Pharmacokinetics and Pharmacodynamics of Methylecgonidine, a Crack Cocaine Pyrolyzate

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Nonstandard Abbreviations: AMB: atropine methyl bromide, MAP: mean arterial pressure

Abstract

Methylecgonidine is formed from cocaine base when smoked and has been identified in biologic fluids of crack smokers. Ecgonidine, a metabolite of methylecgonidine formed via esterase activity, also has been identified in similar samples collected from crack smokers. Methylecgonidine and ecgondine can be used as biomarkers to differentiate smoking from cocaine use via other routes of administration. We determined the pharmacokinetic properties of methylecgonidine and ecgonidine in sheep following intravenous administration of methylecgonidine at doses of 3.0, 5.6 and 10.0 mg/kg employing gas chromatography- mass spectrometric assays. Methylecgonidine clears quickly from blood with a half-life of 18-21 minutes, while ecgonidine has a longer half-life of 94-137 minutes. Since ecgonidine clears more slowly it may be a more effective biomarker of cocaine smoking.

The cardiovascular stimulant effects of cocaine contrast with reported *in vitro* muscarinic agonist effects of methylecgonidine decreasing contractility and stimulating nitric oxide production in cardiac cells and tissues. To test the hypothesis that methylecgonidine produces cardiovascular effects *in vivo* consistent with muscarinic agonism, methylecgonidine was administered to sheep intravenously (0.1-3.0 mg/kg) while monitoring heart rate and blood pressure. Significant hypotension and tachycardia occurred in all three sheep ($p<0.005$). Two of the three sheep demonstrated mild bradycardia 3-5 minutes after methylecgonidine injection ($p<0.005$). Intravenous pretreatment with atropine methyl bromide (15 μg/kg) antagonized methylecgonidine-induced hypotension in all three sheep, supporting the hypothesis that methylecgonidine acts as a muscarinic agonist *in vivo*.

Heating cocaine base (smoking crack) produces the pyrolyzate methylecgonidine (anhydroecgonine methylester). Since methylecgonidine is produced via thermal conversion from cocaine, it can be used as a marker to differentiate between smoking and cocaine use via other routes of administration. In forensic cases, methylecgonidine has been detected in urine (Jacob et al., 1990; Cone et al., 1994; Paul et al., 1999; Riley et al., 2001; Shimomura et al., 2001), blood/plasma (Jenkins and Goldberger, 1997; Toennes et al., 1999; Shimomura et al., 2001; Fandino et al., 2002; Toennes et al., 2003), saliva (Kintz et al., 1997), perspiration (Kintz et al., 1997), hair (Kintz et al., 1995; Kintz et al., 1997), brain and liver (Shimomura et al., 2001) of crack smokers. Though methylecgonidine has been identified in numerous matrices, the number of blood or plasma samples in which it has been detected numbers less than fifty in the literature. The maximum methylecgonidine blood or plasma concentration accurately reported in the literature is 110 ng/mL (Toennes et al., 2003), whereas the maximal urinary concentration is 6340 ng/mL (Riley et al., 2001). We hypothesized that methylecgonidine is rapidly cleared from the central compartment accounting for low blood, plasma and tissue concentrations. Therefore we administered methylecgonidine intravenously to sheep and examined its clearance from blood.

Methylecgonidine is metabolized to ecgonidine (anhydroecgonine) by butyrylcholine esterase activity accounting for the presence of ecgonidine in urine and postmortem brain and liver (Paul et al., 1999; Shimomura et al., 2001; Fandino et al., 2002) (Figure 1, panel A). These investigators and others hypothesized that ecgonidine is the principal metabolite of methylecgonidine, undergoing demethylation analogous to cocaine demethylation forming benzoylecgonine (Jacob et al., 1990; Paul et al., 1999; Shimomura

et al., 2001). Urinary ecgonidine concentrations were greater than methylecgonidine in forensic samples, suggesting that ecgonidine is a major metabolite, having a longer half-life than methylecgonidine, and should be a longer persisting indicator of crack smoking than methylecgonidine (Paul et al., 1999). In our present studies, we analyzed blood samples for methylecgonidine and ecgonidine concentrations to determine their pharmacokinetics, testing the hypothesis that ecgonidine has a longer half-life than methylecgonidine.

Intravenous methylecgonidine administration produces cardiovascular effects that differ from cocaine. Erzouki et al. (1995) reported that 1.0 mg cocaine administered intravenously to anesthetized rabbits increased mean blood pressure and peak heart rate, however 3.0 mg methylecgonidine also administered intravenously decreased mean blood pressure and peak heart rate (Erzouki et al., 1995). These responses to methylecgonidine mimic muscarinic agonsim (Brown and Taylor, 1996). Several *in vitro* studies have demonstrated that methylecgonidine acts as M_2 muscarinic agonist (Huang et al., 1997; Woolf et al., 1997; Yang et al., 2001; Yang et al., 2002). Therefore we wanted to test the hypothesis that the pharmacodynamics of methylecgonidine are consistent with muscarinic agonism in awake sheep. In addition to methylecgonidine's cardiovascular effects we examined whether methylecgonidine could be antagonized by atropine methyl bromide (AMB), a non-specific muscarinic antagonist with limited access to the central nervous system.

Methods

Reagents and Assay Standards Methylecgonidine and ethylecgonidine fumarate, ecgonidine and N-ethyl-N-norecgonidine hydrochloride (Figure 1, panel B) were synthesized as reported previously (Scheidweiler et al., 2000). Atropine methyl bromide was purchased from Sigma Chemical (St. Louis, MO). Methacholine chloride was purchased from Metapharm, Inc. (Brantford, Ontario, Canada). CleanScreen ZSDAU020 columns (United Chemical Technologies, Bristol, PA) were used for solid phase extraction. All solvents were reagent or HPLC grade (Fisher Scientific; Fairlawn, NJ).

