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# Inhibition of aromatic L-amino acid decarboxylase under physiological conditions: optimization of 3-hydroxybenzylhydrazine concentration to prevent concurrent inhibition of monoamine oxidase

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**Abstract**—The activity of the enzyme tyrosine hydroxylase (TH; EC 1.14.16.2) is commonly studied indirectly by quantifying the formation of the product, 3,4-dihydroxyphenylalanine (DOPA), after inhibition of aromatic L-amino acid decarboxylase (AAAD; EC 4.1.1.28), the enzyme which metabolizes DOPA. This study was done to determine if the concentration of the hydrazine derivative 3-hydroxybenzylhydrazine (NSD-1015), a drug frequently used *in vitro* to inhibit AAAD, could be adjusted such that it would inhibit that enzyme, but would not simultaneously inhibit a second, potentially important enzyme, monoamine oxidase (MAO; EC 1.4.3.4). MAO catalyzes the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylglycol (DOPEG) from dopamine (DA) and norepinephrine (NE), respectively. Five concentrations of NSD-1015 in superfusate (0.01 to 20 μM) were tested in strips of canine portal vein superfused and stimulated *in vitro*. DOPA, DA, NE, and DOPEG in superfusate and in the veins after superfusion were quantified by HPLC with electrochemical detection. The efficacy of NSD-1015 in inhibiting AAAD and MAO was determined by examining the levels of DOPA and DOPEG, respectively. NSD-1015, only when applied at 0.1 μM, resulted in the marked augmentation of total DOPA levels, but did not affect levels of DOPEG, which suggests that this concentration of the drug inhibits AAAD, but does not inhibit MAO. Therefore, it is concluded that, of the concentrations of NSD-1015 tested, 0.1 μM is the optimum concentration to use in this preparation for studies designed to examine TH activity by measuring DOPA after the inhibition of AAAD.

Tyrosine hydroxylase (TH)*, together with a pteridine cofactor and molecular oxygen, catalyzes the formation of 3,4-dihydroxyphenylalanine (DOPA) from tyrosine; this is generally considered to be the rate-limiting step in catecholamine biosynthesis in noradrenergic neurons [1]. A second enzyme, aromatic L-amino acid decarboxylase (AAAD), catalyzes the formation of dopamine (DA) from DOPA. AAAD inhibition results in the tissue accumulation and/or increased release of DOPA [2–5]; accordingly, the quantification of DOPA formed in tissues treated with an inhibitor of AAAD is used as an indirect index of TH activity [3, 5]. In other studies, the difference in DOPA formation between tissues not exposed (control) or exposed to the inhibitor is taken as a measure of the amount of DOPA which is directed to catecholamine biosynthesis in the control tissues [4]. Hydrazine compounds are used to inhibit AAAD; a derivative commonly used is 3-hydroxybenzylhydrazine (NSD-1015), and concentrations of 20–1000 μM have been used in *in vitro* preparations [2–5].

Many hydrazines also inhibit monoamine oxidase (MAO) [6, 7], the enzyme which catalyzes the oxidative deamination of monoamines, including norepinephrine (NE). This inhibition results in increased NE levels [6–10]. Because TH activity is modulated in part through feed-back inhibition by NE, the increased intraneuronal levels of NE under MAO inhibition can indirectly decrease TH activity [8]. Furthermore, inhibition of MAO results in an increase in NE that is released from blood vessels [9, 10]. Thus, in studies designed to assess TH activity through inhibition of AAAD by hydrazines, knowledge of the MAO inhibitory properties of the drugs is important.

Therefore the aim of this study was to determine, using an *in vitro* physiological method similar to those used to assess TH activity, if concentrations of NSD-1015 ranging from 0.01 to 20 μM would effectively inhibit AAAD, but would not inhibit MAO in a superfused segment of the canine portal vein. A previous study showed that this vein is rich in NE and DA; that DOPA accumulation is markedly augmented by NSD-1015 treatment; and that these compounds as well as 3,4-dihydroxyphenylglycol (DOPEG), the deamination product of NE, are readily released upon transmural nerve stimulation of isolated vein strips [4]. The four catechols were quantified in superfusates and in the veins after superfusion. Each vein was stimulated at 2 and 10 Hz to induce release of neurotransmitters and related compounds. The total amounts of DOPA and DOPEG measured in the presence of NSD-1015 were used to assess the effectiveness of inhibition of AAAD and MAO, respectively.

