Concurrent Collapse of Keratin Filaments, Aggregation of Organelles, and Inhibition of Protein Synthesis During the Heat Shock Response in Mammary Epithelial Cells

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Abstract. The sequence of heat shock–induced perturbations in protein synthesis and cytoskeletal organization was investigated in primary cultures of mouse mammary epithelial cells (MMEC). Exposure of the cells to 45°C for 15 min caused a marked inhibition of protein synthesis through 2 h after heat. Resumption of protein synthesis began by 4 h, was complete by 8 h, and was accompanied by induction of four major heat shock proteins (HSPs) of 68, 70, 89, and 110 kD. Fluorescent cytochemistry studies indicated that heat shock elicited a reversible change in the organization of keratin filaments (KFs) and actin filaments but had a negligible effect on microtubules. Changes in the organization of KFs progressed gradually with maximal retraction and collapse into the perinuclear zone occurring at 1–2 h after heat followed by restoration to the fully extended state at 8 h. In contrast, actin filaments disappeared immediately after heat treatment and then rapidly returned within 30–60 min to their original appearance. The translocation of many organelles first into and then away from the juxtanuclear area along with the disruption and reformation of polyribosomes were concurrent with the sequential changes in distribution of KFs. The recovery of the arrangement of KFs coincided with but was independent of the resumption of protein synthesis and induction of HSPs. Thermotolerance could be induced in protein synthesis and KFs, but not in actin filaments, by a conditioning heat treatment. Neither protein synthesis nor induction of HSPs was necessary for the acquisition of thermotolerance in the KFs. The results are compatible with the possibility that protein synthesis may depend on the integrity of the KF network in MMEC. Heat shock thus can efficiently disarrange the KF system in a large population of epithelial cells, thereby facilitating studies on the functions of this cytoskeletal component.

THE effects of temperature elevation on cellular physiology have been studied in widely divergent organisms from bacteria to man (58). The dramatic changes in gene programming characterized by the induction of a specific set of proteins, usually referred to as heat shock proteins (HSPs),[1] together with inhibition in the synthesis of most other cellular proteins have been recognized as the main features of the heat shock response (43, 51, 61). This response is thought, at the simplest level, to be homeostatic to protect the cell against the environmental insult and provide it with the capacity to survive the crisis and preserve normal cellular activities. However, the molecular mechanisms responsible for these modifications remain obscure.

Along with the rapid and transient reprogramming of transcription and translation, alterations in cellular morphology and cytoskeletal organization have been documented as additional characteristics of the heat shock response (6, 22, 42, 66–68, 73, 74). Heat shock–induced changes found in the cytoskeleton of mammalian cells have included disruption of actin filaments (22, 67, 68), disassembly of microtubules (42, 67, 68), and aggregation of vimentin filaments around the nucleus (6, 9, 66, 68, 73, 74). However, alterations of keratin filaments (KFs) in epithelial cells as a consequence of heat stress have not been described. In fact, van Bergen en Henegouwen et al. (68) have reported that heat shock had no effect on KFs in rat hepatoma cells.

The dynamic rearrangement and recovery of the cytoskeleton suggest that it might be involved in cellular adaptation to variations in environmental conditions. Several cellular functions such as protein synthesis (7, 38, 53), cellular metabolism (54), and positioning of organelles (5, 14, 19, 45, 49, 70), in addition to cell shape and motility, depend in part upon the cytoskeleton. Moreover, some HSPs appear to be associated with the cytoskeleton (e.g., 37, 50, 52, 56). This has led to the idea that perturbations in cytoskeletal organization are linked to inhibition of protein synthesis, induction of HSPs, and development of thermotolerance, as well as cell toxicity and death precipitated by heat shock (6, 9, 64, 67,

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1. *Abbreviations used in this paper*: AO, acridine orange; HSPs, heat shock proteins; KFs, keratin filaments; MMEC, mouse mammary epithelial cells.

68, 76). However, the exact relationships among these events and the different cytoskeletal constituents have not been clearly defined and may vary depending on cell type.

In the present study, we have investigated the effects of heat shock on the cytoskeleton of mouse mammary epithelial cells (MMEC) growing in primary culture, with particular attention focused on KFs. Unlike most established epithelial cell lines which contain vimentin filaments as well as KFs, MMEC in primary culture have only KFs (2, 3). Our results demonstrate that upon heat shock, the KFs reversibly withdraw from their usual extended state and aggregate around the nucleus. Later, the filaments redistribute to their normal configuration. The kinetics of this sequence parallel the inhibition and subsequent resumption of protein synthesis, the disassembly and reassembly of polyribosomes, and the translocation of several organelles first into and then out of the juxtanuclear region. The response of actin filaments and microtubules to heat implies that they are not associated with these activities.

Materials and Methods

Reagents

All chemicals and drugs, unless otherwise stated, were purchased from Sigma Chemical Co., St. Louis, MO. DME, FBS, Hank's balanced salt solution (HBSS), and gentamicin were supplied by Gibco Laboratories, Grand Island, NY. Ultrapure urea was from Schwarz/Mann, Cambridge, MA. Chemicals and reagents used for PAGE, including mol wt standards, were obtained from Bio-Rad Laboratories, Richmond, CA. [^{35}S]Methionine was from Amersham Corp., Arlington Heights, IL. Rhodamine-conjugated phalloidin and acridine orange (AO) were purchased from Molecular Probes Inc., Eugene, OR. Cytochalasin D was bought from Aldrich Chemical Co., Milwaukee, WI. Rabbit antitubulin and antikeratin antisera were supplied by Polysciences, Inc., Warrington, PA, and Dako Corp., Santa Barbara, CA, respectively. The mouse monoclonal antibody, C4, which recognizes an epitope present on all known actins, was the generous gift of Dr. James Lessard, Children's Hospital Research Foundation, Cincinnati, OH. All FITC-conjugated secondary antibodies were obtained from Cooper Biomedical, Inc., Malvern, PA. Rhodamine 123 was from Eastman Kodak Co., Rochester, NY.

Cell Cultures

Primary cultures of MMEC growing on glass or plastic substrata were prepared from normal mammary tissue as described previously (1). Briefly, mammary tissue was removed from midpregnant BALB/c mice and digested proteolytically to obtain single cells and small clumps of cells. The cells were grown in 55-cm^2 plastic petri dishes either directly on the plastic or on 18-mm^2 glass coverslips. Cultures were incubated at 37°C in DME containing 13% FBS, 18 mM Hepes, and 50 μg/ml gentamicin.

Heat Shock

The medium on subconfluent cells grown for 44–46 h after plating was exchanged with fresh growth medium and allowed to equilibrate to incubator conditions before heat shock. Petri dishes containing the cells were completely sealed with parafilm and heat shocked by immersing in a water bath (model FK-2; Haake Buchler Instruments, Inc., Saddle Brook, NJ) thermoregulated at ±0.1°C for an indicated temperature and time. After heating, cells were postincubated at 37°C as required.

Drug Treatment

Fresh preparations of 10 μg/ml cycloheximide and 50 μg/ml colchicine were made in medium immediately before use. As these chemicals are water soluble, controls received medium without the drugs. Cytochalasin D was first dissolved in methyl sulfoxide and then diluted in medium to 2.5, 5, or 10 μg/ml. Controls received an equivalent amount of methyl sulfoxide in medium. Drug treatments were performed by aspirating the medium and replacing it with medium containing the drug, followed by incubation at 37°C for an indicated time. The drug-containing medium was then washed away with HBSS and replaced with regular medium.

