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Original Article

Thyroid hormone-inducible semicarbazide-sensitive amine oxidase (SSAO) in rat brain cytosol

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Summary The present study was examined that modulators of semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6.) might be present in the brain cytosol and looked for changes in SSAO modulatory activity in brain cytosol. An endogenous inhibitor of SSAO (ESI) was separated by gel filtration from 105,000 g supernate of thyroxine (T4)-treated rat brain cytosol. The inhibition by this inhibitor with SSAO activity for 1 μmol/L benzylamine was concentration dependent. The mode of inhibition was competitive with benzylamine. The molecular weight of this inhibitor was estimated to be 600-700 by gel filtration. The pI value of ESI was determined by isoelectric focusing (IEF)-gel electrophoresis to about 3.6. SSAO inhibition activity was much lower in the cytosol of thyroidectomized, non-T4-treated rats than T4-treated rats, suggesting that this inhibitor is induced by thyroid hormone. SSAO activity in rat brain might be regulated by the level of this inhibitor. The results indicate that this inhibitor in T4-treated rat brain cytosol and that the level of this inhibitor is regulated by thyroid hormone.

Key words : Semicarbazide-sensitive amine oxidase (SSAO); monoamine oxidase (MAO); thyroid hormone; endogenous SSAO inhibitor; thyroidectomized rat

Abbreviations used :

SSAO; semicarbazide-sensitive amine oxidase
MAO; monoamine oxidase
ESI; endogenous SSAO inhibitor

Introduction

Several different amine oxidases are inhibited by carbonyl reagents, such as semicarbazide. The semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6.)[1),2)] are resistant to inhibition by acetylenic inhibitors, such as clorgyline, deprenyl, and pargyline[3)]. SSAO differs from monoamine oxidase (MAO; EC 1.4.3.4) and deaminates various monoamines[4),5)]. SSAO activity is suspected to cause damage, such as diabetes in humans[6),7)]. Despite of widespread tissue distribution this enzyme[8),9)], is physiological role remain far from clear[10)]. This enzyme is highly sensitive to carbonyl reagents. One of the important actions of thyroid hormone is thought to be the regulation of protein synthesis and enzyme activities[11),12)]. Therefore, I hypothesized that modulators of SSAO might be present in the brain cytosol and looked for changes in SSAO modulatory activity in brain cytosol of thyroidectomized rats during thyroid hormone treatment. It is not clear whether thyroid hormones affect the synthesis of SSAO or

act by another mechanism (e.g. Induction of specific modulator). Our results indicate that certain modulators are induced by thyroid hormone administration and that these modulators inhibit SSAO activity by a competitive mechanism.

Materials and methods

Materials

Benzylamine hydrochloride, Thyroxine (T4) sodium salt, subtilisin protease Type III), protease E (protease Type XIV) and trypsin were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The radioactive substrate [7-^{14}C]-benzylamine hydrochloride (1.85-2.29 Gbq/mmol) was obtained from Amersham International (Amersham, England). Servalyte (pH 2-11) was purchased from Serva Fine Biochemical Co. (St. Louis, MO, USA).

Isolation of SSAO Inhibitor

Thyroidectomized rats (Male Wistar), weighing 100-150 g, were used for experiments. In the case of T4 administered rats, T4 (dissolved in saline) was injected subcutaneously to the rats 10 days after from operation at a dose of 200 μg/kg per day for 2 weeks. T4 sodium salt were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The rats were killed by decapitation and brain quickly removed and homogenized in 10 vol. of 10 mmol/L phosphate buffer, pH 7.4 containing 0.32 mol/L sucrose. The homogenates (10 mg protein) was centrifuged at 105,000 g for 60 min, and the supernate (cytosol fraction) was applied on a Sephadex G-25 column (1.0×60 cm), previously equilibrated with 20 mol/L phosphate buffer (pH 7.4). The column was eluted with the same buffer at a rate of 10 ml/hr and the fractions were collected in 2.5 ml each. An aliquot of each fraction was assayed for SSAO inhibition activity, and active fractions were combined and used for further characterization. This fraction is referred to as "endogenous SSAO inhibitor (ESI)". All procedures in dealing with the experimental animals met the guideline principles stipulated by the Physiological Society of Japan and the Animal Ethics Committee of the Oita Medical University, Japan.

