# Vitamin $D_3$ affects expression of thyroid hormone receptor alpha and deiodinase activity in liver of MNU-treated Sprague-Dawley rats

Dana Macejová[1,2], Slavomíra Ondková[2] and Július Brtko[2]

[1] *Office of the Slovak Academy of Sciences, Slovak Academy of Sciences, Štefánikova 49, 81438 Bratislava, Slovakia;*
  *E-mail: ueenmace@savba.sk*
[2] *Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia*

**Abstract** 1α,25-dihydroxyvitamin $D_3$ and its analogue, Seocalcitol (EB1089), are able to reverse or slow the process of carcinogenesis in experimental models and cell cultures. The aim of this study was to investigate the effect of administration vitamin D or Seocalcitol to female Sprague-Dawley rats with 1-methyl-1-nitrosourea (MNU)-induced carcinogenesis of mammary glands on binding characteristics and mRNA levels of thyroid hormone receptors (TRs). Chemopreventive administration of vitamin D caused significant reduction of animal body weight. The expression of TRα mRNA was significantly higher in liver of animals treated with vitamin D after detection of first tumour. In our experiment, administration of vitamin D or Seocalcitol significantly reduced $K_A$ (group MNU+Seo; MNU+D) and increased $B_{max}$ (group MNU+Seo) of thyroid receptors in liver when compared to healthy animals. We show that the activity type I 5'-deiodinase was significantly decreased in livers of animals treated with vitamin D. The data from our *in vivo* experiment has clearly shown, for the first time, that vitamin D but not Seocalcitol i) may affect the body weight of animals, ii) can cause an increase in the expression of TRα in rat liver, remaining the functionality of the TRs unaffected, and iii) is responsible for type I iodothyronine 5'-deiodinase activity decrease in rat liver, remaining the expression of the enzyme unaffected.

**Key words:** Vitamin D — Seocalcitol — Iodothyronine 5'-deiodinase — Nuclear receptor — MNU — Liver

## Introduction

1α,25-dihydroxyvitamin D3 is the main active form of vitamin D. It has an important role in calcium and phosphate homeostasis and in the regulation of cell proliferation and differentiation (Walters 1992; Jones et al. 1998). However, because of its very serious adverse effects (e.g. hypercalcaemia) the treatment with vitamin D is very limited. To sort out these effects and take advantage of vitamin D, the synthetic analogues have been developed. The most usable analogue, Seocalcitol (EB1089), already proved its capability to reduce growth of cancer cells (Colston et al. 1992; Mathiasen et al. 1993) and its effect on calcium metabolism is markedly reduced (Hansen et al. 2000). 1-methyl-1-nitrosourea (MNU)-induced carcinogenesis

of mammary gland in rat is a widely used model for studying the biology of human breast cancer and for developing and evaluating cancer prevention and control strategies. In rats, MNU-induced tumours were found to have some common features with those in human (Berger et al. 1987).

We have already shown that thyroid status of animals can influence progress of MNU-induced carcinogenesis of mammary gland of female Sprague-Dawley rats (Macejova et al. 2005a). The effects of thyroid hormones (3,5,3'-triiodo-L-thyronine; $T_3$) and vitamin D are mediated through thyroid hormone receptors (TRs) and vitamin D receptor (VDR), respectively, which display structural and functional similarities. They form heterodimers with the retinoid-X receptors (RXRs). Furthermore, VDR and TRs can interact with several nuclear co-regulators (co-activators, co-repressors) that play an important part in the control of transcription (Lemon and Freedman 1999; Xu et al. 1999).