Methylecgonidine and Ecgonidine Assay Calibrators for methylecgonidine and ecgonidine were prepared in blank sheep plasma at the following concentrations: 30, 50, 100, 250 and 500 ng/mL. Assay controls; 55, 300, and 2500 ng/mL (3000 ng/mL for ecgonidine) were prepared in blank sheep plasma. Methylecgonidine and ecgonidine were extracted from plasma samples via solid phase extraction using CleanScreen columns following methanol precipitation of protein and analyzed by gas chromatography – mass spectrometry (Scheidweiler et al., 2000).

GC/MS Analysis The GC/MS selected ion monitoring settings for methylecgonidine and ecgonidine assays were detailed previously (Scheidweiler et al., 2000). Methylecgonidine and ecgonidine recovery was greater than 75%. Assay variation was evaluated with methylecgonidine or ecgonidine controls; coefficients of variation were less than 10% for 55, 300 and 2500 (3000 for ecgonidine) ng/mL controls. The

methylecgonidine assay linearity was from 20-2500 ng/mL with a limit of detection of 10 ng/mL. Ecgonidine linearity ranged from 30-3000 ng/mL with a limit of detection of 10 ng/mL. Concentrated study samples were diluted to within assay linearity using distilled water prior to extraction.

Experimental Design

Two separate experiments were conducted for this study, an initial pharmacokinetic experiment and a follow-up pharmacodynamic experiment.

Pharmacokinetic Study Nonpregnant female sheep of mixed breed were purchased from a local vendor and prepared with indwelling femoral catheters (1.3mm ID by 2.3mm OD Tygon® microbore tubing, formulation S-54-HL, Norton Performance Plastics, Akron, OH) while under general anesthesia using aseptic technique. At least one week was allowed for recovery from surgery. Methylecgonidine fumarate was dissolved in sterile saline for injections of 3.0, 5.6 and 10.0 mg/kg as the salt. Evacuated blood collection tubes (part # 366471, Becton-Dickinson, Franklin Lakes, NJ) were used for collecting blood samples (2 to 3mL). Sodium fluoride tubes limit plasma esterase activity and aid stability of methylecgonidine following sample collection (Scheidweiler et al., 2000). Venous blood sampling covered from 5 minutes prior to methylecgonidine injection until at least 55 minutes after methylecgonidine administration. Samples were collected every 30 seconds for first five minutes, every minute until ten minutes, every two minutes until 20 minutes and every five minutes until 55 minutes after administration. Samples were

placed on ice immediately, centrifuged at 1000g for seven minutes; plasma was collected and stored at -80°C until analysis.

Noncompartmental Pharmacokinetic Calculations The venous line, used for both drug administration and blood collection was flushed with five mL saline following drug administration. Inspection of residuals after nonlinear regression fitting of the entire methylecgonidine elimination profiles revealed that datapoints collected two minutes after drug administration were outliers, appearing as falsely elevated methylecgonidine plasma levels. Thus these samples were not included in pharmacokinetic analysis. The terminal linear phase of elimination was fit on the $\log_{10}$-transformed plasma concentrations to the model

$$\log_{10}C = \log_{10}(Ae^{-kt})$$

C is concentration at time t in ng/mL

A is the zero-order intercept in ng/mL

k is the slope of the terminal phase of elimination in minutes^{-1}

t is time in minutes

For all methylecgonidine analysis the terminal linear phase was selected as occurring between 25 to 55 minutes after methylecgonidine administration. Area under the curve was calculated using the trapezoidal rule. Half-life, clearance and volume of distribution were calculated using standard pharmacokinetic formulas (Rowland and Tozer, 1995).

For comparison of methylecgonidine half-life to literature values of cocaine half-life, 95% confidence intervals of half-life were computed by transforming the confidence intervals of the slope estimate. RS/1 (Brooks Automation Inc, Chelmsford MA)

calculates the confidence intervals of the slope estimate using the likelihood method, providing a better confidence estimate of a nonlinear function than linearized confidence intervals (Clarke, 1987; Donaldson and Schnabel, 1987).

Pharmacodynamic Study Solutions of methylecgonidine fumarate and atropine methyl bromide (AMB) were prepared daily in saline. Concentrated methacholine stocks were prepared in saline and stored at -80°C.

Sheep were prepared with indwelling femoral arterial and venous catheters using procedures described above. Sheep were brought to the experimental room and the femoral artery catheter connected to a blood pressure transducer (Abbott, model # 42582-05, North Chicago, IL). The strain gauge coupler was connected to a tachometer and blood pressure processor (Coulbourn models S77-25, S77-26 and S77-34, respectively, Allentown, PA). Heart rate and blood pressure were logged 10 times/second using Biobench version 1.0 (National Instruments, Austin, TX).

Sheep were administered AMB or saline intravenously in a within-animal experimental design (figure 2). Two saline vehicle control volumes were used; one was equivalent to the volume of methacholine delivery (approximately one mL) and a second equivalent to methylecgonidine fumarate delivery volume (3 mL for 0.1, 0.3, and 1.0 mg/kg; 5 mL for 3.0 mg/kg). Methacholine (5 μg/kg iv) served as a positive control for muscarinic agonism. Ten minutes later a dose of methylecgonidine fumarate (0.1, 0.3, 1.0 or 3.0 mg/kg) was administered. Since we hypothesized that methylecgonidine acts as a peripheral muscarinic agonist, we administered AMB, a nonspecific quatenary antagonist with limited central nervous system access. Methacholine and the

methylecgonidine study dose were administered following AMB treatment to examine potential antagonism of methylecgonidine effects on heart rate and blood pressure. Experiments using different methylecgonidine doses were conducted at least 48 hours apart.

