Rapid Loss of Stress Fibers in Chinese Hamster Ovary Cells after Hyperthermia

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ABSTRACT

This study was initiated to characterize the effect of hyperthermia (41-45°) on the distribution of actin stress fibers in Chinese hamster ovary cells using rhodamine-conjugated phalloidin, a probe specific for F-actin. Fluorescent microscopy revealed a rapid loss of stress fibers after immersion in a 45° water bath. After 5-min immersion at 45°, approximately 90% of the cells analyzed did not contain observable stress fibers. Stress fibers were visible after incubation of cells at 37° after heating. The recovery of the appearance of the stress fibers occurred as protein synthesis resumed, and addition of protein synthesis inhibitors following heat treatment blocked the reappearance of these structures. These results support the hypothesis that cytoskeletal components may be a target of hyperthermia, explaining the pleotropic biological effects of heat and, in particular, heat radiosensitization.

INTRODUCTION

The biological effect of hyperthermia (41-45°) on mammalian cells has been extensively studied due to its potential role as an adjuvant to radiotherapy and chemotherapy in the treatment of cancer (18, 23). Although a wide range of biological effects is observed in response to hyperthermia, the molecular mechanisms of heat-induced cell death are not well understood. Based on the variety of observed metabolic changes which occur in cells after hyperthermia, one can easily view heat as a nonspecific agent resulting in many biological responses. Several investigators have concluded that hyperthermia results in both nuclear and cytoplasmic alterations (27).

Previous studies have implicated the plasma membrane as a major target for hyperthermic damage (22, 35). Heat treatment leads to an increase in membrane permeability, as measured by leakage of small polycations, the polyamines (e.g., putrescine, spermidine, and spermine) (16). Concomitantly, addition of exogenous polyamines will sensitize CHO3 cells to the lethal effects of heat, presumably through interaction at the level of the plasma membrane (2, 14). Increased uptake of drugs is also observed following heat (17), and in a wide variety of cell lines, heat-induced proliferative death is inversely proportional to membrane cholesterol content before the heat treatment begins (9). Although the molecular target(s) within the membrane are still speculative, these reports indicate that the plasma membrane is a cellular structure which is modified by hyperthermia.

Nuclear components also have been implicated in hyperthermic damage. A major consequence of heat is an increase in the chromatin protein content. HeLa cells when heated at 45° for 30 min result in an increase in the ratio of protein to DNA of approximately 1.5 over normal chromatin ratios. Since an alteration in the membrane permeability in the absence of heat also can cause an increase in chromatin protein content, the authors postulated that intact cellular structure was critical for the heat-induced increase in the ratios of protein to DNA (28, 29).

A cellular component which is common to the plasma membrane, cytoplasm, and the nucleus is the cytoskeleton, including the microfilaments, microtubules, and intermediate filaments (31, 36). The effect of hyperthermia on cytoskeletal structures in asynchronous cultures has not been extensively characterized. Coss et al. (8) reported that heating CHO cells (45.5°, 6 min) in mitosis disrupts the mitotic spindle, the actin containing contractile ring, and the midbody cytoplasmic bridge complex. In addition, the cortical microfilaments of CHO cells in mitosis are disrupted by heating at 41.5° for 7 hr (7). These studies are interesting, since they suggest that the mitotic structural apparatus is heat labile. The discovery of the cytoskeleton and the development of suitable probes have enabled us to investigate the structural elements of a cell population primarily composed of interphase cells after hyperthermia.

In an attempt to further characterize the effect of heat on cytoskeletal integrity, we have examined the distribution of a component of the actin microfilament system, the stress fibers. The stress fibers consist of bundles of 5-nm actin filaments, and their formation appears to be regulated through plasma-membrane interactions (1, 12, 21).

MATERIALS AND METHODS

Cells and Culture Conditions. CHO cells were maintained in exponential monolayer growth at 37° in McCoy's Medium 5A (Grand Island Biological Co. Laboratories, Santa Clara, CA) supplemented with 10% fetal bovine serum (v/v) (Grand Island Biological Co.), penicillin (100 units/ml), and streptomycin (100 μg/ml). The cultures are routinely maintained in a 5% CO2-95% air atmosphere incubator and checked for pneumonemia-like organism contamination. Under these conditions, the cell culture doubling time was 12 to 14 hr. Cells were harvested by scraping and counted using an electronic particle counter equipped with a Channelizer (Coulter Electronics, Inc., Hialeah, FL).

