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Hydrogen-enriched water restoration of impaired calcium propagation by arsenic in primary keratinocytes

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ABSTRACT

Endemic contamination of artesian water for drinking by arsenic is known to cause several human cancers, including cancers of the skin, bladder, and lungs. In skin, multiple arsenic-induced Bowen's disease (As-BD) can develop into invasive cancers after decades of arsenic exposure. The characteristic histological features of As-BD include full-layer epidermal dysplasia, apoptosis, and abnormal proliferation. Calcium propagation is an essential cellular event contributing to keratinocyte differentiation, proliferation, and apoptosis, all of which occur in As-BD. This study investigated how arsenic interferes calcium propagation of skin keratinocytes through ROS production and whether hydrogen-enriched water would restore arsenic-impaired calcium propagation. Arsenic was found to induce oxidative stress and inhibit ATP- and thapsigaragin-induced calcium propagation. Pretreatment of arsenic-treated keratinocytes by hydrogen-enriched water or beta-mercaptoethanol with potent anti-oxidative effects partially restored the propagation of calcium by ATP and by thapsigaragin. It was concluded that arsenic may impair calcium propagation, likely through oxidative stress and interactions with thiol groups in membrane proteins.

1. Introduction

Arsenic is one of the most abundant metal elements in the Earth crust. People can be exposed to arsenic through inhalation or drinking. Arsenic is a well-documented carcinogen that can lead to several human cancers, including liver, bladder, lungs, and skin, the most common arsenic-induced cancer and one of earliest known carcinogenic responses to arsenic (Tseng et al., 1968). Arsenic-induced Bowen's disease (As-BD), which is the most common arsenical skin cancer, provides a good disease model for the study of the early phase of arsenic carcinogenesis. The pathognomonic features of As-BD include full-layer epidermal dysplasia, epidermal thickening, and individual cell apoptosis, all of which feature aberant keratinocyte differentiation (Yeh et al., 1968).

Physiological epidermal differentiation involves sequential differentiation from the basal layer, granular layer, spinous layer, to outermost corneal layer in epidermis, where the kerationcytes of terminal differentiation become apoptotic and lose their nuclei. In pathological circumstances, abnormal keratinocyte differentiation can lead to abnormal cell proliferation and evasion of apoptotic cells with damaged DNA, resulting in proliferative diseases, including inflammatory skin lesions and benign or malignant skin cancers (Watt, 1989). This epidermal differentiation process is delicately regulated by calcium homeostasis (Hennings et al., 1980; O'Keefe and Payne, 1983), in which the elevation of calcium initiates keratinocyte differentiation. Thus, calcium homeostasis, which tightly regulates keratinocyte differentiation and cell apoptosis, may play a role in skin carcinogenesis, and more specifically, in arsenic skin carcinogenesis. However, how arsenic affects the calcium signaling in primary keratinocytes is seldom addressed. Perez et al. found arsenic decreased terminal differentiation more in control primary keratinocytes than in calcium-treated primary keratinocytes (Perez et al., 2003), suggesting arsenic may interfere with keratinocyte differentiation through calcium regulation. One of our recent studies found that arsenic interferes with ATP-mediated calcium signaling in primary keratinocytes and but not in cancerous keratinocytes (Hsu et al., 2012), further strengthening the possibility that arsenic might contribute to carcinogenesis through calcium signaling and homeostasis.

In addition to calcium, oxidative stress has been presumed to be involved in arsenic carcinogenesis because increased level of 8-hydroxydeoxyguanosine (8-OHdG), an indicator for oxidative stress, has been found in urine from adults and schoolchildren exposed to arsenic (Wong et al., 2005; Yamauchi et al., 2004). In arsenical skin cancers, there is also an increased 8-OHdG expression (Matsui et al., 1999), suggesting that oxidative stress may contribute at least in part to arsenic carcinogenesis as well. In fact, calcium mobilization and signaling play an important role in keratinocyte apoptosis induced by oxidative stresses. Treatment of HaCaT immortalized keratinocytes with the calcium chelator

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BAPTA-AM provides significant cytoprotection against hydrogen-peroxide-induced apoptosis (Bakondi et al., 2003). Hence, calcium signaling may coordinate with the oxidative stress, resulting in the development of pathognomic features in arsenical cancers, including aberrant differentiation and abnormal apoptosis (Bailey et al., 2009; Pi et al., 2003). The interaction of calcium and oxidative stress is further evidenced by the finding that calcium signaling can trigger UVB-induced reactive oxygen species (ROS) generation in HaCaT cells, a cell line of transformed keratinocytes (Masaki et al., 2009). This interaction has been linked to cell proliferation, differentiation, and apoptosis (Goldman et al., 1998). These previous findings led to us to ask whether arsenic affects calcium signaling and induces ROS in primary keratinocytes, and if so, whether abrogation of ROS by reducing agents would restore the arsenic-mediated calcium responses in keratinocytes.

Hydrogen enriched water is defined as having a rich dissolved molecular hydrogen up to thousands of ppb and having a potent reducing activity(Itoh et al., 2009; Ohsawa et al., 2007). It was developed to treat or prevent some ROS-related diseases in cells, animals, and humans (Ohta, 2011). Molecular hydrogen and hydrogen-enriched water have been reported to act as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals for brain ischemia and found to rapidly diffuse across membranes and protect cells from oxidative damages (Ohsawa et al., 2007). In the current study, we measured the calcium propagation and ROS in arsenic-treated keratinocytes, and pretreated the cells with hydrogen-enriched medium to see whether the impaired calcium propagation by arsenic could be restored by replacement of regular medium with hydrogen-enriched medium.

