Mechanism of Action of Methyldopa in the Rat
Role of 3-O-Methylated Metabolites

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SUMMARY We studied the effect of 3-O-methyl-methyldopa (OMMD), the 3-O-methylated metabolite of the antihypertensive drug methyldopa (α-methyldopa, AMD), on blood pressure in the spontaneously hypertensive rat. OMMD lowered blood pressure in a dose-related manner when given orally or intraperitoneally. Its action lasted longer than that of AMD, and daily oral administration produced a cumulative fall in blood pressure. Oral and intraperitoneal OMMD produced similar reductions of blood pressure and similar tissue OMMD levels. After intraperitoneal injection of different doses, levels of OMMD measured in brain, spinal cord, and plasma correlated with the magnitude of the antihypertensive effect. No AMD was detected in tissues after either route of administration, which suggests that the antihypertensive effect was not based on demethylation of OMMD to AMD. Peripheral inhibition of the enzyme, aromatic amino acid decarboxylase (AAAD), failed to suppress OMMD's effect on blood pressure; in contrast, central inhibition of AAAD did decrease OMMD's antihypertensive effect. These observations suggest that 3-O-methylated metabolites may participate in the antihypertensive effect of AMD. Circ Res 45: 684–690, 1979

AN AMINE metabolite acting in the central nervous system (CNS) mediates the antihypertensive effect of methyldopa (α-methyldopa, "Aldomet," AMD), a widely used antihypertensive drug (Dollery and Bulpitt, 1975; Henning, 1975; Van Zwieten, 1973). Considerable evidence supports the view that the active agent is α-methyl-norepinephrine (AMNE), the decarboxylated and β-hydroxylated product of AMD. However, the exact nature of the active agent and the mechanism by which it affects blood pressure remain obscure.

Previous experiments in our laboratory (Lo et al., 1976) demonstrated a dose-related accumulation of O-methylated metabolites in the brains and spinal cords of mice given isotopically labeled AMD. These O-methylated metabolites seem to be produced or stored in catecholaminergic (CA) neurons, since their accumulation is prevented by destruction of CA nerve terminals with 6-hydroxy-dopamine (Lo et al., 1976). Repeated administration of isotopically labeled AMD to mice was associated with a considerable increase in levels of some of these 3-O-methylated metabolites in brains and spinal cords. This evidence raises the possibility that O-methylated metabolites might participate in AMD's antihypertensive effects, especially when multiple doses are given.

Other evidence also supports the possibility that O-methylated metabolites might be involved. Prescott et al. (1966) observed that hypertensive patients taking AMD for 1 month have a 40% increase in the percentage excreted as 3-O-methyl-methyldopa (OMMD), compared with amounts excreted after a single dose. These authors also noted a direct linear correlation between the antihypertensive effect of AMD and the 24-hour urinary excretion of OMMD in chronically treated patients. These results suggest that the O-methylation of AMD can be induced and might assume therapeutic significance after chronic AMD administration. Moreover, the O-methylated metabolites of AMD are structurally similar to normetanephrine and metanephrine, two neurotransmitter metabolites that may be physiologically active as false transmitters (Kopin, 1968) or as α-agonists (Arbilla and Langer, 1978). Similar false-transmitter (Kopin, 1968) and α-agonist (Dollery and Bulpitt, 1975; Henning, 1975) mechanisms have been suggested as the basis of AMD's antihypertensive action. The structural similarity of the metanephrines to AMD's O-methylated metabolites is compatible with the possibility that these AMD metabolites may participate in AMD's antihypertensive action.

We therefore examined the effects of OMMD, a particular O-methylated metabolite of AMD, on blood pressure.

Methods
Male, spontaneously hypertensive rats (SHRs) of the Okamoto strain (Charles River Laboratories)
were exposed to light (300 μW/cm; Vita Lite, Duro Test Corporation) from 7 a.m. to 9 a.m. daily and were given ad libitum access to water and food [Charles River rat-mouse-hamster maintenance formula (24% protein)]. All experiments were begun between 7 a.m. and 8 a.m. daily to compensate for diurnal variation in blood pressure in SHR mouse (Lew, 1976).

Systolic blood pressure was measured by the tail cuff method (Udenfriend, 1976), using a Narco Biomedical system's pneumatic pulse transducer. Rats were prewarmed for 20–25 minutes at 38°C, and at least six blood pressure readings at 30-second intervals were averaged for each time point. Rats were preconditioned by measuring blood pressure on at least four occasions on as many days. Measurement of blood pressure after OMM-D was administered required more care in prewarming than after AMD administration. After OMM-D was administered, slight overwarming occasionally would lead to erratic high blood pressure readings, whereas slight underwarming did not produce adequate tail artery dilation to make adequate readings with our method. Erratic readings were rejected, and the same rat usually could be studied again a few minutes later, after repeating the prewarming procedure. When these precautions were taken, blood pressure readings were reliable and could be reproduced.