Cell Heating. For hyperthermic treatment, CHO cells were seeded at an initial density of 1.5 × 10⁶ cells/sq cm in 35- x 10-mm dishes (Falcon Plastics, Oxnard, CA) containing 2 ml of medium. At 24 hr after seeding the cells, the dishes were sealed with Parafilm (American Can Co., Greenwich, CT) and submerged in a precision controlled circulating water bath (temperature uniform and constant to ±0.05°) at 45° for the appropriate times. The water bath temperature was monitored by a mercury and glass thermometer calibrated by the method of Cetas and Connor (6). Control samples were sealed with Parafilm and incubated at 37°. The temperature of the medium was monitored using a Bailey thermocouple (Bailey Instruments, Saddle Brook, NJ).
Rhodamine-conjugated Phalloidin Staining of F-Actin. Cells to be analyzed for actin stress fibers were processed for treatment as described previously. At varying treatment times, the dishes were removed from the water bath, and the cells were washed twice with phosphate-buffered saline (70 mm NaCl-3 mm KCl-1 mm CaCl2-1 mm MgCl2, pH 7.3), fixed with 3.7% formaldehyde (Fisher Scientific Co., Fairlawn, NJ) at 20°C for 10 min, and permeabilized with absolute acetone at -20°C for 3 to 5 min. The dishes were washed free of acetone with 2 rinses with phosphate-buffered saline and allowed to air dry. Rhodamine-conjugated phalloidin (Molecular Probes, Inc., Junction City, OR) (165 ng/ml in phosphate-buffered saline) was added to the samples and incubated in the dark at room temperature for 30 min. The samples were then washed twice with phosphate-buffered saline and viewed in Zeiss Photomicroscope II (Carl Zeiss, Inc., New York, NY), standard Model, 18 equipped for the fluorescence studies using a mercury light source. Photographs were taken using Kodak Pan-X film (Eastman Kodak Co., Rochester, NY) with an automatic exposure apparatus to insure accurate intensities.

For sample counting, at least 5 random fields from each dish were counted for the presence of stress fibers which spanned the entire cell. The identity of the samples was unknown to the counters. Those cells which contained no discernible fibers or exhibited only barely distinguishable structures were scored as negative (25). The time points were performed in duplicate, repeated at least 3 times, and scored as the percentage of cells containing stress fibers.

Protein Synthesis Determinations. Protein synthesis rates were measured by incubating cells with [3H]leucine (2.5 µCi/ml; 15 Ci/mmol) (New England Nuclear, Boston, MA) for 15 min in McCoy's Medium 5A, modified to contain 10% of the normal leucine concentration, and supplemented with 10% (v/v) fetal bovine serum. Cells were harvested by scraping, and the amount of 10% trichloroacetic acid-insoluble radioactivity was measured (13). Each sample point represented triplicate determinations of at least 1.0 × 10⁶ cells. Cycloheximide and puromycin (Calbiochem/Behring Corp., La Jolla, CA) were used as protein synthesis inhibitors at media concentrations of 20 and 50 µg/ml, respectively. Under these conditions, one can observe a decreased incorporation of radiolabeled leucine into acid-insoluble material by greater than 90% within 10 min (data not shown).

RESULTS

Effect of Hyperthermia on Stress Fiber Distribution. To characterize the effect of hyperthermia on stress fiber integrity, we have used a fluorescent probe (rhodamine-conjugated phalloidin) which is specific for F-actin (33, 37). This probe is advantageous in that nonspecific interaction with G-actin is reduced, allowing optimal visualization of the filaments in intact cells. Asynchronously growing CHO cells were heated by immersion in a water bath at 45°C as described in "Materials and Methods." The samples were fixed and stained, and fields typical of each treatment time were photographed (Fig. 1). Unheated cells show stress fibers which span the entire length of the cell (Fig. 1A). The stress fibers begin to disappear after only 1 min of an immersion in the water bath (Fig. 1B), which is consistent with the micrographs seen in Fig. 1. After 15 min of immersion, no stress fibers are detectable in any sample examined (data not shown). The arrangement of F-actin into stress fibers is equally accessible to the heated and control cells independent of any change in cell size or shape. In addition, the visualization of the stress fibers is independent of cell rounding, since we observed rounded cells containing stress fibers (data not shown) and can observe several focal planes within the cells using the phase-contrast optics.