In Ob17 murine preadipocytes, $T_3$ or vitamin D interfered in each other's adipogenic action and behaved as synergistic

Correspondence to: Dana Macejová, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia
E-mail: ueenmace@savba.sk

agents and then as antagonistic agents when the vitamin D concentration was enhanced (Lenoir et al. 1996; Dace et al. 1997). Both, $T_3$ and vitamin D, were found to markedly down-regulate the total number of TR target expression sites, mainly of the TRα1 type (Schneider et al. 2005). Also, it has been reported that $T_3$ and thyroxine ($T_4$) enhanced vitamin D-induced osteoclast formation (Miura et al. 2002) and that $T_3$ and vitamin D inversely regulate OPG gene in osteoblasts (Varga et al. 2004). These observations indicate an existence of a cross-talk between their respective pathways of activity.

The aim of this study was to investigate the effect of administration vitamin D or its synthetic analogue Seocalcitol on thyroid hormone receptor subtypes mRNA levels, binding characteristics of TRs in liver, and the activity of type I iodothyronine 5'-deiodinase (5'-DI) which play important role in regulation of nuclear TRs and other cellular target sites for thyroid hormone action (Kohrle 1997).

**Materials and Methods**

*Animals*

Female Sprague-Dawley rats were obtained from Anlab farm (Czech Republic) at 40 days of age. Rats were housed 4 *per* cage and maintained at $23 \pm 2$°C, 12 h light/dark cycle. They were fed standard laboratory diet and with access to water *ad libitum*. Rats were given intraperitoneally 50 mg/kg MNU on the 49th and 56th day of age, alternately in the left and the right abdominal part. The MNU was always dissolved freshly in 0.9% NaCl adjusted to pH 4.0 with acetic acid. Solubility of MNU in water at room temperature was 1.4% w/v. Animals were treated with vitamin D or Seocalcitol (7 μg/kg *per* week) either from 60th day of age –chemopreventive administration (groups MNU+D and MNU+Seo) or after the first tumour were observed in animal (approx. 100th day of age) – chemotherapeutical administration (groups MNU+postD and MNU+postSeo). The experiments were terminated on the 216th day of age. Animals in control group received 0.9% NaCl adjusted to pH 4.0 with acetic acid. All animals were euthanized by decapitation and tissues and organs were removed, frozen in liquid nitrogen and stored at –80 °C. Principles of laboratory animal care and all procedures were approved by the Animal Care Committee of the IEE SAS Bratislava, Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

*mRNA analyses*

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. Concentration of RNA was quantified by spectrometry at 260 nm and purity was assessed from the ratio of absorbencies $A_{260nm}/A_{280nm}$. Reverse transcription (RT) was performed with 2 μg of total RNA and the Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Inc., USA) according to the manufacturer's protocol. PCR was performed in a 25 μl total volume consisting of 2 μl RT mixture, 2.5 μl of 10 × PCR buffer, 0.75 μl of 50 mmol/l $MgCl_2$, 0.5 μl 10 mmol/l dNTP, 25 pmol of each specific gene primer set and 0.6 U of DyNAzyme II DNA polymerase (Finnzymes OY, Finland) in buffer provided by the manufacturer. After treatment of samples at 94°C for 3 min to inactivate reverse transcriptase, PCR consisted of 35 cycles of denaturing (95°C, 45 s), annealing (30 s), extension (72°C, 90 s), and a final extension at 72°C for 10 min. The oligonucleotide sequences of primers for TRα, TRβ, 5'-DI and GAPDH used in PCR and corresponding annealing temperatures are described elsewhere (Macejova et al. 2005b). These conditions were proven to be in the log phase for each amplified sequence by us. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The band intensities were measured using the STS 6220I Documentation System (Ultralum, USA) and normalized to the band intensity of PCR product corresponding to the house keeper gene GAPDH.