Pharmacodynamic Data Analysis To minimize the impact of movement artifacts, medians were computed from one-second (sheep 4 and 5) or two-second (sheep 3) intervals; data was collected at a resolution of 10 samples/second. Mean arterial pressure (MAP) was computed as diastolic pressure + 1/3 difference of systolic and diastolic pressure by a blood pressure processor (Coulbourn, Allentown, PA). Smoothed curves were fit to heart rate and MAP (figure 4) (Chambers, 1983).

Effects on heart rate and MAP were expressed as changes from baseline within each experimental session. Baseline MAP was computed as the median of three-minutes of MAP data ending 20 seconds before each drug administration. Baseline heart rate was computed as the median of two-minutes of heart rate data ending 20 seconds before each drug administration.

Cardiovascular effects of methacholine and methylecgonidine were evaluated on four endpoints, which were determined empirically. Bradycardia was defined as the peak decrease in heart rate observed 10-25 seconds following drug administration. Tachycardia was evaluated as the peak increase in heart rate observed 10-90 seconds after drug administration. Delayed bradycardia was characterized by computing the median heart rate of a 3-5 minute window following drug administration. The peak

decrease in MAP was evaluated within the period from 8 to 28 seconds following drug administration.

Effects of saline control injections were compared to those of 5 μg/kg methacholine before antagonist treatment to determine if methacholine produced an effect on each endpoint in each animal. RS/1 (Brooks Automation, Chelmsford, MA) was used to conduct one-way analysis of variance, if the ANOVA were significant at $p<0.05$, then the effects of methacholine before and after 15 μg/kg AMB were examined, using a paired t-test.

ANOVA were conducted to determine if the effects of methylecgonidine on heart rate and mean arterial pressure were dose-related. If two of the three animals showed significant dose-related effects, i.e. an effect consistent with muscarinic agonism, the effect of AMB treatment was evaluated by calculating a *z*-score comparing methylecgonidine effects before (n=3) and after AMB treatment. Similarly, *z*-score comparisons of methylecgonidine effects before AMB treatment (n=3) and following saline treatment (AMB sham, the third saline in Figure 2,) were computed to determine whether tolerance developed to methylecgonidine.

Results

Methylecgonidine quickly achieved maximal plasma concentration (C_{max}) and was subsequently rapidly cleared from blood for all three doses (Figure 3 and Table 1). Samples collected at 24 and 48 hours after methylecgonidine injection never contained detectable concentrations of methylecgonidine.

Ecgonidine appeared in plasma immediately and was always detectable in the first sample collected, ie. 30 seconds to 2 minutes after injection. Ecgonidine concentrations increased as methylecgonidine concentrations decreased (Figure 3). C_{max} was achieved from 30 to 45 minutes after injection and appeared to be dose-related (ecgonidine pharmacokinetic data: panel B, Table 1). In one 10 mg/kg methylecgonidine session, no decline in ecgonidine concentrations was observed before the last time-point (50 mins). Note that ecgonidine carryover from previous experiments occured in four experiments (Table 2). In these cases, the baseline plasma concentrations were subtracted from measured ecgonidine concentrations collected following methylecgonidine administration, and the corrected values were used for all subsequent data analysis. Ecgonidine was present in all 24 hour samples that were collected (Table 3). Ecgonidine elimination was prolonged relative to methylecgonidine with 3-fold increase in half-lives of ecgonidine relative to methylecgonidine.

Effects of Methacholine A representative recording from an experimental session illustrating effects of methacholine on heart rate and mean arterial pressure before and after AMB treatment is presented in panels A and C of Figure 4. Methacholine induced significant, rapid-onset bradycardia occurring 8-20 seconds after administration (panel A

of Figure 5). Intravenous 15 μg/kg AMB treatment 10 minutes before the second methacholine dose significantly attenuated bradycardia in each animal.

Panel B of Figure 5 illustrates the effects of AMB on methacholine-induced changes in mean arterial blood pressure. In all three sheep, 5 μg/kg methacholine iv caused a significant drop in mean arterial pressure, occurring within 8-20 seconds of drug delivery. Comparison of the average from four sessions for maximal decrease in MAP produced by methacholine, before and after AMB treatment, revealed significant antagonism for two of three sheep.

Significant tachycardia occurred within 20-60 seconds of methacholine administration. Tachycardia occurred after the initial bradycardia, and nearly superimposed with hypotension (panel C of Figure 5). This tachycardia was antagonized with AMB in two of the three animals. Methacholine did not produce any significant delayed bradycardia (3-5 minutes after methacholine administration) in any of the sheep (panel D of Figure 5).

Effects of Methylecgonidine A representative recording of the effects of 1.0 mg/kg methylecgonidine on heart rate and mean arterial pressure before and after 15 μg/kg AMB treatment is presented in panels B and D of Figure 4. Methylecgonidine administration produced hypotension and tachycardia. There was no bradycardia immediately following methylecgonidine administration, as there was for methacholine. Analysis of the minimum heart rate 10-25 seconds after methylecgonidine administration demonstrated tachycardia (panel A of Figure 6).

Significant tachycardia occurred in all three sheep 10-90 seconds after methylecgonidine administration. Methylecgonidine tachycardia dose-effect curves exhibited inverted U-shaped responses in two of three sheep (panel B of Figure 6).

