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Dietary levels of acrylamide affect rat cardiomyocyte properties

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ABSTRACT

The toxic effects of acrylamide on cytoskeletal integrity and ion channel balance is well-established in many cell types, but there has been little examination regarding the effects of acrylamide on primary cardiomyocytes, despite the importance of such components in their function. Furthermore, acrylamide toxicity is generally examined using concentrations higher than those found *in vivo* under starch-rich diets. Accordingly, we sought to characterize the dose-dependent effects of acrylamide on various properties, including cell morphology, contraction patterns, and junctional connexin 43 staining, in primary cardiomyocytes. We show that several days exposure to 1–100 μM acrylamide resulted in altered morphology, irregular contraction patterns, and an increase in the amount of immunoreactive signal for connexin 43 at cell junctions. We conclude that dietary levels of acrylamide may alter cellular function with prolonged exposure, in primary cardiomyocytes.

1. Introduction

Acrylamide is commonly used in biological research, but over the past few decades, its abundance in certain starch rich foods (Parzefall, 2008; Tardiff et al., 2010) has been investigated for possible deleterious health effects. Studies have demonstrated that acrylamide is a human mutagen, and rodent teratogen and carcinogen (Bjellaas et al., 2007; Parzefall, 2008; Shipp et al., 2006; Tardiff et al., 2010). Additionally, the organism *Caenorhabditis elegans* exhibits a markedly reduced lifespan in the presence of acrylamide (Hasegawa et al., 2004).

The effects of acrylamide on a range of biological structures from DNA to cells, and from tissues to whole organisms have been examined. For example, acrylamide has been proven to negatively influence the thyroid (Shipp et al., 2006), the spleen and erythrocytes (Beland et al., 2012), smooth muscle (Sakai et al., 2009), and skeletal muscle (Oishi et al., 1996). In addition to being a neurotoxin (Park et al., 2010; Parzefall, 2008; Tardiff et al., 2010), acrylamide may also cause rat hind-leg paralysis (Beland et al., 2012; Oishi et al., 1996), decrease nerve conduction velocity (Shipp et al., 2006), affect cystolic ion balance (LoPachin et al., 1992, 1993), and impact the cytoskeleton, particularly the intermediate filaments (Gold et al., 1985; Strege et al., 2003; Tardiff et al., 2010). However, there has been no systematic examination of low, persistent doses of acrylamide on cardiac tissue.

The effects of acrylamide may be particularly pertinent to cardiac cells. Not only are cardiomyocytes dependent on various ion gradients to maintain regulated contraction, these cells rely on cytoskeletally-stabilized structures, such as the intercalated discs, to synchronize contraction (Sheikh et al., 2009). Acrylamide may alter junctional integrity, disrupt the intercalated discs and redistribute gap junctions (Ferreira-Cornwell et al., 2002; Li et al., 2006). Because gap junctions are imperative for cell–cell communication, their alteration can lead to conduction abnormalities such as arrhythmias and other cardiac pathologies (Kaplan et al., 2004; Saffitz, 2005; Saffitz and Kleber, 2004; Salameh et al., 2010).

High doses of acrylamide have significant effects on cell mechanics, and in particular, on properties related to intermediate filaments (Wei et al., 2013). Doses typically used for such studies are generally far higher (and dosed for shorter periods of time) compared to *in vivo* concentrations acquired through diet. Such doses are presumably required for obvious structural changes to manifest, such as those detected via immunofluorescence staining. Based on related previous work examining desmosomal and gap junctional integrity, we found that significant changes to cell–cell adhesion and the junctional presence of key proteins can be significantly altered without apparent alterations in cytoskeletal structure (Huang et al., 2008). We thus hypothesized that exposure to dietary levels of acrylamide causes downstream alterations in gross cardiomyocyte morphology, contraction properties, and gap junctional distribution, even at doses that do not cause gross, apparent changes to the cytoskeleton. To examine such properties,

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we quantified sarcomere spacing, spontaneous contraction periods and beating patterns, as well as connexin 43 localization at cell junctions under various dosages of acrylamide, including dosages that correspond to levels found in some foods that are cooked at high temperature. We found that cardiomyocytes treated under acrylamide concentrations corresponding to dietary levels significantly alter beating patterns and junctional connexin localization.