Radiolabeling and Gel Electrophoresis

For radiolabeling, the culture medium was replaced with methionine-free medium and 10 μCi/ml [^{35}S]methionine (>800 Ci/mmol) was added. After incubation for 30–60 min as indicated for each experiment, the cells were rinsed three times with cold PBS without calcium and magnesium, harvested by scraping, and then washed twice with cold PBS. Cell pellets were extracted with lysis buffer containing 9.2 M urea, 5 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride, followed by centrifugation at 13,000 rpm (Beckman Instruments Inc. microfuge, Fullerton, CA) for 15 min. The clear supernatants were stored at −30°C before one-dimensional PAGE which was performed using the discontinuous, Tris-glycine system of Laemmli (32) with 12.5% polyacrylamide gels. Samples prepared in urea lysis buffer were mixed with an equal volume of sample buffer containing 2% SDS, 250 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 20% glycerol, and 0.0008% bromphenol blue, and boiled for 2 min. After determination of protein concentrations in each sample (Bio-Rad Laboratories assay), equal amounts of protein were loaded into each well of the gels. Molecular weights were estimated from Bio-Rad Laboratories low and high molecular weight standards. Gels were stained with Coomassie Brillinat Blue. For autoradiography, gels were dried and exposed to Kodak XAR film for 24–48 h.

Cytochemistry

Before staining actin filaments and microtubules, cells on coverslips were rinsed briefly in PBS, fixed in 2% formaldehyde in HBSS for 30 min at room temperature, and then stored in PBS at 4°C. Rhodamine-conjugated phalloidin was used as a direct probe to decorate actin filaments (65). Cells were permeabilized with cold acetone (−20°C) for 5 min, incubated with rhodamine-conjugated phalloidin (10 U/ml) at room temperature for 30 min, rapidly rinsed with PBS, and mounted on a slide in glycerol–PBS. Alternatively, the cells were stained by indirect immunofluorescence with the monoclonal antiactin antibody, C4. To detect microtubules, permeabilized cells were stained with a rabbit antitubulin antiserum by indirect immunofluorescence (2). KFs were visualized in cells that had been fixed in absolute methanol, permeabilized in acetone, and stained by indirect immunofluorescence using a rabbit antiserum prepared against bovine muzzle keratins.

The distribution of mitochondria was monitored with rhodamine 123 (29). Cells were washed to remove medium and then incubated at room temperature with DME (no serum) containing 10 μg/ml rhodamine 123 for 10 min. They were thoroughly washed and mounted in DME for immediate examination and scoring. AO was used as a fluorescent agent to label lysosomes and endosomes (45). Living cells were rinsed with HBSS containing 2 mg/ml BSA and incubated for 1 min at room temperature with HBSS–BSA containing 2 μM AO. They were then washed to remove excess AO and mounted with HBSS–BSA for immediate visualization. Stock solutions of rhodamine 123 (1 mg/ml) and AO (2 mM) were freshly prepared before use.

All stained cells were examined in an Olympus Corp. of America (New Hyde Park, NY) photomicroscope equipped with epifluorescence illumination. Fluorescent micrographs were taken on Kodak Tri-X film using either a 20× or 50× objective.

Electron Microscopy

Cells grown on coverslips were fixed in 1% glutaraldehyde in 50 mM cacodylate buffer (pH 7.4), postfixed in 1% OsO_4, and flat embedded in Epon–araldite. Silver sections were cut parallel to the substratum, stained with 5% aqueous uranyl acetate and Reynold's lead citrate, and examined in a Siemens-Allis Inc. (Cherry Hill, NJ) model 101 electron microscope.

Results

Protein Synthesis After Heat Shock

The effects of heat shock on protein synthesis and the expression of HSPs were examined in MMEC grown in primary culture. Fig. 1 shows the autoradiographic analysis of proteins synthesized after heat treatment and subsequent postincubation at 37°C. A heat shock of 45°C/15 min (Fig. 1 *A*) elicited a characteristic response with marked inhibition and then recovery in the synthesis of normal cellular proteins ac-

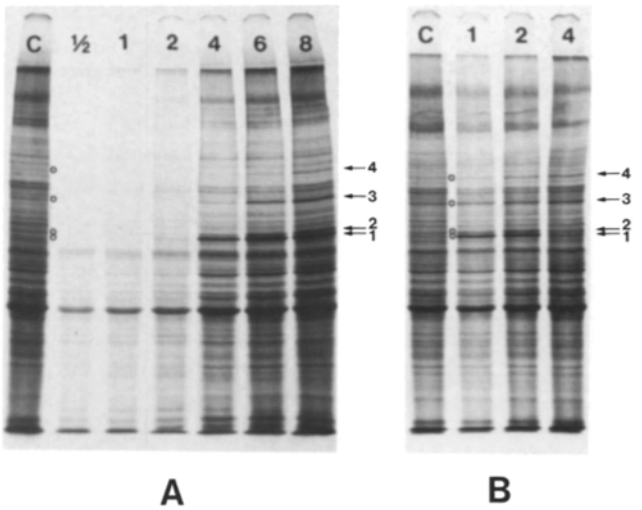

Figure 1. Time-course comparison of the effect of heat shock on protein synthesis in primary cultures of MMEC. Cells were exposed to (*A*) 45°C for 15 min, or (*B*) 43°C for 30 min. *C*, untreated control (37°C). Numbers at the top of lanes indicate hours after heat treatment. Cells were heat shocked and postincubated at 37°C, and labeled with [^{35}S]methionine for 1 h (except the lane of 1/2 h after a 45°C/15 min heat, which was labeled for 30 min with double the amount of [^{35}S]methionine) before extraction. The cells were then lysed and processed for SDS-PAGE and autoradiography. The major HSPs induced at 68, 70, 89, and 110 kD are indicated by arrows *1*, *2*, *3*, and *4*, respectively. Note that these HSPs are also synthesized, but at lower levels, in control cells at 37°C (marked by circles).

companied by induction of HSP synthesis. Four major HSPs of ~68, 70, 89, and 110 kD were induced, although low levels of these proteins were constitutively produced in untreated cells. Kinetic studies indicated that strong inhibition of synthesis of most cellular proteins occurred within 30 min after exposure to heat and continued through 2 h. Thereafter, protein synthesis gradually resumed as seen at 4 and 6 h, with a normal level of protein synthesis attained by 8 h after the 45°C treatment. The induction of HSPs was first observed at 4 h after heat treatment, increased at 6 h, and reached maximum production by 8 h after heat. A similar but less severe response was induced by a 43°C/30 min heat shock (Fig. 1 *B*) with the sequence of events occurring more rapidly. In contrast, a 41°C/30 min treatment did not induce any of the HSPs or disturb protein synthesis (data not shown).

Alterations in Cytoskeletal Organization Induced by Heat Shock

The state of the three major cytoskeleton elements, KFs, actin filaments, and microtubules, in MMEC after exposure to heat was examined by fluorescence microscopy.