In two of three sheep, bradycardia occurred 3-5 minutes after methylecgonidine administration (panel C of Figure 6). However, two of three saline sham control experiment results demonstrated significant differences from methylecgonidine baseline values, so effects of AMB treatment on this endpoint were not evaluated.

In all three sheep, methylecgonidine-induced hypotension (Figure 7). Pretreatment with 15 μg/kg AMB 20 minutes before methylecgonidine administration shifted the dose-response curve to the right, consistent with antagonism. To establish that the AMB antagonism was surmountable (i.e. competitive) in sheep 3 and 4, the AMB dose was decreased to 7 and 4 μg/kg respectively. Saline (sham antagonist) experiments provided no evidence of tolerance to methylecgonidine-induced hypotension.

Discussion

This work comprises the first description of pharmacokinetic data of methylecgonidine clearance from blood, demonstrating thatmethylecgonidine is quickly cleared from blood but has a longer half-life than cocaine in sheep based upon published cocaine values (Khan et al., 1987). The apparent volume of distribution of methylecgonidine is larger than that for cocaine in sheep. Therefore, the low methylecgonidine concentrations in forensic blood specimens, appears largely due to methylecgonidine's rapid distribution and metabolism. Ecgonidine is a metabolite of methylecgonidine, *in vivo*, persists longer than methylecgonidine, and should prove to be a more useful indicator of crack smoking than methylecgonidine. Methylecgonidine induces rapid-onset hypotension that is competitively antagonized by atropine methyl bromide, supporting the hypothesis that methylecgonidine can act as a muscarinic agonist, *in vivo*.

Methylecgonidine is detected infrequently in blood or plasma samples and has never been detected in blood samples collected from crack smokers in controlled settings (Cone et al., 1994; Jenkins et al., 1995). A hypothesis that methylecgonidine is cleared quickly from blood is a possible explanation. Rapid clearance could suggest that proper sample handling and storage conditions are vital in the pharmacokinetic studies reported here. Previous work demonstrated that methylecgonidine is more stable than cocaine in sheep and human plasma; freezing samples at -80°C provides adequate methylecgonidine storage for several months (Scheidweiler et al., 2000; Fandino et al., 2002). Thus, *in vitro* hydrolysis of methylecgonidine to ecgonidine is not likely to occur and would not confound methylecgonidine or ecgonidine pharmacokinetic analysis. Undetectable

methylecgonidine concentrations observed in clinical studies may result from methylecgonidine's rapid clearance from the central compartment, additionally complicated by low levels of exposure to methylecgonidine from smoking in experimental contexts.

It is difficult to determine a standard methylecgonidine exposure from crack smoking, as there is much variability observed in methylecgonidine formation from cocaine and is temperature dependent. Nakahara et al. reported that 68% of cocaine base is converted to methylecgonidine at 320°C and under vacuum, conditions not achieved in a crack pipe (Nakahara and Ishigami, 1991). Heating of cocaine base in a model crack pipe with a Bunsen burner at atmospheric pressure typically demonstrated less than 2% methylecgonidine conversion from cocaine, with a maximum of 5% observed when the cocaine ignited (Wood et al., 1996). Since we wished to determine pharmacokinetic and cardiovascular responses to methylecgonidine, the concentrations of methylecgonidine here are most likely higher than those attained from typical crack smoking.

Both methylecgonidine and cocaine have apparent volumes of distribution larger than the blood volume of sheep, 64.9 mL/kg. This indicates that methylecgonidine, like cocaine, distributes from blood to other tissues. The mean volume of distribution of 4 mg/kg cocaine in sheep is 3.11 L/kg, and the volumes of distribution of 3 mg/kg methylecgonidine for sheep 1 and 2 were 10.0 and 6.6 L/kg, respectively (Khan et al., 1987). Calculation of *z*-scores confirms that both sheep had larger volumes of distribution for methylecgonidine compared to literature values for cocaine ($p<0.001$).

The mean half-lives for 1, 2, and 4 mg/kg cocaine in sheep were 8.9, 10.5, and 10.6 minutes, respectively (Khan et al., 1987). The lower confidence intervals for half-

lives at 3 mg/kg methylecgonidine were 16.3 and 17.8 for sheep 1 and 2, respectively. Therefore, methylecgonidine is cleared quickly but more slowly than cocaine in sheep.

Methylecgonidine produces effects consistent with muscarinic agonism in the awake sheep. Like methacholine, intravenous methylecgonidine administration produces dose-related decreases in mean arterial pressure in conscious sheep that can be antagonized with AMB. Antagonism of methylecgonidine -induced hypotension by AMB was surmountable, consistent with atropine's competitive antagonism of other muscarinic effects (Brown and Taylor, 1996).

There are two possible mechanisms for muscarinic induced hypotension. Hypotension has been linked to stimulation of M_3 receptors in the vascular endothelium, which stimulates nitric oxide synthase to produce nitric oxide thereby relaxing the underlying smooth muscle (Lind et al., 1999; Sawyer et al., 1999). Additionally M_2 receptor stimulation decreases cardiac contractile force via inhibition of adenylyl cyclase and stimulation of guanylyl cyclase, which could also produce a drop in blood pressure (Kelly et al., 1996).

This study was unable to demonstrate significant bradycardia immediately following methylecgonidine administration. This contrasted with methacholine, which produced marked bradycardia. The lack of methylecgonidine -induced bradycardia appears to contrast with reports of *in vitro* M_2 agonist properties in myocardium (Woolf et al., 1997), myocytes (Huang et al., 1997; Yang et al., 2002) and human embryonic lung cells expressing M_2 receptors (Yang et al., 2001). Data from these reports relate to muscarinic signaling pathways involved in decreasing contractility and vasodilation. Muscarinic-induced bradycardia is caused by M_2 stimulation of the acetylcholine-

activated potassium current, K_{ACh}, which delays cardiac depolarization (Yamada, 2002). *In vitro* studies did not show methylecgonidine to possess any agonist effects on K_{ACh} in myocytes (Xiao and Morgan, 1998).