*Isolation of purified rat liver nuclei and extraction of TRs*

The tissue was minced and homogenized in 0.32 mol/l sucrose, 1 mmol/l $MgCl_2$, 0.1 mmol phenylmethylsulfonyl fluoride (PMSF), 1 mmol/l DL-dithiothreitol (DTT). The homogenate was centrifuged at $1000 \times g$, the crude pellet was washed with the same solution and centrifuged repeatedly at $1000 \times g$. The pellet was then mixed with 2.3 mol/l sucrose containing 1 mmol/l $MgCl_2$, 1 mmol/l DTT, 0.1 mmol/l PMSF and treated by isopycnic ultracentrifugation at $220\,000 \times g$, 30 min using the swing-out model SW 40 rotor and the Beckman ultracentrifuge. Liver nuclei were then washed twice in ice-cold SMCT buffer (0.32 mol/l sucrose, 10 mmol/l Tris-HCl (pH 7.4), 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 1 mmol/l DTT, 0.1 mmol/l PMSF), once in the presence of 0.25% Triton X-100 and once in its absence. The nuclear non-histone proteins containing TRs were obtained directly by extracting purified nuclei in a high ionic strength KMTD buffer containing 0.3 mol/l KCl, 1 mmol/l $MgCl_2$, 10 mmol/l Tris-HCl (pH 8.0) and 1 mmol/l DTT at 0°C for 1 h (nuclei obtained from 3 g liver tissue *per* 1 ml) and separated from the mixture of disrupted nuclei by ultracentrifugation at $135\,000 \times g$.

*Binding of $^{125}$I-3,5,3'-L-$T_3$ to nuclear receptors*

The assays on $^{125}$I-$T_3$ specific binding were performed at 22°C in 0.5 ml KMTD buffer (pH 8.0). The samples contain-

ing TRs were incubated with $3.0 \times 10^{-10}$ mol/l of $^{125}$I-$T_3$ for 2 h. Non-specific binding of the labelled ligand was determined by simultaneous incubation with $3.0 \times 10^{-7}$ mol/l of non-labelled $T_3$ *per* sample. After incubation, 0.5 ml of Dowex 1-X8 (80 mg/ml) suspension in KMTD buffer (pH 8.0) at 0–4°C was added to each sample. After short vortexing, the suspension was placed on an ice bath for 10 min, then vortexed and the supernatant was collected after short centrifugation at $1000 \times g$. Then 0.5 ml of the supernatant was decanted and its radioactivity was quantified in a Beckman model 4000 γ-spectrometer (Fullerton, Ca, USA).

*Electrophoretic mobility shift assay*

Electrophoretic mobility shift assay (EMSA) has been performed according to previously described protocol (Schmutzler et al. 1998). Oligonucleotides were labelled with [γ$^{32}$P]-ATP (NEN, U.S.A) using $T_4$ polynucleotide kinase. For protein binding, nuclear extracts obtained in a high-ionic strength buffer (20 mmol/l Hepes (pH 7.9), 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l MgCl$_2$, 0.2 mmol/l EDTA, 0.2 mmol/l PMSF, and 0.5 mmol/l DTT) (Andrews and Faller 1991) were incubated for 15 min on ice in reaction buffer (10 mmol/l Tris-HCl (pH 7.5), 50 mmol/l NaCl, 1 mmol/l EDTA, 5% (v/v) glycerol, 0.2 mmol/l DTT, 0.75 mg/ml BSA) with 0.025 mg/ml poly[d(I/C)] (Pharmacia-Biotech, U.S.A.). As a control of specificity of the process, 100-fold excess of unlabeled competitor oligonucleotide was used in the assay. Approximately 100 000 cpm of labelled oligonucleotide (DR+4 TRE), 250 pg, were used for each binding reaction and the incubation was continued for one hour. Then the reaction mixture was submitted to a vertical polyacrylamide gel electrophoresis, the gel was dried and subsequently used for autoradiography on a KODAK X-OMAT film. The band intensities were measured using the STS 6220I Documentation System (Ultralum, USA).

