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A beating heart cell model to predict cardiotoxicity: Effects of the dietary supplement ingredients higenamine, phenylethylamine, ephedrine and caffeine

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ABSTRACT

Some dietary supplements may contain cardiac stimulants and potential cardiotoxins. In vitro studies may identify ingredients of concern. A beating human cardiomyocyte cell line was used to evaluate cellular effects following phenylethylamine (PEA), higenamine, ephedrine or caffeine treatment. PEA and higenamine exposure levels simulated published blood levels in humans or animals after intravenous administration. Ephedrine and caffeine levels approximated published blood levels following human oral intake. At low or midrange levels, each chemical was examined plus or minus 50 μM caffeine, simulating human blood levels reported after consumption of caffeine-enriched dietary supplements. To measure beats per minute (BPM), peak width, etc., rhythmic rise and fall in intracellular calcium levels following 30 min of treatment was examined. Higenamine 31.3 ng/ml or 313 ng/ml significantly increased BPM in an escalating manner. PEA increased BPM at 0.8 and 8 μg/ml, while 80 μg/ml PEA reduced BPM and widened peaks. Ephedrine produced a significant BPM dose response from 0.5 to 5.0 μM. Caffeine increased BPM only at a toxic level of 250 μM. Adding caffeine to PEA or higenamine but not ephedrine further increased BPM. These in vitro results suggest that additional testing may be warranted in vivo to further evaluate these effects.

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1. Introduction

Higenamine, PEA, and caffeine are currently found in some dietary supplement products marketed for weight loss, enhanced energy, or improved athletic performance. Ephedrine, although banned, is still available online. We wished to study these compounds to compare the effects they produced in vitro with beating cardiomyocytes to their reported cardiac effects documented in the scientific literature. Three criteria were used in selecting these supplement ingredients for study. First, published data suggest they may enhance heart rate. Higenamine and PEA both have been documented to increase heart rate with in vivo experiments (Dunlop and Shanks, 1969; Feng et al., 2012; Liang and Sprecher, 1979; Praman et al., 2012). Ephedrine is known to increase heart rate, and was banned in 2004 because it was associated with deaths and severe cardiac adverse events. It was chosen as an example of a known hazardous dietary ingredient. Caffeine has been suggested to enhance the effect of ephedrine on heart rate (Astrup et al., 1991), and it is found in the majority of supplements advocated for weight reduction, energy, or enhanced athletic performance. Second, all these ingredients are frequently found in dietary supplements. Even a cursory online search found multiple products containing higenamine and PEA in dietary products and in purified forms intended for human consumption. Ephedrine, although banned, is still available on the internet. Third, we also considered FDA adverse event reports for PEA and higenamine. Cases with cardiac-related symptoms (e.g. altered heart rate, palpitations, shortness of breath, etc.) were examined. Beginning five years ago with the first report, there have been 14 cases involving PEA-containing supplements. The first higenamine adverse event report was filed seven months ago, and during the next five months six additional reports were filed on higenamine-containing dietary supplements. These reports, although they do not establish causality, suggested PEA and higenamine should be investigated further. Our ultimate objective was to establish the usefulness of screening these ingredients in vitro

Abbreviations: Amox, amoxicillin; BPM, beats per minute; Caff, caffeine; Eph, ephedrine; Hig, higenamine; i.v, intravenous; NS, not significant; PEA, phenylethylamine.

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for cardiac effects with beating human-induced pluripotent stem cells differentiated into cardiomyocytes (commercially available as iCell cardiomyocytes).

iCells have many relevant electrical, mechanical, and pharmacological properties compared to isolated cardiomyocytes obtained from live animals. The cells have intrinsic pacemaker activity, and will beat synchronously in a well after 1–2 weeks of culture. The cells have functioning ion channels (Ma et al., 2011). The cells respond appropriately to β-adrenergic receptor agonists and antagonists (Sirenko et al., 2013a). They have been reported to be a good model system to study drugs with cardiac effects (Sirenko et al., 2013b).