Keratin Filaments

Indirect immunofluorescence demonstrated an elaborate latticework of KFs coursing throughout the cytoplasm of the MMEC before heat (Fig. 2 *a*). Immediately after subjection of cells to a heat shock of 45°C/15 min, significant retraction of KFs was noted in cells at the periphery of the colonies (Fig. 2 *b*), whereas obvious changes in cells within the colonies were not discernible until ~30 min later (Fig. 2 *c*). Retraction of the filaments reached a maximum at 1–2 h after heat, when the KFs were found in a dense perinuclear aggregate in all epithelial cells examined (Fig. 2, *d* and *e*). Two-dimensional PAGE analysis of cytoskeletal extracts from the MMEC at 2 h after heat shock showed that the set of keratins comprising the KFs was the same as that in unheated cells (data not shown). By 4 h the KFs began to reextend from the juxtanuclear region toward the cell periphery. The dispersal was more obvious at 6 h, and the fully extended state was present by 8 h after heat (Fig.2, *f–h*).

Similar effects on the KFs were induced by a heat shock of 43°C/30 min, but the extent and duration was less than that elicited by 45°C (data not shown). In this case, maximum retraction of KFs occurred at 0.5–1 h after heat with recovery underway by 2 h and redistribution of KFs complete by 4 h. For both of these temperatures, the time and sequence of the changes in the organization of KFs coincided with the inhibition and recovery of protein synthesis in response to the heat shock (e.g., compare Figs. 1 *A* and 2). Furthermore, a treatment of 41°C/30 min had no effect on either KFs or protein synthesis (not shown).

Actin Filaments

In control, unheated cells the actin filaments visualized by rhodamine–phalloidin appeared to crisscross throughout the cytoplasm of each cell (Fig. 3 *a*). Immediately after exposure to 45°C/15 min, actin filaments were no longer detectable (Fig. 3 *b*) and cell–cell boundaries, which were almost completely obscured by the density of actin cables in stained cells before heat, became clear and distinct. The reappearance of actin cables during the recovery period was rapid. By 30 min after the end of the heat treatment, most of the actin filaments were again demonstrable and by 60 min they had returned to their preheated state (Fig. 3 *c*). A similar change occurred after a 43°C heat shock (Fig. 3, *d* and *e*). However, the disruption of actin cables immediately after this lower heat treatment was less severe, and reorganization to their preheated appearance was evident earlier (30 min after heat). Despite the effects on actin filaments, the cells retained a flattened morphology during and after both of these heat treatments. No changes in the arrangement of actin filaments were observed in cells exposed to 41°C (Fig. 3 *f*). The same results were obtained using an antiactin antibody to stain the cells by indirect immunofluorescence (data not shown).

Microtubules

The array of microtubules remained intact during and at least 8 h after heat treatments of 41–45°C (Fig. 4), although slight modifications in their appearance were observed in some cells. In such cells, the microtubules appeared to curve at the cell edge instead of extending directly toward the cell periphery.

Effect of Inhibition of Protein Synthesis on the Organization of KFs

To determine if the inhibition of protein synthesis was responsible for the altered distribution of KFs, the effect of cycloheximide, which blocks protein synthesis, on the organization of KFs in MMEC was studied. Cells were incubated

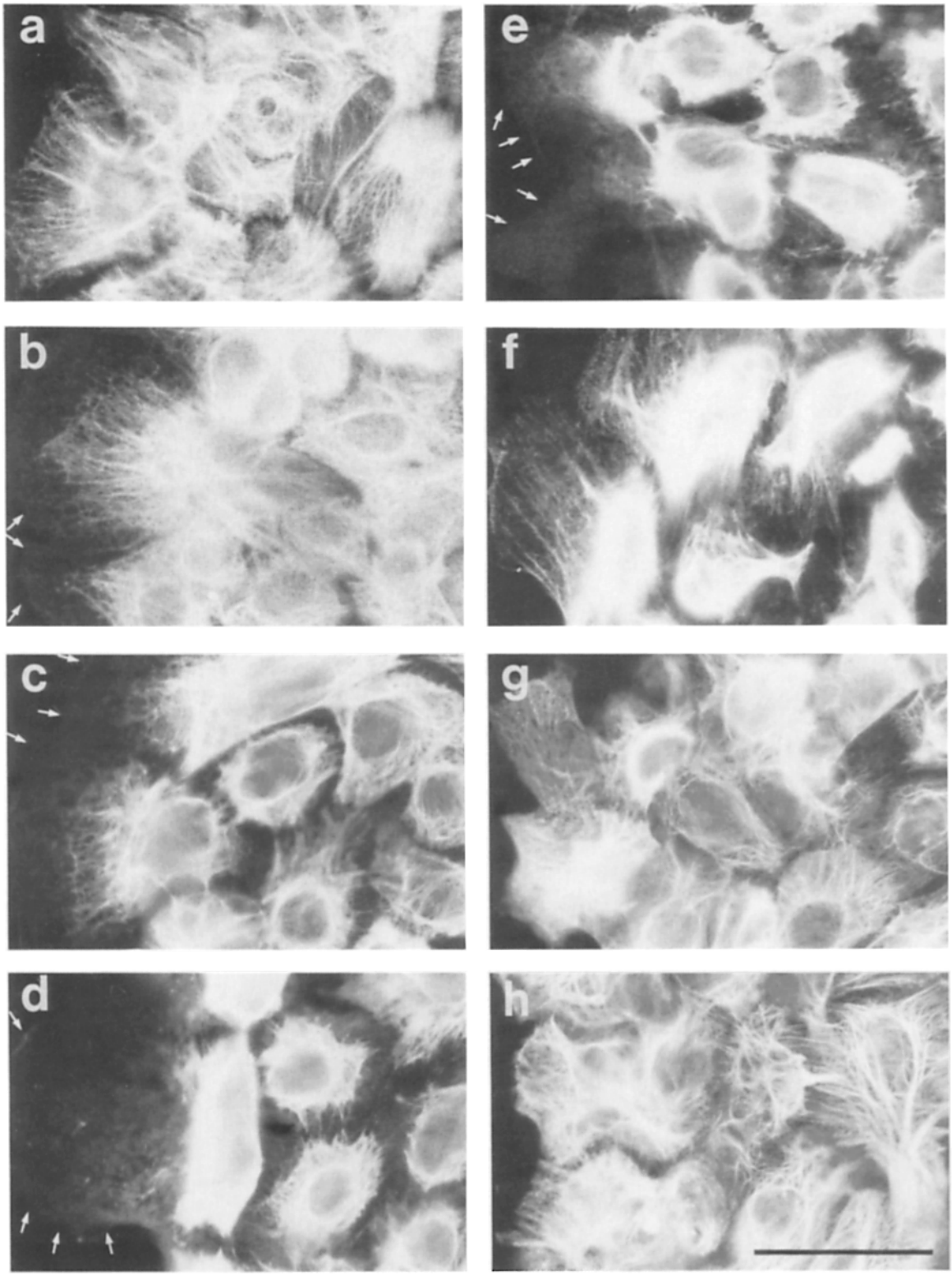

Figure 2. Changes in the arrangement of KFs induced by 45°C/15 min heat shock in MMEC as visualized by indirect immunofluorescence using an antikeratin antiserum. *a*, unheated control; *b*, immediately after heat; *c–h*, 0.5, 1, 2, 4, 6, and 8 h, respectively, after heat. Arrows indicate cell periphery. Bar, 50 μm.

in the presence of cycloheximide for various times and then labeled with [^{35}S]methionine for 30 min before harvesting. As shown in Fig. 5 *A*, strong inhibition in the synthesis of all proteins was induced by the drug, but the extended arrangement of KFs remained undisturbed through at least 4 h of treatment (Fig. 5 *B*). Thus, cycloheximide at the concentration used did not affect the intricate organization of the KF network nor cause its withdrawal from the cortical region of the cell.