Mild bradycardia was present 3-5 minutes after 1.0 and 3.0 mg/kg methylecgonidine administration. However, there was not any observed bradycardia after methacholine at the same time interval. The kinetics of association of methylecgonidine to M_2 muscarinic receptors is unknown. Methylecgonidine might have slower receptor kinetics than methacholine.

Tachycardia was observed following both methacholine and methylecgonidine administration. Methacholine-induced tachycardia may result from muscarinic-mediated bronchoconstriction, as the dose of methacholine consistently induced coughing in two of three sheep. Mild coughing was only observed with the highest dose of methylecgonidine. Tachycardia may also result via baroreceptor compensation to decrease in MAP, as tachycardia did occur with a similar time course as decrease in MAP for methylecgonidine.

A number of cardiovascular disorders have been documented in cocaine users, including hypertension, arrhythmias and infarction (Knuepfer, 2003). This work does support the hypothesis that methylecgonidine may play a role in cardiovascular toxicity associated with crack use (Yang et al., 2002). Our results confirm that methylecgonidine does produce hypotension, an effect consistent with methylecgonidine stimulation of nitric oxide synthase as shown in cell preparations (Yang et al., 2001; Yang et al., 2002). It is possible that methylecgonidine stimulation of nitric oxide synthase leads to the

production of reactive oxygen species that play a role in cardiovascular toxicity resulting from crack use.

Muscarinic receptors play an important role in cognition as evidenced by cognitive impairments observed following treatment with scopolamine, a nonspecific muscarinic antagonist. Scopolamine treatment impairs performance of rats in water and radial-arm mazes (Cassel and Kelche, 1989; Saucier et al., 1996). Impaired working memory was reported in monkeys following scopolamine treatment (Schwarz et al., 1999). Additionally, long-term exposure to muscarinic agonists has been demonstrated to cause downregulation of muscarinic receptors (Haddad el-B and Rousell, 1998).

Some studies have demonstrated cognitive impairments in crack users. Impaired executive function and memory were seen in a group of 30 cocaine users, 29 of whom reported crack as their preferred form of cocaine use (Bolla et al., 1999). DiSclafani et al. also reported impaired executive function after 6 weeks of abstinence in crack, and in crack and alcohol dependent subjects (Di Sclafani et al., 2002); the strongest predictor of neuropsychological impairment in the latter study was dose. If methylecgonidine is acting as a muscarinic agonist *in vivo*, it is possible that repeated methylecgonidine exposure is playing a role in crack-associated cognitive deficits by causing downregulation of muscarinic receptors in brains of crack smokers. However, this work also demonstrates that any consideration of methylecgonidine as a toxicant should be tempered by the understanding that methylecgonidine is rapidly cleared thereby decreasing the amount methylecgonidine exposure. Activity of ecgonidine, the more persistent metabolite is undetermined, as well as what role this compound may play in crack smoking-related disorders.

In summary, these data are the first to demonstrate ecgonidine formation *in vivo*, to characterize kinetics of ecgonidine formation from methylecgonidine and to characterize its elimination from blood. Initially, ecgonidine concentrations increased as methylecgonidine concentrations decreased, indicating that ecgonidine is a metabolite of methylecgonidine. Ecgonidine is a major metabolite of methylecgonidine and should provide a useful biomarker of crack smoking as initially hypothesized by Jacob et al. (Jacob et al., 1990). Ecgonidine persists longer in blood than methylecgonidine, with a half-life more than four times as long as methylecgonidine's. Therefore, as benzoylecgonine and ecgonine methyl ester are more persistent indicators of cocaine use than cocaine itself, ecgonidine is a more persistent indicator of methylecgonidine exposure/crack smoking. Additionally our data is the first to demonstrate muscarinic agonism by methylecgonidine *in vivo*.

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Footnotes:

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Our institutional Animal Care and Use Committee (IACUC) approved these animal studies. Animals were housed following the NIH Guide for the Care and Use of Laboratory Animals.

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Figure Legends

Figure 1 Panel A Cocaine conversion to methylecgonidine and ecgonidine formation from methylecgonidine. Panel B Structures of laboratory synthesized Ethylecgonidine and N-Ethyl-N-norecgonidine, internal standards for methylecgonidine and ecgonidine plasma assays, respectively.

Figure 2 Schematic illustrating the timeline of muscarinic physiologic experimental sessions.

Figure 3 Elimination of methylecgonidine and ecgonidine from plasma.
Note dose-related levels of methylecgonidine and ecgonidine, disregarding initial methylecgonidine points.

Figure 4 Representative data illustrating effects of 5 μg/kg methacholine, i.v. (Panel A) and 1.0 mg/kg methylecgonidine, i.v. (Panel B) on mean arterial pressure (MAP). Panels C and D illustrate effects of methacholine (5 μg/kg) and methylecgonidine (1.0 mg/kg) on mean arterial pressure, respectively. Panels on the left illustrate effects before atropine methyl bromide (AMB) and panels on the right illustrate effects after AMB treatment. Shaded bars indicate time of drug delivery. Curves were fit to the data employing locally weighted regression scatter plot smoothing with factor of 0.25, which uses weighted least squares to fit a line to a scatter plot (Chambers, 1983).

Figure 5 Effects of 5 μg/kg methacholine on heart rate and mean arterial pressure (MAP) (n=4, mean ± s.e.m.). Effects of 15 μg/kg atropine methyl bromide pretreatment 20 minutes before methacholine administration are also shown.

