT expression obtained in the absence of hormone or analogue (Fig. 5). Addition of amiodarone at the same 1 $\mu\text{mol/l}$ concentration was ineffective (less than 10% reduction of the basal CAT activity). Combination of 1 $\mu\text{mol/l}$ DEA with 1 nmol/l T_3 mediated down-regulation of TSH-TRE to 55%.

Discussion

Pharmacological effects of amiodarone on cardiac functions consist of bradycardia, reduction of myocardial oxygen consumption and lengthening of the cardiac action potential and the effective atrial and ventricular refractory periods (24). These effects may be prevented at least in part by administration of T_4 (25). Amiodarone treatment is also associated with an increase in serum cholesterol concentrations, and a reduction in cardiac β -adrenergic receptors (26).

Because these effects resemble those observed in hypothyroidism, it was postulated that a possible

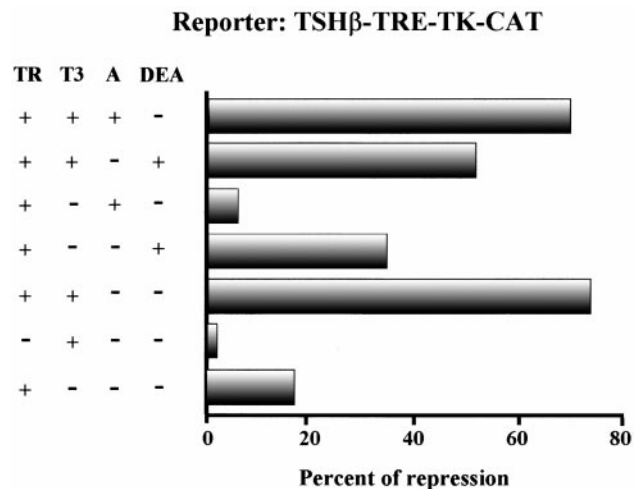


Figure 5 Effect of amiodarone (A) or DEA excess on thyroid hormone-dependent activity of TSH-TRE. Transfections were performed as described in Fig. 3, using a limited amount of RSV-TR β . CAT activity was expressed as percentage of repression of the CAT transcriptional activity obtained in the absence of thyroid hormone, TR or both, which was arbitrarily considered to represent absence of repression. Results represent the average of four independent transfections carried out in duplicate.

mechanism of action of amiodarone might be to induce a local hypothyroidism-like condition (27). However, the serum thyroid hormone pattern in patients chronically treated with amiodarone – increased serum T_4 , reduced serum T_3 and transiently increased serum TSH concentrations, mimics that found in patients with non-thyroidal illness (17). It is generally accepted that this condition, known as Euthyroid Sick Syndrome, is not associated with hypothyroidism, and T_4 treatment is not useful (17). Most of the changes in serum thyroid hormone are due to the inhibitory effect of amiodarone on type I and II 5'-deiodinase (11, 12). However, the inhibition of 5'-deiodinase alone cannot explain all the effects of amiodarone treatment; indeed, treatment with iopanoic acid, which exerts similar inhibitory effects on 5'-deiodinase, reproduces only part of the effects of amiodarone administration (28).

Amiodarone can reduce the expression of thyroid hormone-sensitive genes, such as LDL receptor (29), and of rat pituitary gene (13, 30, 31). Moreover, amiodarone and DEA inhibit the binding of T_3 to its nuclear receptor (32, 33). However, the fact that amiodarone was ineffective in NIH3T3 cells might also be due to the probable absence of its metabolism in this cell line. Our data clearly demonstrate that DEA, but not amiodarone, exerts a direct effect on genes that are either positively or negatively regulated by thyroid hormone: this effect is direct and specific and occurs only in the presence of TR and a TRE. Our results expand those of Bakker *et al.* (19), who found that, in transient transfection assays,

amiodarone was a weak activator of the LDL receptor gene and had an additive effect when used in combination with T₃. This finding was not in keeping with Northern blot results showing that animals fed with an amiodarone-containing diet had a reduction in LDL receptor mRNA levels (20). We showed that DEA antagonizes the action of T₃ at the molecular level when in large excess, in keeping with its weak TR-agonist activity. This effect was evident only when the amount of TR expressed in the transfected cells was limited. When greater amounts of TR-containing expression plasmid were used, a combination of T₃ and DEA induced a greater induction of the reporter plasmid than that observed with T₃ alone. Under these experimental conditions, probably not all TR molecules were bound to T₃ and remained available for DEA binding and activation. Although we could not measure the concentration of TR proteins expressed in the cells after transfection, it is conceivable that lower amounts of transfected plasmids produced lower amounts of TR proteins. This is supported by the fact that the competition by DEA was apparent only with 0.25 µg RSV-TR plasmid. The inhibitory effect of DEA was, at least partially, due to a reduced TR binding to the TRE, as shown by EMSA. However, a conformational change in the TR-DEA complex, leading to less feasible formation of the initiation complex, could not be excluded.

In conclusion, our data demonstrate that DEA binds to TR and activates, although weakly, thyroid hormone-sensitive genes, and that high concentrations of DEA, but not of amiodarone, are functional inhibitory competitors of T₃ binding to TR. These effects might contribute to the hypothyroid-like effect observed in patients receiving treatment with amiodarone.

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