The correlation between the recovery of KFs and the re-

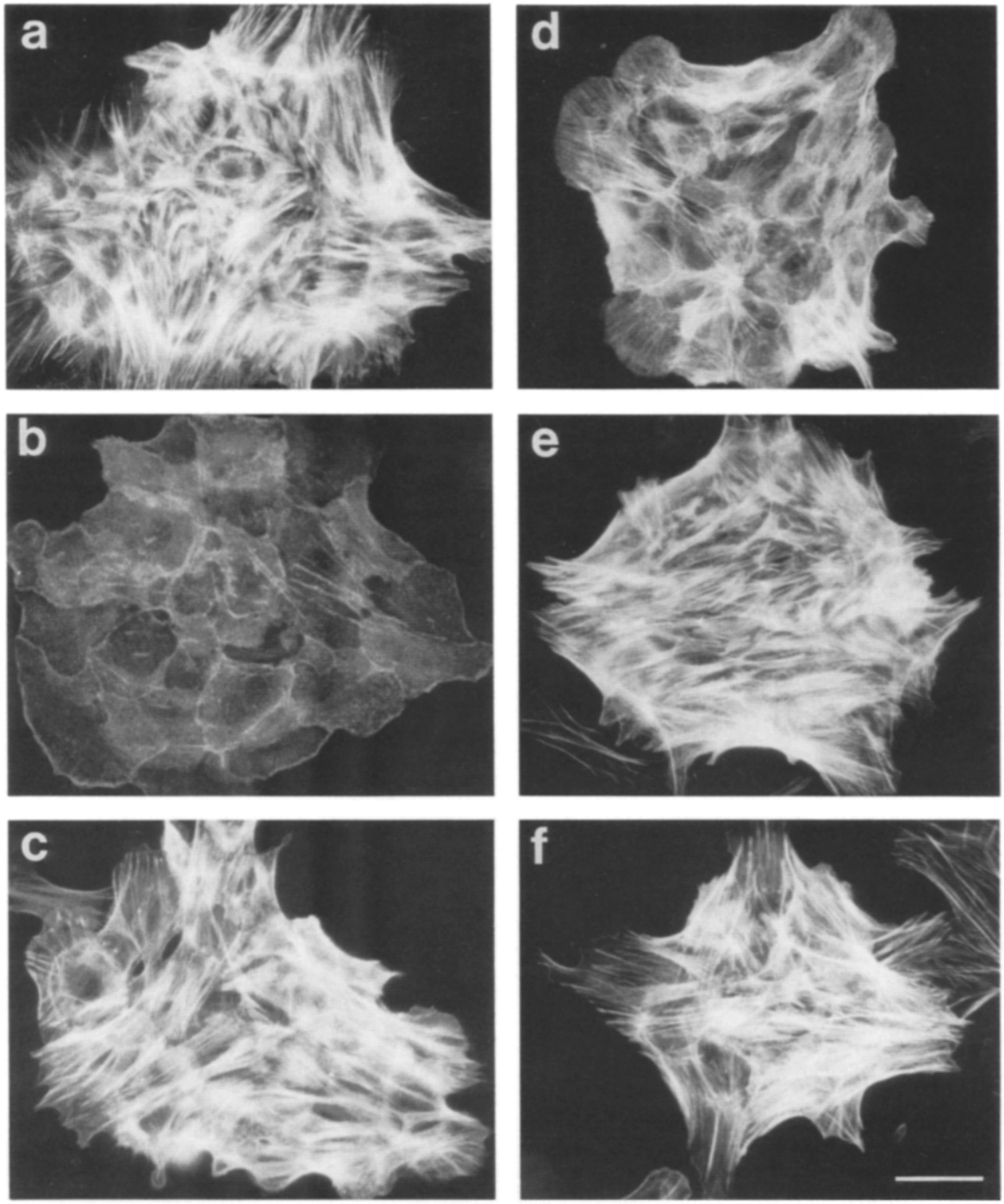

Figure 3. Effect of heat stress on actin filaments in MMEC monitored by staining with rhodamine–phalloidin. *a*, unheated control; *b* and *c*, immediately and 1 h after a 45°C/15 min heat shock. *d* and *e*, immediately and 0.5 h after a 43°C/30 min heat shock. *f*, immediately after a 41°C/30 min heat shock. Note the flattened shape of the cells in *b*. Bar, 50 μm.

sumption of protein synthesis raised the question as to whether protein synthesis was necessary for reextension of KFs. To examine this point, heat-shocked (45°C) cells were postincubated in the presence of cycloheximide and recovery of the KF system was monitored. Although the cycloheximide inhibited both the recovery of protein synthesis and induction of HSPs, the kinetics of reorganization of the KFs were not altered (data not shown), indicating that the recovery of KFs was independent of protein synthesis and the induction of HSPs.

Induction of Thermotolerance in the Cytoskeleton and Protein Synthesis

When cells are subjected to an initial heat stress and allowed to recover, they usually acquire thermotolerance; i.e., they are resistant to the effects of a second heat insult (21, 27). To determine if a conditioning heat treatment could preserve cytoskeletal organization in the MMEC, cells were given an initial heat treatment either at 45°C/15 min with a postincubation at 37°C for 8 h or at 43°C/30 min followed by

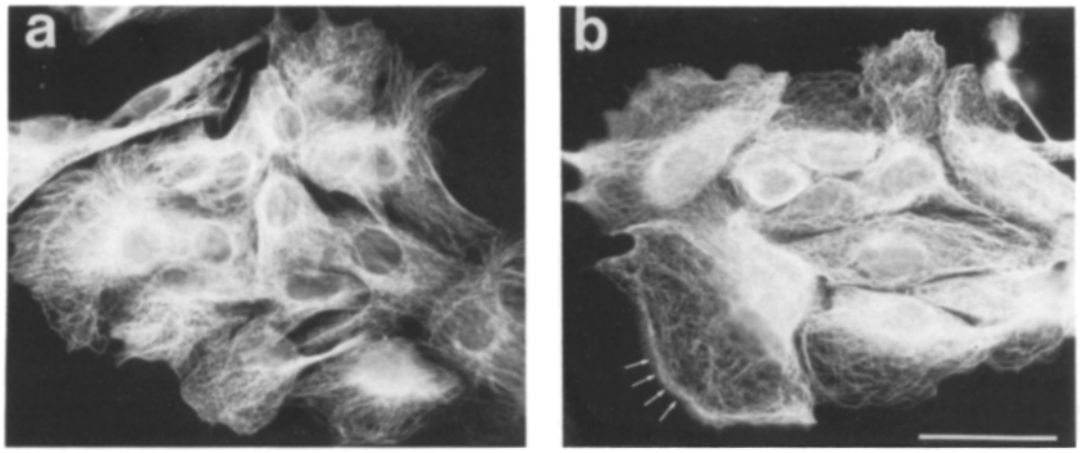

Figure 4. Effect of a 45°C/15 min heat shock on microtubules in MMEC detected by indirect immunofluorescent staining with an antitubulin antiserum. *a*, unheated control; *b*, 1 h after heat treatment. Microtubules appeared unchanged in the majority of MMEC after heat treatment. In a few cells the microtubules appeared to curve at the cell periphery after heat shock, producing a rounded cell edge (indicated by arrows in *b*). Bar, 50 μm.

postincubation at 37°C for 4 h. During these periods the heat-induced changes in the cytoskeletal arrangement were completely restored (refer to Figs. 2 and 3). Unlike KFs in unconditioned cells, the KFs in cells which had been preheated at 43 or 45°C were not disturbed by a challenging heat treatment at 45°C/15 min (Fig. 6), demonstrating that the KFs had acquired thermotolerance and could retain their normal distribution.

