Thermoregulatory Effects of Ergovaline Isolated from Endophyte-Infected Tall Fescue Seed on Rats†

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Ergovaline (EV) was isolated from *Acremonium coenophialum*-infected tall fescue seed. Seed (2 kg) was extracted with 20 L of 5% lactic acid in water/methanol (4:1), filtered, and passed over an Amberlite XAD-2 column. Ergovaline was eluted with MeOH, concentrated, and chromatographed on two reversed-phase C_{18} columns (purity > 95%, 40% overall recovery). It was administered to rats (15 μg/kg of BW, ip) at 22 °C to determine time-related acute effects on thermoregulatory ability. Rectal temperature in EV-treated rats decreased 1.6 °C by 50 min ($p \leq 0.05$), due to reduction in heat production ($p \leq 0.05$) and increase in heat loss across the tail ($p \leq 0.05$) at 10–20 min postinjection. This study provides a rapid, economical method for milligram-scale isolation of ergovaline and defines the thermoregulatory mechanisms responsible for ergovaline-induced hypothermia under nonstress conditions.

INTRODUCTION

Fescue toxicosis in cattle has been studied for over 40 years (Goodman, 1952; Jensen et al., 1956; Maag and Tobiska, 1956). Understanding of the pathophysiological causes of this disorder has been limited by the lack of purified toxic compounds responsible for the syndrome (Porter and Thompson, 1992; Thompson and Stuedemann, 1993). In recent years, progress has been made in linking fescue toxicosis to the presence of an endophytic fungus (*Acremonium coenophialum*; Morgan-Jones and Gams, 1982), which has been shown to produce a number of alkaloids (Bacon et al., 1986; Porter et al., 1981). Recent studies have identified the possible role of ergopeptine alkaloids in endophyte-infected tall fescue toxicosis (Yates et al., 1985; Yates and Powell, 1988; Lyons et al., 1986; Belesky et al., 1988; Osborn et al., 1992; Aldrich et al., 1993a,b; Moubarak et al., 1993). Ergovaline (EV) has been shown to be the most abundant ergopeptine alkaloid produced by the fungus (Porter et al., 1981) and, because it is a blood vessel constrictive agent, is thought to be responsible for fescue toxicosis in cattle (Garner et al., 1990; Cornell et al., 1990). The relationship between physiological effects of EV and reported disorders in cattle [e.g., fescue foot (Garner and Cornell, 1978; Garner et al., 1982), fescue toxicosis (Hemken et al., 1981), and reduced milk production (Hemken et al., 1979; Wallner et al., 1983; Steudemann and Hoveland, 1988)] has not been studied due to lack of an adequate source of purified EV. Small quantities of EV have been isolated in our laboratory (Testereci, 1991) as well as by Moubarak et al. (1989; 1993). One focus of the present study was to develop a rapid, inexpensive method of isolating EV from infected tall fescue seeds in sufficient quantity for biological studies.

The second focus was to use a rat model to determine the effect of EV on thermoregulatory ability. Information derived from this study will be used for comparisons of thermoregulatory responses of rats and cattle to further define the etiology of fescue toxicosis.

MATERIALS AND METHODS

Chemicals. Amberlite XAD-2 was purchased from Sigma Chemical Co. Reversed-phase C_{18} (40 μm) packing material was obtained from J. T. Baker Co. All organic solvents were of ACS grade or better (Fisher Scientific). Ergovaline standard was obtained from Sandos Ltd., Basel, Switzerland.

Endophyte-Infected Tall Fescue Seed. Endophyte-infected tall fescue seed (Kentucky 31) was purchased from Miller Seed Co. (Clinton, MO). All seed was ground to pass a 2-mm screen and stored at −20 °C to prevent decomposition of ergopeptine alkaloids before extraction and purification. Ergovaline concentration was 1.5 mg/kg of seed as determined according to the method of Rottinghaus et al. (1991).

Ergovaline Extraction. Seed (2 kg) was extracted with 20 L of 5% lactic acid in water/methanol (4:1) for 12 h. The extract was filtered through cheesecloth to obtain a clear filtrate and then vacuum filtered through Whatman No. 42 filter paper.

Ergovaline Purification. The filtrate (16 L) was applied to an Amberlite XAD-2 column (40.0 × 8.5 cm) equilibrated with 4:1 water/methanol. The column was washed with 4:1 aqueous methanol (1 L), and EV was eluted with methanol (2 L). The methanol eluant was evaporated to near dryness under vacuum. Reversed-phase flash chromatography was used to isolate EV (Still et al., 1978).