Assay of SSAO activity

Rat brain homogenate fraction was used as a source of SSAO activity. The 10% homogenate was prepared in 0.32 M sucrose with 10 mmol/L phosphate buffer, pH 7.4. SSAO activity was assayed radiochemically as described by Bitsios et al.[13]. Assay mixture contained 20 μL of homogenate fraction (1 mg/ml protein), [^{14}C]-benzylamine (1 μmol/L), in 20 mmol/L phosphate buffer, pH 7.4 in the presence of ESI (0-150 μL). Protein concentrations of the preparations were measured by the method of Lowry et al.[14] with bovine serum albumin as the standard.

Isoelectric focusing (IEF)

Gel isoelectric focusing (IEF) was performed[15]. The final composition of the gel was 5% acrylamide, 0.2% methylene bisacrylamide, 0.75 % Triton X 100, 2% servalyte (pH 2-11), 0.0002% riboflavin, 0.01% ammonium persulfate, 0.05% TEMED (N,N,N'N'-tetramethylenediamine). The gel was mounted on a vertical apparatus containing 0.01 mol/L H_3PO_4 in the upper tank (anode) and 0.02 mol/L NaOH in the lower tank (cathode). The current was at 100 V for the first 1 hr, 200 V for the next 2 hr and then 300 V for 2 hr. After electrophoresis, the gel was cut into 4 mm thick slices and each sliced gel was placed in a test tube, and incubated for 1 hr at room temperature by adding 1 mL of distilled water and bubbled with N_2 gas. After the measurement of slice pH, a minimum amount of 0.5 mol/L H_3PO_4 was added to adjust the pH to 7.4. An aliquot of each slice suspension was then assayed for SSAO inhibition activity with benzylamine as a substrate.

Heat treatment and digestion with pepsin and trypsin

For determination of heat stability of ESI was heat at 100 ˚C for 10 min. The samples were then added to homogenate preparations to determine their effects on SSAO activity. Also ESI were treated with subtilisin or pronase at 37˚C for 12 hr and trypsin at 37˚C for 30 min and trypsin.

Statistical analysis

All values are presented as means±S.E.M. The significance of difference was determined by using ANOVA with Fisher's *post hoc* test. A P value of less than 0.05 was regarded as being statistically significant.

Results

The gel-filtration of T4-treated rat brain cytosol with Sephacryl S-200 column showed that the fractions to

inhibit SSAO activity were eluted in a low molecular weight area (<2000) (data not shown). Therefore, I used Sephadex G-25 column to separate the inhibitor and determine the molecular size (Fig.1). The fractions 19-21 were found to inhibit SSAO activity with 1 μmol/L benzylamine was a substrate. The molecular weight of the inhibitor was estimated to be 600-700. When thyroidectomized, non-T4-treated rats were used (ESI-control), the inhibition activities for 1 μmol/L benzylamine were very low. T4-treatment remarkably enhanced SSAO inhibition activities for substrate. This results suggests that the inhibitor could be induced by T4 treatment. The inhibition curves of SSAO activity by ESI were concentration-dependent, and IC_{50} values for 1 μmol/L benzylamine oxidation was 143 μL (Fig.2). To know the mode of ESI inhibition, the inhibition was investigated with different substrate concentrations. Fig.3 shows Lineweaver-Burk plots of the results with benzylamine as substrate. ESI inhibited SSAO activity competitively. ESI applied at a variety of volumes (50, 100 and 150 μL) increased the Km values for benzylamine from 1.3 to 30.3 μmol/L, but did not alter Vmax values. pl value of ESI was determined by gel isoelectric-focusing. SSAO inhibition activity was found as a single peak at a position 2.0 cm from the top of the gel. The pH measurement after isoelectric focusing revealed that ESI has a pI value of about 3.6 (Fig.4). When ESI were treated at 100 ˚C for 10 min, the inhibition activity was not changed. Also, treatment of ESI with subtilisin or pronase at 37 ˚C for 12 hr did not affect the inhibition activity of ESI. These results suggest that ESI is a non-peptide inhibitor. Moreover, ESI was inactivated with trypsin at 37 ˚C for 30 min digestion.