In contrast, no thermotolerance was developed in actin filaments by a conditioning heat treatment. A rapid disappearance of actin cables was noted immediately after a challenging treatment in preheated cells (Fig. 6), indicating that the actin filaments were not protected.

A conditioning heat treatment did confer thermotolerance on protein synthesis upon subsequent challenging heat stress (Fig. 6). The strong inhibition of protein synthesis observed at 1 and 2 h after a primary 45°C/15 min heat shock alone (refer to Fig. 1) did not occur in preconditioned cells subjected to the same challenge (Fig. 6), confirming that the translational machinery, like the KFs, was protected by the initial heat conditioning.

Heat stress induces both HSPs and thermotolerance, and protein synthesis is thought to play an important role in the development of thermotolerance (36, 61, 62). We therefore conducted experiments to determine if there was a connection between these two events in the conditioning-induced preservation of the organization of KFs. When cycloheximide was present during the recovery period after the conditioning heat treatment to inhibit both the resumption of normal protein synthesis and the induction of HSPs, thermotolerance of KFs still developed comparable to that observed in cells postincubated without the drug (Fig. 6). Thermotolerance was not induced in the KFs by cycloheximide treatment alone without a conditioning heat stress (not shown).

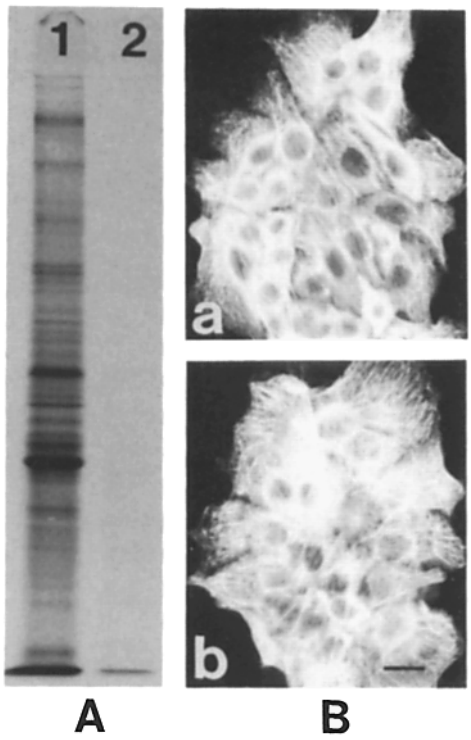

Figure 5. Effect of cycloheximide on (*A*) protein synthesis and (*B*) organization of KFs in MMEC. Cells were treated with 10 μg/ml cycloheximide for 4 h, and protein synthesis was analyzed by labeling with [^{35}S]methionine for the last 30 min. (*A*) Autoradiogram of proteins separated by PAGE. (*B*) Indirect immunofluorescence using an antikeratin antiserum. Lane *1* and *a*, untreated control; lane *2* and *b*, samples exposed to the drug for 4 h. Despite the inhibition of protein synthesis induced by the drug (*A*), the arrangement of KFs remained unaltered (*B*). Bar, 50 μm.

Effects of Colchicine and Cytochalasin D on Protein Synthesis

Although heat shock–induced inhibition of protein synthesis was concurrent with the collapse of KFs while microtubules were relatively undisturbed, we wanted to verify the status of protein synthesis in the absence of microtubules. In MMEC, colchicine affected microtubules but not KFs (Fig. 7), similar to other epithelial cells (18, 63). Cells were therefore incubated with colchicine and protein synthesis was analyzed at different times by radiolabeling with [^{35}S]methionine for 1 h. The results demonstrated that the drug did not inter-

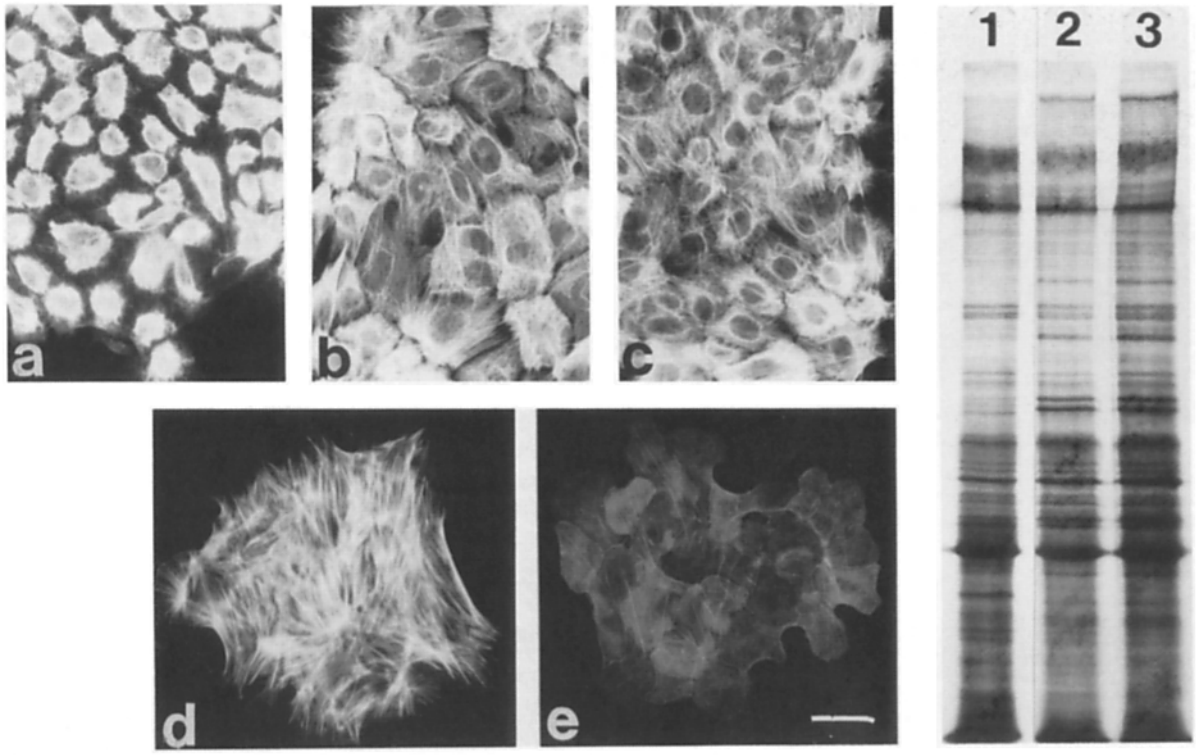

Figure 6. Demonstration of thermotolerance in KFs and protein synthesis in MMEC. (*a–c*) Cells stained by indirect immunofluorescence with an antikeratin antiserum; (*d* and *e*) cells stained with rhodamine-phalloidin. (*a*) Unconditioned cells at 2 h after a 45°C/15 min heat shock; (*b*) preconditioned cells 2 h after a challenging 45°C/15 min heat shock; (*c*) same as *b* except the cells were exposed to cycloheximide (10 μg/ml) during the recovery period after the conditioning heat treatment; (*d*) unheated control cells; (*e*) preconditioned cells immediately after a challenging 45°C/15 min heat shock. The same results were obtained when the MMEC were preconditioned by exposure to 43°C/30 min. Lanes *1*, *2*, and *3*, autoradiogram of proteins labeled with [^{35}S]methionine and separated by PAGE. Lane *1*, control, unheated cells; lane *2*, 1 h; and lane *3*, 2 h after a challenging heat treatment of heat-conditioned cells. The strong inhibition of protein synthesis observed in unconditioned cells (refer to Fig. 1) did not occur in conditioned cells (lanes *2* and *3*). See Fig. 1 for details of radiolabeling. Bar, 50 μm.