Initial C_{18} Column Chromatography. A column (12.0 × 4.0 cm) was packed with reversed-phase C_{18} material in methanol and equilibrated with water. Water (0.3 L) was added to the concentrate above and filtered through Whatman No. 42 filter paper under vacuum to remove precipitated material. The filtrate was applied to the C_{18} column, and the column was eluted with 20, 30, 40, and 50% methanol/water (0.6 L, eluted at a flow rate of 10 mL/min each) followed by 60% methanol/water (1.2 L) under air pressure. Fractions were collected in 0.1-L aliquots for HPLC analysis. Ergovaline fractions were combined and evaporated to approximately 20 mL, and a 30-fold excess of water was added.

Final C_{18} Column Chromatography. The concentrated EV fraction from above was applied to a second flash reversed-phase C_{18} column. The column (20.0 × 2.0 cm) was packed with C_{18} material and washed with an additional 0.3 L of methanol followed by double-distilled water (0.5 L). Ergovaline was separated from other alkaloids by eluting the column with 20–60% methanol under air pressure at a flow rate of 3.0 mL/min. Fractions of 0.6 L were collected for each aqueous methanol combination, and 1.2 L was collected for the 60% MeOH/H_2O combination.

Detection and Quantitation of Ergovaline. Ergovaline purity and quantitation was performed by the HPLC method described by Rottinghaus et al. (1991). Analyses were performed on a Perkin-Elmer 250 LC pump equipped with a silica gel

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saturator column, a Rheodyne Model 7125 loop injector valve (20-μL capacity), and a Perkin-Elmer 8.3-cm C_{18} cartridge column (3-μm particle size), with detection by a Hitachi F-1200 fluorescence spectrophotometer (excitation wavelength 250 nm; emission wavelength 420 nm. The mobile phase was 33% acetonitrile in a 200 mg/L solution of ammonium carbonate in distilled water with a flow rate of 1 mL/min. The purity of EV was also determined by tandem mass spectrometry (MS/MS).

Thermoregulatory Effects of Ergovaline. *Rats.* Sprague-Dawley rats (n = 22) were assigned to control and experimental groups at 50 days of age (199.27 ■ 2.92 g body mass). Each animal was acclimated for 8 days to partial restraint in Plexiglas stocks (Shimada and Stitt, 1983). Rat chow and water were provided for *ad libitum* intake between test and acclimation sessions, and lights were on from 7:00 a.m. to 8:00 p.m. daily.

Experimental Procedure. Each rat was weighed to 0.1 g prior to the study. A sterilized polyethylene catheter (PE 10; 0.28 mm i.d., 0.61 mm o.d.; Becton Dickinson and Co., Parsippany, NJ) was inserted into the peritoneal cavity using a 22-gauge syringe needle inserted into the abdominal wall as a slide tube. After insertion of the catheter into the cavity, the needle was withdrawn and the catheter attached to the animal using cyanoacrylate glue. Thermocouple wires (40-gauge copper–constantan), with a melting ice bath reference junction, were inserted 20–30 mm beyond the anal sphincter to measure rectal temperature (T_{re}), and attached to the mid-dorsal surface of the tail to measure tail temperature (T_{tail}). Thermocouples were attached to the skin using a thin film of flexible collodion, which does not significantly alter skin temperature (Spiers and Adair, 1986). Each rat was individually tested in a horizontal cylinder (31-cm length × 10.5-cm diameter), surrounded by a water-jacketed Plexiglas metabolic chamber (Spiers and Adair, 1986; Spiers, 1988). Cylinder wall and air temperatures were controlled independently of each other at 22.0 ± 0.3 °C and averaged to derive an estimate of individual test temperature.

An open air flow system was used to determine oxygen consumption rate of each animal. Dried room air was pumped through the cylinder at a controlled rate of 560 mL/min. Oxygen content of influent and effluent air was measured with a calibrated Beckman paramagnetic oxygen analyzer (Model 755, La Habra, CA). Each value was adjusted to STPD conditions, and metabolic heat production was estimated using a respiratory quotient of 0.83. Metabolic rate was expressed relative to whole body weight (W/kg). Each animal was allowed a minimal 1-h equilibration to test conditions before injection of either EV (purified or standard EV; 15 μg/kg of body mass in 1.0% lactic acid solution) or vehicle (1.0% lactic acid). No animal was tested before body temperatures or metabolic rate had reached a steady resting level. All measurements were recorded 5 min prior to injection and for 120 min postinjection using a computerized data acquisition system (Acro Systems 900, Analogic Corp., Peabody, MA).