Food was removed during experiments, but water was available ad libitum (except during prewarming and blood pressure measurement), and rats were maintained at room temperature. At least 6 days passed between consecutive experiments on a given rat. OMM-D and AMD, suspended in 0.05 N HCl or water (2.5 ml/kg), were given i.p., using a 20-gauge needle, or orally, in the same volumes (5 ml/kg), using a metal stomach tube.

Merck, Sharp, and Dohme generously supplied the following compounds: L-α-methyl-3,4-dihydroxyphenylalanine (methyldopa, AMD), L-α-methyl-4-hydroxy-3-methoxydihydroxyphenylalanine (3-O-methyl-methyldopa, OMM-D), and S-α-methyl-L-dopa-hydrizine (carbidopa, MK486). Hoffmann-LaRoche, Inc., generously supplied N,N-4-seryl-N-4-(2,3,4-trihydroxybenzyl)-hydrazine (benserazide, RO4-4602). All other chemicals were reagent grade.

AMD and OMM-D were measured fluorimetrically after ion exchange chromatography. Rats were decapitated and blood was collected from the neck in ice-cold tubes containing 100 U of heparin. Blood was centrifuged at 1000 g for 10 minutes, and 1–2 ml of plasma was aspirated and immediately added to 10 ml of ice-cold 0.4 N perchloric acid. Brains and spinal cords were removed, immediately frozen on dry ice, weighed, and homogenized in perchloric acid. All homogenates were then centrifuged in a Sorvall centrifuge at 30,000 g for 20 minutes at 4°C. Supernatant fluids were frozen overnight at −20°C.

The next day, the supernatant fluids were adjusted to pH 2.0 and passed over 4.5-cm columns of Dowex 50W-X4 (Carlson and Waldeck, 1964; Lo et al., 1976). The columns were washed with 10 ml of 0.1 M citrate-phosphate buffer (pH 2.5); then 10 ml of water, and buffer and water were discarded. The amino acid fraction, containing both AMD and OMM-D, then was eluted with 0.1 M citrate buffer (pH 4.5); the 1st ml was discarded, and the next 4 ml were collected and frozen overnight at −20°C.

AMD was measured fluorimetrically by the trihydroxyindole method of Dominic and Moore (1971). An appropriate volume of the Dowex eluate, with and without internal standards, was assayed. Ten micrograms of AMD were added to several columns to determine recoveries, which averaged 80%. A standard curve was run as part of each assay to demonstrate that the assay was linear and was working properly at the time. Fluorescence readings (A395/E505) were taken with an Aminco-Bowman spectrophotofluorimeter 45 minutes after oxidation, only after thorough vortexing of oxidation solutions, since streaming of the viscous oxidation solutions interfered with fluorescence readings. Results were calculated using internal standards to compensate for variable quenching. This assay was linear from 50 to 1000 ng (P < 0.01, as demonstrated by regression analysis). OMM-D, even in very large amounts, did not interfere with our AMD assay.

OMM-D was measured by a modification of the method used by Fahn et al. (1972) for measuring O-methyl-l-dopa (OMD), the O-methylated metabolite of l-dopa. This assay was done on the same Dowex eluates used to assay AMD. A 0.5-ml sample (or appropriate standard) was mixed with 0.5 ml of 10 M ammonium hydroxide in an oxidation tube. The tubes then were heated for 1 minute in an oil bath at 100°C to destroy DOPA, which interfered with the assay (Fahn et al., 1972). Freshly prepared potassium ferricyanide (0.1 ml, 0.01% wt/vol) then was added to each tube, which was vortexed. Exactly 4 minutes later, cysteine (0.1 ml, 0.078% wt/vol) was added to the tube, which then was vortexed to stop the reaction. Tissue blanks were prepared by reversing the order of addition of the last two reagents. Twenty micrograms of OMM-D were added to columns to determine recovery, which averaged 72%. A standard curve was run as part of each assay, to confirm the assay’s reliability and linearity. Assays were linear from 50 to 2500 ng (P < 0.01 by regression analysis). Results were calculated using internal standards to compensate for variable quenching. AMD, even in very large amounts, did not interfere with this assay.

Statistical analysis was done using variance and regression analyses. Calculations were done with a Wang 2200 desktop computer.