2. Materials and methods

2.1. Cell culture

Cardiomyocytes were isolated from one day old Wistar rat hearts (Charles River, Massachusetts) based on previously established protocol for cardiac cell isolation (Michaelson et al., 2012; Shen et al., 2013). Briefly, hearts were minced and sequentially dissociated using trypsin and pancreatin, then filtered and plated to remove cardiac fibroblasts. Subsequently, cardiomyocytes were cultured in collagen-coated 35 mm plastic or MatTek glass-coverslip-bottomed dishes, in M199 media (Invitrogen) supplemented with epinephrine and bromodeoxyuridine, calf serum at 10% (Sigma) for the first 24 h, then 5% thereafter, at one of 0 μM, 1 μM, 10 μM, or 100 μM of acrylamide (Sigma). Plating cell density was 3 million cells per dish. Animal work was done in accordance with the Columbia IACUC guidelines and protocols.

2.2. Acrylamide dosing

In certain western diets, average daily consumption of acrylamide can be as high as 4 μg/kg of body weight (Parzefall, 2008; Tardiff et al., 2010). Assuming the chemical is fully miscible in blood (Bjellas et al reports that the acrylamide consumed is typically 92% bioavailable in the plasma (Bjellaas et al., 2007)) and that an adult male weighing 80 kg has about 5 L of blood, the molar blood concentration of acrylamide would be around 1 μM.

$$\frac{4\ \mu g}{kg\ body\ weight} * \frac{1\ mol}{71.08\ g} * 80\ kg * \frac{1}{5\ L} = 0.9\ \mu M \approx 1\ \mu M$$

This value is supported in several studies. Calleman et al. studied human populations with plasma concentrations of 0.92–2.00 μM as a control population when studying the carcinogenic effects of acrylamide (Calleman et al., 1994). Tolerable daily intake levels of acrylamide are estimated to be around 40 μg/kg body weight (Tardiff et al., 2010). Using the same approximation, 10 μM should have no obvious impact on the tissues. We additionally tested 100 μM, which is higher than the amounts deemed safe to the thyroid, reproductive system, and nervous system (Shipp et al., 2006; Tardiff et al., 2010), and higher than the amounts shown to be carcinogenic (Besaratinia and Pfeifer, 2003) or to decrease the lifespan of C. elegans (Hasegawa et al., 2004). Thus, we would expect to see at least some effects on cardiac myocyte physiology at 100 μM.

2.3. Sarcomere staining

Following three days of culture, cardiomyocytes were fixed in 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton X (Sigma), washed again and then incubated in Alexa 594 Phalloidin (Invitrogen, 1:400) for 1 h. Samples were imaged using an Olympus IX-81 inverted fluorescence microscope at 60x/NA 1.42. Sarcomeres were identified within each sample and at least three different fields of view were imaged for each condition, with some brightness or contrast adjustment to more clearly display sarcomeres. Sarcomere spacing was quantified for cells exhibiting at least five visible sarcomeres, and at least 15 cells were analyzed for each condition. Using a custom MATLAB program, the spacing between sarcomeres was quantified by measuring the peak-to-peak distances. Briefly, a line was drawn perpendicularly to the sarcomeres, and the intensity of the fluorescence stain was plotted along the line. Peak-to-peak distances were then measured using the intensity plot.

2.4. Contraction imaging

Fluorescent 0.5 μm diameter polystyrene beads (Invitrogen) were introduced into the media two days after plating and were incorporated into the cardiomyocytes by endocytosis overnight. Cells were then imaged following a media wash. Image sequences 100 frames long were captured at 60x/NA 1.42 at 36 frames per second, with at least four beads present in each field of view, for at least 5 different fields of view per acrylamide concentration. The image sequences were converted to AVI files and analyzed using a custom Matlab program used for particle tracking (Huang et al., 2008, 2001). The period and amplitude of bead movement over time was then extracted to assess contraction patterns of the myocytes. These values were normalized to the control sample average to account for variation between different cell isolations. Abnormalities of contraction patterns, such as irregular spacing of contraction or double-contractions, were determined visually. Contraction patterns with extended periods were excluded from abnormal contraction analysis since there were insufficient numbers of contractions to assess irregularity with certainty. At least 18 cells per condition (per acrylamide concentration per time point) were analyzed.

