Ethionamide Alters Thyroid Receptor Gene Expression in Rats' Muscle

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Abstract

BACKGROUND: Ethionamide usage as one of the drug regimens still becomes a challenge due to high numbers of patients developing hypothyroid. Ethionamide had been associated with the inhibition of thyroid hormone (TH) synthesis and interestingly, ethionamide (C$_8$H$_{10}$N$_2$S)-induced hypothyroidism is supported by its similar structure with thioamides, propylthiouracil (C$_7$H$_8$N$_2$S). However, hypothyroidism is not solely caused by its production, it could be caused by signaling alteration. Therefore, knowing that important TH action is determined via genomic pathway, alteration of this receptor could bring serious clinical problem. Unfortunately, there is limited study about the regulation of ethionamide and its connection on TH genomic signaling especially thyroid hormone receptor (TR) gene expression in soleus, gastrocnemius and cardiac muscle.

METHODS: Thirty-eight rats were divided into control, ethionamide and propylthiouracil groups. After 12-week treatment, rat were sacrificed, then gastrocnemius, soleus and cardiac muscles were dissected out, snap freezed using liquid nitrogen, and stored in -80°C until use. RNA was extracted and run for reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: In soleus muscle, ethionamide stimulated TR mRNA expressions and deiodinase compared to control group. In contrast, $TR\alpha_1$ gene expression was not affected by ethionamide administration. In gastrocnemius muscle, only $TR\beta_1$ gene and $Dio2$ gene expressions that were significantly increased compared to control group. In cardiac muscle, ethionamide significantly stimulated all the thyroid hormone receptor isoform and iodothyronine deiodinase gene expression compared to the control group.

CONCLUSION: Long ethionamide treatment upregulates TR gene expressions and deiodinase in soleus and cardiac muscle, there is different expression pattern of soleus, gastrocnemius and cardiac muscle after ethionamide stimulation.

KEYWORDS: ethionamide, hypothyroid, $TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$, $TR\beta_2$

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Introduction

Ethionamide (C_{12}H_{10}N_{2}S) is bacteriostatic anti-tuberculosis agent which derives from isonicotinic acid and act as oral second line drugs treatment regimen for multi drug resistance tuberculosis (MDR-TB).(1) Ethionamide are pro drugs that require enzymatic activation by mycobacterial EthA and convert ethionamide into InhA competitive inhibitor of Mycobacterium tuberculosis mycolic acid synthesis.(2) The most frequently observed side effects of ethionamide include gastrointestinal disturbance, psychiatric disorder, arthralgia, hepatitis, peripheral neuropathy, epileptic seizures, dermatological effects, ototoxicity, nephrotoxicity and hypothyroidism.(3)

Hypothyroidism is a known complication of MDR-TB treatment, which using ethionamide as on of its regimen.(4,5) Interestingly, 28-49% of patients have developed hypothyroidism during MDR-TB treatment, which may indicate the importance of understanding the complete mechanism.(6) Studies revealed that ethionamide have direct effect to thyroid hormone (TH) synthesis, which induced hypothyroidism.(7) Aside from alteration of TH synthesis, the molecular mechanism of ethionamide effects on regulating thyroid hormone receptor (TR) gene expression remains unclear.

TH is essential for the regulation of metabolic processes throughout the body, which deficiency of this hormone alter normal body function.(8) TH synthesis in thyroid gland is regulated by the level of thyroid stimulating hormone (TSH) and thyrotropin releasing hormone (TRH). TSH and TRH level are regulated by the level of thyroxine (T4) and triiodothyronine (T3) via binding to TSH-β2 and TRH-β2 receptor. TH form in the serum are T3 and T4 form, T3 is active form of TH and T4 needs to be converted to T3 form by deiodinase enzyme activity in tissues. Deiodenase act as enzyme for activating or deactivating. Iodothyronine deiodinase (Dio)1 and Dio2 are activating enzyme which catalyze conversion of T4 into T3 in circulation, and Dio3 act as deactivating enzyme which catalyze conversion of T4 into reverse T3 (rT3) and the conversion of T3 to T2. TH action in molecular level works in genomic and nongenomic pathway, which T3 and T4 serum level, and TH receptor mechanism are essential for normal cells function throughout the body. There are 2 TR genes, namely TRα and TRβ.(9,10) TRα1 is mostly expressed in cardiac muscle, skeletal muscle, and central nervous system. TRβ1 is highly expressed in the liver and kidney, while TRβ2 is highly expressed in retina, and cochlea.(11)

Unfortunately, there is limited study about ethionamide effects on TR gene expression. Thus, exploring the effects of ethionamide on TR gene expression might be beneficial in the development of a new ethionamide post effect treatment which induce hypothroid.

