Policosanol Inhibits Cholesterol Synthesis in Hepatoma Cells by Activation of AMP-Kinase

Dev K. Singh, Li Li, and Todd D. Porter
Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky
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ABSTRACT
Policosanol is a mixture of long-chain primary alcohols that has been shown to decrease serum cholesterol in animals and in humans. The hypocholesterolemic effect results from a decrease in cholesterol synthesis by suppression of HMG-CoA reductase activity, but the mechanism of this suppression and the active components of policosanol have not been established. In the present study, we investigated the ability of policosanol and its principal components to inhibit cholesterol synthesis in cultured rat hepatoma cells. Maximal inhibition by policosanol yielded a 30% decrease in [14C]acetate incorporation without evidence of cellular toxicity. Octacosanol (C28; the major constituent of policosanol), heptacosanol (C27), and hexacosanol (C26) yielded smaller and statistically insignificant decreases in cholesterol synthesis, whereas triacontanol (1-hexacosanol; C30) replicated the inhibition obtained with policosanol. At pharmacological concentrations (≤5 μg/ml), policosanol and triacontanol decreased [14C]acetate incorporation into cholesterol without affecting the incorporation of [14C]mevalonate, indicating that these compounds act at or above HMG-CoA reductase. Policosanol and triacontanol did not directly inhibit HMG-CoA reductase, and incubation of these compounds with hepatoma cells did not affect reductase enzyme levels. However, reductase activity was decreased by up to 55% in lysates prepared from these cells, suggesting that HMG-CoA reductase activity was down-regulated by policosanol treatment. Consistent with this hypothesis, a 3-fold increase in AMP-kinase phosphorylation was noted in policosanol-treated cells. Because AMP-kinase is activated by phosphorylation and is well established to suppress HMG-CoA reductase activity, these results suggest that policosanol or a metabolite decreases HMG-CoA reductase activity by activating AMP-kinase.

Policosanol, a mixture of very long-chain alcohols isolated from sugarcane, at doses of 10 to 20 mg/day has been shown to lower total and LDL cholesterol by up to 30%, equivalent to low-dose statin therapy (Gouni-Berthold and Berthold, 2002). In both short-term (≤12-week) and long-term (up to 2-year) randomized, placebo-controlled, double-blind studies, policosanol lowered LDL-cholesterol in normocholesterolemic patients by an average of 33%, and in hypercholesterolemic patients by 24% (for review, see Gouni-Berthold and Berthold, 2002; Varady et al., 2003). In normocholesterolemic patients, policosanol caused a small and generally insignificant increase in high-density lipoprotein-cholesterol, whereas in seven clinical studies of dyslipidemic patients high-density lipoprotein-cholesterol was increased by an average of 17%. Policosanol is also effective in rabbits and monkeys, where it lowers blood cholesterol and reduces the development of atherosclerotic plaques (Arruzazabala et al., 1994; Rodriguez-Echenique et al., 1994; Menendez et al., 1997), but it was found not to be effective in hamsters (Wang et al., 2003).

The major components of policosanol are the primary alcohols octacosanol (C28; ~60%), triacontanol (C30; 12–14%), and hexacosanol (C26; 6–12%), with lesser amounts of other alcohols with chain lengths of 24 to 34 carbons. The product has no evident toxicity and is available over-the-counter in many outlets. The active component(s) has not been established, but it has been shown that very long-chain alcohols can undergo oxidation to fatty acids with subsequent peroxisomal β-oxidation, which also yields chain-shortened metabolites (Singh et al., 1987). D-003, a mixture of very long-chain saturated fatty acids, also purified from sugarcane, similarly lowers LDL and total cholesterol in normocholesterolemic patients (Castano et al., 2002) and in normocholesterolemic and casein-induced hypercholesterolemic rabbits, and a more rapid onset of effects suggests that oxidation of policosanols to very-long-chain fatty acids may be necessary for their hypocholesterolemic actions (Menendez et al., 2004).

