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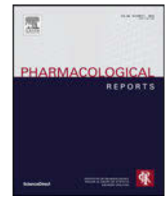

Original article

Expression levels of interferon-γ and type 2 deiodinase in patients diagnosed with recurrent depressive disorders

Elżbieta Gałecka[a,*], Monika Talarowska[b], Michael Maes[c,d,e], Kuan-Pin Su[f,g], Paweł Górski[a], Anna Kumor-Kisielewska[a], Janusz Szemraj[h]

[a] Department of Pneumology and Allergy, Medical University of Łódź, Łódź, Poland
[b] Department of Adult Psychiatry, Medical University of Łódź, Łódź, Poland
[c] Deakin University IMPACT Strategic Research Centre, Deakin University, School of Medicine, Barwon Health, Geelong, Australia
[d] Department of Psychiatry, Chulalongkorn University, Bangkok, Thailand
[e] Health Sciences Graduate Program, Health Sciences Center, State University of Londrina, Brazil
[f] Graduate Institute of Neural and Cognitive Sciences, School of Medicine, China Medical University, Taichung, Taiwan
[g] Department of Psychiatry and Mind-Body Research Center (MBI-Lab), China Medical University Hospital, 404, Taichung, Taiwan
[h] Department of Medical Biochemistry, Medical University of Łódź, Łódź Poland

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ABSTRACT

Background: Thyroid hormones (TH) are involved in modulation of the immune system and inflammation. TH dysregulation is associated with depressive disorders. The iodothyronine deiodinases (DIOs), the key enzymes for TH synthesis, can be affected and induced by pro-inflammatory cytokines. We aimed to investigate the levels of and correlation between type 2 DIO (DIO2) and interferon-gamma (IFN-γ) in patients with recurrent depressive disorders (rDD).
Methods: Data from 91 rDD patients and 105 healthy controls were analyzed. The diagnoses are based on the ICD-10 criteria (F33.0-F33.8). Expression levels of DIO2 and IFN-γ were estimated using the method based on the polymerase chain reaction and the enzyme-linked immunosorbent assay (ELISA).
Results: The DIO2 expression on mRNA/protein levels in rDD patients (both female and males) was reduced as compared with the control subjects. No correlation between DIO2 and IFN-γ expression was observed.
Conclusion: This is the first study to reveal that one may cautiously suggest that DIO2 may be involved in the development and/or progression of rDD. The mechanisms of TH regulation on depression, however, need further investigation.
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Introduction

Thyroid hormones (TH) are key factors affecting proper functioning of almost every tissue, organ and system of a human body. The primary mechanism of action of TH involves an interaction with widely expressed receptors for thyroid hormones (THR), the specific aim of which is gene promotion and substantially gene expression or reduced transcription [1,2]. The thyroid gland is predominantly responsible for TH synthesis; nevertheless, sites of action and metabolism are under control of numerous other molecules, including TH transporters and three types of iodothyronine deiodinases 1,2,3 (DIO1, DIO2, DIO3) [3,4]. DIO1 and DIO2 generate triiodothyronine (T3) from thyroxine (T4), while DIO3 inactivates TH by converting T4 into reversed T3 (rT3), and T3 into diiodothyronine (T2) [5].

Pathways of synthesis and levels of TH are influenced and affected by many mechanisms and factors including pro-inflammatory cytokines such as: interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-gamma(IFN-γ) [6]. An inverse correlation is observed between serum T3 levels and IL-6 concentration [7]. Sato et al. found that all the cytokines mentioned above decrease iodothyronine secretion [8]. Yu and Koenig observed that IL-1, IL-6 reduce expression of DIO1 in HepG2 cells [9]. The results regarding DIO2 are varied and depend on the tissue.

For example, the expression of DIO2 in pituitary cell goes up after incubation with cytokines [10], as well as after treating rat and mouse brain with lipopolysaccharide (LPS) [11], while the

* Corresponding author.
E-mail address: galeckaela@wp.pl (E. Gałecka).