*Type I iodothyronine 5'-DI activity*

The tissues were homogenized in ice-cold homogenization buffer containing 0.25 mol/l sucrose, 20 mmol/l Hepes (pH 7.4), 1 mmol/l DTT, 1 mmol/l EDTA, centrifuged at $1500 \times g$ for 15 min at 4°C and sonificated (three times for 5 s). 5'-DI activity was determined according to Leonard and Rosenberg (Leonard and Rosenberg 1980) by the release of $^{125}$I$^-$ from [$^{125}$I]-3,3',5'-triiodo-L-thyronine (rT$_3$) using nonradioactive rT$_3$ and 40 mmol/l DTT in the absence or presence of 0.1 mmol/l PTU. The fraction of iodide release blocked by PTU was assigned to the 5'-DI activity. Specific activity of the 5'-DI was expressed as pmol of $^{125}$I$^-$ released *per* minute and *per* milligram of

protein. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

*Statistical analysis*

Data are expressed as mean ± SD. Statistical significance was assessed using an unpaired Student's *t*-test.

**Results and Discussion**

Experimentally MNU-induced model of breast carcinogenesis in the rat is widely used for studying the biology of breast cancer and for developing and evaluating cancer prevention and control strategies (Macejova and Brtko 2001a). Recent investigations confirmed that 1α,25-dihydroxyvitamin $D_3$, i.e. vitamin D, is able to reverse or slow the process of carcinogenesis in experimental models and cell cultures (Colston and Hansen 2002; Ondkova et al. 2006). Thus, it becomes a part of the group of notable and perspective substances which in combination with other chemotherapeutical agents can significantly contribute in therapy not only breast cancers but also other organs cancers. However, because of its very serious adverse effects (e.g. hypercalcaemia) the treatment with vitamin D is very limited. To sort out these effects and take advantage of vitamin D, the synthetic analogues have been developed. The most usable analogue, Seocalcitol (EB1089), already proved its capability to reduce growth of cancer cells (Colston et al. 1992; Mathiasen et al. 1993) and its effects on calcium metabolism are markedly reduced (Hansen et al. 2000). It has been shown that administration of vitamin D to hyperthyroid patients with untreated Graves' disease has beneficial effects on serum levels of free $T_3$, free $T_4$ as well as $T_3$, $T_4$ and TSH (Kawakami-Tani et al. 1997). Therefore, the aim of this study was to investigate the effect of administration vitamin D or its synthetic analogue Seocalcitol to female Sprague-Dawley rats with MNU-induced carcinogenesis of mammary glands on binding characteristics and mRNA levels of TRs.