To quantitate the loss of stress fibers in a population, at least 5 representative microscopic fields were viewed from each time point, and cells were scored for the presence of the structures. Due to the rapid effect of hyperthermia on the distribution of stress fibers, we analyzed the cells at various times of immersion at 45°C and measured the temperatures of the monolayer at each time point. These data are shown in Chart 1. Under the conditions used in our assay, at 1 min of immersion, the monolayer temperature is 43°C and has reached an equilibrium at 45°C within 3 min (Chart 1B). We observe a decrease in stress fibers detectable in the cell population after 1 min with approximately 8% of the cells showing stress fibers at 5 min, and these data correspond to the micrographs seen in Fig. 1. After 15 min of immersion, no stress fibers were detectable in any sample examined (data not shown). The arrangement of F-actin into stress fibers is exquisitely sensitive to heat, since the cell population is depleted of these structures as the cells reach 45°C. Other mammalian cell lines were also analyzed for stress fiber distributions, and all exhibited a disappearance of these structures with hyperthermia, although the kinetics of the disappearance was different (data not shown).
Recovery of Stress Fibers after Hyperthermia. After observing the rapid loss of the stress fibers in response to heat, we analyzed the reversibility and recovery of the structures. The cells were immersed for 15 min at 45°C to ensure the complete disappearance of stress fibers, removed from the water bath, and incubated at 37°C. At times after removal from the heat treatment, the cells were processed and scored for stress fiber appearance. Again, the appearance of stress fibers is judged according to the number of cells in the population which show discernible structures. The recovery of the stress fibers is seen within 4 hr of incubation at 37°C after a 45°C, 15-min immersion treatment. At 5 hr after the heating, approximately 50% of the cell population contains distinct stress fibers (Chart 2). At 20 hr after returning to 37°C, greater than 90% of the cells in a population show detectable stress fibers (data not shown). The stress fibers that reappear are microscopically indistinguishable from those present in untreated cells.

Concomitant with these studies, protein synthesis rates of identically treated samples were performed by measuring the incorporation of [3H]leucine into 10% trichloroacetic acid-insoluble fractions. These data are shown in Chart 1. The recovery of protein synthesis rates parallels the reappearance of stress fibers. To test whether newly synthesized proteins were needed for the reformation of the stress fibers, the protein synthesis inhibitors cyclohexamide and puromycin were added to the samples after heat, and cells were analyzed for stress fibers. These data are shown in Table 1. At 6 hr after heat treatment, approximately 80% of the untreated populations displayed stress fibers, while those treated with the protein synthesis inhibitors only showed approximately 20 to 30% recovery. These data indicate that newly synthesized proteins are being utilized for the reformation of these structures, and studies are continuing to define the specific proteins utilized for this reformation.

Table 1

<table>
<thead>
<tr>
<th>hr after heating</th>
<th>Heat alone</th>
<th>Heat plus cyclohexamide</th>
<th>Heat plus puromycin</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>36.7 ± 14.1*</td>
<td>34.1 ± 6.9</td>
<td>22.2 ± 2.8</td>
</tr>
<tr>
<td>6</td>
<td>91.1 ± 4.4</td>
<td>24.4 ± 8.4</td>
<td>19.8 ± 6.8</td>
</tr>
<tr>
<td>8</td>
<td>93.3 ± 1.9</td>
<td>32.6 ± 8.4</td>
<td>38.9 ± 10.6</td>
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* Mean ± S.D. of at least 3 determinations.

DISCUSSION

The results presented here demonstrate that, when asynchronously growing CHO cells are exposed to elevated temperatures (45°C), there is a disappearance of stress fibers. This effect is rapid, in that visible alterations in the distribution are evident within 1 min of immersion at 45°C (Fig. 1). Complete loss of detectable stress fibers is seen after 15 min of immersion in a water bath, which corresponds to a 12-min incubation at 45°C (Chart 1). The effect is also reversible. When the cells were returned to 37°C, one sees no gross detectable change in the overall distribution of stress fibers as compared with unheated cells. The recovery of the stress fibers in the heated population occurs with the resumption of normal protein synthesis rates as measured by [3H]leucine uptake (Chart 2). The reformation of stress fibers is dependent upon newly synthesized proteins, since the addition of protein synthesis inhibitors following heat significantly reduces the reappearance of these structures (Table 1).