Statistical Design. One-way ANOVA was used to detect significance between purified EV, standard EV, and lactic acid control groups. Scheffe's t-test was used to assess significance between any two groups. Repeated-measure ANOVA was used to determine significant levels in each treatment group between different treatment periods. Significance level was preset at $p \leq 0.05$ for all groups.

RESULTS AND DISCUSSION

The extraction and purification of EV was a modification of the procedure of Testereci (1991). Ergovaline was readily extracted and stabilized by the addition of lactic acid to the aqueous extract. Moubarak et al. (1993) also used 5% lactic acid to extract EV from seed and removed the EV from the aqueous extract by mixing with SM-2 Bio-Beads. This was followed by centrifugation and elution of EV from the SM-2 with methanol. Although it was a very effective procedure, the Bio-Beads were not economically feasible for large-scale separations. The most economic and effective extraction solvent proved to be 5% aqueous lactic acid with 20% methanol, which reduced filtration time and gave a cleaner filtrate while at the same time did not affect the extractability or stability of EV.

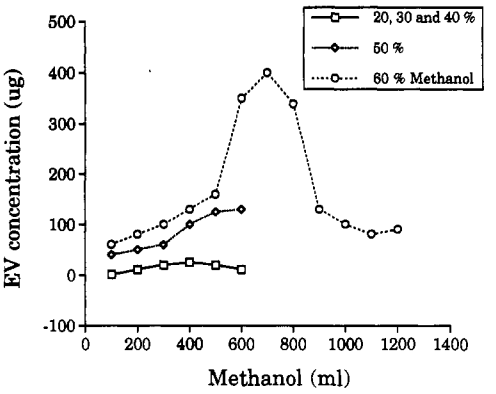

Figure 1. Ergovaline elution from initial C_{18} column.

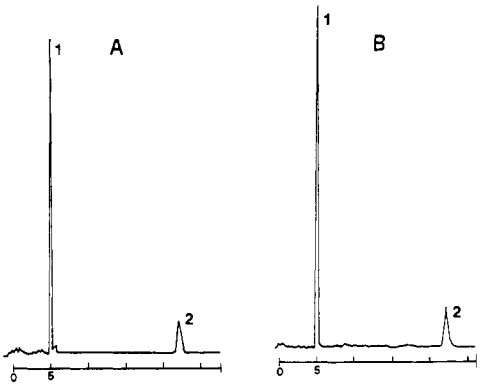

Figure 2. HPLC chromatograms of ergovaline standard (A) from Sandos Ltd. and and isolated ergovaline (B) from endophyte-infected fescue seed: 1, ergovaline; 2, ergovalinine.

Amberlite XAD-2, a cheaper grade of SM-2 Bio-Beads, proved to be as effective for removing EV from the filtrate, but at a much reduced cost. Methanol elution quantitatively removed the EV from the XAD-2, allowing the XAD-2 to be reused many times. Testereci (1991) used a single reversed-phase C_{18} column for EV purification, while Moubarak et al. (1993) used an initial reversed column cleanup followed by semipreparative HPLC reversed-phase chromatography for final purification. In the described study, rapid reversed-phase flash chromatography cleanup was used to remove pigments and oils with little attempt to separate EV from other alkaloids present. The column was eluted with a step gradient of 20–60% methanol/water to ensure pigments were removed before EV. As shown in Figure 1, at 50% methanol, some EV was eluted from the column. Ergovaline was eluted with 60% methanol in fractions between 500 and 900 mL. Final purification was achieved using reversed-phase flash chromatography to readily separate EV from other alkaloids present and allow processing of relatively large quantities of material in a relatively short time. Column fractions were monitored by HPLC and the EV fractions combined. The purified EV was free of other alkaloid components as determined by HPLC (see Figure 2) and tandem mass spectroscopy. Purity was estimated at greater than 95% with none of the imine isomer present, but the isomer was formed if EV was stored for more than 10 days (see Figure 2). To increase stability, the purified EV was stored as an aqueous 1% lactic acid solution (Garner et al., 1990).