2.5. Connexin staining and immunoblot

Cardiomyocyte immunostaining was performed based on existing protocol (Kwong et al., 1998). Cells were simultaneously blocked and permeabilized for 60 min in 3% goat serum, 1% w/v BSA, and 0.15% Triton X-100 in PBS, followed by an overnight incubation at 4 °C with mouse-monoclonal anti-connexin-43 antibody (Invitrogen; 1:2000 in blocking buffer). Samples were subsequently incubated with secondary antibody (goat-anti-mouse AlexaFluor 594 from Invitrogen; 1:2000 in PBS) for 2 h at room temperature. The immunostained cultures were imaged at 40x/1.3 NA using an Olympus IX-81 inverted confocal microscope. Each image was masked to isolate cell junctions, allowing for discrete quantification of junctional and cytoplasmic immunoreactive signal. Images were analyzed as previously described, using quantitative confocal microscopy (Huang et al., 2008; Saffitz et al., 2000). Briefly, lower and upper thresholds were set based on control cells to remove background signal from each image and to maximize the signal at cell junctions, respectively. Each image was masked by hand in ImageJ to separate cell junctions from the cytosol, and a threshold was applied to discretely quantify the junctional and cytoplasmic immunoreactive signal. The fraction of pixels above threshold divided by the total number of pixels in the image space was reported. At least four fields of view were analyzed for each sample, for at least four samples per condition.

Connexin immunoblotting was performed by lysing cultured NRVM in standard RIPA lysis buffer containing 500 nM phenylmethanesulfonyl fluoride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and protease inhibitor cocktail. Samples were solubilized in LDS sample buffer supplemented with 5% v/v 2-mercaptoethanol. Total protein concentration was determined by Bradford assay (Sigma). Soluble fractions containing equal amounts of total protein were separated using SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, MA). Immunoblotting was performed using the following mouse anti-connexin antibody (Invitrogen 1:1000) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Biorad 1:1000). Blots were developed with ECL reagents (Perkin Elmer, MA) and imaged using a FUJI imaging unit (Fujifilm, CT). Relative band intensities were quantified using ImageJ, with values normalized to GAPDH as a loading control. At least 3 samples were processed for each condition.

2.6. Statistical analysis

Samples were compared using ANOVA with either Dunn's (for non-parametric data) post test or Tukey (for normally distributed data) post test applied on significance. For comparing the percentage of abnormal beats, a Chi-squared contingency table test was used, and standard error was estimated using standard error = sqrt($a(1-a)/n$), where a represents the fraction of abnormal beats and n represents the sample size. A p-value less than 0.05 was considered significant. Data in plots are shown as mean ± standard error.

3. Results

3.1. Cardiomyocytes cluster with prolonged acrylamide exposure

Cardiomyocytes cultured in different concentrations of acrylamide were observed daily for overt morphological differences. For the first three days of exposure, no differences were observed. Starting at day four, the cardiomyocytes exposed to 100 μM acrylamide appeared to separate into clusters with thin strands connecting the clusters (Fig. 1). However, lower concentrations of acrylamide did not have an obvious morphological effect at day four. By day six, myocytes in the control condition exhibited some clustering, but all acrylamide-exposed cardiomyocytes exhibited far more clustering, with higher concentrations showing tighter cluster formations (Fig. 1). These cell clusters remained overtly viable as they continued to contract and did not exhibit significant detachment over the 6 days of experiments. They appeared merely to be a more three-dimensional, compact group of cells that primarily differed in their lack of the usual two-dimensional monolayer formation.