## *Materials and Methods*

These studies were approved by the Institutional Animal Care and Use Committee. Catechol standards were purchased from the Sigma Chemical Co. (St. Louis, MO). NSD-1015 was purchased from Research Biochemicals, Inc. (Natick, MA).

Helical strips of canine portal vein were superfused at 2 mL/min with Krebs–Ringer (37°) as described previously [4, 11]. The strips were superfused for 60 min before superfusate collection began; thereafter, three consecutive 10-min collections of superfusate were made, followed by a 30-min period during which no collection was made, and finally, four additional 10-min collections. Each strip was stimulated continuously (9 V, 0.2 msec), via platinum wire electrodes, at 2 Hz during collection period 2 and at 10 Hz during collection period 5. Six sets of vein strips were used.

* Abbreviations: AAAD, aromatic L-amino acid decarboxylase; DA, dopamine; DOPA, 3,4-dihydroxyphenylanine; DOPEG, 3,4-dihydroxyphenylglycol; MAO, monoamine oxidase; NE, norepinephrine; NSD-1015, 3-hydroxybenzylhydrazine, and TH, tyrosine hydroxylase.

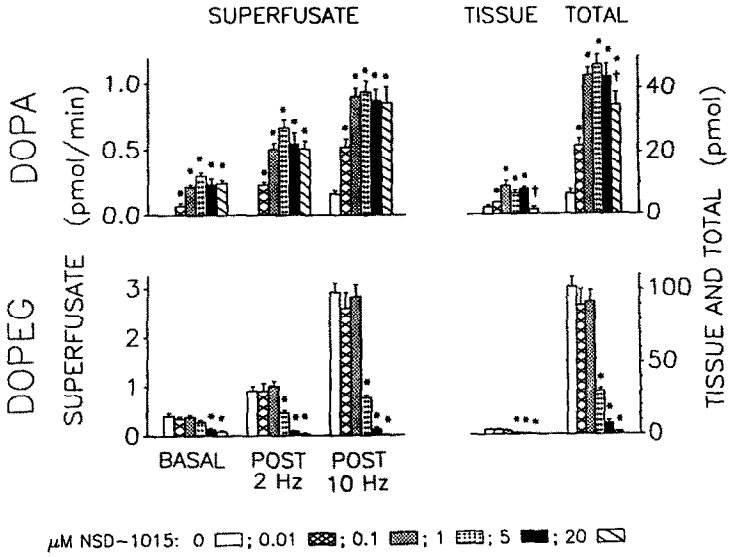


Fig. 1. Effect of various NSD-1015 concentrations on overflow into superfusate, tissue content, and total measured of DOPA (upper panel) and DOPEG (lower panel) in isolated canine portal vein strips superfused and stimulated *in vitro*. Total = overflow during all 7 collection periods + amount in tissue after superfusion. The protocols for the superfusion, and for the isolation and quantification of DOPA and DOPEG are described in Materials and Methods. For clarity, overflow into superfusate is shown only for collection periods 1 (basal), 3 (post 2 Hz), and 6 (post 10 Hz). Values are means ± SEM of 5–8 determinations each and are adjusted to 100 mg tissue wet weight. Key: (*) significantly different ($P < 0.05$) from control, no NSD-1015; and (†) significant difference ($P < 0.05$) between DOPA value in 20 μM NSD-1015-treated vein vs each corresponding value in 0.1, 1, and 5 μM NSD-1015-treated veins.

Except for control (no NSD-1015) strips, NSD-1015 (0.01, 0.1, 1, 5, or 20 μM) was added to the superfusate of each strip 20 min before the first collection began, and was maintained throughout the remainder of the superfusion.

For each strip of vein, DOPA, DA, NE, and DOPEG in the superfusate from each of the seven collection periods and in the tissue after superfusion were isolated on Sep-Pak Plus C-18 cartridges (Waters Instruments, Milford, MA) and quantified by HPLC with electrochemical detection [4]. The model 5100 dual analytical cell (ESA, Inc., Bedford, MA) was used in the REDOX mode; the compounds of interest were first oxidized at +0.12 V on a conditioning cell, then back-reduced on a second, or analytical cell maintained at −0.40 V. The output from the second cell was monitored on a chart recorder (Recordall Series 500, Fisher Scientific Co., Springfield, NJ). For each analyte, the amount which overflowed into the superfusate during each period, the total overflow (periods 1 through 7), the tissue content, and the total measured (total overflow + tissue content) were determined. The effect of each NSD-1015 concentration on the overflows of the four catechols over the course of the superfusion (periods 1 through 7) was assessed using repeated-measures ANOVA. Where ANOVA indicated significant difference, the two-tailed *t*-test was then used to identify differences related to drug concentration in the catechol content for each individual time period. Significance of differences between the catechol content in tissues after superfusion was determined by the two-tailed *t*-test. Differences were considered significant at $P < 0.05$. Analyte levels were corrected for recovery and adjusted to 100 mg tissue wet weight. Recoveries were run with each concentration of NSD-1015 in Krebs–Ringer used; however, no concentration-related differences were observed. The average recoveries of DOPA, DA, NE, and DOPEG were 76.3 ± 1.9, 93.0 ± 1.4, 93.4 ± 0.5, and 81.5 ± 1.1%, respectively, in superfusate (N = 36 each), and 79.7 ± 2.3, 94.0 ± 1.8, 93.9 ± 1.3, and 93.3 ± 2.8%, respectively, in tissue (N = 9 each). The average tissue weight was 87.6 ± 2.3 mg (N = 39).