Here we examine, at pharmacologically-achievable levels, the effects of higenamine, ephedrine, and PEA in iCell beating cardiomyocytes. Cells were monitored by a rapid-response fluorescence measurement system with appropriate software to capture the beating rate and other parameters associated with beating. Caffeine and ephedrine have been suggested to have a synergistic effect on heart rate (Astrup et al., 1991), but this has not been replicated in all studies (e.g. Haller et al., 2005). We wished to see if we could replicate this reported effect in vitro, as well as examine whether caffeine might exert a similar effect with higenamine and PEA.

2. Materials and methods

2.1. Cell culture methods

Two types of cells were used: iCell cardiomyocytes (Cellular Dynamics, Inc., Madison, WI, USA) and H9c2 rat cardiomyoblasts (ATTC, Manassas, VA, USA).

Human induced pluripotent stem cell cardiomyocytes (iCell cardiomyocytes) were used to produce a 96-well plate of synchronously beating cardiomyocytes. The production of the cells is a proprietary process, but has been described in general terms elsewhere (Ma et al., 2011 and Babiarz et al., 2012). Cardiomyocytes were initially seeded at a density of 2×10^5 cells per ml into gelatin-coated black 96-well plates with transparent bottoms. The different media for plating and subsequent maintenance were provided by Cellular Dynamics. Cells were placed in a humidified air incubator with 5% CO_2 at 37 °C. After 48 h, the surface of the cell layer within the wells was gently washed by slowly aspirating media up and down with a multichannel pipetter to suspend non-adherent cells. Plating media was removed and replaced with warm maintenance media. Maintenance media was changed every other day. Cells were maintained for 10 to 15 days post plating, which was usually the time required for the cells to beat synchronously in all of the wells.

H9c2 rat cardiomyoblasts were initially seeded into T75 flasks and expanded. The initial growth media was DMEM (high glucose) with 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS and 1% antibiotic/antimycotic solution. All components were obtained from Life Technologies (Frederick, MD, USA), except for FBS, which was obtained from Thermo-Fisher (Waltham, MA, USA). After propagation in growth media, cells were removed by treatment with a proprietary cell dissociation solution (TrypLE, Life Technologies) and plated at 2×10^5 cells per ml in either white or black 96-well plates with transparent bottoms. On the day after plating, the cells were differentiated by replacing the growth media with differentiation media. The differentiation media was the same composition as growth media, with 2% adult goat serum (Thermo-Fisher) replacing FBS and addition of 20 nM each of all-trans and 9-cis retinoic acids. Cells were grown seven days in differentiation media (changed every two days) prior to use in cell viability assays.

2.2. Chemicals

Caffeine, (1R,2S)-(-) ephedrine, phenylethylamine hydrochloride and DMSO were obtained from Sigma-Aldrich (St. Louis, MO, USA). Each was 98% pure or better. Higenamine (99.2% purity) was obtained from the National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS, USA. Amoxicillin, used as a no effect control in the beating iCells, was obtained from Sigma-Aldrich. Isoproterenol hydrochloride, used as a positive chronotropic agent, was obtained from Molecular Devices LLC, Sunnyvale, CA, USA.

2.3. Preliminary cytotoxicity screening in H9c2 cells

H9c2 cells were used to screen the test compounds for overt cytotoxicity, prior to their use in beating cells. Cells were exposed to concentrations from 10 to 1000 μg/ml. The intent was to establish limits of exposure to avoid overt cytotoxicity during later testing with the beating iCells. Cytotoxicity was assessed using kits for ATP content and LDH release (Promega, Madison, WI, USA) according to the manufacturer's instructions, except for prior rinsing of wells for the ATP assay to avoid interference of the test compounds with light production (Calvert and Vohra, 2013). Concentrations of higenamine and PEA ranged from 10 to 1000 μg/ml. Caffeine concentrations tested were 10 μM, 50 μM, 250 μM, and 400 μM. For ephedrine, test concentrations were 0.5, 1.0, 1.5, 10, and 100 μM.

2.4. Concentrations for testing compounds in iCells

Caffeine and ephedrine were tested at concentrations designed to mimic human blood levels after oral dosing. For caffeine the lowest level was based on the C_{max} blood levels associated with consumption of one cup of coffee (10 μM), a high dose caffeine-containing dietary supplement (50 μM), and an excessive dose (250 μM), (Carrillo and Benitez, 2000). Caffeine was tested alone and as an additive to each of the other compounds at a level of 50 μM. For ephedrine, dose levels were based on the serum level associated with consumption of 25 mg taken once (0.5 μM), 50 mg taken once (1.0 μM) both reported in Schier et al. (2003), and an arbitrarily selected higher dose of 5.0 μM. For reference, human lethal doses have been reported to result in serum levels ranging from 17.5 to 100 μM (Garriott et al., 1985).