Discussion

In the present study, I have demonstrated the presence of ESI in rat brain cytosol fraction of thyroid hormone inducible modulator, which inhibit SSAO activity. To my knowledge, this is first report on SSAO modulator in rat brain. It is well established that one of the action of thyroid hormones is thought to be the regulation of protein synthesis and enzyme activities[16]. The fact that the inhibitory activity in the hyperthyroid state suggests the possibility that thyroid hormone may regulate the SSAO activity by induction of this inhibitory modulator. However, it is not known whether such as a decrease in SSAO activity in hyperthyroid state is a direct effect of thyroid hormone or mediated through specific mechanism. I found an endogenous SSAO modulator of a low molecular weight (600-700) in brain cytosol of T4 treated rats (Fig.1). As far as I known, this is the first isolation of the endogenous inhibitor of SSAO from the T4-treated rat brain. The inhibitory effects of this inhibitor was non-linear concentration-dependent (Fig.2). When thyroidectomized, non-treated rats were used (ESI control), inhibition activities for benzylamine were very low. T4-treatment remarkably enhanced SSAO inhibition activities for benzylamine.

Dilution experiments showed that the dilution of the reaction mixture restored the activity to the level at final concentration of the inhibitor (data not shown), suggesting that the inhibition is reversible. This may indicates that ESI was a reversible inhibitor of SSAO. A kinetical study showed that ESI increased the Km values for benzylamine but did not alter Vmax values (Fig.3). The mode of ESI inhibition was competitive. The competitive kinetics were obtained at high benzylamine concentration (100 μmol/L) associated with MAO enzyme, not at the low (1 μmol/L) concentration of SSAO[8]. Therefore, when ESI increased in the hyperthyroid state, thyroid hormone not only SSAO, but also may regulate the MAO activity by induction of this inhibitory modulator. From these results, it can be concluded that the rat brain contains low molecular weight materials which act like SSAO inhibitor drugs. ESI may be a low molecular weight compound such as certain monoamines or their metabolites, β-carboline[17] or tribuline[18]. The pI value of ESI was determined by IEF-gel electrophoresis to about 3.6 (Fig.4). This result indicate that ESI is an acidic compound. Although the nature of this inhibitor is not clear at present, the present study showed that this compound is heat-stable and resists to protease, subtilisin and trypsin treatment. My findings suggested that modulators induced by thyroid hormone may have an important role in SSAO activity. The observations that T4 did not affect SSAO activity directly *in vitro* and that treatment with T4 did not directly affect the level of SSAO indicate that thyroid hormone induced

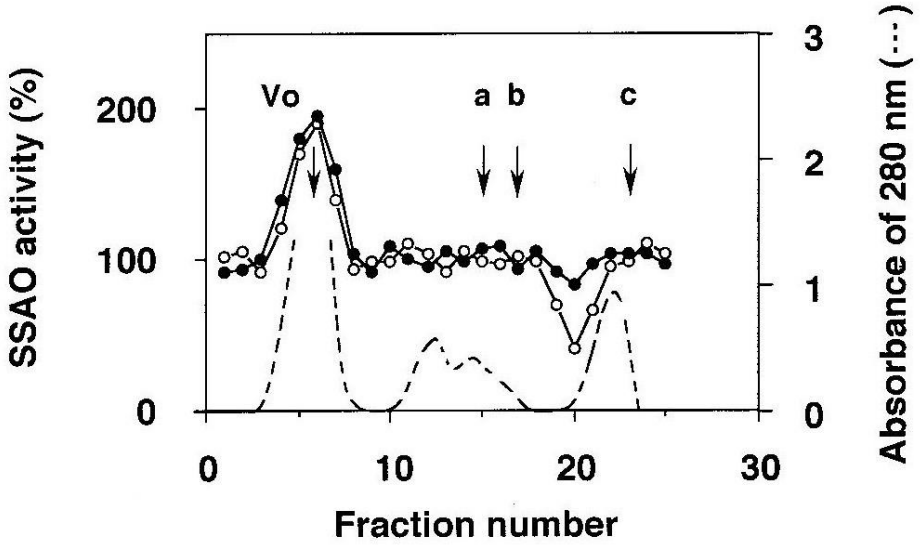

Figure 1

Sephadex G-25 chromatography of cytosolic fraction from T4-treated rat brain. Cytosolic fraction (10 mg protein) was applied to a Sephadex G-25 column (1.0×60 cm). ESI control (closed circle) or ESI-T4 (open circle) was obtained from thyroidectomized rat or thyroidectomized, T4 treated rat, respectively, as described in the text. The activity of SSAO in homogenate was assayed with 1 μmol/L benzylamine as a substrate. Arrows indicate the position of marker compounds. The molecular for markers used were as follows: a, cyanocobalamine (MW, 1,355); b, FAD (MW, 786); c, DNP-Alanine (MW, 255). Vo; dextran (MW, 2,000,000). The broken line shows the absorbance at 280 nm.