Rats in both isolated and pure EV-injected groups exhibited rapid reductions in rectal temperature from 37.9 to 36.7 °C and from 37.7 to 36.5 °C, respectively, over the first 30 min postinjection (Figure 3). Mean rectal temperature in both treatment groups was significantly

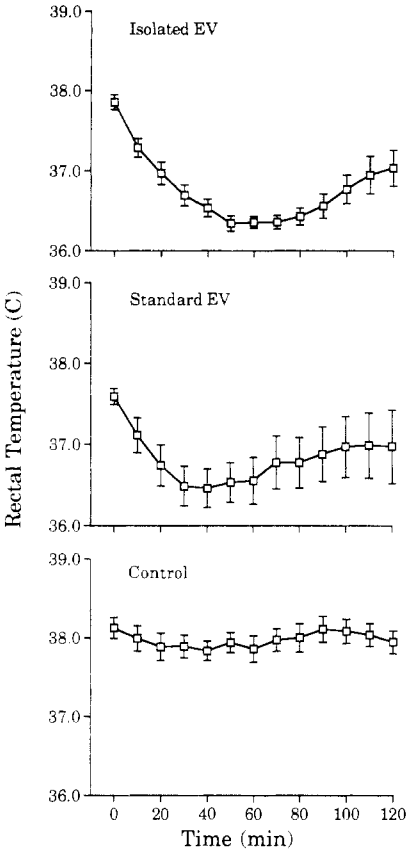

Figure 3. Rectal temperature of rats administered isolated EV (top), standard EV (middle), and vehicle solutions (bottom). Error bar = SE.

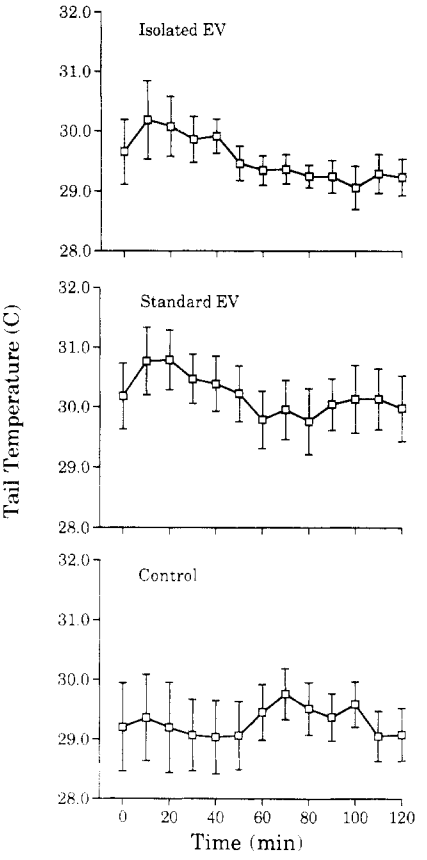

Figure 4. Tail temperature of rats administered isolated EV (top), standard EV (middle), and vehicle solutions (bottom). Error bar = SE.

different from control value ($p \leq 0.05$) at 10 to 80 min postinjection. Peak reductions in rectal temperature were 1.5 °C (50 min) and 1.2 °C (40 min) for purified and standard EV-injected groups, respectively. Rectal temperature began to return toward normal after 60 min postinjection but without complete return during the 120-min test period (Figure 3). There was no significant change in rectal temperature for rats injected with lactic acid ($p > 0.05$) (Figure 3). Although it has never been reported that EV injection (ip) decreases rectal temperature in rats at thermoneutrality (22 °C), Neal and Schmidt (1985) noted that rats fed 50% endophyte-infected seed in the diet for 15 days exhibited a significant reduction in rectal temperature from 37.7 to 36.8 °C over ambient temperatures ranging from 24 to 32 °C. Intravenous ergopeptine administration (0.26 and 0.46 mg/kg) in rabbits decreased rectal temperature 0.5 and 1 °C, respectively (Loew et al., 1978). Roberts et al. (1949) reported that hypothermic response to ergotoxine in young Wistar rats (28 °C ambient temperature) was associated with decreased oxygen consumption, with no explanation of response mechanisms. Loew et al. (1978) suggested that ergot alkaloid-induced hypothermia is mediated by either peripheral or central α-adrenergic blockade. Roberts et al. (1949) suspected hypothermia may be due to unselective inhibition/stimulation of heat-production and heat-loss centers, with the latter predominating in young rats after ergot alkaloid injection. This could be the reason for the rapid reduction in body temperature in rats.