Results

OMM-D Time Course

OMM-D, given i.p. at a dose of 100 mg/kg, lowered blood pressure significantly (Fig. 1). OMM-D produced a significant antihypertensive effect at 2
hours, with a peak at 6–7 hours; this effect persisted for up to 15 hours. In contrast, AMD had little effect at 2 hours, a peak effect at 4–5 hours, and no significant effect at 8 hours. The peak antihypertensive effect of OMMD is approximately half that of a similar dose of AMD (Zavisca and Wurtman, 1978) administered i.p.

OMMD Dose-Response Curve

Orally administered OMMD caused a dose-related fall in blood pressure (Fig. 2). Its effect after i.p. administration was similar in magnitude. The lowest dose tested (10 mg/kg) produced a small but insignificant effect; higher doses produced significant effects, with maximal falls in blood pressure occurring at 100 mg/kg. The effect of this dose was about half the maximal effect of AMD (Zavisca and Wurtman, 1978).

Daily Oral OMMD

No fall in blood pressure was noted 24 hours after rats received a single oral dose of OMMD (Fig. 3). The next day, 24 hours after the second 100 mg/kg dose, blood pressure was depressed significantly ($P < 0.005$); a larger daily dose (250 mg/kg) produced an even more significant change ($P < 0.001$) in blood pressure. Subsequently, blood pressure was reduced significantly until day 7, 1 day after therapy was discontinued. On day 8, 2 days after the last dose, the blood pressure in all rats had returned to baseline.

OMMD Levels and Blood Pressure Change after Different Doses of OMMD

We examined the relationship between tissue OMMD levels and the blood pressure response to an OMMD dose. We were interested particularly in spinal cord levels, since it is possible that AMD or OMMD may act at descending monoaminergic synapses (Chalmers and Wurtman, 1971; Lo et al., 1976). Relatively high doses of OMMD (50 and 100 mg/kg) were used to obtain readily measurable antihypertensive responses and tissue levels of OMMD (and, if present, AMD). Blood pressure was measured initially and again at 6 hours; rats were killed and levels of OMMD were measured in brain, spinal cord, and plasma. In preliminary experiments, we used either the AMD or the OMMD assay and demonstrated that no significant native fluorescence was present in tissues.

The effects of OMMD administration on OMMD levels in plasma, brain, and spinal cord were dose-related, as was the fall in blood pressure (Table 1). No AMD was detected in any of the samples.

A correlation analysis of each rat's fall in blood pressure vs. its brain OMMD level (Fig. 4) showed a highly significant linear correlation between the two parameters ($P < 0.001$). Significant linear correlations also were noted between OMMD's anti-

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**Figure 1** Time course of blood pressure change after OMMD, 100 mg/kg, i.p. SHR's weighing 240–270 g were injected i.p. with OMMD, 100 mg/kg suspended in 0.05 N HCl ($n = 4$) and vehicle alone ($n = 3$), immediately after baseline blood pressures were obtained. Blood pressures then were measured at 1-hour intervals for 7 hours. The change in blood pressure from the baseline at 0 time for each rat was calculated for each time point. Each point represents the mean ± SEM of the change in blood pressure from the baseline for the drug or vehicle groups at each time point. Data were analyzed by two-way analysis of variance with repeated measures, followed by the Scheffe test.

**Figure 2** Dose-response curve for oral OMMD. SHR's weighing 320–370 g were given oral OMMD (10, 25, 50, 100, and 250 mg/kg, suspended in 0.05 N HCl) or vehicle alone, immediately after a baseline blood pressure was obtained. Blood pressure was measured again 6 hours later. Percent fall in blood pressure at 6 hours compared to the baseline was calculated for each rat. Each point represents the mean ± SEM (from 6 to 15 rats, from one to three experiments) of the percent change for one dosage. Data were analyzed by one-way analysis of variance, followed by the Scheffe test.
hypertensive effects and its levels in spinal cord (P < 0.05) and in plasma (P < 0.05). Multiple linear regression analysis of blood pressure changes vs. OMMD levels in brain, spinal cord, and plasma showed an even stronger linear correlation (P < 0.005).