*Results*

The overflows of DOPA and DOPEG, as well as the corresponding tissue and total amounts are shown in Fig. 1. For clarity, the overflows are shown only for periods 1, 3, and 6. These periods were selected to be representative of periods during which relatively low (basal), moderate (post 2 Hz) and high (post 10 Hz) levels, respectively, of the two compounds were measured in control veins. As was the case in a previous study employing a similar protocol in portal vein, the overflows of both DOPA and DOPEG were frequency dependent, and peaked during the post-stimulation periods [4]. Over the course of the superfusion, DOPA overflow was increased significantly by all concentrations of NSD-1015 tested (ANOVA). The drug was maximally effective in increasing DOPA overflow at 0.1 μM in that no differences in overflow were seen among veins treated with 0.1, 1, 5 or 20 μM NSD-1015. For each individual collection period, the DOPA overflow was increased significantly by 0.01 μM NSD-1015 (by two-tailed *t*-test); the four higher concentrations of the drug were usually equally effective in further augmenting (approximately doubling) this increase. The tissue DOPA as well as the total DOPA followed a similar pattern,

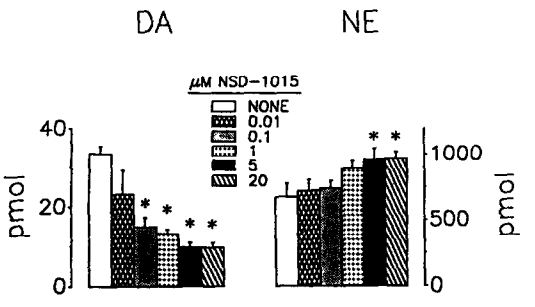


Fig. 2. Effect of various NSD-1015 concentrations on total DA (left panel) and total NE (right panel) measured in isolated canine portal vein strips superfused and stimulated *in vitro*. Total refers to overflow into superfusate during all 7 collection periods + amount in the vein strip after superfusion. The protocols for superfusion, and for isolation and quantification of DA and NE are given in Materials and Methods. Values are means ± SEM of 5–8 determinations each and are adjusted to 100 mg tissue wet weight. Key: (*) significantly different ($P < 0.05$) from control, no NSD-1015.

except that in these cases there was significant decrease below the plateau levels in the 20 μM NSD-1015-treated veins (Fig. 1, upper panel).

The overflow, tissue content, and total measured for DOPEG were unaffected, as compared to controls, by 0.01 or 0.1 μM NSD-1015; however, at the higher drug concentrations tested (1, 5 and 20 μM), there was a graded decrease in these amounts (Fig. 1, lower panel).

In the control veins DA overflow into superfusate was detectable only during the 10 Hz stimulation period (212.3 ± 127.9 fmol/min). This overflow was reduced by 0.01 μM NSD-1015 (80.8 ± 50.1 fmol/min), and was further reduced by the 0.1 μM concentration (34.8 ± 22.6 fmol/min); no overflow (< 26.7 fmol/min) was detected in the 1, 5, or 20 μM NSD-1015-treated veins. The content of DA in the veins after superfusion was reduced in a graded manner from 31.37 ± 1.49 pmol in controls to 14.61 ± 2.25 pmol in the 0.1 μM NSD-1015-treated veins; the higher concentrations of the drug did not induce further decreases. Thus, the total DA was reduced by NSD-1015 (Fig. 2, left panel).

