Desethylamiodarone interferes with the binding of co-activator GRIP-1 to the β_1-thyroid hormone receptor

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Abstract Ligand binding to the thyroid hormone nuclear receptor β1 (TRβ$_1$) is inhibited by desethylamiodarone (DEA), the major metabolite of the widely used anti-arrhythmic drug amiodarone. Gene expression of thyroid hormone (triiodothyronine, T_3)-regulated genes can therefore be affected by amiodarone due to less ligand binding to the receptor. Previous studies have indicated the possibility of still other explanations for the inhibitory effects of amiodarone on T_3-dependent gene expression, probably via interference with receptor/co-activator and co-repressor complex. The binding site of DEA is postulated to be on the outside surface of the receptor protein overlapping the regions where co-activator and co-repressor bind. Here we show the effect of a drug metabolite on the interaction of TRβ$_1$ with the co-activator GRIP-1 (glucocorticoid receptor interacting protein-1). The T_3-dependent binding of GRIP-1 to the TRβ$_1$ is disrupted by DEA. A DEA dose experiment showed that the drug metabolite acts like an antagonist under 'normal' conditions (at 10^{-7} M T_3 and $5\times10^{-6}\rightarrow10^{-3}$ M DEA), but as an agonist under extreme conditions (at 0 and 10^{-9} M T_3 and $>10^{-4}$ M DEA). To our knowledge, these results show for the first time that a metabolite of a drug which was not devised for this purpose can interfere with nuclear receptor/co-activator interaction.

Key words: Desethylamiodarone; Co-activator glucocorticoid receptor interacting protein-1; Thyroid hormone receptor

1. Introduction

Hormones can influence transcription via binding to their specific nuclear receptors. Occupancy of binding sites with ligand results in conformational changes of the receptor protein, allowing dissociation of co-repressors and association of the hormone–receptor complex with nuclear co-activators. This sequence of events ultimately leads to modulation of the transcription rate of the hormone-responsive genes [1]. The role of nuclear co-repressors and co-activators in transcriptional regulation has only recently become apparent. Their biological relevance is highlighted by the finding that particular mutations in the human thyroid hormone receptor β_1 (hTRβ_1) disturb the interaction between co-activator and co-repressor interactions and thyroid hormone receptor (TR), resulting in thyroid hormone resistance [2]. Desethylamiodarone (DEA), the major metabolite of the powerful anti-arrhythmic and anti-anginal drug amiodarone, is a non-competitive inhibitor of the binding of triiodothyronine (T_3) to the thyroid hormone receptor β1 (TRβ_1) protein [3], its binding site is postulated to be on the outside surface of the receptor protein overlapping the regions where co-activator and co-repressor bind [4]. Here we report that DEA can also affect the binding of co-activator glucocorticoid receptor interacting protein-1 (GRIP-1) to the ligand–receptor complex.

