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More methemoglobin is produced by benzocaine treatment than lidocaine treatment in human *in vitro* systems

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ABSTRACT

The clinical use of local anesthetic products to anesthetize mucous membranes has been associated with methemoglobinemia (MetHba), a serious condition in which the blood has reduced capacity to carry oxygen. An evaluation of spontaneous adverse event reporting of MetHba submitted to FDA through 2013 identified 375 reports associated with benzocaine and 16 reports associated with lidocaine. The current study was performed to determine the relative ability of benzocaine and lidocaine to produce methemoglobin (MetHb) *in vitro*. Incubation of 500 μM benzocaine with whole human blood and pooled human liver S9 over 5 h resulted in MetHb levels equaling 39.8 ± 1.2% of the total hemoglobin. No MetHb formation was detected for 500 μM lidocaine under the same conditions. Because liver S9 does not readily form lidocaine hydrolytic metabolites based on xylidine, a primary metabolic pathway, 500 μM xylidine was directly incubated with whole blood and S9. Under these conditions MetHb levels of 4.4 ± 0.4% were reached by 5 h. Studies with recombinant cytochrome P450 revealed benzocaine to be extensively metabolized by CYP 1A2, with 2B6, 2C19, 2D6, and 2E1 also having activity. We conclude that benzocaine produces much more MetHb in *in vitro* systems than lidocaine or xylidine and that benzocaine should be more likely to cause MetHba *in vivo* as well.

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1. Introduction

Benzocaine is a local anesthetic often used topically. It is frequently employed to anesthetize the oropharynx for procedures such as trans-esophageal echocardiography, bronchoscopy, and esophagogastroduodenoscopy. Although benzocaine is generally well tolerated, it is known to cause methemoglobinemia (MetHba) in a small proportion of patients (Chowdhary et al., 2013; Guay, 2009; Kane et al., 2007). MetHba is a condition where the ferrous iron in hemoglobin is converted to the $^{+}3$ oxidation state, forming "methemoglobin (MetHb)" which cannot carry oxygen effectively (Coleman and Coleman, 1996; Mansouri, 1985; Skold et al., 2011; Umbreit, 2007). MetHb is normally present in the blood at levels of less than one percent. Levels of MetHb less than 10% of total hemoglobin are generally asymptomatic; greater than 10% causes cyanosis; greater than 20% causes exercise intolerance; greater than 30% causes headaches and dizziness; lethargy and stupor occur at over 50%; and levels over 65% can be life-threatening (Coleman and Coleman, 1996; Skold et al., 2011). MetHb forms naturally from normal oxygen exchange in the red cells and can be produced by some exogenous compounds; this MetHb can be reduced back to hemoglobin by two distinct methemoglobin reductase systems present in the erythrocytes, one using NADH and the other using NADPH as electron donors. These recovery mechanisms make most cases of MetHba self-limiting; in serious cases methylene blue can be administered to greatly facilitate the reduction of MetHb by stimulating NADPH methemoglobin

Abbreviations: acetylbenzocaine, ethyl 4-acetamidobenzoate; CYP, cytochrome P450; FDA, United States Food and Drug Administration; MetHba, methemoglobinemia; MetHb, methemoglobin; S9, liver homogenate postmitochondrial fraction; xylidine, 2,6-xylidine.

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reductase (Coleman and Coleman, 1996; Mansouri, 1985; Skold et al., 2011). MetHba is associated with use of a number of medications, most notably amyl nitrite, dapsone, sulfonamides, and many local anesthetics such as prilocaine, benzocaine, and lidocaine (Coleman and Coleman, 1996; Skold et al., 2011). For most of these compounds, their active metabolites are responsible for the production of MetHb (Bradberry, 2003).

The clinical incidence of MetHba from benzocaine has been estimated in retrospective studies to be in the range of 0.005–0.25% when used to anesthetize the airway for various diagnostic procedures (Chowdhary et al., 2013; Kane et al., 2007). Peak MetHb levels of around 40% of total hemoglobin are frequently reported when MetHba is found with benzocaine use but levels over 60% are possible (Ash-Bernal et al., 2004). In these cases cyanosis is generally evident within 30–60 min of application (Spiller et al., 2000) and may persist over 20 h (Rodrigues et al., 1994). Lidocaine is also used in these procedures, and in one study fewer cases of MetHba were seen with lidocaine than with benzocaine (Chowdhary et al., 2013). Although MetHba associated with lidocaine treatment can be observed with procedures such as infiltration anesthesia (Neuhaeuser et al., 2013), levels of MetHb from 14–37% have been reported for topical administration (Karim et al., 2001). In these cases of topical administration of lidocaine, MetHba occurred within 60 min. The observation that topical benzocaine seems to cause more MetHba than lidocaine should be tempered with the observation that more patients are treated with benzocaine spray than with lidocaine delivered as a spray in inpatient hospital settings (IMS Health, 2013) so it may be inaccurate to rely solely on number of cases reported.