NE was detectable in all superfusate samples, and as in previous studies using blood vessels [4, 9, 11], the overflow was frequency dependent (data not shown). The total overflow of NE (periods 1–7) was increased significantly, compared to controls, only in the 20 μM NSD-1015-treated veins (71.71 ± 4.71 pmol vs 104.96 ± 6.19 pmol, respectively). Also, NE remaining in the veins after superfusion was increased significantly in both the 5 and the 20 μM-treated veins as compared to controls (e.g. 602.78 ± 104.37 pmol and 858.79 ± 59.51 pmol in control and 20 μM NSD-1015-treated veins, respectively). The total NE measured was increased by 5 and by 20 μM NSD-1015 as compared to controls (Fig. 2, right panel).

*Discussion*

The results of the present study illustrate two important aspects of the action of NSD-1015 in isolated portal vein: (1) concentrations of 0.1 to 5 μM NSD-1015 in superfusate are equally and apparently maximally effective in inhibiting AAAD, and (2) at concentrations of 0.1 μM and below, NSD-1015 does not inhibit the formation of DOPEG by MAO. We conclude therefore that, of the concentrations of NSD-1015 tested, the optimum concentration to apply in this preparation to study TH activity is 0.1 μM. The application of this concentration of NSD-1015 resulted in an increase in total DOPA; further increases in drug did not result in further increases in DOPA, which suggests that DOPA metabolism by AAAD had been effectively inhibited. This assessment of AAAD inhibition, derived by physiological means, is close to the reported $K_i$ of 0.086 μM for AAAD inhibition by NSD-1015 in rat brain slices [2]. Thus, the drug may be slightly more effective in inhibiting AAAD in portal vein although it is difficult to draw an exact parallel between the two types of study. The marked reduction or abolishment in the amounts of DOPEG, the product of the deamination of NE, was taken as an indication of the effective inhibition of MAO.

Use of NSD-1015 at the 0.1 μM concentration permits the quantification of DOPA to be used as an index of TH activity [3, 5], and, when compared to DOPA formation in untreated tissues, also indicates the amount of DOPA which is normally directed to the catecholamine biosynthetic pathway [4]. Importantly, since 0.1 μM NSD-1015 apparently does not inhibit MAO, it would not directly affect TH activity [8] or catecholamine release [9] via the mechanisms described. That the MAO inhibitory capacity of NSD-1015 may have been a factor affecting TH activity through feedback inhibition by NE is suggested by the small but significant decrease in total DOPA measured in the presence of the 20 μM concentration of the drug as compared to the level seen with lower concentrations, and by the corresponding increases in NE, compared to controls, at the two highest NSD-1015 concentrations tested. This effect may be more pronounced in other tissues if differences in feedback inhibition of TH exist which are related to the inherent differences in the neuroplasmic levels of catecholamines (including NE, DA and epinephrine) between tissues, or between noradrenergic and dopaminergic neurons. A previous study indicated that inhibition of MAO results in increases not only in NE contained within the vesicles, but also in neuroplasmic NE (i.e. in NE that could exert feedback inhibition of TH) [7]. It should be noted also that other mechanisms exist for the modulation of TH activity, e.g. phosphorylation of the enzyme through calcium-dependent [12] and cyclic AMP-dependent [13] protein kinases.

Interestingly, NSD-1015 essentially abolished the overflow, but not the tissue content of DA. It dose-dependently reduced, by about 50%, the DA in the vein when applied at concentrations up to 0.1 μM; however, the higher concentrations of the drug did not result in further decreases. These findings suggest that there may be two pools of DA in the portal vein: a readily-releasable pool of newly synthesized DA, the formation of which is inhibited by NSD-1015, and a second pool of DA which is stored as vesicular reserves and used as substrate for NE biosynthesis. The existence of two comparable pools of DA in noradrenergic neurons of the mesenteric artery has been proposed by Soares-da-Silva [14].

The inhibition of MAO, and thus the inhibition of one of the two major pathways of NE catabolism in blood vessels, likely resulted in the increases in NE observed in the NSD-1015-treated veins in this study. Previous studies have shown that inhibition of MAO by hydrazines [6, 7] as well as by the MAO type A-specific inhibitor clorgyline [9, 10] and the non-specific MAO inhibitor pargyline [8, 10] results in increased NE levels.

In summary, this study suggests that NSD-1015, when applied at 0.1 μM to superfused portal vein segments, inhibits AAAD but does not inhibit MAO. This concentration of the drug is well below the concentrations of 20–1000 μM used previously to examine TH activity in brain slices [2, 3, 5] and in portal vein segments [4]. When applied at 0.1 μM, NSD-1015 provides a useful tool to assess TH activity and catecholamine biosynthesis in portal

vein, and may be relevant to the study of TH in other isolated tissues as well.

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