Brain OMMD Levels after Oral or Intraperitoneal Administration

Because OMMD produced similar antihypertensive effects after oral and i.p. administration, we compared brain OMMD levels after OMMD admin-

![Graph showing blood pressure changes over days for different doses of OMMD.](image)

**Figure 3** Effects of daily oral OMMD on blood pressure. SHRs weighing 310–370 g were given oral OMMD (25, 100, or 250 mg/kg, suspended in water) or water alone, immediately after a baseline blood pressure was taken. This procedure was followed for 5 days, beginning at approximately 7 a.m. each day. After the last dose, daily blood pressures were taken for an additional 3 days. Percent of change in blood pressure compared to the baseline on day 1 was calculated for each rat for days 2–9. Each point represents the mean ± SEM from five rats from each treatment group on a certain day. Data were analyzed by two-way analysis of variance with repeated measures, followed by the Scheffe test.

![Graph showing correlation between brain levels of OMMD and blood pressure changes after OMMD.](image)

**Figure 4** Correlation between brain levels of OMMD and blood pressure changes after OMMD. SHRs weighing 340–400 g were injected with OMMD (50 or 100 mg/kg, suspended in 0.05 N HCl) immediately after a baseline blood pressure was obtained. Blood pressure was measured again at 6 hours, the rats were killed, and brain levels of OMMD were measured (as described in Methods). Percent of change in blood pressure at 6 hours, compared to the baseline, was calculated for each rat. OMMD levels are expressed as μg/g for brain. Each point represents the percent of change in blood pressure from one rat and the level of OMMD in the brain of the same rat. Data were analyzed by linear regression. ○ = 50 mg/kg dose; X = 100 mg/kg dose.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>% Blood pressure change</th>
<th>Brain OMMD</th>
<th>Spinal cord OMMD</th>
<th>Plasma OMMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>-12.6 ± 0.78 (8)</td>
<td>6.79 ± 1.61 (7)</td>
<td>5.93 ± 0.79 (8)</td>
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<tr>
<td>100</td>
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<td>15.48 ± 2.55 (6)</td>
<td>11.90 ± 2.65 (5)</td>
<td>14.74 ± 2.23 (6)</td>
</tr>
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P

SHRs, weighing 340–400 g, and with baseline blood pressures between 188 and 219 torr, were injected with OMMD (50 or 100 mg/kg, suspended in 0.5 N HCl) immediately after a baseline blood pressure was obtained. Blood pressure was measured again at 6 hours; the rats were killed, and the levels of OMMD were measured (as described in Methods) in brain, spinal cord, and plasma. Percent of change in blood pressure at 6 hours, compared to the baseline, was calculated for each rat. OMMD levels are expressed as μg/g for brain and spinal cord and as μg/ml for plasma. Values are expressed as means ± SEM for the number of rats noted in parentheses. For each parameter, the t-test was used to compare the results after the two different doses.

* Blood pressure taken by tail cuff method.

† P = Probability of differences between two groups.
istration by both routes. Groups of rats received OMMD (50 mg/kg), orally or i.p., and were killed after 6 hours. The two treatments caused identical falls in blood pressure (−13.1 ± 1.2 mm Hg vs. −13.4 ± 1.2 mm Hg) and similar brain OMMD levels (5.4 ± 0.3 mg/g vs. 6.8 ± 1.0 mg/g). Again, no AMD was detected in any of the samples. Thus, OMMD seemed to be absorbed well when orally administered.

Decarboxylase Inhibition Studies

Studies using inhibition of the enzyme aromatic amino acid decarboxylase (AAAD) support the hypothesis that an amine metabolite of AMD is the active agent mediating AMD's antihypertensive action. AMD-induced antihypertensive action was essentially unaffected by the inhibition of AAAD in the periphery, but not in the CNS, by drugs like carbidopa. However, inhibition of AAAD, in both the CNS and the periphery, by drugs like benserazide (RO4-6062) decreases or abolishes the AMD-induced fall in blood pressure (Dollery and Bulpitt, 1975; Henning, 1975). These results are consistent with the view that an amine metabolite of AMD [such as α-methyladapamine (AMDA) or AMNE], formed in the CNS, lowers blood pressure. However, they are also consistent with the possibility that these amines are O-methylated. We therefore tested the effect of decarboxylase inhibition on the blood pressure response to OMMD.

Carbidopa alone did not affect blood pressure, whereas benserazide alone, as shown previously (Day et al., 1973), moderately lowered blood pressure (Fig. 5). Carbidopa did not modify the antihypertensive response to OMMD, whereas benserazide significantly decreased it. These results are consistent with an O-methylated metabolite mediating the fall in blood pressure produced by OMMD or AMD.

Discussion

These studies demonstrate that OMMD lowers blood pressure and suggest that O-methylated metabolites may be involved in AMD's antihypertensive action.