Higenamine administered intravenously in a human clinical study, resulted in a C_{max} level of 31.3 ng/ml, producing an average pulse rate of 126 bpm (Feng et al., 2012). This concentration, plus 10 times lower or higher concentrations (3.13 and 313 ng/ml), were used in the present study. PEA concentrations were 0.8, 8 and 80 μg/ml. The two lower doses were selected based on published studies of intravenous administration in rats (Wu and Boulton, 1975) or dogs (Liang and Sprecher, 1979). The highest dose tested in our system was 2.5 times lower than the lowest dose of PEA causing cytotoxicity in H9c2 cells. Ten micromolar isoprel was used as a positive chronotropic control, while 5.9 μg/ml amoxicillin was used as a no effect control. The level of amoxicillin was based on a published therapeutic level (Lode et al., 1974).

2.5. Instrumentation

A FLIPR Tetra instrument (Molecular Devices LLC) was used for all measurements with the beating cells. The FLIPR Tetra is a rapid fluorescence plate reader, capable of taking rapid exposures of all wells of a 96-well plate simultaneously. Signal detection was based on changes in fluorescence, measured with an excitation wavelength of 470–495 nm, and an emission wavelength of 515–575 nm. The principle of the instrument is based on the binding of calcium to a fluorescent dye once it enters the cytoplasm from extracellular sources and the sarcoplasmic reticulum. As calcium increases, a parallel increase in fluorescence occurs, which then declines back to baseline. Calcium movement corresponds to cardiomyocyte contraction, each peak representing one beat. Software supplied with the instrument was used to calculate beats per minute (BPM), peak rise time, peak decay time, and peak width.

2.6. Experimental protocol

Filter-sterilized solutions of the test materials were prepared one day prior to administration to the iCells. The vehicle used for higenamine was DMSO, while caffeine, PEA and ephedrine were dissolved in distilled water. The DMSO concentration for the maximal concentration of higenamine was 0.02%. For caffeine, ephedrine, and PEA the maximal water concentrations in the cell media were 0.3125%, 0.018%, and 0.08%, respectively. Each plate had a series of vehicle control wells, no-effect controls (amoxicillin) and positive chronotropic controls (isoproterenol). All stocks were diluted in iCell maintenance media prior to use.

After a pre-determined two hour incubation with a calcium binding dye (from the EarlyTox Cardiotoxicity kit, Molecular Devices), 96-well plates of beating iCells were placed in the FLIPR Tetra instrument for reading. After baseline readings were obtained, the cells were returned to the incubator for 15 min. Test compounds were then added, utilizing the automated pipetter in the FLIPR Tetra to add the compounds to all 96-wells simultaneously. Cells were then returned to the incubator for 30 min, based on use of this exposure interval in a study of iCell cardiomyocytes exposed to pharmaceuticals (Sirenko et al., 2013b). Following the 30 min exposure to treatment, plates were read again to measure the effect of the compounds. PEA, because of its reported short half-life (Wu and Boulton, 1975) was also examined after 10 min of exposure. Results following a 10 min exposure were essentially the same as with the 30 min exposure. All experiments were done in triplicate.

Because of differing beat rates pre-treatment, all wells were normalized by dividing the post-treatment value by the pre-treatment value to yield a ratio. All values from a plate were standardized by division by the mean control value for that plate. Data from all plates were then combined for analysis. A three-way ANOVA test was used to adjust for both experimental date and plate variability to identify actual treatment effects. All values were compared to the control, with p-values adjusted according to Dunnett's test. In addition, the effect of added caffeine was evaluated by comparing the middle dose group of each compound alone vs. the middle dose plus 50 μM caffeine.

3. Results

3.1. Cytotoxicity

The lowest dose causing cytotoxicity for each test compound was 100 μM for ephedrine, 100 μg/ml for higenamine, and 200 μg/ml for