fere with protein synthesis during a 2–4-h treatment (not shown), indicating that the disruption of microtubules, which had no effect on KFs, had no effect on translational efficiency. The effect of colchicine treatment on the recovery of protein synthesis after heat was also examined. Heat-shocked cells were postincubated in the presence of colchicine from 1 to 8 h after heat. The resumption of protein synthesis, induction of HSPs, and reextension of KFs were not altered by the drug (data not shown).

To determine if disruption of actin filaments would interfere with protein synthesis, the MMEC were incubated with cytochalasin D for 2 h and radiolabeled with [^{35}S]methionine during the second hour of treatment. Despite the drastic disruption of the actin filament system and distortion of the arrangement of the KF network (Fig. 7), protein synthesis remained unchanged after exposure to the drug at concentrations through 10 μg/ml (data not shown). The KFs were aggregated into large bundles, although they still appeared to extend to the cell periphery and were not collapsed around the nucleus (Fig. 7).

Aggregation of Organelles After Heat Shock

The cytoskeleton has been implicated in maintaining the distribution of organelles in the cell (5, 14, 19, 45, 49, 70). Moreover, it has been suggested that mitochondria may be primary targets of the effects of heat shock (4, 35). We therefore examined the effect of heat shock on the positions of mitochondria, lysosomes, and endosomes. Living cells were stained with rhodamine 123 to label the mitochondria. In control, unheated cells the stained structures were distributed randomly throughout the cytoplasm (Fig. 8 *a*). A 45°C/15 min heat shock resulted in a concentration of mitochondria in the juxtanuclear region. This was apparent by 0.5 h and continued through 2 h after heat (Fig. 8 *b*). Thereafter, the clustered mitochondria began to move away from the cell center and redistribute in the cytoplasm. The transposition into dispersed locations was observed by 6–8 h after heat (Fig. 8 *c*). Marked morphological changes in the mitochondria were also evident during the latter stages. The mitochondrial conformation became grossly elongated as compared to the more rounded, compact shape seen in unheated cells or cells at 2 h or earlier after heat shock (Fig. 8).

Lysosomes and endosomes were monitored during the heat shock response by staining with AO. As compared with control cells (Fig. 8 *d*), significant aggregation of the AO-labeled structures into the perinuclear zone was induced within 2 h after exposure to a 45°C/15 min heat shock (Fig. 8 *e*). By 4–6 h, these structures began to move away from the cell center with restoration to their original distribution complete by 8 h after heat (Fig. 8 *f*). The dynamic changes in the positions of mitochondria, lysosomes, and endosomes induced by heat thus coincided with the aggregation and reextension of KFs during the heat shock response.

In contrast to the effects of heat, treatment of the MMEC

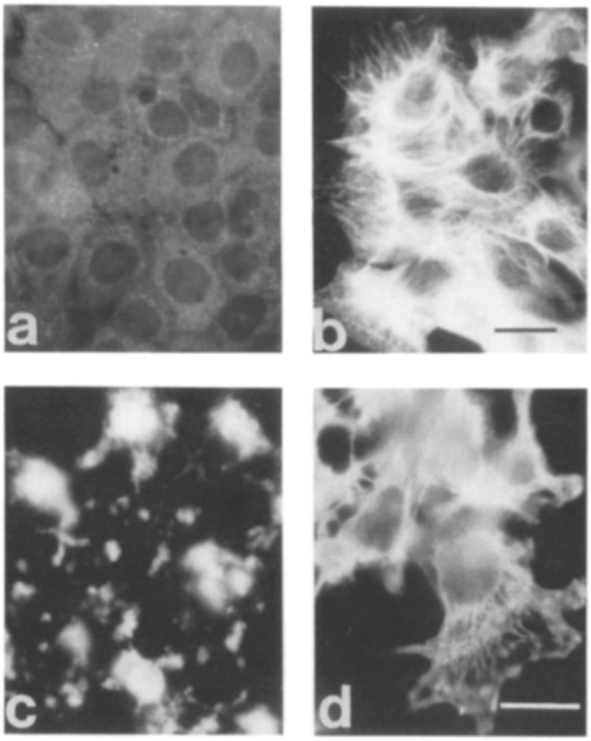

Figure 7. The effects of colchicine and cytochalasin D on the cytoskeleton in MMEC. (*a* and *b*) Cells incubated with colchicine for 2 h and then stained by indirect immunofluorescence with (*a*) antitubulin or (*b*) antikeratin antiserum. (*c* and *d*) Cells treated with 10 μg/ml cytochalasin D for 2 h and then stained either (*c*) with rhodamine-phalloidin or (*d*) by indirect immunofluorescence with an antikeratin antiserum. Note that in *d*, the KFs appear aggregated into bundles but still extended to the cell periphery. Bars: (*a* and *b*) 25 μm; (*c* and *d*) 50 μm.

with colchicine at concentrations which completely disrupted microtubules produced no change in the location of organelles (data not shown). The effects of cytochalasin D on organelle positions were difficult to assess because of the dramatic change in cell shape induced by the drug.

Consistent with the fluorescent cytochemistry results, electron microscopy confirmed the presence of numerous bundles of 8-nm filaments along with an abundance of organelles clustered next to the nucleus at 1–2 h after a 45°C/15 min heat treatment (Fig. 9). The elongation of mitochondria at 6–8 h after heat was also evident. In addition, as observed in other cell types (46), polyribosomes disappeared and later reappeared in the stressed MMEC (Fig. 9). The time course for the latter alterations was concomitant with those in protein synthesis and KFs. Only single ribosomes were scattered throughout the cytoplasm through 2 h after heat, whereas polyribosomes were first apparent by 4 h and were plentiful by 8 h (Fig. 9).