Amiodarone is widely used in the treatment of a variety of cardiac diseases. It is a peculiar drug in view of its extensive tissue accumulation (notably in the liver, lung and adipose tissue) and very long elimination half-life. Characteristic structural features are its resemblance to thyroxine and its high iodine content. The release of pharmacological quantities of iodine during biotransformation of the drug results in iodine-induced thyrotoxicosis and hypothyroidism in a minority (about 16%) of patients. In contrast, in almost every patient treated with amiodarone, hypothyroid-like effects are observed in the expression of various T_3-responsive genes. Examples are the decrease in β-adrenoreceptor density and Na,K-ATPase activity in the heart, and the decrease of low density lipid (LDL)) receptor density in the liver as evident from studies in humans and in experimental animals [5–8]. These hypothyroid-like effects can be reversed by the administration of T_3, and are explained from a decreased transcription of involved genes [9]. For example, the increase in serum LDL cholesterol observed in amiodarone-treated patients [10] is caused by a decreased expression of the hepatic LDL receptor protein, which is due to a decreased transcription of the gene encoding the LDL receptor. The decrease in transcription might well be caused by a lower T_3 receptor occupancy as amiodarone substantially decreases the generation of T_3 out of T_4 in the liver by inhibition of type I deiodinase [11]. Furthermore, DEA has been shown to be a competitive inhibitor of the binding of T_3 to thyroid hormone receptor α1 (TRα_1) [12] and a non-competitive inhibitor with respect to TRβ_1 [3]. Further insight into this antagonistic effect of DEA on T_3 receptor binding was obtained by evaluating the changes in the molecular constitution of either the drug or the receptor. We have previously reported the results of competition studies with amiodarone analogues: the bulky iodine atoms, the hydrophobicity, the electric charge and the overall size of the analogues markedly influenced the nature and potency of their inhibition on T_3 receptor binding [13]. These studies, however, did not provide further information on the localization of the DEA binding site on the receptor. Therefore we studied the effect of naturally occurring and artificial mutations in TRβ_1 which were known to decrease T_3 affinity. The results of these studies demonstrated that amino acids E457 and R429 are at or near the DEA binding site on the receptor [4]. Since these residues may be involved in co-acti-

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vator (E457) [14] or co-repressor (R429) [15,16] interaction, this finding led us to hypothesize a possible disruption of the TRβ$_1$–co-activator interaction by DEA. The aim of the present study was to verify this hypothesis by studying the influence of DEA on the T_3-dependent binding of co-activator GRIP-1 to hTRβ$_1$.

2. Materials and methods

2.1. Chemicals

T_3 was obtained from Henning (Berlin, Germany). DEA from Sanofi Recherch (Montpellier, France) was used as a 10^{-2} M stock solution in ethanol.

2.2. Protein expression

Co-activator GRIP-1 was expressed as a glutathione-*S*-transferase (GST)-GRIP-1-nuclear receptor interacting domain (NID) fusion protein containing the NID of the co-activator GRIP-1 attached to GST. This fusion protein was expressed in *Escherichia coli* as described previously [17].

TRβ$_1$ (amino acids 153–461) was cloned with a hemagglutinin epitope-tag (HA-TRβ$_1$) at the N-terminus which did not interfere with the hormone binding domain at the C-terminus. The HA-TRβ$_1$ was expressed in *E. coli* and isolated as described before [17,18].

2.3. Pull-down assay

To study the receptor co-activator interaction in the presence of DEA, we used a recently developed non-radioactive 'pull-down' assay [18]. In short, GST-GRIP-1-NID bound to glutathione–Sepharose beads was resuspended in binding buffer (20 mM HEPES, pH 7.9, 80 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol (DTT), 0.1% NP-40, 0.1% Triton X-100; Complete (EDTA free) protease inhibitor (Roche)). HA-TRβ$_1$-containing lysate (8 μl) was diluted to 20 μl with binding buffer containing 20 μl/ml bovine serum albumin and preincubated with or without T_3 and in the absence or presence of DEA for 30 min at 4°C. Thereafter GST-GRIP-1-NID beads were added and the incubation continued for 90 min at 4°C whilst shaking. The samples were washed, dried and resuspended in protein loading buffer containing 5 mM DTT. They were run on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis gel blotted onto PVDF membrane (Roche) using a transfer buffer without SDS and lowered methanol (15%).

For detection of the HA-tag, the blot was incubated with a high affinity monoclonal anti-HA antibody conjugated with peroxidase (POD) (anti-HA 3F10-POD). Using Lumi-Light^{plus} substrate (Roche), the chemiluminescent signal was detected and quantified using a Lumi Imager (Roche). Average exposure time was 2 min.