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activity of the enzyme in human skeletal muscle is lower after incubation with TNF-α and IL-6 [12]. There are also results demonstrating that TH may modulate the immune system and affect inflammatory processes by influencing phagocytosis, chemotaxis, oxidative stress or cytokine production. In addition, the above-mentioned processes are found to be altered in hypothyroidism and hyperthyroidism [13].

Depressive disorder is a disease with multifactorial components, including disturbances in the hypothalamic-pituitary-thyroid (HPT) axis and TH concentration, the presence of immune activation and inflammation [14–16]. Depressed patients are characterized by TRH-mRNA downregulation, elevated peripheral free T4 levels [17,18], and increased rT3 concentrations in CSF [19], but not in serum [20]. In addition, hyper- and hypothyroid states are often observed in depressed patients, may be involved and in the development of a depressive disorder. 8.8% of major depressed subjects with melancholic features show some degree of subclinical hyperthyroidism, but no hormonal signs of hypothyroidism [20]. Depressive disorder is sometimes regarded as the low T3 syndrome with low T3 and increased rT3 levels [21,22]. Results of a recent study on an animal model have demonstrated that defects in DIO2 expression in brain astrocytes could result in mood and behavioral disorder[23]. The prominent features of the immune-inflammatory activity include, increased levels of cytokines (e.g. IL-1, IL6 and IFN-γ), and a higher activity and number of immune cells [16]. Interferons take part in many immune reactions and regulate innate and adaptive mechanisms during viral infections [24]. IFN-γ is secreted by T lymphocytes, natural killer cells, macrophages and dendritic cells, and has immunoregulatory effects on various immune cells, including pro-inflammatory effect. The secretion of different molecules, for example other cytokines, but also TH metabolism-related molecules, such as deiodinases, is also under the influence of IFN-γ [25].

Available data demonstrate the interaction between molecules and signs of inflammatory processes and thyroid hormone-related molecules [26]. For example, a significant inverse association between baseline TSH levels and increased haptoglobin, an indicant of inflammation, has been described in depressed patients [26]. These results suggest that the immune-inflammatory process in depression may modulate HPT-axis function.

Considering all above described data, we aimed to investigate DIO2 and IFN-γ on mRNA/protein levels in the patients diagnosed with recurrent depressive disorders (rDD).

Materials and methods

Subjects

A group of 91 patients (53 females; 58.24%), diagnosed with and treated due to rDD, were recruited to this study. The mean age in that group was 47.24 ± 11.82 years (mean ± SD). The number of depressive episodes and the duration of the disease were assessed for each individual. The average duration of the disease was 6.43 ±7.72, while the number of depressive episodes totaled 4.44 ±5.32, number of hospitalization was 2.09 ± 1.61 (mean ± SD). A diagnosis of rDD was made according to the ICD-10 (1992) criteria (F33.0-F33.8). A medical history was obtained in all cases and assessed with the use of the standardized Composite International Diagnostic Interview (CIDI) form [27]. The Hamilton Depression Rating Scale (HDRS) was used to estimate the severity of depressive symptoms. The HDRS baseline value (before treatment) was 22.79 ± 6.14 and 6.88 ± 4.36 final (after treatment). The group of control subjects comprised selected healthy community individuals (n = 105, 69 females; 65.71% and 38 males) aged; 28.89 ± 8.69 (mean ± SD), invited to take part in the study based on the absence of diagnostic criteria of the psychiatric CIDI interview. We excluded both patients and controls with other psychiatric axis I and II diagnoses from the study. The exclusion criteria were as follows: (auto)immune-inflammatory diseases and thyroid diseases. All the patients were native inhabitants of central Poland and were unrelated to one another. Peripheral blood samples were drawn from analyzed subjects in the morning between 7.00 and 9.00 after all night fasting. All the procedures were reviewed and approved by the Local Bioethics Committee. Written informed consent was obtained from all the participants of the study.