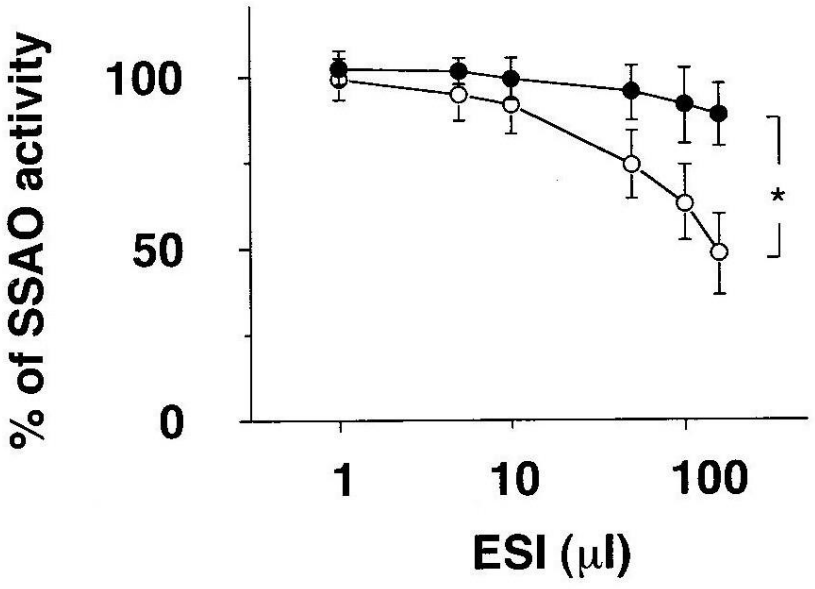

Figure 2

Concentration-dependent effect of ESI without preincubation. Inhibition of SSAO activity by ESI-control (closed circle) and ESI-T4-treated rat (open circle) were tested. In the presence of ESI, SSAO activity in rat brain homogenate was determined 1 μmol/L benzylamine as substrate at 37 ˚C for 10 min. The control activity for 1 μmol/L benzylamine was 0.14±0.03 nmol/min/mg protein. The values represent the mean percentage (±SE) of SSAO activity. Values are means±S.E.M. for six animals. *$P<0.05$ significant difference between the data connected by bracket (ANOVA and Fisher test).

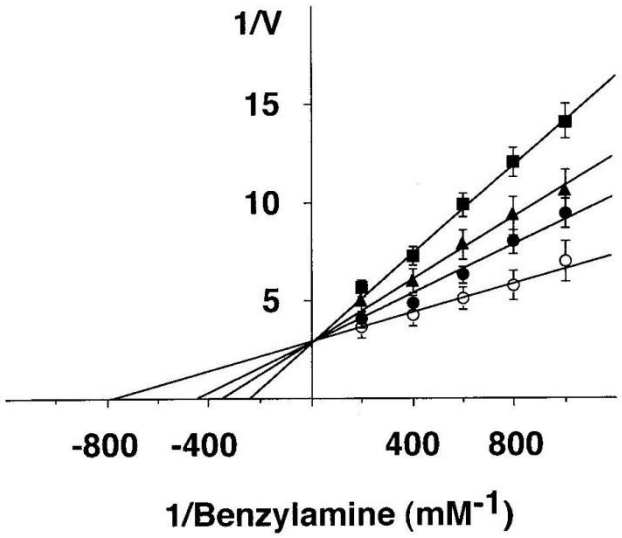

Figure 3

Lineweaver-Burk plots of the reciprocal of the initial velocity of benzylamine oxidation against reciprocal of the substrate concentration in the presence of ESI without preincubation. Sample assayed for activity in the absence (open circle) and presence of 150 μL (closed circle), 100 μL (triangle) and 50 μl (square) ESI, with benzylamine as substrate. Abscissa, 1/substrate concentration in mM (mmol/L); ordinate, 1/initial velocity in nmol/min/mg protein. Each point represents the mean SSAO activity (±SE) assayed in triplicate determinations.