To investigate whether DEA had an effect on this protein–protein interaction two sets of experiments were performed. First the dose effect of T_3 was studied in the absence and presence of a constant amount of DEA. TRβ$_1$ was preincubated with thyroid hormone (T_3) over a concentration range of 3×10^{-10} up to 10^{-7} M T_3 in the absence and presence of 10^{-5} M DEA. In the second set of experiments, the dose-dependent effect of DEA was studied. TRβ$_1$ was preincubated with DEA over a concentration range of 10^{-6} up to 10^{-3} M DEA in the absence of T_3 and in the presence of 10^{-9} M and 10^{-7} M T_3.

3. Results

The results of the T_3 dose effect experiments indicate a T_3 dose dependency of the binding of GRIP-1 to the TRβ$_1$, which was observed previously [19]. The binding of GRIP-1 to the TRβ$_1$ increased at higher T_3 and was lower in the presence of 10^{-5} M DEA at all T_3 concentrations tested (Fig. 1a) ($P<0.0001$; ANOVA, two way with replication; $n=3$). Individual points of the binding curves differ significantly at 3×10^{-8} M ($P<0.01$; $n=5$) and at 10^{-7} M ($P<0.05$; $n=4$) (Fig. 1b). The HA-TRβ$_1$ protein binds T_3 with wild type affinity (K_D 0.3×10^{-9} M).

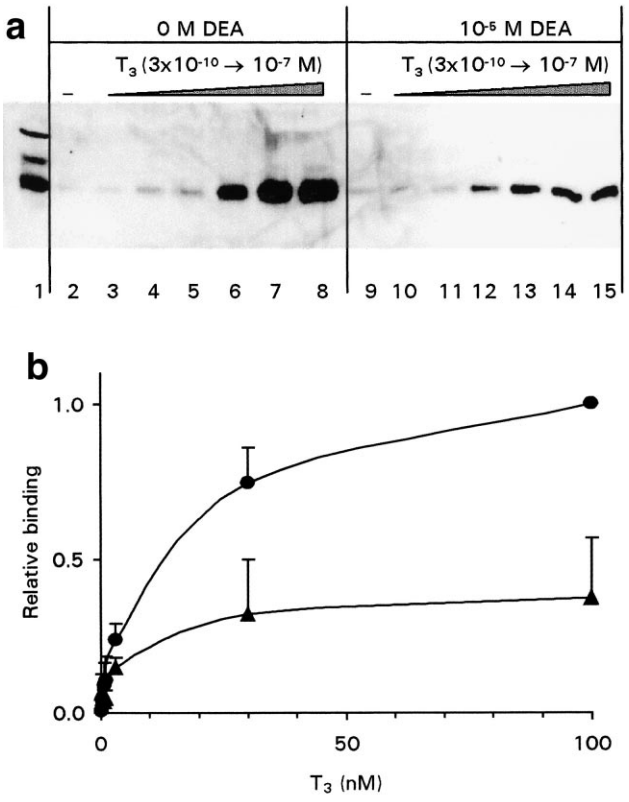

Fig. 1. a: Western blots of HA-TRβ$_1$, depicting the dose-dependent effect of T_3 on the binding of TRβ$_1$ to GRIP-1 in the absence and presence of 10^{-5} M DEA. Lane 1: 10% of HA-TRβ$_1$ input. Lanes 2–8: binding in the presence of 0; 3×10^{-10}; 6×10^{-10}; 10^{-9}; 3×10^{-9}; 3×10^{-8}; 10^{-7} M T_3, respectively, in the absence of DEA. Lanes 9–15: binding in the presence of 0; 3×10^{-10}; 6×10^{-10}; 10^{-9}; 3×10^{-9}; 3×10^{-8}; 10^{-7} M T_3, respectively, in the presence of 10^{-5} M DEA. HA-TRβ$_1$ was detected by anti-HA-POD and chemiluminescent substrate. b: Dose–response effect of T_3 on the binding of TRβ$_1$ to GRIP-1 in the absence (●) and presence (▲) of 10^{-5} M DEA. Quantification and plot of the light units from Western blot (a), measured by the Lumi Imager. The relative binding is the ratio of the observed binding of TRβ$_1$ to GRIP-1 to the (maximal) binding at 10^{-7} M T_3 in the absence of DEA.