Several studies have demonstrated that policosanol inhibits cholesterol synthesis in laboratory animals and cultured cells, and it is thought that this is the principal mechanism to lower cholesterol levels.
by which it lowers blood cholesterol levels. Policosanol reduced the incorporation of tritiated water into sterols in hypercholesterolemic rabbits (Menendez et al., 1997) and decreased \(^{14}\)Cacetate incorporation into cholesterol in human fibroblasts (Menendez et al., 2001a). In the latter study, policosanol did not affect the incorporation of \(^{14}\)Cmevalonate into cholesterol, indicating that policosanol was acting at or above mevalonate synthesis. However, policosanol did not inhibit HMG-CoA reductase (mevalonate synthase) when added to cell lysates, arguing against a direct interaction with this enzyme. The ability of policosanol to prevent the up-regulation of HMG-CoA reductase activity in these cells in response to lipid-depleted media suggested that policosanol suppresses HMG-CoA reductase synthesis or enhances enzyme degradation. Similar results were obtained with D-003 (Menendez et al., 2001b), although neither study measured HMG-CoA reductase protein levels. The present studies were undertaken to further explore the mechanism by which policosanol inhibits cholesterol synthesis and to identify the active component(s) of this natural product.

**Materials and Methods**

**Chemicals.** Policosanol 10-mg tablets were manufactured by Source Naturals, Inc. (Scotts Valley, CA) and purchased from a local health food store. Hexacosanol, heptacosanol, octacosanol, triacontanol (1-hydroxytriacontane), mevalonolactone, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADH, lactate dehydrogenase, pyruvate, Triton X-100, ketoconazole, squalene, lanosterol, and mevalonolactone were purchased from Sigma-Aldrich (St. Louis, MO). Durbecco's modified Eagle's medium (DMEM), penicillin-streptomycin-glutamine (100×), fetal bovine serum, lipoprotein-depleted serum, and trypsin were purchased from Invitrogen (Carlsbad, CA). Terbinafine was from TCI America, Inc. (Portland, OR). \(^{14}\)CAcetate, sodium salt (56 mCi/mmol), and \(^{14}\)CMevalonolactone, dibenzylethylene diamine salt (65 mCi/mmol) were purchased from GE Healthcare (Piscataway, NJ). \(^{14}\)CMevalonolactone (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

**Preparation and Analysis of Policosanol.** Two 10-mg policosanol tablets were dissolved in 10 ml of absolute ethanol with a mortar and pestle. The insoluble excipients were removed by low-speed centrifugation, and the supernatant was aliquoted and stored at \(-20°C\) and added directly to media or buffer upon use. For the mass spectrometric analysis, the tablets were dissolved in methylene chloride, fractionated by gas chromatography (Trace, Waltham, MA), and quantified by ion-trap mass spectrometry (PolarisQ; Thermo Electron Corp., Waltham, MA) at the University of Kentucky Mass Spectrometry Facility (Lexington, KY).

**Cytotoxicity Assays.** McArH7777 rat hepatoma cells (American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with 10% fetal bovine serum and 1.0% penicillin-streptomycin-glutamine in six-well plates at 37°C under a humidified atmosphere of 5% CO\(_2\). The cells were used between passages 1 and 40. After 48 h, the media were replaced with fresh media with the addition of policosanol (2 mg/ml in ethanol) or a long-chain alcohol (1 mg/ml in ethanol), and incubation was continued for 3 h. Control cells received an equal volume of ethanol. The cells were detached with trypsin, suspended in DMEM containing 0.2% trypsin blue, and counted in a hemocytometer. Leakage of lactate dehydrogenase from cells was determined by measuring NADH oxidation from added pyruvate spectrophotometrically at 340 nm and compared with total lactate dehydrogenase activity from cells lysed with 0.1% Triton X-100.