Methods

Animal Model and Treatment

All animal procedure is approved by Ethical Committee of Faculty of Medicine, Universitas Padjadjaran (No. 1462/UN6.KEP/EC/2019). Thirty-eight, male, wistar rats, aged 20 weeks old, were bred in PT. Bio Farma, Bandung, Indonesia. Rats were kept in comfort room at 24°C temperature, 68% humidity. Rats were given food and water ad libitum for 12 weeks per day in the Animal Laboratory, Faculty of Medicine, Universitas Padjadjaran.

Rats were divided into 3 groups, which were negative control group (no treatment), treatment group (ethionamide treatment), and positive control group (propylthiouracil treatment). Each group were given its respective treatment for 12 weeks. Ethionamide (CAS Number: 536-33-4; E6005, Sigma, North Liberty, USA) was diluted in water accordingly and specific rat dose were counted (1.66 mg/200 gram body weight) and given orally by using soft gavage for 12 weeks consecutively. We used isofluran anaesthesia 1-3% inhalant during sacrifice. Gastrocnemius, soleus, and cardiac muscle were removed, dip frozen into liquid nitrogen and stored in -80°C until use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Examination

Soleus, gastrocnemius, and cardiac muscle tissue were collected, weighted for 25 mg into microtube and used for RNA isolation using TRIsure reagent (Invitrogen, Carlsbad, California, USA). Total RNA were quantified using Multimode Microplate Reader at 268/280 nm absorbance spectrophotometry (Tecan, Männedorf, Switzerland). Specific primer were used in this study for TRα1, TRα2, TRβ1, TRβ2, Dio1, Dio2, Dio3 and GAPDH as internal controls for normalization (Table 1). Primers were purchased from Sigma (Genetika Science, Singapore) and Onestep RT-PCR Kit by Bioline (London, UK). Twenty-five μL reaction mixture contained 10x reaction buffer, 200 μM deoxynucleoside triphosphates (dNTPs), 1 μL of each oligonucleotide primer at 20 mM (0.8 mM each primer), and 300 ng of RNA and 1 unit of Taq polymerase enzyme. All samples were amplified with a Thermal
Results

Table 1. Specific primers used in this study.

| Gene  | Primer Sequence                          | Product Size (bp) | Tm (°C) | Cycle |
|-------|------------------------------------------|-------------------|---------|-------|
| TRα1  | F 5'-TGAGCACTACGTCACAACCACC-3'          | 136               | 590     | 29    |
|       | R 5'-GGTGCGGACTGCTCGACCATATTTT-3'       |                   |         |       |
| TRα2  | F 5'-AGTTTTTCTCTTCTGGCTTGCC-3'          | 265               | 570     | 29    |
|       | R 5'-TGCGGACTGCTCGACTCTTATCAT-3'        |                   |         |       |
| TRβ1  | F 5'-ACTCGGTATCAAGGCTGCTC-3'            | 284               | 590     | 29    |
|       | R 5'-TCACTATGGGCAATTACAGGG-3'           |                   |         |       |
| TRβ2  | F 5'-ACTGATGGCTCTTTGTCCG-3'             | 183               | 600     | 31    |
|       | R 5'-GACGCGGCTCCATGAAAC-3'              |                   |         |       |
| Dio1  | F 5'-TCCTTCTGCTCGACGATGGATG-3'          | 197               | 600     | 31    |
|       | R 5'-GCCCCACCTATGAGCTACGT-3'            |                   |         |       |
| Dio2  | F 5'-TACCTGTTGTTCAAGGTCCAC-3'           | 123               | 600     | 31    |
|       | R 5'-TGCACAGCAGATGTTG-3'                |                   |         |       |
| Dio3  | F 5'-TTCCTCCAGCTCTGACTGT-3'             | 144               | 600     | 31    |
|       | R 5'-GCACCCAGAGCCTTCC-3'                |                   |         |       |
| GAPDH | F 5'-CCATTCTCAGCTCGACTGT-3'             | 177               | 590     | 24    |
|       | R 5'-TCTGCGCTGATGGCGCCG-3'              |                   |         |       |

Cycler following PCR protocol repeated for 31 cycle; final elongation at 72°C for 7 min; then cooling to 4°C.

