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Acrylamide induces immunotoxicity through reactive oxygen species production and caspase-dependent apoptosis in mice splenocytes via the mitochondria-dependent signaling pathways

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ABSTRACT

Introduction: Acrylamide (AA), a well-known food neo-contamination, can be produced during food preparing at high temperature. The immunotoxicity of AA have been revealed in the experimental animals. In this study, we explored the molecular mechanism responsible for the immunotoxicity of AA.
Methods: The mice splenocytes exposed to AA concentrations (0,5,10 and 25 mM) and apoptosis cell death was measured through Annexin V/Propidium Iodide staining by flow cytometry method. The role of extrinsic and intrinsic pathways were evaluated respectively by activity of caspase-8 and-9. Furthermore, the spleen mitochondria were obtained using differential centrifugation from mice and mitochondrial toxicity endpoints were determined after AA exposure.
Results: Exposure of splenocytes to AA increased the splenocytes' apoptotic cell death. Also, increased activation of both caspase-8 and-9 were observed in mice splenocytes after AA exposure. Treatment of isolated mitochondria with AA lead to disturbance in activity of complex I and III of mitochondrial electron transfer chain that result in increased reactive oxygen species (ROS) production, lipid peroxidation and glutathione oxidation. These events were accompanied by mitochondrial membrane swelling, collapse of mitochondrial membrane potential and significant falling of mitochondrial activity.
Conclusion: AA-mediated mitochondrial dysfunction along with mitochondrial oxidative damage seems to be critical events leading to activation of caspase cascade and apoptotic cell death in spleen that finally can attenuate immune system's function.

1. Introduction

Acrylamide (AA) is an important industrial compound. The polymers of acrylamide (polyacrylamide) are extensively used in industry for water flocculation, soil coagulation and grouts [1]. Recently, the different level of AA contamination was reported in national dietary patterns of many countries. Preparation of carbohydrate- rich foods (such as potato products, bakery products, chips, roasted cereals and coffee) at high temperature (>120) leads to reaction between amino acid named asparagine and the carbonyl group of glucose that produces AA [2].

The wide usage of AA in the industry and the finding of AA in food made it become a concern in public health and attracted attentions to studying its pathogenic mechanisms [3].

Several adverse effects were reported after AA exposure in animal and human models. Many researches demonstrated acrylamide can induce reproductive toxicity, genotoxicity and carcinogenicity in cell line and animal models [4]. Similarly, the neurotoxicity of AA was clearly demonstrated in human [5] and the immunotoxicity of AA was recently gained attention by scientists. Fang et al. showed AA can cause an inhibitory effect on cellular and humeral immunity of mice, But its exact toxicity mechanism is not well known [6].

Several possible molecular mechanisms were proposed for the toxicity of AA. One of the most important mechanism is oxidative stress-induced apoptosis, whose involvement in acrylamide induced neurotoxicity is well-known [7].

Apoptosis, the natural programmed cell death procedure, is essential for the homeostasis of all multicellular organisms [8]. Too much apoptotic cell can lead to the biological disorders such as ischemic heart disease, AIDS and neurodegenerative diseases like Alzheimer and Parkinson [9].

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Apoptosis is mediated by a signaling cellular pathway that consists in the caspase family[10]. There are both extrinsic and intrinsic apoptotic pathways involved in the activation of caspases. In general, the activation of caspase 8 initiates extrinsic pathway while activation of caspase 9 is an initiator for intrinsic pathway [10]. The intrinsic pathway, known as mitochondria depended pathway, starts from the mitochondria. When the cells affect by an internal stimulus, mitochondrial trans membrane potential was decreased and the release of cytochrome c to cytosol are promoted. These events lead to activation of caspase-9 [8]. Therefore, the mitochondria play an important role in apoptotic cell death especially via intrinsic pathway.

On the other hand, mitochondria are the main source of reactive oxygen species (ROS) in many mammalian cell types. In normal condition, mitochondria are responsible for the generation of 60–80% of ROS in cells which can be raised in pathological conditions [11]. Several experimental studies have confirmed that excessive ROS generation directly leads to apoptosis [12]. Activation of caspase proteins, a biochemical hallmark of apoptotic cell death, are sensitive to ROS [10].

Therefore, toxic agent-induced mitochondrial dysfunction leads to increased generation of ROS and subsequently activation of caspase cascade and apoptosis [9].

The spleen is the largest secondary lymphoid organ that contains about 25% of the body's lymphocytes and plays a key role in immune system homeostasis and functions [13,14]. The spleen's damage can usually cause immune disorders through humoral and cell-mediated pathways [15].

Furthermore, the effects of AA on the induction of apoptosis in splenocytes and underlying detailed mechanisms have not been clarified to date. In this study, we planned to investigate the cellular and molecular mechanism underlying immunotoxic events induced by AA in splenocytes via assessment the mitochondrial oxidative damage as the main initiator of the apoptosis in cells. Also, the role of intrinsic and extrinsic pathway in AA-induce apoptosis were evaluated by measurement of activity of the main executive caspases in splenocytes.