Detection of gene expression using real-Time PCR reaction

Total RNA isolation from blood of the patients was carried out using a modified Chomczyński method [28]. The absorbance of isolated RNA was measured using a spectrophotometer (Picodrop, Hinxton, United Kingdom) at λ = 260 nm to determine total RNA concentration. Isolated RNA was stored at a temperature of −70 °C. The quality of total RNA was checked with the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara CA, USA) in accordance with the manufacturer's recommendations. 1 μl of RNA 6000 Nano dye was added to a test tube containing 65 μl of Agilent RNA 6000 Nano gel matrix and then centrifuged (10 min, 13000 x *g*). The gel-fluorescent dye mixture was applied on the surface of a Nano chip placed in a workstation. Then, 5 μl of RNA Nano marker was added to selected pits. Isolated samples of RNA and RNA size marker were subject to denaturation (2 min, 70 °C), and then 1 μl of the sample was pipetted to selected pits of the Nano chip and mixed (1 min, 2400 rpm). The quality of isolated RNA was checked using the 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA).

An RT reaction was carried out with the use of the TaqMan® RNA Reverse Transcription Kit (Applied Biosystems) based on the manufacturer's recommendations. The samples were incubated (30 min, 16 °C, and 30 min, 42 °C) in a thermocycler (Biometra, Gottingen, Germany). Reverse transcriptase was inactivated (5 min, 85 °C) and the obtained cDNA was stored at a temperature of −20 °C.

Real-Time PCR reaction was conducted using the TaqMan® Universal PCR Master Mix, No UNG (Applied Biosystems, Foster City, CA, USA), according to the protocol provided by the manufacturer using Hs00988260_m1, Hs00989291_m1, Hs04194366_g1 probes specific, respectively, for DIO2, INF gamma and RPL13A genes, delivered by Applied Biosystems. To calculate expression of the analyzed genes on the mRNA level, the Ct comparative method was applied [29]. The level of DIO2, INF gamma gene expression was normalized in relation to the RPL13A reference gene. Each target probe was amplified in a separate 96-well plate. All the samples were incubated at 50 °C for 2 min and at 95 °C for 10 min, and then cycled at 95 °C for 30 s, at 60 °C for 30 s and at 72 °C for 1 min; 40 cycles were performed in total. Fluorescence emission data were captured and mRNA levels were quantified using the critical threshold (Ct) value. The analyses were performed with ABI Prism 7000 (SDS Software, Applied Biosystem). Controls without RT and with no template cDNA were performed with each assay. The threshold cycle (Ct) was calculated for each sample. The RT-PCR amplification of the *DIO2* and *INF* gene was compared to that of *RPL13A*, a house-keeping reference gene and ΔCt were determined (ΔCt = Ctgene-Ct*RPL13A*). in each sample patients and controls. The results were analyzed according to the $2^{-\Delta ct}$ method [29].

Determination of protein concentration with enzyme-linked immunosorbent assay (ELISA)

Human enzyme-linked immunosorbent assays were used to detect the levels of IFN-γ and DIO2 in serum.

Serum was separated from peripheral blood using clot activating tubes. Next, the samples were allowed to clot for 30 min and then centrifuged for 15 min at approximately 1000 x *g*. After the centrifugation, the serum was removed and stored aliquot at −80 °C. IFN-γ and DIO2 was measured using commercially available Human IFN-gamma Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) and Human DIO2 ELISa Kit (MyBiosourse, San Diego, CA, USA). Calculations of results were performed according to the instructions and protocols provided by the manufacturers. The absorbance of the samples was measured using the Multiskan Ascent Microplate Photometer (Thermo Labsystems, Waltham, MA, USA) at λ = 450 nm. Analytical curves for the analyzed proteins were made with the aim of determining protein concentration. Serum IFN-γ levels was presented as pg/ml while DIO2 levels as ng/ml.