**Determination of Sterol Synthesis.** Hepatoma cells were cultured for 48 h in six-well plates, at which time the media were replaced, and appropriate concentrations of the test substances (policosanol, 2 mg/ml in ethanol, or long-chain alcohol, 1 mg/ml in ethanol) were added along with 1 μCi of \(^{14}\)Cacetate or \(^{14}\)Cmevalonate. Incubations were carried out for 3 h, after which time the cells were washed twice with phosphate-buffered saline, harvested by trypsinization or scraping, resuspended in 20 ml Tris buffer, pH 7.4, containing 0.1% Triton X-100, and lysed by sonication (Sonics Dismembrator; Fisher Scientific, Pittsburgh, PA) at medium setting on ice with 10 8-s pulses, separated by 30 s each. Lipids were extracted into 5 ml of chloroform/methanol (2:1), the solvent was removed by evaporation at \(60°C\) under nitrogen, and the lipids were suspended in 50 μl of chloroform/methanol and spotted onto silica thin layer plates (Whatman, Florham Park, NJ). Chromatography was carried out in petroleum ether/ethyl ether/acetic acid (60:40:1). Cholesterol was identified by cochromatography of an authentic standard visualized by iodine vapor staining and quantified by electronic autoradiography (Packard Instant Imager). Further confirmation of identity was obtained by scraping the corresponding region of non-radiolabeled samples into chloroform/methanol (2:1), derivatizing the samples with trimethyl silane, and submitting them to mass spectrometric analysis as described above for policosanol.

**Determination of Squalene and Lanosterol Synthesis.** For the determination of squalene and lanosterol synthesis, cells were incubated as described above for cholesterol synthesis with the inclusion of 60 μM terbinafine (100× in ethanol), an inhibitor of squalene monoxygenase (for the determination of squalene), or 10 μM ketoconazole (100× in methanol), an inhibitor of lanosterol demethylase (for the determination of lanosterol). Lipids were saponified by addition of 0.5 ml of 10% methanolic potassium hydroxide and incubated at 80°C for 1 h. For the determination of squalene the neutral lipids were extracted into 5 ml of petroleum ether, the solvent was removed by centrifugal evaporation, and the samples were resuspended in 50 μl of petroleum ether and resolved by silica thin layer chromatography in 5% ethyl acetate in hexane. Lanosterol was determined as described for cholesterol. Authentic standards for squalene and lanosterol were visualized by iodine vapor staining. Further confirmation of these products was obtained by scraping the corresponding region of non-radiolabeled samples into chloroform/methanol (2:1) and submitting them to mass spectrometric analysis as described above for policosanol.

**Determination of HMG-CoA Reductase Activity in Microsomes.** The microsomal fraction (100,000g pellet resuspended at \(-15\) mg of protein/ml) was prepared by standard procedures from the livers of untreated male Harlan Sprague-Dawley rats (\(-200\) g). HMG-CoA reductase activity was determined by the procedure of Brown and Goldstein (1974) as follows: Microsomes (100 μg) were incubated at 37°C in a final volume of 200 μl containing 0.1 M potassium phosphate buffer, pH 7.5, 20 mM glucose 6-phosphate, 2.5 mM NADP\(^+\), 1 unit of glucose 6-phosphate dehydrogenase, 5 mM dithiothreitol, and 0.2 μCi of \(^{14}\)Cmevalonolactone. The reaction was stopped after 3 h by the addition of 25 μl of 6 M HCl. Mevalonate was converted to the lactone by standing at 37°C for 30 min, extracted into 5 ml of ethyl acetate, and brought to dryness by evaporative chromatification. The sample was redissolved in 50 μl of ethyl acetate and fractionated with silica thin layer chromatography with benzene/acetone (1:1). Mevalonolactone was identified by comigration with authentic mevalonolactone visualized by iodine vapor staining and quantified by electronic autoradiography.

**Determination of HMG-CoA Reductase Activity in Cell Culture.** Policosanol or a long-chain alcohol was added as indicated in fresh media to 48-h hepatoma cell cultures and incubated for 3 h. Cells were lysed by sonication, and HMG-CoA reductase activity was determined with 100 μg of total cell lysate protein as described above.

**Immunoblot Analysis of HMG-CoA Reductase Expression in Cell Culture.** Policosanol or a long-chain alcohol was added as indicated in fresh media to 48-h hepatoma cell cultures and incubated for up to 12 h. Cells were lysed by sonication and 50 μg of total cell protein was fractionated by SDS-polyacrylamide gel (10%) elec-
trophoresis. The proteins were electroblotted to nitrocellulose, and the membrane was blocked with 0.5% Tween 20 and 5% defatted milk and then incubated with rabbit antibody to HMG-CoA reductase (1:2000; Upstate Biotechnology, Lake Placid, NY). The immunoblot was developed with a secondary antibody conjugated to alkaline phosphatase and visualized by bromochloroindolyl phosphate/nitro blue tetrazolium staining.