A DEA dose-dependent effect of the binding of GRIP-1 to the TRβ$_1$ was also observed. In the absence of T_3, the binding of GRIP-1 to TRβ$_1$ remains very low at DEA concentrations up to 10^{-5} M (Fig. 2a); however, a 10-fold increase is observed in the presence of 10^{-3} M DEA. It appears therefore that DEA acts as an agonist with respect to the unoccupied TRβ$_1$. In the presence of 10^{-9} M T_3, the DEA-induced increase in binding is less pronounced and significantly different from that obtained in the absence of T_3 ($P<0.001$; ANOVA, two-factor with replication; $n=4$) (Fig. 2b). Interestingly, at 10^{-7} M T_3, a reversal of the dose–response pattern is seen and the binding curve differs significantly from the other two ($P<0.001$; ANOVA, two-factor with replication; $n=4$). At a T_3 concentration of 10^{-7} M, the highest binding of GRIP-1 to TRβ$_1$ is observed in the absence of DEA, and the binding decreases in the presence of DEA in a dose-dependent manner (Fig. 2a,b).

4. Discussion

From the above results, we conclude that DEA can act as a

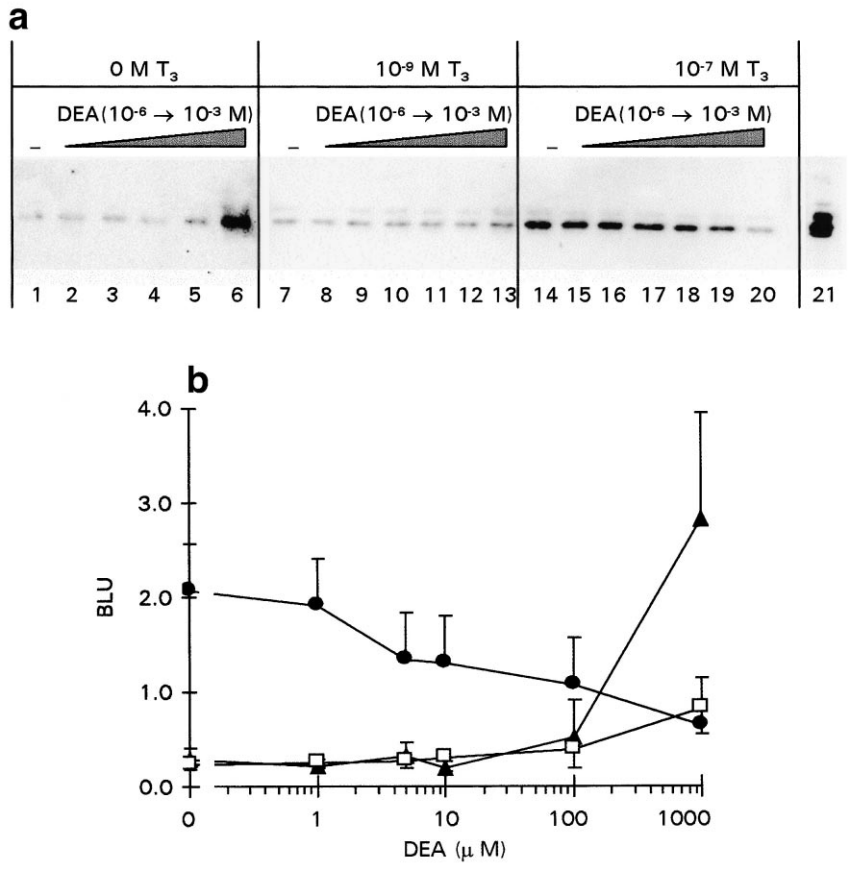

Fig. 2. a: Western blots of HA-TRβ_1, depicting the dose-dependent effect of DEA on the binding of TRβ_1 to GRIP-1 in the presence of 0, 10^{-9} or 10^{-7} M T_3. Lanes 1–6: binding in the absence of T_3 and in the presence of 0; 10^{-6}; 5×10^{-6}; 10^{-5}; 10^{-4}; 10^{-3} M DEA, respectively. Lanes 7–13: binding in the presence of 10^{-9} M T_3 and in the presence of 0; 10^{-6}; 5×10^{-6}; 10^{-5}; 5×10^{-5}; 10^{-4}; 10^{-3} M DEA, respectively. Lanes 7–13: binding in the presence of 10^{-7} M T_3 and in the presence of 0; 10^{-6}; 5×10^{-6}; 10^{-5}; 5×10^{-5}; 10^{-4}; 10^{-3} M DEA, respectively. Lane 21: 10% of HA-TRβ_1 input. b: Dose–response effect of DEA on the binding of TRβ_1 to GRIP-1 in the absence of T_3 (▲) and in the presence of 10^{-9} M T_3 (□) and 10^{-7} M T_3 (●). Quantification and plot of the light units from Western blot (a), measured by Lumi Imager. The light units are expressed as BLUs (Boehringer Light Units). The crossing of the lines at high and low T_3 concentration is indicative for partial antagonism.

partial antagonist: the actual mode of action depends on the ambient T_3 concentration. This phenomenon can be explained from the non-competitive nature of the inhibition of T_3 binding to TRβ_1 by DEA. This non-competitiveness allows the formation of both the complexes DEA/TRβ_1 and DEA/T_3/TRβ_1, since the binding sites of T_3 and DEA are not the same. Only in the case of DEA/T_3/TRβ_1 will there be antagonism, i.e. there must be some T_3 present for DEA to act as an antagonist.