OMMD deserves further study as a prototype for antihypertensive drugs because of several of its characteristics: its effects are cumulative (Fig. 3), which may make daily administration practical; it is absorbed well and its effects are dose-related (Figs. 2, 3); and it gradually lowers blood pressure after daily administration (Fig. 3). OMMD's toxicity would be expected to be no greater than AMD's, since OMMD is normally produced in patients taking AMD (Prescott et al., 1966).

The observation that OMMD causes a prolonged, cumulative fall in blood pressure (Fig. 1) is consistent with the hypothesis that O-methylated metabolites, accumulating in the nerve terminals after repeated AMD administration (Lo et al., 1976), may participate in the fall in blood pressure caused by AMD. Indeed, our dose-response experiments (Fig. 2) and the studies of OMMD levels (Table 1) demonstrate that relatively small doses of OMMD [which produce measurable tissue levels of OMMD and, presumably, of O-methylated amines, in the range expected after AMD administration (Lo et al., 1976)] can lower blood pressure. These observations, along with the existence of a linear correlation between OMMD tissue levels and the fall in blood pressure (Fig. 4), strongly suggest that these O-methylated metabolites are not physiologically inert.

Prescott et al. (1966) noted a 40% increase in the
percentage of OMMED excretion in the urine of hypertensive patients taking AMD for 1 month compared with the percentage seen after one dose. This finding suggests that O-methylation may assume more importance after chronic than after acute therapy. This hypothesis was supported by a linear correlation of OMMED excretion with standing (but not supine) blood pressures in chronically treated patients (Prescott et al., 1966). Thus, OMMED may have more effects on cardiovascular reflexes than on resting blood pressure. AMD depresses cardiovascular reflexes less than do many other antihypertensive agents (Dollery and Bulpitt, 1975), allowing patients to maintain some reserve in the event of stress, while still lowering basal blood pressure. O-methylated metabolites may have a greater effect on cardiovascular reflexes than on sympathetic tone.

It is conceivable, but unlikely, that OMMED acts by being demethylated to AMD. A similar relationship was proposed between oral OMD and L-dopa (Bartholini et al., 1974; Scriabine et al., 1976; Tyce, 1976; Tyce et al., 1976). O-demethylation of OMD may occur in red blood cells (Tyce, 1976; Tyce et al., 1976) and, by bacterial action, in the gut (Chalmers et al., 1972). In patients with Parkinsonism on chronic DOPA therapy, the time course of long-term improvement after abrupt discontinuation or reinitiation of therapy closely parallels changes in plasma OMD levels (Muenter et al., 1972). Isotopically labeled OMD (Bartholini et al., 1974) produces distributions of labeled dopamine and noradrenaline in rat brain similar to those seen after labeled DOPA administration. (The absolute concentrations of the labeled catecholamines after OMD administration were, of course, much lower, and declined more slowly, than after DOPA was administered.) This finding is consistent with slow demethylation of OMD to DOPA, which would then be metabolized normally.

By structural analogy to OMD, slowly declining levels of AMD, formed by slow demethylation of OMMED, might account for some of OMMED’s effects on blood pressure. The concentrations of AMNE and AMDA after OMMED would be lower (accounting for less potency), and would persist longer, than after AMD. Thus, OMMED might form a storage pool of slowly released AMD. However, the lack of any measurable AMD in the tissues of our rats given i.p. OMMED suggests that significant demethylation does not occur. Similarly, the lack of AMD in brain after oral OMMED suggests that AMD is not formed in significant amounts by demethylation via gut bacteria (Chalmers et al., 1972), and that i.p. and oral OMMED cause a fall in blood pressure by identical mechanisms. Demethylation of the O-methylated amines (OMAMDA and OMAMNE) to the non-O-methylated amines (AMDA and AMNE) could occur also. However, the absence of any measurable AMD makes it unlikely that significant demethylation of related amines occurs.

Measurements of OMMED in the spinal cord yielded observations similar to those made on brain samples (Fig. 4, Table 1). Lo et al. (1976) noted similar patterns of OMMED and its labeled metabolites in spinal cords and brains of mice given AMD. The spinal cord contains terminals of descending monoaminergic neurons, thought to be important in blood pressure control (Chalmers and Wurtman, 1971). For example, an increase in norepinephrine release within the cord is associated with a rise in blood pressure. AMD metabolites, especially O-methylated metabolites (Lo et al., 1976), could accumulate in the cord and, acting as false neurotransmitters, lower blood pressure.

References


Udenfriend S (Chairman) (1976) Committee on care and use of spontaneously hypertensive rats (SHR). In Spontaneously hypertensive rats (SHR): Guidelines for breeding, care, and use. ILAR News 18: G1–G2a