**Immunquantitation of AMP-Kinase.** Hepatoma cells (rat McARH7777 or human HepG2) were cultured in lipoprotein-deficient media overnight, after which 20 μg of policosanol was added, and cells were incubated for 1 or 3 h. Cells were washed once with phosphate-buffered saline, pelleted by low-speed centrifugation, and lysed by two cycles of freeze-thawing in 0.25 M Tris buffer, pH 7.5, containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). For the metformin comparison studies, metformin (0.5–2 mM; Sigma-Aldrich) or policosanol (10 or 20 μg/ml) was added, and incubation continued for 3 to 6 h, following the protocol of Zang et al. (2004). The lysates were fractionated by centrifugation (20,000g; 15 min at 4°C), and the supernatant was collected and stored at −80°C. Twenty-five micrograms of protein was fractionated by SDS-polyacrylamide gel (10%) electrophoresis and electroblotted to nitrocellulose. The membrane was blocked with 0.05% Tween 20 and 5% defatted milk and then incubated in this same buffer with rabbit antibody to total AMP-kinase (anti-AMPKα-pan, 1:2000; Upstate Biotechnology) or to phosphorylated AMP-kinase (anti-phospho-AMPKα, 1:1000; Upstate Biotechnology). The immunoblot was developed with a secondary antibody conjugated to horseradish peroxidase and visualized and quantified by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL) on a Kodak Image Station (Eastman Kodak, Rochester, NY).

**Results**

Policosanol is a commercial mixture of very long-chain alcohols composed primarily of octacosanol (~60%), triacontanol (~13%), and hexacosanol (~6%). To evaluate the composition of our commercial preparation, we extracted the alcohols into methylene chloride and subjected the extract to gas chromatography. Five major peaks were isolated and identified by mass spectrometry (Fig. 1). Peaks A and B correspond to short-chain carboxylic acid excipients used to prepare the tablets; peaks C, D, and E correspond to hexacosanol (16%), octacosanol (60%), and triacontanol (19%), respectively. Minor components (not identified) comprise approximately 5% of the material eluting between 14 and 30 min and may include C$_{27}$ (heptacosanol) and C$_{29}$ (nonacosanol) alcohols.

The addition of policosanol to McARH7777 rat hepatoma cells resulted in a dose-dependent decrease in cholesterol labeling from [14C]acetate (Fig. 2). Maximal inhibition (~30%) was obtained at 25 μg/ml, and higher concentrations, up to 50 μg/ml did not yield greater inhibition. Policosanol at concentrations up to 50 μg/ml did not affect cell viability as assessed by trypan blue exclusion or the release of lactate dehydrogenase into the media. Triacontanol was similarly effective, whereas hexacosanol yielded a lesser (~20%) and statistically insignificant decrease in cholesterol labeling. Heptacosanol, which was not present in our preparation, decreased cholesterol synthesis by less than 10%, and octa-
cosanol, the most abundant alcohol in policosanol, yielded only a 15% decrease in cholesterol labeling at 25 μg/ml. The combination of triacontanol with hexacosanol and octacosanol was slightly more inhibitory than triacontanol alone at the same concentration (5 μg/ml). None of the alcohols proved toxic to the cells at concentrations up to 50 μg/ml.

The possibility that policosanol was decreasing [14C]acetate uptake was examined by measuring total [14C] incorporation into cells after 30-min incubations with policosanol and the radiolabel. [14C]Acetate uptake was decreased substantially at concentrations of 5 μg/ml and above (Fig. 3A), and cholesterol labeling was similarly decreased at these concentrations. In contrast, acetate uptake was not affected by policosanol concentrations below 5 μg/ml, whereas cholesterol synthesis was decreased by ~20% between 0.5 and 5 μg/ml (Fig. 3B). Triacontanol similarly decreased acetate uptake (data not shown). In contrast to the effect of policosanol on acetate uptake, policosanol did not affect mevalonate uptake into hepatoma cells (Fig. 3C). At concentrations above 5 μg/ml, policosanol decreased the incorporation of [14C]mevalonate into cholesterol by as much as 50%, whereas at concentrations below 5 μg/ml, policosanol had no effect on cholesterol synthesis from mevalonate (Fig. 3D). Triacontanol had a similar effect on cholesterol synthesis, decreasing the incorporation of [14C]acetate, but not [14C]mevalonate, into cholesterol by ~20% at 5 μg/ml and decreasing the incorporation of both acetate and mevalonate by up to 35% at 25 μg/ml (data not shown). Taken together, these results suggest that policosanol inhibits cholesterol synthesis at or before mevalonate synthesis at concentrations below 5 μg/ml.