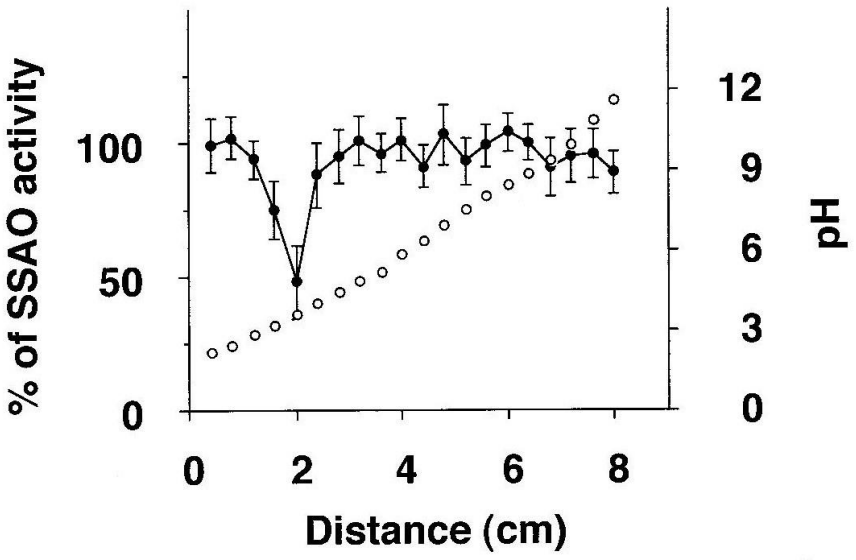

Figure 4

Estimation of pl value of ESI-T4 in rat brain cytosol by gel isoelectric focusing. ESI-T4 fraction was collected and was solubilized with 0.75% Triton X-100. Isoelectric focusing was performed as described in Materials and Methods. After IEF-gel electrophoresis, the gel was cut into 4-mm slices, and the pH (open circle) of each gel slice was determined. After adjusting pH to 7.4, the SSAO activity for 1 μmol/L benzylamine was determined in the presence of each gel slice (closed circle). The control activity for benzylamine was 0.14±0.03 nmol/min/mg protein. Each point represents the mean percentages (±SE) on the control SSAO activity in triplicate experiments.

SSAO inactivation does not occur in the absence of modulator.

Conclusion

In the present study, I found a new SSAO inhibitory modulator in T4-treated rat brain cytosol and that the level of this modulator is regulated by thyroid hormone. Although the physiological role of this inhibitor still remains unclear, I consider that this modulator may play some role in regulating the SSAO activity in rat brain. While the role of these modulators is no doubt important, their mechanisms remain to be elucidated.

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甲状腺ホルモンにより誘発するラット脳の細胞質中の セミカルバザイド感受性アミン酸化酵素（SSAO）

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要 旨

本研究はラット脳の細胞質中にセミカルバザイド感受性アミン酸化酵素（ＳＳＡO; EC 1.4.3.6.）の活性を変化させる調節器が存在するか否かについて調べた。遠心分離によりチロキシン（Ｔ４） 処理したラット脳の 105,000 g 分画から内因性ＳＳＡO阻害剤（ＥＳＩ）を分離した。この阻害剤は１μmol/L ベンジルアミンを基質とするＳＳAO活性は濃度依存的に阻害した。阻害モードはベンジルアミンに対して競合的であった。ゲル濾過法により分子量を求めると 600-700 位で、等電 （ＩＥＦ）電気泳動法によりＥＳＩの pI 値はおよそ 3.6 でした。ＳＳAO阻害活性は甲状腺ホルモンにより誘発されるＴ４処理ラットよりＴ４未処理の甲状腺摘出ラットは非常に小さかった。ラット脳中のＳＳAO活性はこの阻害剤のレベルによって調節されているに違いない。以上の結果はＴ４処理したラット脳細胞質中の阻害剤のレベルは甲状腺ホルモンにより調節されていることを示している。

キーワード：セミカルバザイド感受性アミン酸化酵素（ＳＳAO）、モノアミン酸化酵素（MAO）、甲状腺ホルモン、内因性ＳＳAO阻害剤、甲状腺摘除ラット