The trend for tail temperature response in EV-treated groups was a slight rise (0.5 °C) within 10 min, followed by a 0.7 °C reduction over the next 60 min (Figure 4). Tail temperature of the rat is a reflection of both ambient temperature and heat transfer from the core of the animal to the temperature site. There is no evaporative water loss from the rat tail, so this would not be a determinant of tail temperature. At a steady ambient temperature, any change in tail temperature is an indicator of shifting heat flow and possibly blood flow to this region. A reduction in core temperature without a comparable decrease in tail temperature, as occurred in this study, would suggest an increase in blood flow to the tail. Thermal circulation index (TCI) has been used as an estimate of regional differences in peripheral heat or blood flow under steady environmental conditions (Burton and Edholm, 1955)

$$\mathrm{TCI} = \frac{T_{\mathrm{skin}} - T_{\mathrm{a}}}{T_{\mathrm{re}} - T_{\mathrm{skin}}}$$

where T_{skin}, T_{a}, and T_{re} represent skin, ambient, and rectal temperatures, respectively. The advantage of the TCI estimate is that it allows for incorporation of both rectal and skin temperatures into a single value. An increase in tail TCI occurred in both EV treatment groups ($p \leq 0.05$) (Figure 5), with a peak time of 10–20 min, to suggest that EV produced an initial increase in blood flow to this region.

Metabolic rate decreased during the first 20 min following injection of purified and standard EV (0.075 and 0.085 W kg^{-1} min^{-1}, respectively), with no changes in vehicle-treated rats (Figure 6). The overall change in metabolic rate of experimental and control groups reached significance ($p \leq 0.05$) at 20–40 min postinjection and was as pronounced as the rectal temperature change. The nadir in metabolic rate was 20 min after EV injection, with return to preinjection level at 70 and 100 min postinjection of pure and isolated EV solutions, respectively. Similar changes in metabolic rate have been reported to occur in

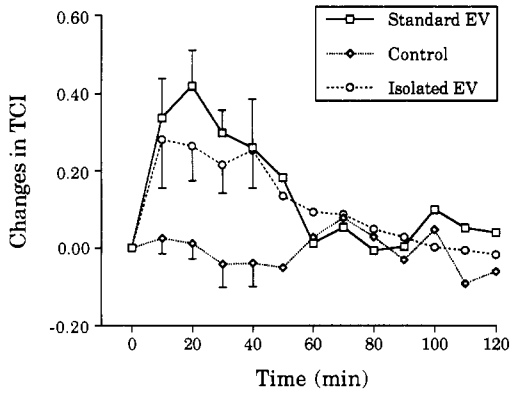

Figure 5. Changes in thermal circulation index (TCI) for the tail of rats administered isolated EV, standard EV, and vehicle. Error bar = SE.

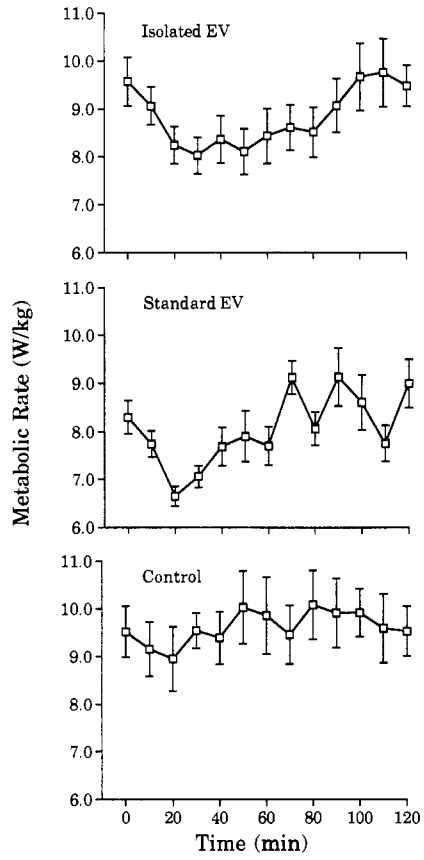

Figure 6. Metabolic rate of rats administered isolated EV (top), standard EV (middle), and vehicle solutions (bottom). Error bar = SE.

16-day-old Wistar rats injected with ergotoxine at 28–32 °C, with a concomitant change in rectal temperature (Roberts et al., 1949).

The rapid reduction in rectal temperature that occurs in rats following injection of pure or isolated EV is accompanied by simultaneous shifts in metabolic rate and TCI. The decrease in heat production and possible increase in heat loss across the tail are considered to be responsible for the reduction in rectal temperature.

In conclusion, the present study provides a rapid, inexpensive method for EV purification, using 5% lactic acid in water/methanol (4:1) for extraction followed by XAD-2 and C_{18} flash chromatography. A comparison of thermoregulatory responses to purified and standard ergovaline solutions showed similar directions of change in body temperature control mechanisms and magnitudes of response. Further studies are needed to compare the responses of rats and cattle to ergovaline under these conditions.

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