In general, the application of MNU didn't cause any significant reduction of body weight of animals – already shown in our previous experiments (Macejova et al. 2005a). However, chemopreventive administration of vitamin D to animals with MNU-induced carcinogenesis caused significant reduction of animal body weight when compared to healthy animals ($p < 0.01$) as well as with MNU animals ($p < 0.05$) (group MNU) without any treatment (Fig. 1). The administration of Seocalcitol had no effect on body weight of animals. Concerning toxicological effects of the compounds on liver, the MNU itself increased the liver/body ratio and even significant increase in this ratio
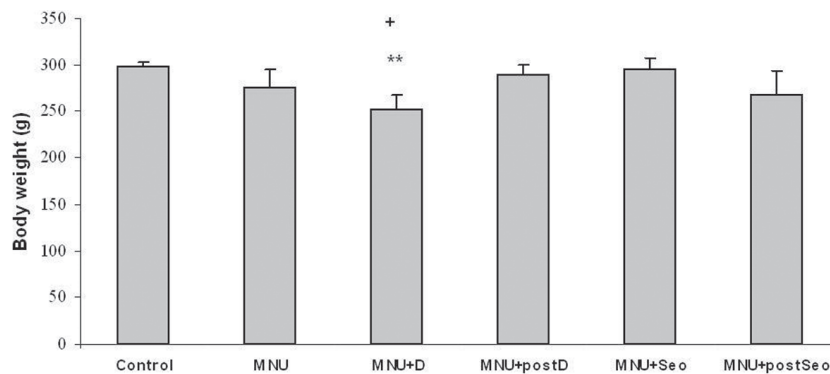
**Figure 1.** Body weight of animals. Results are expressed as mean ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$. $^+ p < 0.05$ *vs.* MNU; $** p < 0.01$ *vs.* Control.
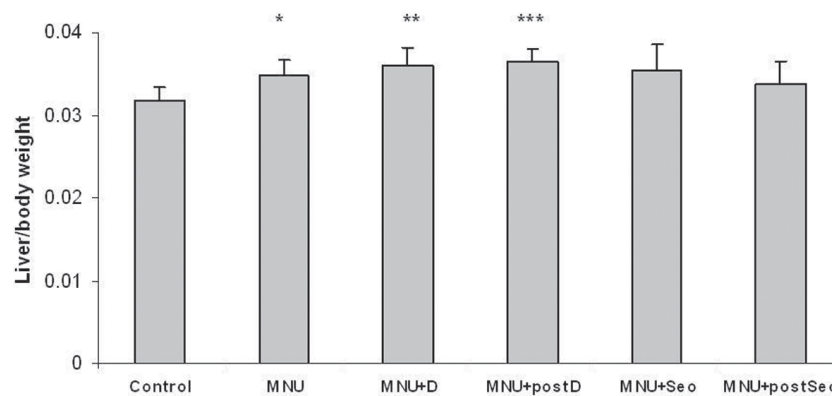
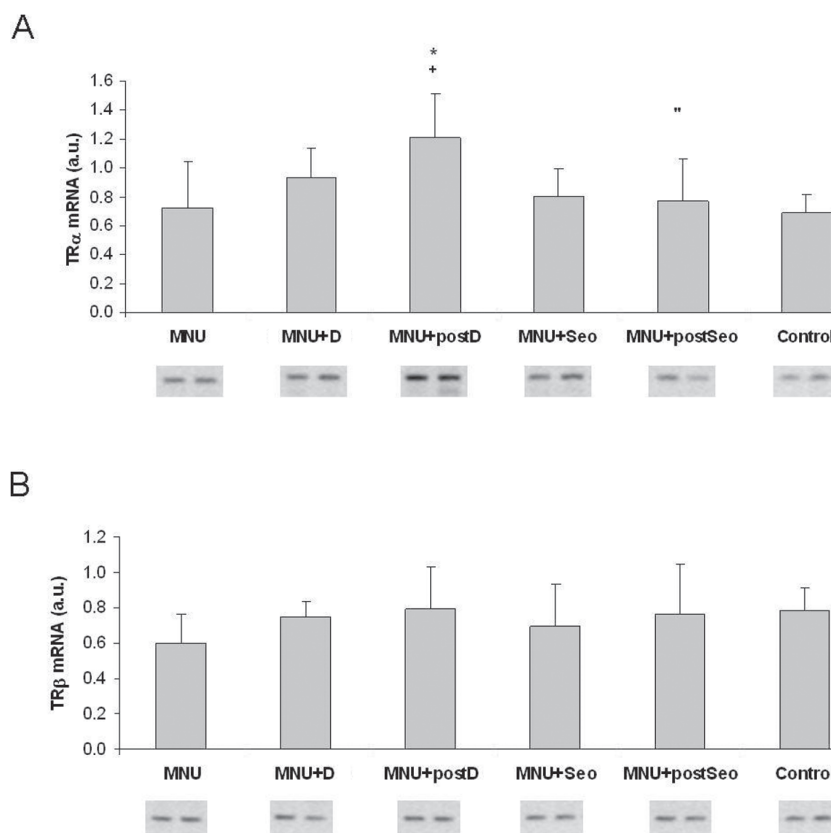

**Figure 2.** Liver/body weight ratio. Results are expressed as mean ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$. $* p < 0.05$ *vs.* Control; $** p < 0.01$ *vs.* Control; $*** p < 0.001$ *vs.* Control.