Statistical analysis

A statistical analysis of the collected material included calculation of both descriptive and inferential statistics. The results are presented as percentages (%) or means (M) with standard deviations (±SD). The chi-square test and Mann-Whitney U- test was used to compare demographic variables (gender and age) between patients and controls. To compare nonparametric variables in the test groups, the Whitney *U* test for two independent groups was used. To evaluate the relationships between the analyzed variables, Pearson's correlation coefficients were estimated. For all the analyses, statistical significance was defined as $p < 0.05$. All the data analyses were calculated using STATISTICA PL, version 12.

Results

No significant differences were found between the rDD patients and the controls with respect to gender ($\chi 2 = 1.16$, $p = 0.28$). The groups were gender-matched but varied significantly with respect to age distribution ($Z = 8.65$, $p < 0.0001$). There were no significant differences in IFN-γ expression between the patients and the controls on both the mRNA and protein levels ($p > 0.05$). The obtained results indicated that DIO2 expression levels were significantly lower in the patients diagnosed with rDD when compared to the controls. Results are presented in Table 1.

Table 1 In addition, the significant differences on both the mRNA and protein level were also observed between group of males with rDD and healthy males and between females with rDD and healthy females. Results can be found in Table 2.

Table 2 There were no differences between rDD males and rDD females and between healthy females and males (Mann-Whitney U − test $p > 0.05$). No correlation was found between the IFN-γ and DIO2 expression on mRNA/protein levels and between both IFN-γ, DIO2 and the clinical variables tested ($p > 0.05$).

Significant relationships were observed between the expression of IFN-γ on the mRNA and protein levels in both the patients as well as the controls ($r = 0.887911$, $r = 0.924115$; $p < 0.05$, respectively), between DIO2 gene and protein expression similarly in both group ($r = 0.954134$, $r = 0815538$; $p < 0.05$, respectively).

Discussion

To our knowledge, this is the first study to investigate DIO2 expression levels in the patients diagnosed with rDD and the correlation between the molecule and IFN-γ.

In our study, we did not observe and confirm the role of IFN-γ during depressive disorders. The obtained data are not in line with other studies. The small sample size cannot be excluded and investigation should be performed on larger sample in the future. Increased levels of IFN-γ in depressed patients, were for example reported by Schmidt et al. [30]. In addition, the latter authors observed elevated levels of IFN-γ in depressed and obese patients, which may suggest and partially explain the co-morbidity between depression and obesity as a result of immune dysfunctions that may be a common feature. Similarly, higher levels of IFN-γ, were observed in the depressed patients with acute heart failure exacerbations [31]. The increased level of IFN-γ was found in depressed patients and correlated with total cholesterol and LDL levels [32], suggesting involvement of immune-inflammatory molecules which may affect total cholesterol and lipid metabolism as a factor taking part in the pathophysiology of depression. The higher level of peripheral IFN-γ was presented by Dahl et al. in free medication patients diagnosed with major depressive disorders; however, after 12-week treatment the levels decreased and were similar to the concentration in healthy subjects [33]. It cannot be excluded that other mechanisms, including different molecules, epigenetic regulation and/or long non-coding RNAs, may participate in IFN-γ transcription [34,35]. Regarding the relationship between IFN-γ and DIO2 levels, we did not find an association between the molecules in the group of patients. Similarly, there are findings confirming that there is no relationship between serum IFN-γ and T3 levels in subjects with normal and/or subnormal serum TH concentration [36]. On the contrary, studies in animal models showed that IFN-γ injection can decrease T3 concentrations in a dose-dependent manner, but does not affect DIO1 expression [37]. Such results may indicate that influencing DIO2 levels is one of the mechanisms based on which IFN-gamma can lower T3. Other inflammatory-related factors and/or mechanisms cannot obviously be excluded. As described by Wajner and Maia, IL-1β, IL-6 or TNF-α may influence TH metabolism as well as affect deiodinase levels and/or activity [38]. A close correlation is observed between IL-1β and DIO2 upregulation during an acute and chronic inflammation in macrophages and monocytes, but independently of serum T3 [39].