Five µL of PCR products were combine with run on a 1.2 % agarose gel TAE, stained with green dye. Electrophoresis HU10 SCIE-PLAS from Scie-Plas Ltd. (Cambridge, United Kingdom) were used in this research. Gel was visualized using blue pad and density of PCR band products in the photograph were analyzed using Image J software (NIH, Bethesda, USA).

Statistical Analysis

All data were analysed using Graphpad Prism software version 7.00 for windows (GraphPad Software, San Diego, USA). Results were tested with One Way ANOVA test, Kruskal Wallis and followed by Mann Whitney or Tukey post hoc test with 95% confidence interval (p<0.05).

Discussion

Physiological changes are due to ethionamide share similar structure with thioamides, for example, propythiouracil (C,H,N,S) and methimazole (C,H,N,S). Its similar structure induce inhibition of peroxidase enzyme which decrease the iodine conversion to iodide, and inhibit organization of
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Iodide into moniodothyronine (MIT) and diiodothyronine (DIT), which reduce TH synthesis. (5, 12) This inhibition mechanism induce lower T3 and T4 in circulation.

In this present study, ethionamide upregulates significantly in TR and iodothyronine deiodinase gene expression on soleus and cardiac muscle, however this effect seem does not affect it in gastrocnemius muscle tissue (Figure 1). Our data showed that 12 weeks of ethionamide treatment altered the gene expression and might affect the genetic modulation of TH via TR. TH action works in genomic and nongenomic pathway. In genomic pathway, TR works with thyroid hormone response element (TRE), heterodimeric partner, coregulator, and deiodinase to regulate RNA transcription in TR-DNA complexes. TRE are transcription factors that bind to TR and TH-regulated genes. (13, 14) Heterodimeric partner, like retinoid X receptor, peroxisome proliferator-activated receptor, and vitamin D receptor increase TR to TRE binding. (15, 16) Coregulator regulate TR-DNA complexes transcription by two mechanism, corepressor and coactivator. Corepressors, consist of silencing mediator of retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR), repress transcription by binding with TR-DNA complexes. In the presence of ligand, coactivators consist of steroid receptor, Trip-1, and TR-associated protein coactivator complex will replace corepressor in TR-DNA complexes.

Figure 1. Representative electrophoresis image of thyroid hormone receptor (TRα1, TRα2, TRβ1 and TRβ2) and iodothyronine deiodinase (Dio1, Dio2, Dio3) mRNA expression in soleus, gastrocnemius and cardiac muscle.

Figure 2. Relative gene expression level of TRα1, TRα2, TRβ1, TRβ2, Dio1, Dio2, and Dio3 in soleus muscle. Data were normalized with GAPDH and presented as relative ratio values (average mean±SEM). *p<0.05; ***p<0.001 were considered as significant. Results were tested with One Way ANOVA test, Kruskal Wallis and followed by Mann Whitney or Tukey post hoc test with 95% confidence interval.
complexes which will stimulate transcription. (14,17) TR gene expression are expressed specifically in various organ, in this case is upregulating gene expression TR is expressed as different isoforms (TRα1, TRβ1, and TRβ2) differentially expressed in various tissues. (18)

In skeletal muscle, TR binds to thyroid response elements in genes regulated by thyroid hormones, forming heterodimers with the retinoid X receptor. Subsequent formation of a protein complex including NCoR2 co-repressor and histone-modifying enzymes causes repression of transcription. (14,17) When T3 binds to the TR, repression is reduced and transcription is further stimulated by the recruitment of co-activators to the complex. Among proteins whose expression is regulated transcriptionally by T3 in muscle are sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 1a, SERCA2a, uncoupling protein (UCP)3, glucose transporter (GLUT)4, malic enzyme (ME) 1, mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), and myosin-7. (19-22) This is process that reflect a genomic regulation of thyroid hormone.