($p < 0.05$) was observed after the treatment with studied compounds when compared to healthy animals (Fig. 2). Chemopreventive (group MNU+D) or chemotherapeutical (group MNU+postD) administration of vitamin D to MNU animals resulted in maintaining of increased liver/body weight ($p < 0.01$ and $p < 0.001$, respectively) in comparison with healthy animals (group Control). We did not observe any toxicological effect on liver/body weight ratio of MNU animals treated with Seocalcitol when compared to healthy animals and MNU animals as well. Our observation confirmed reduced adverse effects of Seocalcitol on general metabolism of animals.

Carcinogenesis is characterized by an increased and compartmentalized energetic expenditure. We have already shown that thyroid status of animals can influence progress of MNU-induced carcinogenesis of mammary gland of female Sprague-Dawley rats (Macejova et al. 2005a). Since TRs and VDR belongs to the steroid/thyroid/retinoid nuclear receptors family, they display struc-

tural and functional similarities. They heterodimerise with the retinoid-X receptors (RXRs). Furthermore, VDR and TRs can interact with several nuclear co-regulators (co-activators, co-repressors) that play an important part in the control of transcription (Lemon and Freedman 1999; Xu et al. 1999).

In liver, vitamin D increases the amount of NADPH-dependent cytosolic $T_3$-binding protein, regulating the cellular $T_3$ translocation (Hashizume et al. 1991) and many others parameters (reviewed in Segura et al. 1999). In Ob17 murine preadipocytes, $T_3$ or vitamin D interfered in each other's adipogenic action and had synergistic as well as antagonistic effects after increase in vitamin D concentration (Lenoir et al. 1996; Dace et al. 1997). Both, $T_3$ and vitamin D, was found to markedly down-regulate the total number of TR target expression sites, mainly of the TRα1 type (Schneider et al. 2005). Also, it has been reported that $T_3$ and $T_4$ enhanced vitamin D-induced osteoclast formation (Miura et al. 2002). These observations indicate an

A



B



**Figure 3.** Relative levels of TRα mRNA (A) and TRβ mRNA (B). Results are expressed as mean ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$. + $p < 0.05$ *vs.* MNU; * $p < 0.05$ *vs.* Control; " $p < 0.05$ *vs.* MNU+postD. Bands shown below are representative samples of each tested groups.

existence of a cross-talk between their respective pathways of activity.

In our experiment, the expression of TRα mRNA was significantly higher in liver of animals treated with vitamin D after detection of first tumour (group MNU+postD) when compared to healthy animals (group Control) and MNU animals (group MNU) as well (Fig. 3A). On the other hand, the expression of TRα mRNA in liver of animals treated with Seocalcitol chemotherapeutically was, in comparison with animals treated with vitamin D in the same way, significantly lower. This observation indicates that Seocalcitol did not affect the expression of TRα mRNA at all when compared to vitamin D. The mRNA levels of TRβ receptor were unaffected (Fig. 3B). These observations indicate that opposite effect of administration of vitamin D and not its analogue Seocalcitol to animals in advanced mammary gland carcinogenesis on TRα receptor mRNA level when compared to murine preadipocytes (Schneider et al. 2005).

Marshall's *in silico* modelling (Marshall 2008) indicates that vitamin D also has a strong affinity for other nuclear

receptors, suggesting that at high levels it can interfere with their activity. This author has shown that vitamin D has a very high affinity for TRα. Normally, the levels of T$_3$ keep vitamin D out of the nuclear thyroid hormone receptor binding pocket. In our experiment, administration of vitamin D or Seocalcitol significantly reduced K$_A$ (group MNU+Seo; MNU+D) and increased B$_{max}$ (group MNU+Seo) of thyroid receptors in liver when compared to healthy animals (group Control) (Tab. 1). We suggest that this dysregulation of thyroid hormone receptor physicochemical properties due to vitamin D or Seocalcitol administration might be responsible for changes in both affinity and maximal binding capacity of TRs in liver. However, the ability of TRs to bind to appropriate DNA sequence (TRE*) remained unaffected (Fig. 4).