Discussion

The present studies reveal that upon exposure to heat, dramatic reversible alterations in KFs and actin filaments are induced in MMEC. These changes include the retraction of KFs from the plasma membrane, followed by their collapse into a perinuclear aggregate, and rapid disappearance and subsequent reappearance of actin filaments. In contrast, only slight changes in the network of microtubules were noted. Of particular interest was the finding that the kinetics of the sequential derangement and recovery in the organization of KFs, but not actin filaments, closely paralleled the inhibition and resumption of protein synthesis in the cells. The differences in time course and acquisition of thermotolerance im-

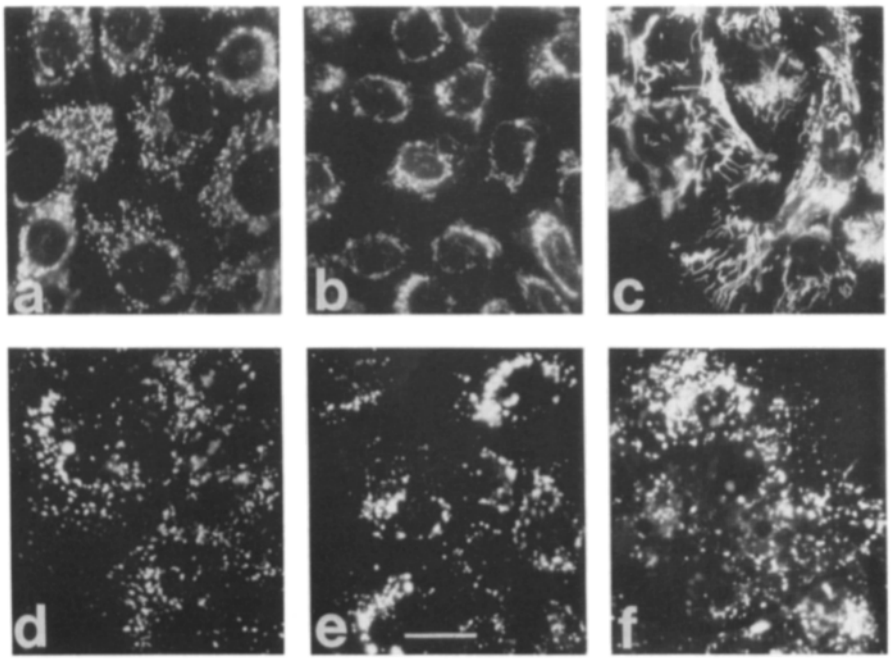

Figure 8. The distribution of mitochondria, lysosomes, and endosomes in living MMEC after a 45°C/15 min heat shock. (*a–c*) Cells stained with rhodamine 123. (*d–f*) Cells stained with AO. (*a* and *d*) Unheated control; (*b* and *e*) 2 h after heat shock; (*c* and *f*) 8 h after heat shock. Bar, 25 μm.

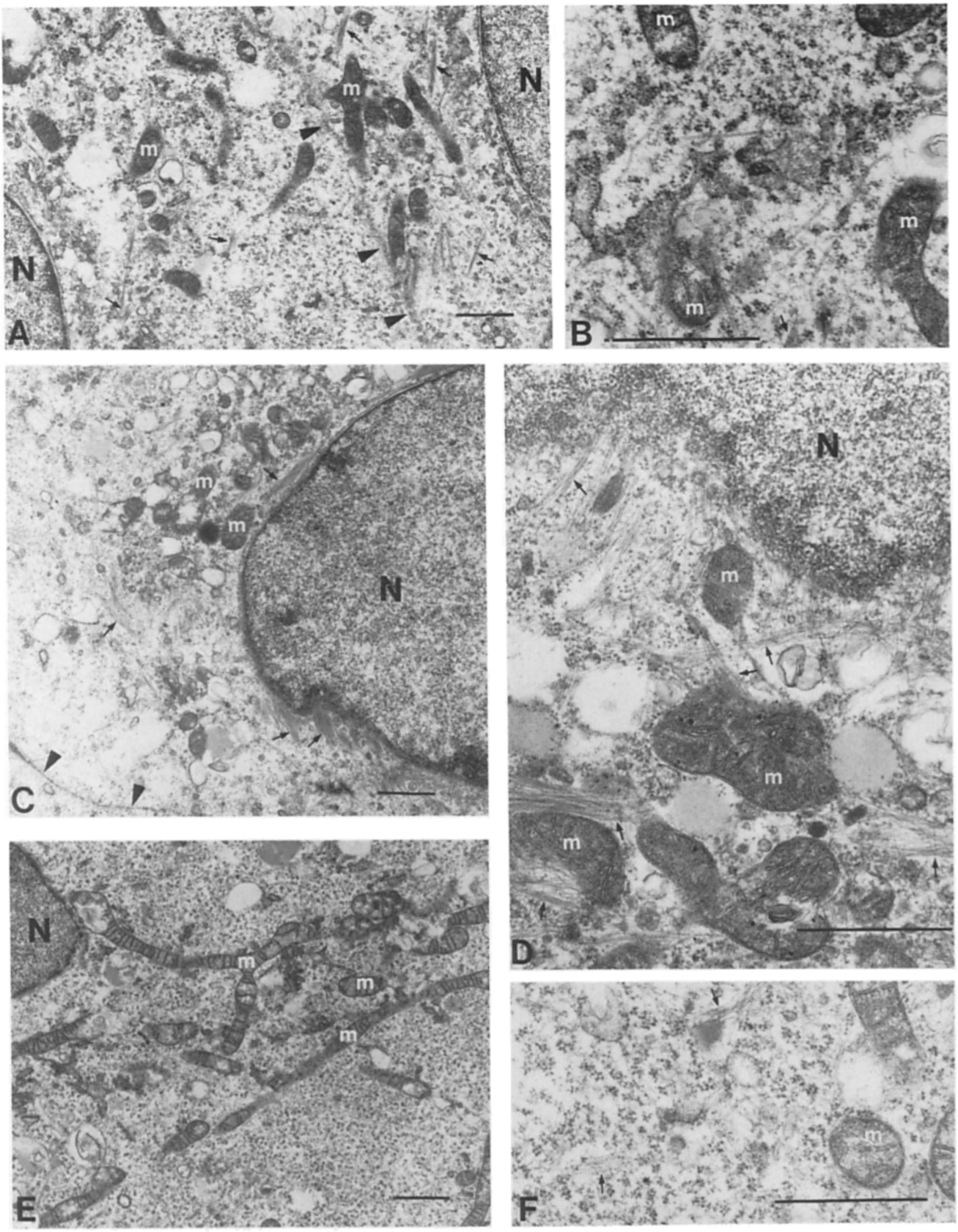

Figure 9. Electron micrographs of control and heat-stressed MMEC. Cells were either (*A* and *B*) untreated; or subjected to a 45°C/15 min heat shock, and then fixed and processed for electron microscopy (*C* and *D*) 2 h or (*E* and *F*) 8 h later, respectively. *N*, nucleus; *m*, mitochondrion; *arrows*, bundles of 8-nm filaments; *arrowheads*, cell–cell border. Note the lack of polyribosomes in *C* and *D* as compared to *A*, *B*, *E*, and *F*, and the 8-nm filaments coursing among the polyribosomes in *B* and *F*. *B* and *F* illustrate regions of the cortical cytoplasm as compared to the perinuclear zone in *D*. Bars, 1 μm.

plied that the effects of heat shock on KFs and actin filaments were independent events.