2. Material methods

2.1. Cell culture and exposure to acrylamide concentrations

The splenocytes (obtained from mouse spleen based on the procedures approved by the ethical standards of Animal experimentation of Mazandaran university of medical sciences committee, Sari, Iran) were kept in DMEM (supplemented with 10% FBS, 2 mM L-glutamine and 100 mg/ml Pen/Strep) under humidified atmosphere of 95% air, 5% CO_2 at 37 °C. The cell count and viability were determined by trypan blue staining and a density of 1×10^6 cells/ml divided to several parts for evaluation of cytotoxicity, apoptosis and caspases activity tests. Then, AA was dissolved in DMEM and the splenocytes were exposed to different concentrations of AA (0, 5, 10 and 25 mM) for 2 h at 37 °C [16]. As usual, for determination toxic concentrations of AA, we exposed a range of concentrations to splenocytes (based on previous studies) and assayed the cytotoxicity by MTT test and then proper ranges of concentrations were chosen. Same cell counts (1×10^6 cells) were used for all experiment tests.

2.2. Detection of apoptosis in splenocytes

The annexin V assay is based on the ability of the protein annexin V to bind to phosphatidylserine (PS) exposed on the outer membrane leaflet in apoptotic cells. In viable cells, PS is located in the inner membrane leaflet, but after induction of apoptosis it is trans located to the outer membrane leaflet and becomes available for annexin V binding [17]. The annexin V assay was performed following the manufacturer's instructions (Annexin V-FITC kit, eBioscience, cat number: 88–8005). Briefly, 1×10^6 cells were washed with PBS and resuspended in binding buffer. Then 100 μl of cell suspension were mixed with 5 μl Annexin V-FITC and incubated for 10 min in the dark at room temperature. The cells were washed with binding buffer, resuspended in 200 μl binding buffer and then added 5 μl of propidium iodide staining solution and analyzed by flow cytometry (Partec, Deutschland) with the Flomax software (version 2.4) within 4 h.

2.3. Measurements of caspase-8 and −9 activities

Caspase-8 and −9 activities were determined using Caspase Apoptosis ELISA assay Kit (ZellBio GmbH, Germany) according to the manufacturer's suggestions. In brief, treated cells washed and diluted in PBS (pH = 7.2–7.4) to 1×10^6 cells/ml. the cell suspensions were damaged through repeated freeze- thaw cycles to let out the inside components. Then the cells centrifuged at 2000 RPM for approximately 10 min. the supernatants carefully collected and the caspase activity was performed as follows: 40 μl of supernatant + 10 μl caspase-Ab was mixed with 50 μl streptavidin- HRP reagent in pre-coated plate (with anti-caspase monoclonal antibody) and incubated in 37 °C for 60 min. the plate washed and 50 μl of chromogen A and B added. After 10 min incubation in 37 °C, stop solution was added and absorbance measured at 450 nm.

2.4. Mitochondrial dysfunction

2.4.1. Mitochondrial preparation

Mitochondria were prepared from mouse spleen using differential centrifugation. The spleen were minced and homogenized with glass handheld homogenizer. The homogenate suspension were centrifuged at 2000 × *g* for 10 min at 4 °C and the pellet was discarded. The supernatant was subjected to a further centrifugation at 10,000 × *g* for 10 min and the superior layer was carefully discarded. The mitochondrial pellet was washed and gently suspending in the isolation medium and centrifuged again at 10,000 × *g* for 10 min. Final mitochondrial pellets were suspended in Tris buffer at 4 °C, except for mitochondria used to assess ROS production (that respiratory buffer was used). The isolation of mitochondria was confirmed by the measurement of succinate dehydrogenase. Mitochondria were prepared fresh for each experiment and used within 4 h. All steps were strictly operated on ice to guarantee the isolation of high-quality mitochondrial preparation. the isolated mitochondria were divided to same density parts (0.5 mg protein/ml) for evaluation of oxidative damage parameters [11].

2.4.2. Evaluation of mitochondrial function

To investigate the effects of AA on mitochondrial activity, succinate dehydrogenase activity was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazoliumbromide) test. MTT is a substrate reduced by mitochondrial succinate dehydrogenase and generates a chlorophor product foramazan. Briefly, after mitochondrial isolation we added different concentrations of AA (0, 5, 10 and 25 mM) to mitochondria for 1 h. Then 0.4% of MTT was added to them and incubated at 37 °C for 30 min. Foramazan crystals were dissolved in 100 μl DMSO and the absorbance was measured at 570 nm with an ELISA reader (Tecan, Rainbow Thermo, Austria) [18]. Mitochondrial function was expressed as a percentage relative to the untreated control mitochondria.