Although the inhibition of cholesterol synthesis at low concentrations of policosanol (<5 μg/ml) seemed to take place at or above mevalonate synthesis, higher concentrations of policosanol seemed to act downstream of mevalonate synthesis. To try to identify this site of inhibition, the labeling of lanosterol, a midpoint intermediate in the cholesterol biosynthetic pathway, was determined in the presence of various concentrations of policosanol (Fig. 4A). As with cholesterol synthesis, lanosterol synthesis from [14C]acetate was decreased at very low concentrations of policosanol, consistent with it...
acting early in the biosynthetic pathway. When \[^{14}\text{C}]\text{mevalonate}\) was supplied as the precursor, lanosterol synthesis was not impaired until the policosanol concentration reached 10 \(\mu\text{g/ml}\), as seen with cholesterol synthesis from mevalonate. A similar result was obtained when squalene synthesis was measured in the presence of various concentrations of triacontanol (Fig. 4B). The decrease in labeling of squalene and lanosterol from mevalonate suggests that higher concentrations of policosanol act somewhere between HMG-CoA reductase and squalene synthase to block synthesis. Alternatively, the greater inhibition of HMG-CoA reductase at higher concentrations of policosanol or triacontanol may lead to a decrease in mevalonate synthesis that cannot be overcome by the exogenously supplied \[^{14}\text{C}]\text{mevalonate}.

To determine whether policosanol and triacontanol directly inhibit HMG-CoA reductase, these compounds were added to rat liver microsomes, and the conversion of \[^{14}\text{C}]\text{HMG-CoA}\) to mevalonate was measured. As shown in Fig. 5A, neither compound affected HMG-CoA reductase activity, indicating that they did not act as direct inhibitors of this enzyme. However, when hepatoma cells were incubated for 3 h with these compounds and HMG-CoA reductase activity was measured in the cell lysates, both policosanol and triacontanol reduced enzyme activity by as much as 55% (Fig. 5B). These results suggest that either policosanol requires metabolism to form the active inhibitor or that policosanol reduces the expression of HMG-CoA reductase by decreasing transcription and translation or by enhancing degradation of the enzyme. To determine whether policosanol alters HMG-CoA reductase expression, levels were evaluated by immunodetection. As shown in Fig. 5C, policosanol and triacontanol did not decrease the amount of HMG-CoA reductase protein over the 3-h period of the experiment, despite a marked decrease in the activity of this enzyme.

Because HMG-CoA reductase protein levels were not changed by policosanol treatment, we considered the possibility that policosanol inactivates HMG-CoA reductase by promoting its phosphorylation by one of three protein kinases shown to inactivate HMG-CoA reductase (Beg et al., 1987a). As shown in Fig. 6A, policosanol increased the amount of phosphorylated AMP-kinase in cells by more than 3-fold after 3 h of treatment. Similar results were obtained with the human hepatoma cell line HepG2 (Fig. 6B), indicating that this is not a species-specific effect; metformin, which is known to promote the phosphorylation of AMP-kinase (Zhou et al., 2001), was similarly effective. AMP-kinase is well established to be a regulator of HMG-CoA reductase activity in response to changes in cellular energy levels, and it is activated by phosphorylation by one or more upstream kinases, including LKB1 (Hawley et al., 2003; Shaw et al., 2004). These results suggest that policosanol decreases HMG-CoA reductase activity by activating AMP-kinase.

