EFFECT OF AGING ON TRYPTOPHAN HYDROXYLASE IN RAT BRAIN: IMPLICATIONS ON SEROTONIN LEVEL

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ABSTRACT:

Tryptophan hydroxylase (TrpH) catalyzes a rate-limiting step in the biogenesis of serotonin. The main objective of this study is to investigate the effect of aging on the activity of TrpH in serotonergic-enriched brain regions such as midbrain, pons, and medulla. TrpH activity was monitored by incubating various concentrations of tryptophan in a fixed amount of brain homogenate from midbrain, pons, and medulla of 2-month (young), 12-month (mature), and 24-month (old) rats (pH 7.4, 37°C). The product 5-hydroxytryptophan was quantitated using a reversed phase HPLC equipped with an electrochemical detection system. Michaelis-Menten constants, Km and Vmax, were calculated using the Lineweaver-Burk plot. The affinity (Km) of the enzyme significantly declined in midbrain and pons of old rats (141.1 ± 2.6, 126.0 ± 10.8 μM) relative to mature rats (22.4 ± 7.7, 38.2 ± 4.7 μM). However, no change was observed in medulla of old rats. The Vmax of TrpH in pons of all three age group rats was fairly constant. However, the Vmax of midbrain was significantly elevated, whereas that of medulla was reduced in old rats relative to mature rats. Clearance formation, a ratio of Vmax to Km, of 5-hydroxytryptophan declined significantly in midbrain, pons, and medulla of old rats relative to mature rats. A combined effect of inefficient phosphorylation and oxidative damage of TrpH enzyme may be responsible for lower TrpH activity in aging brain. Such alterations in TrpH activity may reduce the level of serotonin in brain, which may be linked to late-life depression and other brain disorders, such as Alzheimer and Parkinson diseases.

A variety of human physiological functions have been shown to decline with aging (Shock, 1957). This decline in physiological functions may be associated with malfunctioning of the various autonomic systems in the body. One such system is the central nervous system in which aging causes a diminished function accompanied by changes in various neurotransmitter levels. Such variations in neurotransmitter levels may lead to various behavioral changes. It has been reported that aging causes a decrease in the concentration of various neurotransmitters in the brain (Meek et al., 1977; Sparks et al., 1985). Alterations in the brain concentration of serotonin can produce behavior abnormalities such as aggression, insomnia, suicidal or criminal behavior, loss of sex drive, despair, and/or misery often observed in elderly individuals (Frazer and Hensler, 1994). Reduction of serotonin level in aging may serve as a susceptibility factor in the development of late-life depression (Lerer et al., 1995). The prevalence of major depression is estimated at 1% to 10% of population aged 60 years or older, whereas depression may persist in up to 20% of the elderly population. Furthermore, the suicidal rate in this age group is higher than at any other stage of life (Casey, 1994). One possible reason for decreased serotonin level could be the decreased transport of precursor amino acid (tryptophan) across the blood-brain barrier.

Another possibility may be the diminished activity of the anabolic enzymes and/or enhanced activity of catabolic enzymes, which could lower the neurotransmitters level in aging brain (Scheme 1). Transport of tryptophan, an amino acid precursor for serotonin, was found to decline in old rats (Tang and Melethil, 1995). A strong correlation between age-related mental disorders and brain neurotransmitters concentrations exists according to several studies that have reported reduced brain neurotransmitter levels associated with aging (Brizée, 1975; Sanatiago et al., 1988). Very little mechanistic information is available regarding age-related changes in brain serotonin levels. In this article we have described the effect of aging on tryptophan hydroxylase (TrpH) activity, a rate-limiting step involved in the biogenesis of serotonin in rat brain. The enzyme is localized in serotonergic neurons, which convert tryptophan to 5-hydroxytryptophan (5-HTP). TrpH is highly localized in midbrain, pons, and medulla (Frazer and Hensler, 1994), and we studied its kinetics in three different age group Fisher 344 BNF1 rats, i.e., aged 2 months (young), 12 months (mature), and 24 months (old).

Experimental Procedures

Chemicals. Tryptophan, 5-HTP, dithiothreitol, t-6-methyl-5,6,7,8-tetramethyl-ethyl hydroperoxide, and catalase were all obtained from Sigma Chemicals (St Louis, MO). Citric acid, EDTA disodium, and HPLC grade methanol were obtained from Fisher Scientific (St Louis, MO). Fisher 344 BNF1 rats were obtained from Harlan NIA (Indianapolis, IN).