The FDA has been evaluating the risk of MetHba with benzocaine and lidocaine used for topical anesthesia. An evaluation of spontaneous adverse event reports reported to FDA's Adverse Event Reporting System through 2013 identified a total of 375 cases of MetHba reported in association with benzocaine, including 8 cases of MetHba that resulted in death, and 16 cases of MetHba reported in association with lidocaine that resulted in no deaths. In some cases, MetHba has occurred even when these anesthetics were administered according to the manufacturer's recommendations. To address the limited information about the relative ability of benzocaine and lidocaine to produce MetHb, our laboratory has conducted *in vitro* studies to assess the relative rate of MetHb production from benzocaine and lidocaine with and without human liver S9 fractions to provide metabolic activation. Also, in an attempt to account for the rare occurrence of MetHba with benzocaine, a study using recombinant cytochrome P450 (CYP) enzymes was performed to see if known CYP polymorphisms might be involved.

2. Materials and methods

2.1. Materials

Human material was obtained from commercial sources using anonymized donors. Human whole blood was obtained from Lampire Biologicals (Pipersville PA) with Citrate Phosphate Dextrose Adenine (CPDA-1) used for anticoagulation. Subjects were asked to abstain from prescription and over-the-counter medications for 48 h before the blood was drawn. Human liver postmitochondrial fraction (S9, lot SUW) was obtained from Celsis-IVT (Baltimore, MD). Recombinant human cytochrome P450 containing microsomes (Supersomes) were obtained from BD Biosciences (San Jose, CA). Dapsone, benzocaine, lidocaine hydrochloride, 2,6-xylidine (xylidine), 4-aminobenzoic acid, 4 acetamidobenzoic acid, nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ammonium chloride and zinc dust were obtained from Sigma–Aldrich (St. Louis, MO). Ethyl 4-nitrobenzoate was obtained from Fisher Scientific (Pittsburg, PA). Phosphate buffered saline was obtained from Life Technologies (Grand Island, NY). Ethyl 4-acetamidobenzoate (acetylbenzocaine) was obtained from Combi-Blocks (San Diego, CA). All other chemicals were obtained from commercial sources.

2.2. Synthesis of benzocaine hydroxylamine

Benzocaine hydroxylamine was synthesized by a modification of the method of Shintani and Fu (2013). Briefly 6.5 g zinc powder was added over 60 min to a stirring mixture of 10 g ethyl 4-nitrobenzoate and 3.2 g ammonium chloride in 65 ml ethanol/32 ml water under a nitrogen atmosphere. The reaction mixture was stirred for an additional hour without addition of heat and the precipitate was removed by filtration. The ethanol was evaporated from the filtrate under a stream of dry nitrogen until a yellow oil separated. Off-white needle-like crystals of benzocaine hydroxylamine formed from the mother liquor when it was chilled to 0 °C. Purity and identity were checked by mass spectrometry and NMR. The product was approximately 95% pure with the major impurity being benzocaine present at a level of about 3%.

2.3. MetHb incubations and measurement

Incubations to assay the extent of MetHb production in whole blood were based on the work of Coleman (Coleman and Taylor, 1997). Dapsone 500 μM was used as a positive control because the mechanism of MetHb production by this compound has been examined extensively and dapsone has been shown to require metabolic activation to produce MetHb (Clement et al., 2005; Ganesan et al., 2010). Incubations consisted of 2.2 ml whole blood with 250 μl phosphate buffered saline or S9 (final concentration 2 mg S9 protein/ml), 25 μl NADPH generating system (282 mg glucose-6-phosphate, 84 mg NADP, 120 units glucose-6-phosphate dehydrogenase per ml; final concentration 10 mM glucose-6-phosphate, 1 mM NADP per ml), and 25 μl benzocaine, lidocaine, xylidine, or specific benzocaine metabolites in ethanol (final ethanol concentration 1%). Study compounds were used at a final concentration of 500 μm unless otherwise specified. Incubations were run in 20 ml glass scintillation vials; blood was maintained at 37 °C on a shaking waterbath for 60 min before adding other reagents to allow for full oxygenation. Incubations were performed in triplicate and were started by the addition of drug. Samples of 100 μl were taken at time zero and periodically thereafter, generally every 30 min for 5 h. Samples were analyzed for MetHb immediately after collection using an Avoximeter 4000 whole blood CO-oximeter (ITC, Edison, NJ) following the manufacturer's instructions. In incubations using recombinant human microsomes, the S9 was replaced with recombinant human CYP representing a single isoform to a final concentration of 15 pmol CYP/ml. These incubations were also performed for 5 h. Benzocaine was added as an acetonitrile stock solution in the recombinant CYP studies (final concentration acetonitrile 1%). All data is plotted as the mean of three determinations ± the standard deviation as determined by Microsoft Excel.

3. Results and discussion

3.1. In vitro generation of MetHb by test compounds

In the presence of S9, incubation of whole blood with 500 μM dapsone led to the production of MetHb which progressed over time in an approximately linear fashion to 27% total hemoglobin at 300 min, consistent with literature values (Coleman and