A.Methacholine effect on peak decrease in heart rate 8-28 seconds after administration. B. Effect on peak decrease in mean arterial pressure 10-25 seconds after administration. C. Effect on peak heart rate occurring 30-90 seconds after administration. D. Effect on median heart rate occurring 3-5 minutes after administration. *, ** indicate significant differences by paired t-test ($p<0.05$ and $p<0.005$, respectively).

Figure 6 Effects of methylecgonidine on heart rate. A. Effect of methylecgonidine on minimum heart rate observed 10-25 seconds after methylecgonidine administration. B. Methylecgonidine -induced tachycardia occurring 10-90 seconds after methylecgonidine administration. C. Effect of methylecgonidine on median heart rate occurring at 3-5 minutes after methylecgonidine administration. All effects were computed as changes from baseline within each session. Median of two minutes of baseline heart rate data was used for each comparison. Appropriate vehicle control injections conducted at the beginning of each session are included (solid squares, mean of at least three experiments ± s.e.m.); 3 mL (equivalent for 0.1 to 1.0 mg/kg methylecgonidine injection volume) and 5 mL (equivalent for 3.0 mg/kg methylecgonidine). Open squares are for methylecgonidine studies conducted prior to atropine methyl bromide (n=3 for each dose, ± s.e.m.). *, ** indicate significant differences from appropriate saline control ($p<0.05$ and $p<0.005$, respectively).

Figure 7 Methylecgonidine-induced hypotension. Hypotension was computed as change from baseline within each session. Median of three minutes of baseline MAP was used for each comparison to maximum drop in MAP occurring 8-28 seconds after methylecgonidine. Appropriate vehicle control injections conducted at the beginning of each session are included (solid squares, mean of at least three experiments ± s.e.m.); 3 mL (equivalent for 0.1 to 1.0 mg/kg methylecgonidine injection volume) and 5 mL (equivalent for 3.0 mg/kg methylecgonidine). Open squares are for methylecgonidine studies conducted prior to atropine methyl bromide (n=3 for each dose, ± s.e.m.). Open circles are for methylecgonidine experiments 20 minutes after AMB treatment. Filled circles are for methylecgonidine experiments 20 minutes after saline treatment (the third saline in figure 2), evaluating methylecgonidine tolerance. Filled triangles indicate experiments with sheep 3 and 4 using lower AMB doses to demonstrate surmountability of AMB antagonism. *, ** indicate significant differences from methylecgonidine pre-AMB ($p<0.05$ and $p<0.005$, respectively).

Table 1 Noncompartmental pharmacokinetic parameters of methylecgonidine (MEG) and ecgonidine following intravenous methylecgonidine administration

A. Methylecgonidine Parameters

MEG dose	3.0 mg/kg		5.6 mg/kg		10.0 mg/kg	
Sheep	**1**	**2**	**1**	**2**	**1**	**2**
C_{max}	917.8	1663.9	1239.6	3794.6	2960.4	8570.0
t_{max}	2.0	2.0	2.5	2.0	2.5	2.0
k	-0.034	-0.033	-0.035	-0.039	-0.035	-0.036
$t_{1/2}$	20.3	20.7	19.8	17.7	19.7	19.2
$t_{1/2}$ CI	16.3 - 26.8	17.8 - 24.7	14.0 - 33.5	15.7 - 20.2	14.3 - 31.7	14.2 - 29.5
AUC_{total}	8771	13683	11,000	23,285	21,549	33,661
CL	342.0	219.2	509.1	240.5	464.0	297.1
V_d^{term}	10.0	6.6	14.5	6.1	13.2	8.2

B. Ecgonidine Parameters

MEG dose	3.0 mg/kg		5.6 mg/kg		10.0 mg/kg	
Sheep	**1**	**2**	**1**	**2**	**1**	**2**
C_{max}	1753.0	2218.0	3386.6	2805.7	5949.3	5113.7
t_{max}	45	30	40	45	50[a]	40
k	-0.006	-0.007	-0.005	-0.005		-0.007
$t_{1/2}$	113.5	94.4	135.6	137.3		98.0
$t_{1/2}$ CI	83.4 - 175.6	59.13 - 233.8	90.4 - 271.2	84.4 - 367.8		70.5 - 161.0
AUC_{total}	336,465	332,591	635,015	645,846		897,224

[a] No decline in ecgonidine levels

C_{max}= maximum concentration in ng/mL, t_{max}= time in minutes that maximum concentration occurred, k= slope of the line fit to the terminal phase of elimination in minutes^{-1}, $t_{1/2}$= half-life of elimination in minutes, $t_{1/2}$ CI= 95% confidence intervals of half-life, AUC_{total}= area under the curve in ng x min x mL^{-1}, CL= clearance in mL x kg^{-1} x min^{-1}, V_d^{term}= volume of distribution of the terminal phase in L x kg^{-1}

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Table 2 Ecgonidine (EC) carryover from previous methylecgonidine (MEG) experiments

Previous MEG experiment	Sheep	EC 50 min[a] (ng/mL)	EC carryover (ng/mL)	MEG experiment[b] (mg/kg)
5.6 mg/kg, 2 days prior	1	3071.4	262.42	10.0
5.6 mg/kg, 2 days prior	2	2579.2	327.70	3.0
10.0 mg/kg, 4 days prior	1	5949.3	100.48	5.6
10.0 mg/kg, 4 days prior	2	5106.0	167.22	3.0

[a] Ecgonidine concentration of the sample collected 50 minutes after methylecgonidine bolus, 2 or 4 days previous to collection of baseline sample containing ecgonidine

[b] Methylecgonidine experiment refers to the experimental session for which the baseline sample contained ecgonidine.