An additional possible mechanism of regulation of TRs by vitamin D can also include regulation through epidermal growth factor receptor (EGFR) since vitamin D also stimulates EGFR levels by a posttranslational mechanism that is associated with an increase in receptor autophosphorylation and tyrosine kinase activity (Ethier et al. 1993), and at the
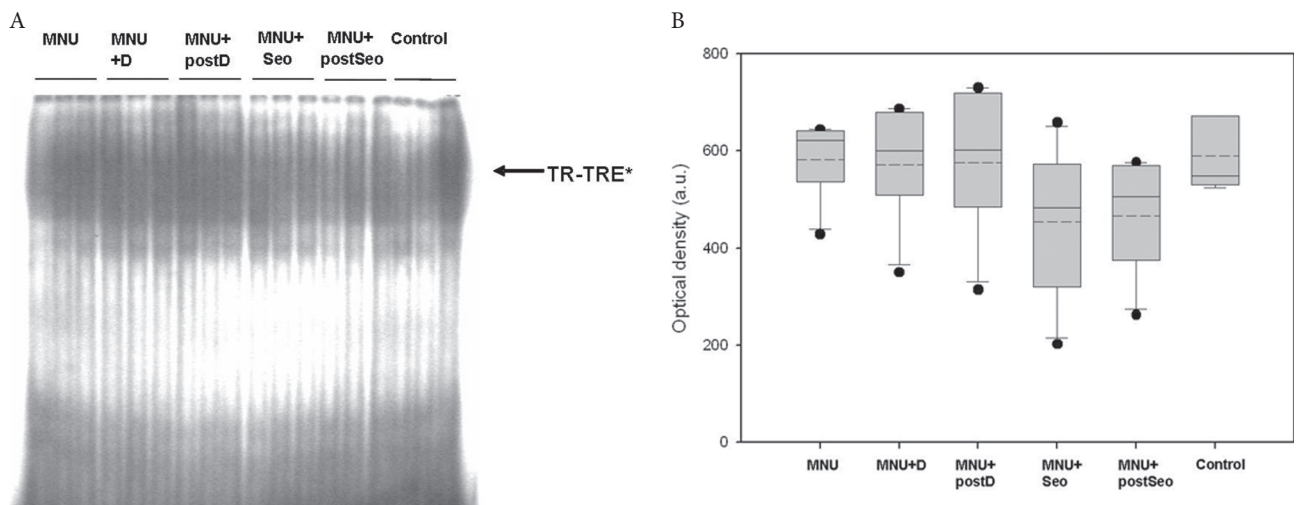
**Figure 4. A.** A representative autoradiogram of the functional DNA binding of thyroid hormone receptors. TR-TRE* represents complex TRs-TRs responsive elements. **B.** Data evaluation of the EMSA analyses. Medians ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$.

**Table 1.** $K_A$ and $B_{max}$ characteristics of binding ligand to thyroid hormone receptors

|  | MNU | MNU+D | MNU+postD | MNU+Seo | MNU+postSeo | Control |
|---|---|---|---|---|---|---|
| KA | 2.085625 | **1.9553333 | 2.1605 | **1.988333 | 2.687571 | 3.07275 |
| SD | 1.18296 | 0.4893619 | 1.5254023 | 0.662273 | 1.07446 | 0.325179 |

|  | MNU | MNU+D | MNU+postD | MNU+Seo | MNU+postSeo | Control |
|---|---|---|---|---|---|---|
| $B_{max}$ | 0.622125 | 0.5271667 | 0.663333 | *0.4665 | 0.445286 | 0.3605 |
| SD | 0.3408864 | 0.1737543 | 0.458168 | 0.093095 | 0.119444 | 0.011121 |

Results are expressed as mean ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$. * $p < 0.05$ *vs.* Control; ** $p < 0.01$ *vs.* Control.

same time it has been shown that epidermal growth factor decreased TRs and inhibited thyroid hormone response in GH4C1 cells (Kaji and Hinkle 1987). Yen and co-workers demonstrated significant VDR : TR cross-talk as they suggest, VDR may modulate $T_3$-mediated transcription in target genes in cells that express both receptors such as bone, gut, and skin (Yen et al. 1996).