Methods. TrpH assay was adapted from a published procedure with minor modification (Sugden et al., 1989). Briefly, male Fisher 344BNF1 rats were
decapitated and midbrain, pons, and medulla were surgically isolated. Pooled brain regions were then homogenized in 5 volumes of 0.05 M Tris-HCl, pH 7.4, containing 2 mM dithiothreitol. The homogenate was centrifuged at 40,000g at 4°C for 30 min. Aliquots of supernatant was then preincubated with 1 mM NSD 1015 (decarboxylase inhibitor), 0.5 mM DL-6-methyl-5,6,7,8-tetrahydropterine (cofactor), and 1500 units of catalase at 37°C for 15 min. After preincubation, tryptophan (10–300 μM) was added and the mixture was incubated at 37°C for 45 min. The reaction was terminated by 1 N perchloric acid. Precipitated proteins were removed by centrifugation at 15,000g at 4°C for 10 min. The metabolite (5-HTP) formed was then analyzed by HPLC using electrochemical detection. Protein estimation was done using the Lowry method (Lowry et al., 1951).

**HPLC Conditions.** The chromatographic separation was achieved by a reversed phase octadecyl silane column (Microsorb, particle size 5 μm; 150 × 4.6 mm i.d.). A short precolumn (60 × 5 mm i.d.) packed with octadecyl silane was used as a guard column. A mobile phase containing (0.05 M citric acid, 0.05 M sodium acetate, and 0.13 mM EDTA disodium (pH 6.0) with 5% v/v methanol as the organic modifier was employed. The flow rate was adjusted to 0.5 ml/min. Metabolite 5-HTP was detected using an electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Glassy carbon was used as a working electrode. The working potential was set to +0.5 V with Ag/AgCl as the reference electrode. Using these chromatographic conditions, a clear separation of 5-HTP from other endogenous compounds was obtained. Tryptophan could not be detected at this potential, because it is oxidized at +0.9 V.

**Results**

The enzyme kinetic parameters of TrpH were obtained using the Lineweaver-Burk plot. Kinetic constants $K_m$ and $V_{max}$ of three brain regions, the midbrain, pons, and medulla, were calculated for young, mature, and old rats from Figs. 1, 2, and 3, respectively.

A variable trend with the age-related changes in the affinity of TrpH was observed in all three brain regions. In midbrain the affinity of the enzyme remained fairly unchanged in young to mature rats and then declined in old rats ($P < .01$) by almost 7-fold (Table 1). A biphasic phenomenon was also observed in pons. The affinity of TrpH was significantly elevated from young to mature rats ($P < .05$) and then declined significantly ($P < .05$) in old rats (Table 1). In medulla the affinity of the enzyme increased significantly from young to mature rats ($P < .05$) and then remained unchanged in the old animals. In all three age groups studied, the affinity of the enzyme in midbrain was significantly more than in pons and medulla except in old rats, where the affinity of the enzyme decreased drastically and leveled off in the pons and medulla.

An interesting phenomenon was observed in the midbrain of old rats. Contrary to our expectations, a 2-fold increase ($P < .01$) in the $V_{max}$ of old rats was noted relative to young and mature rats (Table 2). No statistical significant difference was observed in midbrain of young and mature rats. Pons was found to be the only region studied, where aging did not alter $V_{max}$. No significant changes ($P > .05$) were observed in the $V_{max}$ values of pons among rats of various age groups.

A biphasic phenomenon was observed in medulla with a significant $V_{max}$ increase ($P < .05$) from young to mature rats followed by a significant decline ($P < .05$) in old rats (Table 2).

Clearance formation ($Cl_t$) of 5-HTP was calculated from the ratio of $V_{max}$ to $K_m$. No significant change in $Cl_t$ was observed in midbrain and pons of young rats relative to mature rats (Table 3). However, in the same brain regions almost a 3-fold decline in the $Cl_t$ of 5-HTP was observed in old rats in comparison to mature rats. A biphasic phenomenon was observed in medulla with an initial rise in $Cl_t$ from young to mature rats ($P < .05$) and then significant decline in old rats ($P < .01$). The magnitude of decline in $Cl_t$ of 5-HTP from mature to old rats in all three brain regions was approximately 3-fold.

**Discussion**

TrpH is the initial and rate-limiting enzyme involved in the biogenesis of the neurotransmitter serotonin (Jequier et al., 1967). It is also involved in the biogenesis of melatonin in the pineal gland. The present study provides evidence that TrpH activity is affected during the aging process, leading to a decreased level of serotonin in old rats (Meek et al., 1977; Sparks et al., 1985). TrpH from different regions of brain have different biochemical properties, suggesting that there are several isoforms of the enzyme. The basis of these biochemical
Reciprocal values of mean velocity ± S.D. of medulla are plotted against the reciprocal of corresponding tryptophan concentrations. $K_m$ and $V_{\text{max}}$ values are calculated from the $x$ and $y$ intercepts, respectively.

Statistical significance: $^a$ as compared with 2 months, $P < .05$; $^b$ as compared with 12 months, $P < .05$; $^c$ as compared with 12 months, $P < .01$; $^d$ as compared with 12 months, $P < .005$; $^e$ as compared with 12 months, $P < .001$.