Note: Methylecgonidine was not detected in any baseline samples

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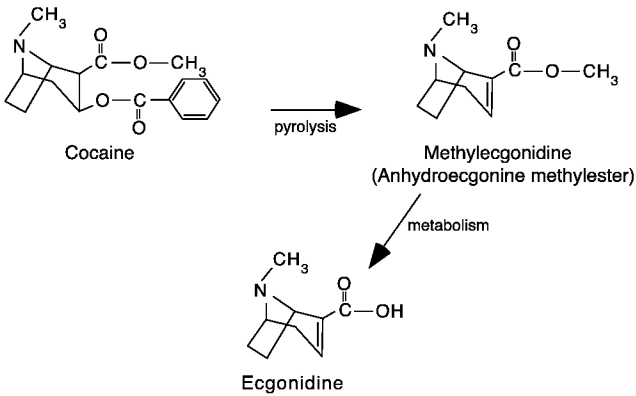
Table 3 Ecgonidine (EC) plasma concentrations 24 and 48 hours after methylecgonidine (MEG) injection

Animal	MEG dose (mg/kg)	EC at 1 hour (ng/mL)	EC at 24 hours (ng/mL)	EC at 48 hours (ng/mL)
1	3.0	1663.5	60.37	ND
1	5.6	2882.8	253.27	ND
2	10.0	4638.9	462.60	233.61

ND: ecgonidine concentration was less than the level of detection (10 ng/mL)

Note: methylecgonidine was not detected in any samples at 24 or 48 hours

A.


CH3
N
O
C—O—CH3
O—C
O
Cocaine
pyrolysis
CH3
N
O
C—O—CH3
Methylecgonidine
(Anhydroecgonine methylester)
metabolism
CH3
N
O
C—OH
Ecgonidine

B.

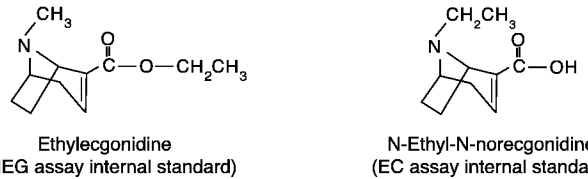

CH3
N
O
C—O—CH2CH3
Ethylecgonidine
(MEG assay internal standard)
CH2CH3
N
O
C—OH
N-Ethyl-N-norecgonidine
(EC assay internal standard)

Figure 1

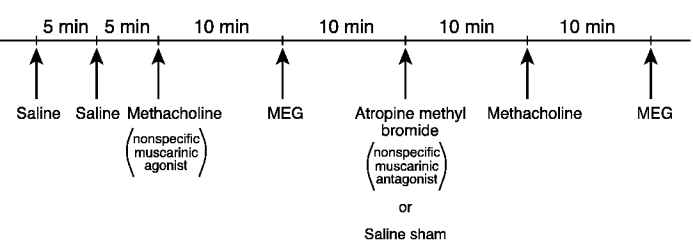

5 min
5 min
10 min
10 min
10 min
10 min
Saline
Saline
Methacholine
(nonspecific muscarinic agonist)
MEG
Atropine methyl bromide
(nonspecific muscarinic antagonist)
or
Saline sham
Methacholine
MEG

Figure 2

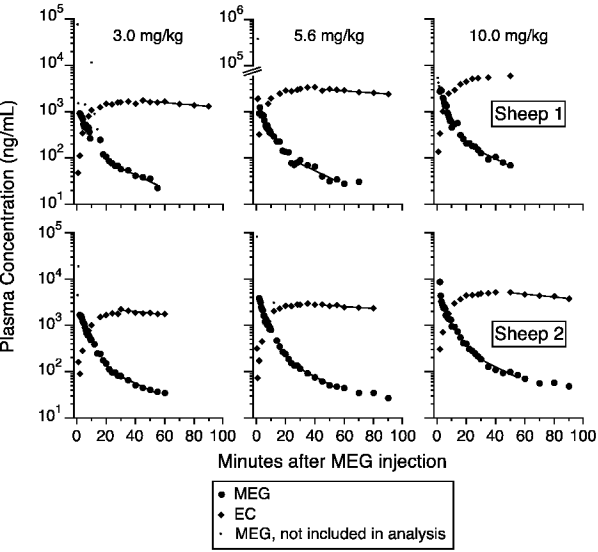

3.0 mg/kg
5.6 mg/kg
10.0 mg/kg
Sheep 1
Sheep 2
Plasma Concentration (ng/mL)
Minutes after MEG injection
MEG
EC
MEG, not included in analysis

Figure 3

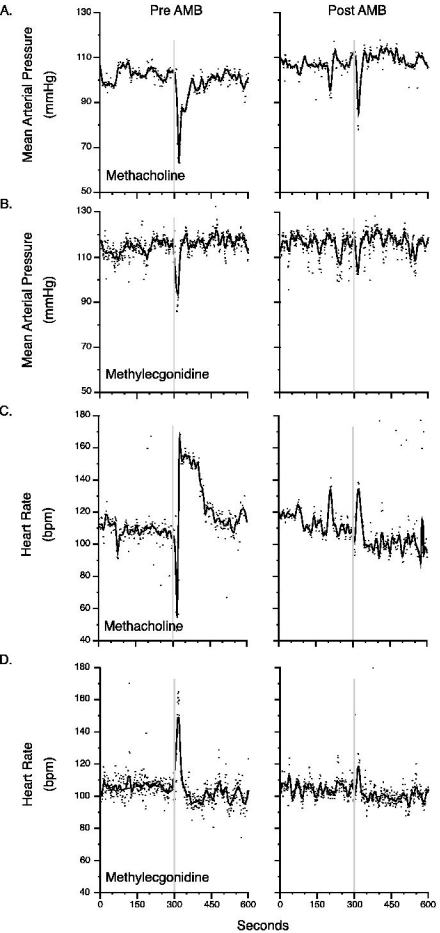

A.
Pre AMB
Post AMB
Mean Arterial Pressure (mmHg)
130
110
90
70
50
Methacholine
B.
Mean Arterial Pressure (mmHg)
Methylecgonidine
C.
Heart Rate (bpm)
180
160
140
120
100
80
60
40
Methacholine
D.
Heart Rate (bpm)
Methylecgonidine
0
150
300
450
600
Seconds