In order to relate these in vitro findings to the in vivo situation, T_3 and DEA concentrations in our experiments must be compared with tissue levels in vivo. In human serum, T_3 values are in the order of 10^{-9} M and DEA levels in the order of 2×10^{-6} M [20] in patients on long-term amiodarone treatment. Rat liver T_3 and DEA levels are reported to be about 10^{-8} mol/kg [11] and 1.2×10^{-5} mol/kg [21], respectively. In human liver, DEA concentration can reach 4×10^{-3} mol/kg [22]. A thousand times higher DEA concentration in liver and other tissues compared to T_3 is therefore possible. The actual intracellular T_3 and DEA levels are not known, but taking into account the above mentioned serum and tissue concentrations, our in vitro model may well represent the in vivo situation in the human cell. DEA is a very lipophilic drug and accumulates substantially in tissues during amiodarone treatment. The drug also inhibits T_4 5′-deiodination into T_3 resulting in a substantial decrease of intracellular T_3 levels and lower TR occupancy [11]. At a 'normal' receptor occupancy of about 50%, the effect of DEA will be limited. However, at higher DEA concentrations and especially in the case of a decreased T_3 occupancy of the binding sites, DEA will act as an antagonist, resulting in lower co-activator binding.

Simplified, T_3-dependent gene regulation can be seen as an on/off switch (with or without ligand), whereas in the fine tuning of this process many other proteins are involved. This complex regulation system thus forms a balance between hormone, receptor, co-activators, co-repressors and other proteins involved in the transcription machinery. DEA interferes in this balance in three ways. First, by inhibiting deiodination of T_4 to T_3 in the liver [11]. Second, by a non-competitive inhibition of the binding of T_3 to the TRβ_1 [3]. And third, by disrupting the binding of co-activator GRIP-1 to the TRβ_1 (this study).

Overall we postulate that not amiodarone itself, but its stable major metabolite DEA interferes with T_3-dependent gene expression [23] by interfering with the binding of T_3 to the thyroid hormone receptor and disrupting the co-activator/TR binding. This mechanism of action would explain the T_3

antagonistic effects of DEA observed in patients on long-term amiodarone treatment.

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