Reciprocal values of mean velocity ± S.D. of pons are plotted against the reciprocal of corresponding tryptophan concentrations. $K_m$ and $V_{\text{max}}$ values are calculated from the $x$ and $y$ intercepts, respectively.

Statistical significance: $^a$ as compared with 2 months, $P < .05$; $^b$ as compared with 12 months, $P < .01$; $^c$ as compared with 12 months, $P < .01$; $^d$ as compared with 12 months, $P < .005$.

Effect of age on the $V_{\text{max}}$ of tryptophan hydroxylase in different brain regions

Values of mean $V_{\text{max}}$ ± S.D. were obtained for midbrain, pons, and medulla of different age groups (2, 12, and 24 month) rats from the Lineweaver-Burk plot as described under Experimental Procedures. Data were analyzed by ANOVA.

<table>
<thead>
<tr>
<th>Regions</th>
<th>2 months</th>
<th>12 months</th>
<th>24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>0.253 ± 0.084</td>
<td>0.237 ± 0.014</td>
<td>0.567 ± 0.031$^a$</td>
</tr>
<tr>
<td>Pons</td>
<td>0.273 ± 0.068</td>
<td>0.205 ± 0.013</td>
<td>0.182 ± 0.020</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.156 ± 0.012$^c$</td>
<td>0.281 ± 0.011</td>
<td>0.199 ± 0.074$^d$</td>
</tr>
</tbody>
</table>

Effect of age on the $K_m$ of tryptophan hydroxylase in different brain regions

Values of mean $K_m$ ± S.D. were obtained for midbrain, pons, and medulla of different age groups (2, 12, and 24 month) rats from the Lineweaver-Burk plot as described under Experimental Procedures. Data were analyzed by ANOVA.

<table>
<thead>
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<th>Regions</th>
<th>2 months</th>
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<th>24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>30.5 ± 5.99</td>
<td>22.4 ± 7.7</td>
<td>141.1 ± 2.6$^a$</td>
</tr>
<tr>
<td>Pons</td>
<td>97.6 ± 12.9$^c$</td>
<td>38.2 ± 4.7</td>
<td>126.0 ± 10.8$^d$</td>
</tr>
<tr>
<td>Medulla</td>
<td>124.3 ± 8.42$^c$</td>
<td>56.3 ± 0.1</td>
<td>95.6 ± 33</td>
</tr>
</tbody>
</table>

Effect of age on the $Cl_f$ of 5-hydroxytryptophan in different brain regions

Values of mean $Cl_f$ ± S.D. were obtained for midbrain, pons, and medulla of different age groups (2, 12, and 24 month) rats from the ratio of $V_{\text{max}}$ to $K_m$ of tryptophan hydroxylase as described under Experimental Procedures. Data were analyzed by ANOVA.

<table>
<thead>
<tr>
<th>Regions</th>
<th>2 months</th>
<th>12 months</th>
<th>24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>0.0082 ± 0.0012</td>
<td>0.0107 ± 0.0032</td>
<td>0.0040 ± 0.0001$^e$</td>
</tr>
<tr>
<td>Pons</td>
<td>0.0028 ± 0.0004</td>
<td>0.0054 ± 0.0004</td>
<td>0.0014 ± 0.0001$^b$</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.0012 ± 0.0001$^c$</td>
<td>0.0050 ± 0.0001</td>
<td>0.0020 ± 0.0001$^c$</td>
</tr>
</tbody>
</table>

Found to be identical (Kim et al., 1991). These results support the hypothesis that tissue-specific differences in the properties, including TrpH activity, result from different post-translational modifications rather than pretranslational ones.

Among the brain regions we have studied, affinity of TrpH in midbrain is markedly higher than in brain stem, despite higher activity in the latter. This observation suggests that different forms of TrpH exist in the two tissues (Dumas et al., 1989). However, cDNA has been characterized from both tissues and found to be identical (Kim et al., 1991). These results support the hypothesis that tissue-specific differences in the properties, including TrpH activity, result from different post-translational modifications rather than pretranslational ones.
activation of adenylate cyclase to generate second messenger cAMP. This second messenger, once formed, stimulates protein kinase A, which in turn phosphorylates TrpH. Any alteration in the phosphorylation cascade can subsequently modulate enzyme activity. Studies have shown that the activity of adenylate cyclase enzyme is enhanced in aging animals from young to mature rats (Araki et al., 1995) and subsequently diminished in old rats (Nomura et al., 1984; Hoskins and Ho, 1986). This initial increase and subsequent decrease of adenylate cyclase activity can be translated into variations in efficiency of phosphokinase A. This is consistent with our observations in pons and medulla, where the affinity of TrpH increased from young to mature rats and then decreased in old rats. The basal level of cAMP is maintained by its degradation with phosphodiesterases (3',5'-cAMP-nucleotide hydrolase). A significant increase in the high-$K_m$ cAMP phosphodiesterase activity in the various brain regions has been reported in old rats relative to mature rats (Stancheva and Aloba, 1991). This increase in the phosphodiesterase activity coupled with a decrease in the adenyl cyclase activity may alter the phosphorylation cascade of TrpH, leading to loss of TrpH activity with aging. TrpH is also phosphorylated by calcium/calmodulin-dependent protein kinase at serine 260 and serine 443 residues (Darmon et al., 1988). Several studies have reported that the levels of calmodulin, a calcium-binding protein, diminishes in various brain regions of old rats in comparison with young and mature rats (Teolato et al., 1983; Hoskins and Ho., 1986). Decreased levels of calmodulin in old rats can also indirectly affect the phosphorylation of TrpH by calcium/ calmodulin-dependent protein kinase.