The molecular mechanisms for the formation of the microfilament bundles are not well known (21, 24). Many proteins in vitro have been shown to facilitate the polymerization of G-actin into microfilaments (21, 31). The generation of stress fibers has also been observed in vitro, and recently, Brown et al. (5) have purified an integral membrane protein which induces the "bundling" effect. In cell culture, attachment of cells to a substrate appears to be critical for the formation of nucleating centers from which stress fibers are generated (21). The directed nucleation requires transmembrane linkages (1) presumably through interaction with vinculin (12). Hyperthermia is known to induce plasma membrane alterations (22, 29, 35), and the loss of stress fibers in response to this treatment might be a reflection of changes in the plasma membrane. Ca2+ ion concentrations in vitro can also regulate the formation of stress fibers (10, 21), and rapid changes in ion distribution in response to heat could lead to a disassembly of the bundles. It is also probable that hyperthermia could cause the degradation of proteins involved in the formation of stress fibers. More detailed studies should determine the molecular mechanisms involved in the alterations of these structures. Due to the rapid effect of heat on the integrity of the stress fibers, heat may be a useful tool for the study of the formation of these structures in vivo.

Our results support and expand upon the observation of Coss et al. (8), who reported that treatment of mitotic CHO cells at 45.5°C led to a loss of the contractile ring and mitotic spindle. Our findings also could explain the 11-hr mitotic delay observed after heat treatment in asynchronously growing CHO cells of 45.5°C for 10 min reported by Westra and Dewey (38). The actin microfilaments play a role in cytokinesis and may interact in the process of chromosomal segregation (5, 30), and if the integrity of these structures is destroyed by heat treatment, cells would not be able to progress through mitosis. The loss of the contractile ring as shown by Coss et al. and the interphase stress fibers reported here in response to hyperthermia indicated that similar control mechanisms for formation of these 2 functionally
distinct structures exist. Transmembrane linkages may be important in the formation of the contractile ring during cytokinesis, and further studies need to confirm the functional regulation between stress fibers and the contractile ring.

Possible conflicting results have been described by Thomas et al. (34), who analyzed actin distributions in HeLa cells grown under heat-shock conditions. They reported changes in overall cell morphology and an increased distribution of actin filaments in response to culturing the cells in medium containing L-azetidine-2-carboxylic acid, a proline analogue. It is difficult to compare our observations with those of Thomas et al., since in their studies, the heat-shock conditions are approximated by treatment with a proline analogue, and another probe (anti-actin antibody) was used to identify the stress fibers.

Roti Roti and Winward have reported that, after the heat treatment of HeLa cells in vitro, there is a significant increase in the ratio of protein to DNA of isolated chromatin (27, 28). This effect was also observed after membrane disruption by a nonionic detergent and was not seen when isolated nuclei were heated. The authors concluded that a component of cellular structure was involved in mediating the heat-directed increase in chromatin protein content. We suggest a reasonable candidate for mediating this effect is the disruption of the actin stress fibers, a cellular component which extends throughout the cell. Our results show that hyperthermia can cause a rapid change in the distribution of stress fibers, which may affect the organization of subcellular structures. It is evident that the cytoplasm and nucleus are highly ordered structures and that major metabolic events (RNA, DNA, protein synthesis) are coupled to ordered subcellular structures. It is evident that the cytoplasm and nucleus are highly ordered structures and that major metabolic events (RNA, DNA, protein synthesis) are coupled to ordered subcellular structures. With the increasing availability of probes to analyze the cytoskeleton, it would be of interest to analyze in greater detail the effects of hyperthermia on these structures in intact cells. It should also be stressed that our analysis is limited to only one component of the complex cytoskeleton system and, in particular, to only one subset of the microfilament system, the stress fibers. Nuclear microfilament proteins have been described (11, 30), and if these are destroyed by hyperthermia, it might explain the reported nuclear alterations in response to heat (e.g., DNA repair blocks, chromosomal aberrations).

When biological effects are analyzed in response to a cytotoxic agent, it is useful to correlate the observed phenomenon with cell killing. Our data suggest that a direct correlation between the loss of stress fibers in CHO cells with cell death does not exist. After 15 min of immersion (12 min of incubation at 45°), there are no detectable stress fibers in the cell population, yet there is a 30% cell survival as determined by a colony-forming assay (20). However, to prove the relationship between the stress fiber disappearance and the cytotoxicity of hyperthermia will require detailed time and temperature studies.

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REFERENCES


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Fig. 1. Disappearance of actin stress fibers in response to hyperthermia. CHO cells were immersed in a 45° water bath and analyzed for the presence of stress fibers by immunofluorescence microscopy. Cells were either maintained at 37°C (A) or treated with hyperthermia for 1 min (B), 5 min (C), or 15 min (D).
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