In soleus muscle, most of TR and iodothyronine deiodenase were significantly upregulated. Dio1, Dio2, and Dio3 which act as converting enzyme in thyroid hormone-skeletal muscle activation, were found significantly upregulated. TRα2, TRβ1, TRβ2 were also significantly upregulated. However, TRα1 was not upregulated significantly in soleus muscle (Figure 2). In gastrocnemius muscle, most of thyroid hormone receptor gene expression and iodothyronine deiodenase were not significantly upregulated. TRβ1 and Dio2 were found significantly upregulated. On the contrary, TRα1, TRα2, TRβ2, Dio1, and Dio3 weren’t significantly upregulated (Figure 3). The upregulation of TR and iodothyronine deiodenase gene expression may indicate compensation of hypothyroid

![Gastrocnemius Muscle](image1)

**Figure 3.** Relative gene expression level of TRα1, TRα2, TRβ1, TRβ2, Dio1, Dio2, and Dio3 in gastrocnemius muscle. Data were normalized with GAPDH and presented as relative ratio values (average mean±SEM). *p<0.05; ***p<0.001 were considered as significant. Results were tested with One Way ANOVA test, Kruskal Wallis and followed by Mann Whitney or Tukey post hoc test with 95% confidence interval.

![Cardiac Muscle](image2)

**Figure 4.** Relative gene expression level of TRα1, TRα2, TRβ1, TRβ2, Dio1, Dio2, and Dio3 in cardiac muscle. Data were normalized with GAPDH and presented as relative ratio values (average mean±SEM). *p<0.05; ***p<0.001 were considered as significant. Results were tested with One Way ANOVA test, Kruskal Wallis and followed by Mann Whitney or Tukey post hoc test with 95% confidence interval.
environment in soleus and gastrocnemius muscle. Alteration of these mechanism may decrease contractility rate, growth, and metabolism of skeletal muscle. Thus, further research is still needed to understand the effects on tissue function.

Cardiac is one of the major target organ of TH action. T3, an active form of thyroid hormone binds to the TR in the same process as skeletal muscle, repression relieved and transcription is further stimulated by the recruitment of co-activators to the complex. TH in the cardiac muscle regulates the myosin heavy chain (MHC) genes which encode two contractile protein isoforms of the thick filament in cardiomyocyte. Triiodothyronine has positive effect on the transcription of the MHCα gene and negative effect on the MHCβ gene expression.(23-26) TH also regulates SERCA2a which is responsible for the calcium reuptake during the diastole, which important for systolic and diastolic function.(27-29) Understanding this important regulation of TH in cardiac muscle, our data had shown that TRα1, TRα2, TRβ1, TRβ2, Dio1, Dio2, and Dio3 were all significantly upregulated in cardiac muscle by ethionamide (Figure 4). Ethionamide could affect TRs signaling direct or indirectly. Level of thyroxine and triiodothyronine in serum might alter the TRs and iodothyronine deiodenase gene expression as compensation of hypothyroid environment in cardiac muscle. Alteration of these mechanism might decrease contractility, systolic, and diastolic function capability of the cardiac muscle.(30) However, further study is compulsory to understand the complete molecular mechanism on tissue function. TRs RNA expression could be altered by many external factor like exercise (31), medicine (32), environmental compounds like PCB (33), and also caused by internal factor like genetic mutation (34). The ethionamide might also alter the expression of Deiodinase gene expressions, this gene is important for activating enzyme and deactivating enzyme of TH. Deiodinase 1, 2 and 3 mRNA expression were significantly upregulated in soleus and cardiac muscle. In gastrocnemius muscle, only Dio2 was significantly upregulated (Figure 2-4). Iodothyronine deiodinase 1 and 2 (Dio1 and Dio2) are activating enzyme which catalyze conversion of T4 into T3 in circulation, and iodothyronine deiodinase 3 (Dio3) act as deactivating enzyme which catalyze conversion of T4 into reverse T3 (rT3) and the conversion of T3 to T2.(35,36) Different response and expression pattern might caused by different type of skeletal muscle fiber (red and white fibers). The alteration of TR gene expression in this research may lead to new perspective about thyroid hormone related study and tuberculosis treatment in the future. Although TR gene expression significantly upregulated in soleus muscle and cardiac muscle tissue, it is not confirmed whether it effects the function of the cells, hence needs to be further studied. However, specific alteration of TRs and Deiodinase gene expression might indicate that ethionamide could alter TH genomics signaling via modulating TRs expression. In the future, ethionamide treatment for MDR-TB patient should consider its side effect induce hypothyroidism. Alteration of these gene expression might still need further research to understand its mechanism on tissue function.

**Conclusion**

In summary, ethionamide significantly upregulate thyroid hormone receptor gene expression in soleus and cardiac muscle tissue but not in gastrocnemius muscle. The upregulation of TRS expression caused by ethionamide treatment should increase our awareness of ethionamide effect to TH signaling for TB treatment in the future.

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