DIO2 plays a role in an inflammatory and immune response; DIO2 and TH influence respiratory burst, inducible nitric oxide (iNOS) activation and cytokine secretion [40]. Recently, Wen et al. examined DIO2 gene expression in peripheral blood mononuclear cells in patients with the Kashin-Beck disease – a type of osteoarthritis during which development inflammation plays a

Table 1
Expression on the protein level and on the level of mRNA for IFN-γ and DIO2 in the examined group.

Variable	rDD n = 91 M (±SD) Median	Controls n = 105 M (±SD) Median	Statistical analysis
IFN-γ mRNA ($2^{-\Delta Ct}$)	0.19 (0.08) 0.18	0.18 (0.07) 0.17	$p = 0.42$; Z = 0.0.81
IFN-γ protein (pg/ml)	4.79 (0.74) 4.75	4.75 (0.81) 4.66	$p = 0.81$; Z = 0.24
DIO2 mRNA ($2^{-\Delta Ct}$)	0.12 (0.019) 0.12	0.28 (0.06) 0.27	$p < 0.0001$; Z = −12.05
DIO2 protein (ng/ml)	0.36 (0.04) 0.36	0.67 (0.13) 0.65	$p < 0.0001$; Z = −11.75

Z – Mann –Whitney *U* test IFN –γ – interferon – γ; DIO2–deiodinase type 2;n – number of subjects; rDD – recurrent depressive disorders; M – mean; SD – standard deviation, *p* – level of statistical significance.

Table 2
Expression on the protein level and on the level of mRNA for DIO2 in females and males with rDD and controls.

variable	rDD females n = 53	Control females n = 69	Statistical analysis	rDD males n = 38	Control males n = 36	Statistical analysis
	M (±SD) Median	M (±SD) Median		M (±SD) Median	M (±SD) Median	
DIO2 mRNA ($2^{-\Delta Ct}$)	0.12 (0.02) 0.12	0.29 (0.06) 0.27	$p < 0.001$ $Z = -9.44$	0.12 (0.02) 0.11	0.27 (0.06) 0.26	$p < 0.001$ $Z = -7.38$
DIO2 protein (ng/ml)	0.36 (0.04) 0.36	0.68 (0.13) 0.67	$p < 0.001$ $Z = -9.16$	0.35 (0.04) 0.35	0.65 (0.13) 0.64	$p < 0.001$ $Z = -7.25$

Z – Mann-Whitney *U* test; interferon – γ; DIO2–deiodinase type 2; n – number of subjects; rDD – recurrent depressive disorders; M – mean; SD – standard deviation, *p* – level of statistical significance.

significant role [41] – and found significantly higher mRNA levels of DIO2 in patients [42]. In addition, suppression of mRNA of DIO2 significantly increases COX-2 gene expression – both basal and IL-1β induced, which may, on the other side, suggest anti-inflammatory properties of DIO2 [43].

The important objective of our study was to compare the expression levels of DIO2 between control subjects and patients. We observed that DIO2 levels were significantly lower in rDD patients as compared with the healthy controls. Our results are similar to those presented by Bocca et al. [23], who found a relationship between lower expression of DIO2 gene and mood and behavioral diseases on an animal model. Previously we showed that functional polymorphisms within the DIO2 gene has an impact on the risk of rDD [44]. The polymorphisms are known to affect expression levels. There is a possibility that lower expression is a result of a genetic variant. Our data are not in line with results suggesting that higher levels of DIO2 could be considered a compensatory mechanism for the lower levels of DIO1 as the expression and function of DIO1, and TH metabolism and synthesis, may be inhibited by pro-inflammatory cytokines [45,46]. We did not confirm that the involvement of pro-inflammatory molecules in the development and course of depression may explain the higher levels of DIO2 and its inducible nature. Similarly, decreased expression of DIO2 was found in the muscles of septic patients and in mice affected by *S. pneumoniae* [47,48]. Nevertheless, there are studies which show that TH-related signals are crucial elements of the defense mechanism [49] and that NF-kB – the most crucial factor involved in the expression of numerous inflammatory response genes – is important for the expression of DIO2 [50]. The inflammatory-related induction of DIO2 and the role of DIO2 in the regulation of inflammatory processes is also supported by the fact that high expression and activity of the enzyme is observed after stimulation with LPS [11]. Inflammation-induced DIO2 expression was confirmed in macrophages in mice during an inflammation [51], and DIO2 is upregulated in response to a ventilator induced lung injury [52].