TrpH has been shown to be highly homologous with tyrosine hydroxylase (Grenett et al., 1987). A recent study has revealed that tyrosine hydroxylase in substantia nigra is inactivated byoxidation in old rats (De la Cruz et al., 1996). A similar effect may also be expected to cause a decline in TrpH activity along with inefficient phosphorylation in old rats. TrpH when incubated with catalase, which degrades $H_2O_2$, caused a significant elevation in the TrpH activity, suggesting that the enzyme is prone to oxidation (Friedman et al., 1972). Furthermore, in a recent study TrpH has been shown to be susceptible to oxidative damage by reactive oxygen species (Cash., 1998). Studies are currently in progress in our laboratory to investigate the effect of oxidation on TrpH. We have also found that the activities of antioxidant enzyme, e.g., superoxide dismutase, glutathione peroxidase and catalase, undergo a significant age-related decrease in midbrain, pons, and medulla (AMH and AKM, unpublished results). A decrease such as this may lead to an increase in reactive oxygen species concentrations, which in turn may cause oxidative damage to TrpH.

No age-related changes were found in the $V_{\text{max}}$ of TrpH in the pons, indicating a fairly constant activity of enzyme. However, an increase in the $V_{\text{max}}$ of medulla from young to mature rats, which remains constant in old rats, suggests that the activity of TrpH increases in the prematuration phase. We found a 2-fold increase in $V_{\text{max}}$ associated with the midbrain of old rats relative to mature rats. This may be due to a positive feedback mechanism, which is attempting to compensate for the 7-fold decrease in the affinity of the enzyme in old rats compared with mature rats. The other explanation could be a decline in the protein turnover rate by the proteolytic enzymes. Decrease in proteolytic enzyme susceptibility is known to occur due to formation of cross-linked aggregates of the denatured protein (Davies et al., 1987). A similar event can also be speculated in the midbrain of old rats. TrpH, being prone to oxidation as described earlier, can undergo aggregation and become less susceptible to protein turnover, subsequently causing increased $V_{\text{max}}$. Because the catalytic domain of the tryptophan has been shown to occur at the C terminus (Yang and Kaufman, 1991), aggregation by partial unfolding of the hydrophobic core might not result in total loss of activity. Furthermore, TrpH has been reported to exist in its catalytically active tetrameric form (Nakata and Fujisawa, 1982).

To comprehend the overall effect of the aging process on TrpH, $C_L$ of 5-HTP formation can be calculated from kinetic parameters, $V_{\text{max}}$ and $K_m$. The ratio of $V_{\text{max}}$ to $K_m$ generates the intrinsic clearance of tryptophan by TrpH. Tryptophan, being intrinsically cleaved by the TrpH enzyme, may be considered as clearance formation of 5-HTP. In all three brain regions the $C_L$ of 5-HTP declined in old rats in comparison with mature rats. This decline in $C_L$ of 5-HTP can be translated into decreased levels of serotonin in aging animals, because the 5-HTP decarboxylation step is not the rate-limiting step. A 3-fold decrease in the $C_L$ of 5-HTP in midbrain, pons, and medulla in old rats was revealed. This decline in the formation of 5-HTP may be adequate to affect the basal levels of serotonin, which in turn can be a major cause for late-life depression and other brain disorders. Although a clear relationship between the serotonergic system and Alzheimer disease has not yet been established, there is considerable evidence of a decreased brain level of serotonin in patients with Alzheimer disease (Reinikainen et al., 1990).

In conclusion, TrpH is significantly affected by aging in all three brain regions, i.e., midbrain, pons, and medulla, which can drastically affect the levels of serotonin in brain. A combined effect of inefficient phosphorylation and oxidative damage of TrpH is suggested as the probable cause of diminished activity in old rats. Further experiments are warranted to support this hypothesis.

References