Figure 4

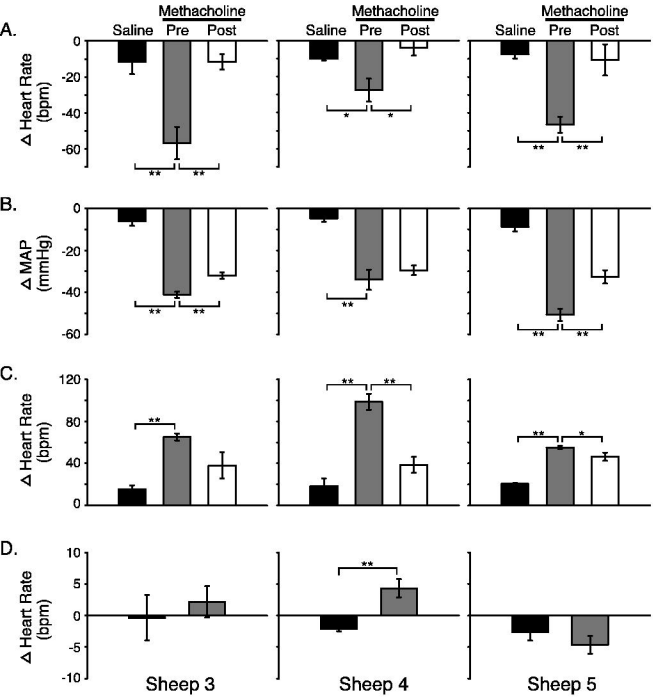

A.
Methacholine
Saline
Pre
Post
Δ Heart Rate (bpm)
0
-20
-40
-60
B.
Δ MAP (mmHg)
C.
120
80
40
D.
10
5
-5
-10
Sheep 3
Sheep 4
Sheep 5

Figure 5

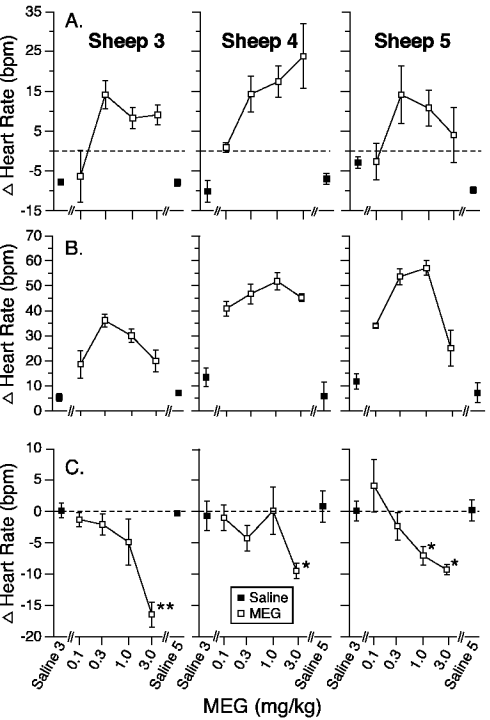

A.
Sheep 3
Sheep 4
Sheep 5
Δ Heart Rate (bpm)
B.
C.
Saline
MEG
Saline 3
0.1
0.3
1.0
3.0
Saline 5
MEG (mg/kg)

Figure 6

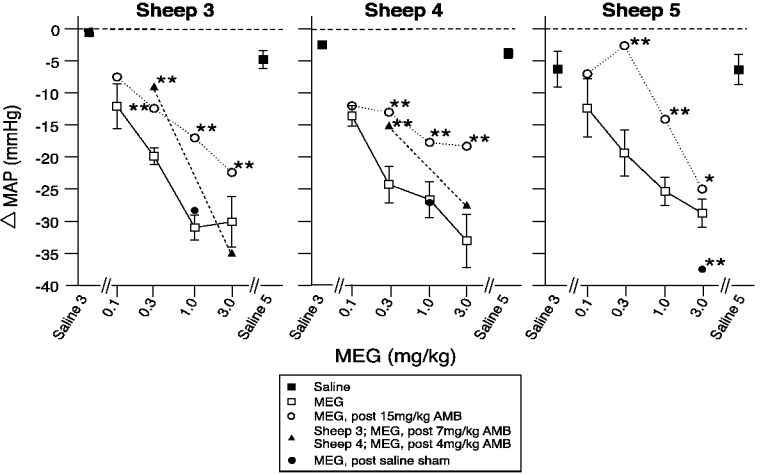

Sheep 3
Sheep 4
Sheep 5
△ MAP (mmHg)
0
-5
-10
-15
-20
-25
-30
-35
-40
Saline 3
0.1
0.3
1.0
3.0
Saline 5
MEG (mg/kg)
Saline
MEG
MEG, post 15mg/kg AMB
Sheep 3; MEG, post 7mg/kg AMB
Sheep 4; MEG, post 4mg/kg AMB
MEG, post saline sham

Figure 7