The effects of heat stress on microtubules and actin filaments vary depending on cell type, with disruption occurring in some cells and not in others (11, 42, 68, 73, 74). The molecular basis for the dynamic disappearance and reappearance of actin filaments in the MMEC in unknown. Although disruption of the actin filaments may be related to interruption of protein synthesis, filament recovery occurred long before protein synthesis resumed. A similar disruption of stress fibers by heat was found in Chinese hamster ovary (CHO) and neuroblastoma cells (22, 68). However, reformation of the stress fibers in CHO cells coincided with and depended upon the return of protein synthesis. The most obvious explanation for the MMEC is that the microfilaments underwent rapid disassembly and reassembly because recovery of the filaments occurred while protein synthesis was still inhibited. The assembly and disassembly of actin filaments within cells is governed by a complex set of factors, such as transmembrane linkage (44), calcium ion concentration (48), and a spectrum of actin-binding proteins (55). As hyperthermia has been shown to induce structural and functional alterations in the plasma membrane (39) and cause a rapid change in the distribution of calcium ions within cells (12), the perturbation of actin filaments immediately after heat may be a consequence of these events. It is also possible that some actin-binding proteins are modified or degraded during heat shock.

The heat shock–induced retraction of KFs in MMEC from a fully extended state to a perinuclear location is similar to the changes observed in vimentin filaments after heat stress (6, 9, 66, 68, 73, 74). Under other conditions, however, vimentin filaments are affected but KFs are not. For example, the perinuclear aggregation of vimentin filaments induced by microtubule inhibitors (18) and by cycloheximide (60) does not occur with KFs in epithelial cells (18, 63, and present data). The mechanism by which KFs in heat-shocked cells become rearranged is not clear but probably does not involve microtubules, because no disruption of microtubules occurred in MMEC after heat and colchicine neither disturbed the normal distribution of KFs nor prevented reextension of KFs during recovery after heat shock.

As electrophoretic analysis indicated that the composition of the KFs was unaffected by temperature elevation, the altered organization of KFs might result from chemical or physical modifications of the keratins in the filaments by heat or changes in a certain factor(s) required for stabilizing the normal distribution of KFs. For example, Eckert and Yeagle (15) recently found that treatment of epithelial cells with acrylamide, which also causes perinuclear aggregation of KFs (13, 14), results in marked dephosphorylation of keratins. A similar effect may accompany heat shock and this possibility is under investigation.

The coincidence of the sequential changes in the organization of KFs and the inhibition and recovery of protein synthesis during the heat shock response suggested that these events are related. Two finding argue against a role for protein synthesis in the configuration of the KF network. The organization of KFs in MMEC remained intact during cycloheximide inhibition of total cellular protein synthesis, and the KFs reextended normally in the presence of cycloheximide after heat shock. Therefore, the changes in the organization of KFs are independent of protein synthesis. The converse possibility that protein synthesis may depend on the integrity of the KF system in MMEC is compatible with all of our current data. Involvement of the cytoskeleton in the regulation of protein synthesis has been hypothesized from studies showing that (*a*) mRNA, polyribosomes, initiation factors, and aminoacyl-tRNA synthetases required for protein synthesis are associated with the cytoskeleton (7, 28, 38, 47, 53, 69); (*b*) mRNAs are translated primarily when bound to the cytoskeletal network (7, 53, 69); and (*c*) changes in the cytoskeleton-associated mRNA population correlated with changes in protein synthesis (7, 53, 69).

Biessman et al. (6, 16) suggested that heat shock–induced inhibition of protein synthesis might be linked to aggregation of vimentin filaments. However, in other studies disarrangement of vimentin filaments in fibroblasts and neuroblastoma cells had no effect on protein synthesis (66, 68, 71). Epithelial cells have not been examined in this regard. Our data suggest that protein synthesis in MMEC may depend on KFs but not actin filaments or microtubules. Thus, only those heat shock conditions (i.e., 45 and 43°C) which induced changes in the KF network altered protein synthesis. The sequential changes in the KF network after heat were concurrent with inhibition and recovery of protein synthesis and the loss and reformation of polyribosomes. Protein synthesis was unaffected by agents which disrupt microtubules or microfilaments but do not cause collapse of KFs. Both KF integrity and protein synthesis were preserved in thermotolerant cells after a challenging heat treatment. Moreover, we have recently found that acrylamide, which also effects perinuclear aggregation of KFs (13, 14), produces a marked inhibition of protein synthesis (Asch, B. B., and H. L. Asch, manuscript in preparation). It must be emphasized that the associations between KF integrity and protein synthesis are temporal and have not been proved to be causal.

One widely accepted model for thermotolerance is that induction of HSPs provides protection as measured by various parameters (33, 41, 57, 59, 62, 67), including preservation of cytoskeletal components (67, 72, 76). However, no direct evidence for a causal relationship between these two phenomena exists. In fact, thermotolerance of protein synthesis (26, 75), vimentin filaments (67), and KFs (Fig. 6) can develop even when HSP induction and total protein synthesis are inhibited. We cannot rule out the possibility, as discussed by Welch and Mizzer (72), that the HSPs present in cells before heat shock are mediating the thermotolerance seen in the absence of protein synthesis. Unlike vimentin filaments in neuroblastoma cells (67), however, the KFs in the MMEC did not become thermotolerant upon treatment with cycloheximide alone (data not shown).

Information concerning the exact function(s) of KFs has been difficult to obtain due to the dearth of methods available to perturb these structures. The ability to alter the KF system by heat provides another approach for investigating the role of this cytoskeletal element in epithelial cells. Collapse and aggregation of KFs can also be elicited by acrylamide (13, 14). Other treatments have different effects on KFs. Simultaneous disruption of microtubules and microfilaments converts KFs from their normal array into a latticework of bundles with focal attachment sites on the plasma membrane (31). Microinjection of antikeratin antibodies into cells causes retraction, collapse, and fragmentation of the KFs

into granular aggregates (30). The latter experiments established that KFs are not necessary for cell shape, mitosis, organization of microtubules or microfilaments, saltatory motions of intracellular particles, position of the nucleus, membrane ruffling, or cell motility.

Numerous studies have implicated the cytoskeleton in the distribution and movement of organelles in cells (e.g., 5, 8, 10, 14, 18, 19, 45, 49, 70, 73). Correlation of the redistribution of mitochondria, lysosomes, and endosomes with the cytoplasmic shifts of KFs in MMEC is consistent with other studies wherein organelles aggregated in the juxtanuclear region along with intermediate filaments (8, 14, 49, 73). Some investigators have reported that microtubules, not intermediate filaments, are critical in determining the location of organelles in cells (5, 10, 45). This apparent discrepancy may be explained by the proposal of Eckert (14) that organelles move along microtubules but must associate with intermediate filaments in order to maintain position. His work also suggests a role for intermediate filaments in stabilizing the orientation of the nucleus (14).

The apparent links between intermediate filaments and several major macromolecular components, including the nuclear envelope (17, 20, 23), plasma membrane (17, 20, 23, 24), organelles (5, 10, 14, 19, 49, 73), prosomes (25), and perhaps polyribosomes, support the concept that intermediate filaments form a scaffold to organize and integrate intracytoplasmic space (34). Accordingly, epithelial cell functions dependent upon precise placement of organelles, e.g., those requiring polarization of intracellular components, such as production and secretion of milk by mammary epithelium, would be severely affected by an absence or abnormal distribution of KFs. KFs could provide a conduit for signal transduction among these various structures in response to external and internal stimuli (23). Unified cell function is a hallmark of epithelia, and the connection of KFs to desmosomes, which link all cells in an epithelium, may help coordinate cell activities. KFs, then, are ideally situated to serve as intracellular and intercellular orchestrators of epithelia.

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