Practically, no data are available in the literature concerning the direct effect of vitamin D on type I iodothyronine deiodinase activity in liver. Selenoenzyme 3,5,3'-triiodo-L-thyronine

5'-DI type I converts prohormone L-thyroxine to biologically active thyroid hormone 3,5,3'-triiodo-L-thyronine, a ligand of the thyroid nuclear receptors. In our experiment, in spite of the fact that the levels of 5'-DI mRNA was unaffected (Fig. 5), we show for the first time that the activity of this enzyme was significantly decreased in livers of animals treated with vitamin D (group MNU+D and MNU+postD), while in groups of animals treated with Seocalcitol, we didn't observed any change when compared to group MNU (Tab. 2). The data from this experiment suggest that vitamin D but not Seocalci-

**Table 2**. Activity of type I iodothyronine 5'-DI

|  | MNU | MNU+D | MNU+postD | MNU+Seo | MNU+postSeo | Control |
|---|---|---|---|---|---|---|
| 5'-DI | 3.53 | *2.816667 | *2.52 | 3.345 | 3.595 | 3.175 |
| SD | 0.471932 | 0.353535 | 0.611741 | 0.478445 | 0.263344 | 0.525516 |

Results are expressed as mean ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$. * $p < 0.05$ *vs.* MNU.
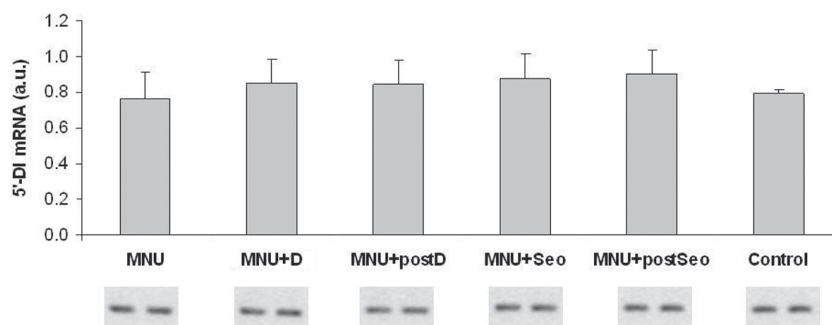
**Figure 5:** Relative levels of 5'-DI mRNA. Results are expressed as mean ± SD. MNU, MNU+D, MNU+postD, MNU+Seo, MNU+postSeo: $n = 6$; Control: $n = 4$. Bands shown below are representative samples of each tested groups.

tol may be responsible for decreased monodeiodination of T$_4$ in liver. We have already shown that the type I 5'-DI activity was markedly increased in MNU-induced mammary gland tumours, but in contrast, it was nearly negligible in non-lactating mammary glands of mock treated virgin female rats (Macejova et al. 2001b). Markedly increased 5'-DI activity in mammary tumours indicates a possible role for the increased monodeiodination of T$_4$ into T$_3$ to support high energetic expenditure during carcinogenesis. Also, it has been shown that vitamin D induced the dose- and time-dependently mRNA expression of type II iodothyronine deiodinase in primary osteoblastic cells, which is responsible for maintaining local T$_3$ concentration (Miura et al. 2002).

In conclusion, our data support the previous findings showing that human breast cancer could be accompanied by a variety of thyroid disorders (Rasmusson et al. 1987; Giani et al. 1996).

Evidence from epidemiologic studies has also revealed a possible association between thyroid dysfunction and breast cancer (Kuijpens et al. 2005; Saraiva et al. 2005). The data from our *in vivo* experiment has clearly shown for the first time that vitamin D, but not Seocalcitol i) may affect the body weight of animals, ii) can cause an increase in the expression of TRα in rat liver, remaining the functionality of the TRs unaffected, and iii) is responsible for type I 5'-DI activity decrease in rat liver, remaining the expression of the enzyme unaffected.

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