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Bacterial lipopolysaccharide induces cytoskeletal rearrangement in small intestinal lamina propria fibroblasts: actin assembly is essential for lipopolysaccharide signaling

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Abstract

Cytoskeletal proteins are major components of the cell backbone and regulate cell shape and function. The purpose of this study was to investigate the effect of lipopolysaccharide (LPS) on the dynamics and organization of the cytoskeletal proteins, actin, vimentin, tubulin and vinculin in human small intestinal lamina propria fibroblasts (HSILPF). A noticeable change in the actin architecture was observed after 30 min incubation with LPS with the formation of orthogonal fibers and further accumulation of actin filament at the cell periphery by 2 h. Reorganization of the vimentin network into vimentin bundling was conspicuous at 2 h. With further increase in the time period of LPS exposure, diffused staining of vimentin along with vimentin bundling was observed. Vinculin plaques distributed in the cell body and cell periphery in the control cells rearrange to cell periphery in LPS-treated cells by 30 min of LPS exposure. However, there was no change in the tubulin architecture in HSILPF in response to LPS. LPS increased the F-actin pool in HSILPF in a concentration-dependent manner with no difference in the level of G-actin. A time-dependent study depicted an increase in the G-actin pool at 10 and 20 min of LPS exposure followed by a decrease at further time intervals. The F-actin pool in LPS-treated cells was lower than the control levels at 10 and 20 min of LPS exposure followed by a sharp increase until 120 min and finally returning to the basal level at 140 and 160 min. Further ^{35}S-methionine incorporation studies suggested a new pool of actin synthesis, whereas the synthesis of other cytoskeletal filaments was not altered. Cytochalasin B, an actin-disrupting agent, severely affected the LPS induced increased percentage of 'S' phase cells and IL-6 synthesis in HSILPF. We conclude that dynamic and orchestrated organization of the cytoskeletal filaments and actin assembly in response to LPS may be a prime requirement for the LPS induced increase in percentage of 'S' phase cells and IL-6 synthesis

Keywords: Lamina propria fibroblast; Lipopolysaccharide; Actin; Vimentin; Vinculin; Tubulin

1. Introduction

Bacterial lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria triggers a plethora of signals in both myeloid and non-myeloid cells [1,2]. Among the non-myeloid cells, fibroblasts are not only major cellular components of in-

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flammatory responses, but they also secrete various growth factors thereby supporting the growth, differentiation and activation of inflammatory cells [3,4]. Our previous reports suggested that LPS induced cell proliferation and collagen synthesis by downregulating nitric oxide in human small intestinal lamina propria fibroblasts (HSILPF) [5]. Further, LPS was also found to induce potent proinflammatory cytokines and adhesion molecules production in lamina propria fibroblasts [6]. Evidence suggests that LPS could affect the cellular physiology by perturbing the cytoskeletal arrangement [1]. The cytoskeleton is an integrated network of microfilaments, intermediate filaments and microtubules and there are structural and functional interactions between these cytoskeletal components [7,8]. LPS has been shown to induce a disassembly of actin microfilaments in both murine macrophages [9] and rat mesangial cells [10]. These combined data suggest a potential role of cytoskeletal filaments in highly coordinated series of intracellular responses to LPS in various cell systems. Hence, it was of immense interest to investigate the cytoskeletal architecture in human small intestinal lamina propria fibroblasts (HSILPF) in response to LPS.

In this study, we describe the reorganization of the principal cytoskeletal elements: actin, vimentin and vinculin and the dynamics of the F- and G-actin pool in HSILPF in response to LPS incubation. Our results show that upon LPS treatment actin undergoes an extensive margination at the cell periphery. The F-actin pool was increased over a period of time with little or no effect on the G-actin pool, with an increase in the de novo actin synthesis. Vinculin plaques were redistributed from the cell body to the cell periphery. Extensive bundling of the vimentin was observed upon LPS stimulation. However, the tubulin network remained unchanged. Inhibition of actin filament formation by cytochalasin B severely disrupted the ability of HSILPF to secrete IL-6, a multifunctional cytokine and decreased the percentage of cells in DNA synthesis phase ('S' phase).

2. Materials and methods

All the chemicals, reagents and antibodies were from Sigma unless mentioned.

2.1. LPS purification

LPS was purified from *Escherichia coli* O55:B5, as described previously [5].

2.2. Cells and cell culture

Human small intestinal lamina propria fibroblasts (HSILPF) were prepared from fetus samples (18–22 weeks) and used for the study as described previously [5]. For immunofluorescent labeling of the cells, the cells were grown on ethanol cleaned glass coverslips for 24 h followed by addition of the test component for various time periods.

2.3. Fluorescent labeling of the cells

Cells on the coverglass were processed as described in the following sections and examined using a Zeiss fluorescence microscope and photographed using 400 ASA films (Konica).