Expression levels may be obviously under the influence of many factors and mechanisms (i.e. transcriptional and post-transcriptional); they are tissue-specific, depend on the type of illness and inflammatory processes, and may be also time and cell-specific.

DIO2 is an important extrathyroidal source of T3 [5]. The decreased DIO2 levels in current study may explain that lower T3 might be involved in the development and course of depression [53,54]. Moreover, DIO2 and TH are very important for brain development – both during development and after achieving full maturity [15]. In addition, studies on animal models indicate lower cortical serotonin concentration, sensitization of serotonin receptors and inhibition of cortical and hippocampal serotonin release in hypothyroid conditions [14]. Lowered DIO2 levels may explain the TH-related disturbances characteristically found in depression such as fatigue and psycho-motor speed, attention, concentration and memory impairment [55–57]. Insufficient levels of DIO2 and TH may explain the efficiency of T3 supplementation in the treatment of depression [58]. We did not examine brain expression of DIO2; however, expression in peripheral cells may reflect that in the brain [59].

The lack of information about the sera TH (T3, T4) levels is considered a limitation of this study. We are aware of the fact that no such data are demonstrated. Castagna et al. [60] observed an association between free T3 (FT3) values, DIO2 enzymatic activity and polymorphic variant within *DIO2* gene. Protein stability and FT3 levels were significantly lower in the subjects carrying mutated Ala allele. The results are in line with a study that found higher levels of T3 in subjects carrying Thr allele [61]. In our previous investigation we observed that Ala allele may serve as a marker for a lower risk of rDD (Gałecka et al., 2015). Regarding the next results by Castagna et al. [60], Ala mutant of DIO2 producing less T3 caused less apoptosis. Protective effects of this allele may be related to reduced damage of brain cells. The fact is worth to be mentioned as DIO2 is an important source of T3 in the brain [5], while disruption of brain cells is involved in depression development [62]. The presentation of T3 and T4 levels could be valuable; obviously, it could expand the knowledge about various links, particularly between DIO2 and the status of TH. We did not eliminate the role of DIO2 as a significant regulator of TH levels [5]. We cannot exclude that DIO2 may be involved in different processes related to depression. The main aim of the study was to assess the expression levels of DIO2 and IFN-γ in the patients suffering from rDD. We would like to explain that we decided to investigate DIO2 and IFNγ as molecules involved in inflammation and immune activation processes characteristically observed in depressive disorders [16] rather than factors affecting T3 and T4 levels. There are results indicating that DIO2 levels are changed during inflammatory processes [11,51]. In addition, some data indicate an interaction between DIO2 and immune-inflammatory molecules [43]. Another limitation of this study is associated with the fact that the patients were under antidepressant treatment, which may affect expression levels [63,64]. To our best knowledge, there is no available information in public domain demonstrating the impact of antidepressants on DIO2 and IFN-γ levels. Some results confirm observed circadian changes in cytokine expression, including IFN-γ [65], while other data provide information that DIO2 expression is under circadian control [5] We want to emphasize that the blood samples were collected at the same time from all subjects included in the study.

Conclusions

In summary, the results of our study show that DIO2 expression levels in peripheral cells are reduced during depression. Based on the obtained data, one may cautiously suggest that DIO2 levels seem to be associated with the mechanisms that underpin the pathophysiology of rDD and may play a role in the processes involved in the course of depression. Our results maysuggest the role of the factors involved in TH metabolism in the etiology of the

said disease. More investigations are obviously required to explore and explain the possible significance of the molecule in depression.

Conflict of interest

The authors declare no conflict of interest in association with this manuscript.

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