**Discussion**

Policosanol is an effective inhibitor of cholesterologenesis in hepatoma cells at concentrations that are readily obtainable in vivo: a single 20-mg oral dose in a 70-kg man would yield a total body water concentration of up to 0.5 \(\mu\text{g/ml}\); and in the present study, this concentration of policosanol reduced cholesterol synthesis by \(-15\%\) (Fig. 3B). Moreover, because oral dosing is likely to result in significant hepatic exposure, the effective concentration therein is likely to be substantially higher; indeed, Kabir and Kimura (1995) reported that radiolabeled octacosanol is predominantly located in the liver after oral dosing. Policosanol reduces LDL-cholesterol by 20 to 30% and increases hepatic LDL-receptor expression (Menendez et al., 1997), both of which are consistent with down-regulation of hepatic cholesterologenesis. Numerous clinical studies have documented the ability of policosanol to reduce serum cholesterol in both normocholesterolemic and hypercholesterolemic patients (for review, see Gouni-Berthold and Berthold, 2002; Varady et al., 2003; see also Castano et al., 2005; Wright et al., 2005), although a very recent clinical study was unable to find a significant cholesterol-lowering effect (Berthold et al., 2006).

Despite the evidence that policosanol reduces cholesterol
Electrophoresis and transferred to nitrocellulose for immunodetection. Lysates were prepared, and a low-speed supernatant was fractionated by policosanol for 1 h (lanes 2 and 5) or 3 h (lanes 3 and 6), after which cell [14C]acetate, but not from [14C]mevalonate, indicating a site pharmacological concentrations (Menendez et al., 1997; present study), D-003, a mixture of very long-chain fatty acids that similarly inhibits cholesterol synthesis in cultured cells (Menendez et al., 2001b), contains a significant proportion of fatty acids greater than 30 carbons in length. This may contribute to its suggested greater effectiveness in lowering blood cholesterol levels (Menendez et al., 2004).

Suprapharmacological concentrations of policosanol impaired the uptake of [14C]acetate into these cells. Thus, at higher policosanol concentrations the decrease in cholesterol labeling from [14C]acetate could not exclude decreased uptake of label as a cause. However, we suspect that the decrease in uptake of [14C]acetate may actually reflect decreased incorporation into fatty acids in the presence of high levels of policosanols, because fatty acid synthesis in untreated cells represents a significant “sink” for radiolabeled acetate. If policosanol acts via the AMP-kinase pathway, it would be expected that fatty acid synthesis would also be suppressed, because AMP-kinase regulates acetyl-CoA carboxylase, the first and regulatory step in fatty acid synthesis (Hardie and Pan, 2002). Mevalonate uptake was not affected by policosanol, consistent with its more limited role in cellular biochemistry; however, higher concentrations of policosanol decreased mevalonate incorporation into cholesterol, suggesting the possibility of additional sites of inhibition downstream of HMG-CoA reductase. Thin layer chromatographic analysis did not reveal the accumulation of sterol precursors or intermediates that would indicate additional sites of inhibition; the decrease in mevalonate incorporation at higher policosanol levels may reflect decreased mevalonate synthesis by HMG-CoA reductase, thereby limiting overall flux through the pathway in the presence of subsaturating levels of radiolabeled mevalonate. We conclude that suppression of HMG-CoA reductase activity is the principal mechanism by which policosanol decreases cholesterol synthesis.
The observation that policosanol activates AMP-kinase is reminiscent of the mechanism of metformin, a drug widely used to treat type II diabetes. Metformin (Glucophage) acts through AMP-kinase to reduce blood glucose levels, enhance glucose uptake into skeletal muscle, decrease circulating lipids, and inhibit hepatic gluconeogenesis (Zhou et al., 2001). Metformin requires the presence of LKB1, the kinase that activates AMP-kinase, for its anti-diabetic effects (Shaw et al., 2005). A comparison of policosanol to metformin in their ability to stimulate AMP-kinase phosphorylation in the human hepatoma cell line HepG2 revealed that policosanol at 25 μg/ml was as efficacious as metformin, while being considerably more potent: 20 μg/ml (~8 μM triacontanol) was equivalent to 265 μg/ml (1.6 mM) metformin in this in vitro assay (Fig. 6B; data not shown). These results, taken in total, suggest that an evaluation of the ability of policosanol to moderate blood glucose levels in type II diabetes may be warranted.

References


Address correspondence to: Dr. Todd D. Porter, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082. E-mail: tporter@email.uky.edu