2. Materials and methods

2.1. Reagents and culture of primary keratinocytes

Sodium arsenate, β-mercaptoethanol, ATP, and thapsigargin were all obtained from Sigma–Aldrich (St. Louis, MO). Fluo-4-AM and MitoSox were purchased from Molecular Probes (Eugene, OR). Normal human keratinocytes were obtained from adult foreskins obtained through routine circumcisions. The method for keratinocyte cultivation is described in a previous report (Lee et al., 2011). Briefly, skin specimens were washed with phosphate-based saline (PBS), cut into small pieces, and incubated in medium containing 0.25% trypsin (Gibco, Grand Island, NY) overnight at 4 °C. The epidermal sheet was lifted from the dermis using fine forceps. The epidermal cells were centrifuged (500 g, 10 min) and the pellets dispersed into individual cells by repeated aspiration. The keratinocytes were gently resuspended in keratinocyte-SFM (serum-free medium) (Gibco) containing 25 μg/ml bovine pituitary extract (BPE) and 5 ng/ml recombinant human epidermal growth factor (rhEGF). The medium was changed every two days. Keratinocytes at the third passage were then grown in keratinocyte-SFM medium free of supplements 24 h before the experiments. Written informed consent was obtained from all participants. The protocol for this study was approved by the Institutional Review Board at Kaohsiung Medical University Hospital (KMUH-IRB-990440) and adheres to guidelines set forth in the Declaration of Helsinki.

2.2. Calcium propagation and MitoSox staining

To induce calcium mobilization, we treated primary keratinocytes with ATP and thapsigaragins, both known to induce calcium propagation. They induce calcium mobilizations through different mechanisms. ATP induces calcium mobilizations by binding to purinergic receptors in the cell membrane, while thapsigaragin raises the intracellular calcium by blocking the ability of the cell to pump calcium into the endoplasmic reticulum (Yokokawa and Takeyasu, 2011 and Lee et al., 2012). To measure calcium propagation, a calcium imaging system was equipped with a perfusion system was used to measure the calcium propagation. Before the experiments, the cells were stained with 1 μM Fluo-4-AM at 37 °C for 20 min and then washed three times with BSS buffer containing 5.4 mM KCl, 5.5 mM D-glucose, 1 mM $MgSO_4$, 130 mM NaCl, 20 mM HEPES pH 7.4, and 2 mM $CaCl_2$. Intracellular calcium concentrations were estimated based on the intensity ratio of the fluorescence emitted by 488-nm light excitation at a resolution of 1376 × 1038 pixels with Olympus CellR-IX81 fluorescence microscope (Olympus, Essex, UK) equipped with an MT 20 illumination system (Olympus) and UPLanApo 10X objective lens. We then averaged the signal intensity from 20 selected cells (Fig. 1 inset). The differences in the peak and bottom intensity were then measured and analyzed by publically accessible software, NIH image J, to measure the signal intensity within a region of interest (ROI).

To measure mitochondrial oxidative stress, 1.0–2.0 mL of 5 μM MitoSOX™ reagents was used to cover cells adhering to the coverslip. Cells were incubated for 10 min at 37 °C and protected from light. Cells were then washed gently three times with warm buffer and then imaged. The images were also analyzed by NIH image J.

2.3. Preparation of hydrogen-enriched water

Hydrogen was dissolved in phosphate-based saline (PBS) or keratinocyte culture medium (keratinocyte-SFM) for two hours under high pressure (0.4 MPa) of gaseous hydrogen until saturated based on a similar hydrogen-rich saline-producing apparatus as designed by Ohta's lab (Ohsawa et al., 2007). Briefly, O_2 was dissolved into a second keratinocyte-SFM by bubbling O_2 gas at the saturated level (42.5 mg/l), and CO_2 into a third keratinocyte-SFM by bubbling CO_2 gas. All three media (H_2 medium:O_2 medium:CO_2 medium) were maintained at atmospheric pressure and combined at a proportion of 75%:20%:5% (vol/vol/vol). For the nitrogen control, we combined N_2 medium:O_2 medium:CO_2 medium at proportion of 75%:20%:5% (vol/vol/vol). The saturated hydrogen-rich medium was stored under atmospheric pressure at 4–8 °C in an aluminum bag with no dead volume. The final concentration of hydrogen water was saturated and maintained for 24 h as previously described (hydrogen more than 0.3 mM with pH = 7.4 ± 0.1) (Kamimura et al., 2011).

2.4. Statistical analysis

Statistical analysis was performed using the SigmaPlot, version 12.0 (SPSS, Chicago, IL) and Microsoft Excel 2010 (Microsoft, Redmond, WA). The data are presented as means ± SD of the results obtained from three independent experiments. The significance level of the difference between the control and the experimental groups was determined by the student's *t*-test using SigmaPlot. Trend analysis was performed using Microsoft Excel. Statistical difference was considered significant when *P*-value was less than 0.05.

3. Results

3.1. Impaired calcium propagation in cultured keratinocytes

The result first confirmed a previously well-known phenomenon in which both ATP and thapsigaragin induce calcium mobilizations in keratinocytes (Fig. 1a). Interestingly, although ATP and