2.4. Actin, vimentin, vinculin and tubulin

Cells were fixed in PFA (paraformaldehyde) and extracted with 0.1% Triton X-100 for 10 min and blocked by 1% BSA for 2 h. mAbs to actin (1:200), vimentin (1:200 dilution), vinculin (1:400) and tubulin (1:500) (all antibodies were obtained from Sigma, MO, Louis, USA) were added to the cells and incubated for 1 h. Cells were washed with phosphate-buffered saline (PBS) 5 times and incubated with FITC-conjugated anti-mouse IgG for 30 min. Finally, the cells were washed with PBS and mounted on coverglass with glycerin and PBS (1:1). The cells were examined under a Zeiss fluorescent microscope and photographed.

2.5. F-actin quantitation by spectrofluorometry

F-actin was fluorometrically measured as described previously [11]. HSILPF were seeded at a density of 5×10^5 cells into the wells of 6-well plates and cultured for 24 h. The monolayers were exposed to various concentrations of LPS for different time periods. Following LPS exposure, the monolayers were washed with buffer A (KCl 75 mM, $MgSO_4$ 3 mM, EGTA 1 mM, imidazole 10 mM, DTT 0.2 mM,

aprotinin 10 μg/ml and PMSF 0.1 mM) and fixed with formaldehyde 4% for 10 min. Monolayers were permeabilized with Triton X-100 and stained with NBD-phallicidin for 20 min and extracted with ice-cold methanol at −20°C (overnight). Extracts were harvested into cuvettes and fluorescence was measured in a Perkin–Elmer spectrophotometer at 465 nm (10 nm slit) and 535 emission (10 nm slit) and expressed in arbitrary fluorescence units per mg total protein.

2.6. G-actin quantitation by DNAse inhibition assay

G-actin was measured as described previously [12]. HSILPF in 6-well plates were exposed to various concentration of LPS and extracted for G-actin quantitation. The extraction procedure involved removal of medium, two washes with HBSS and extraction with 0.5 ml of lysis buffer (HBSS with 1% Triton X-100, 2 mM $MgCl_3$, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT and 0.1 mM PMSF) for 5 min. Extracts were centrifuged at $15\,000 \times g$ and chilled on ice. Fifty microliters of the extract and 25 μl of actin depolymerization buffer containing 1.5 M guanidine hydrochloride were mixed and left standing for 20 min on ice. Seven hundred and fifty microliters of 60 pg/ml of DNAse I was added and mixed. Reaction was initiated by adding 50 μl of 20 OD DNA and incubated for 10 min at 37°C and transferred onto ice and 200 μl of 60% perchloric acid was added and left for 30 min on ice. Reaction mixtures were clarified by centrifugation. Optical densities at 260 nm of supernatants were recorded. Bovine skeletal muscle actin was used as standard.

2.7. Total protein concentration

As F-actin and G-actin measurements involved fixation, permeabilization and extraction, parallel plated cells were used for determining total protein concentration using the BCA kit (Pierce). Total HSILPF protein was used to standardize for G or F actin.

2.8. Immunoprecipitation of actin, vimentin, vinculin and tubulin from ^{35}S-methionine-labeled HSILPF

HSILPF monolayers in 6-well plates were metabolically labeled with 200 μCi/ml of ^{35}S-methionine (Bhaba Atomic Research Center, Bombay, India) and stimulated by LPS for 2 h. The cells were lysed in situ with 1 ml of ice-cold lysis buffer (3% SDS, 1% 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0), 10 mM PMSF, 10 μg/ml leupeptin and 1 mM EDTA). Cells extracts were clarified by centrifugation and subjected to immunoprecipitation. Protein concentration of the same extracts were measured as described before prior to immunoprecipitation.

Lysates were incubated with antibody to actin, vimentin, vinculin and tubulin for 18 h at 4°C. The complexes were incubated with precleared and prewashed.

Protein A-Sepharose beads (Pharmacia) for 30 min on ice and microfuged.The supernatants were decanted and the pellets were resuspended in Tris-buffered saline. The pellets were transferred to scintillation vials into Optifluor scintillation fluid and β-counts obtained. ^{35}S-methionine incorporation into the cytoskeletal filaments was expressed as dpm per mg total protein.

2.9. Analysis of 'S' phase cells

Labeling of cells with bromodeoxyuridine (Brdu) was done as described previously [5]. In brief, cells plated on glass coverslips were exposed to LPS (10 μg/ml) and 10 μmol Brdu for 2 h, fixed and stained with antibody to Brdu (Boehringer Mannheim 1:10 dilution) followed by FITC-anti-mouse IgG (1:120 dilution). Coverslips were observed under the fluorescence microscope for Brdu-positive cells and 50-microscopic fields were randomly counted for total cells and Brdu-positive cells and the results were expressed as % 'S' phase cells. In some experiments, cells were pretreated with cytochalasin B (2.5 μg/ml, Cyto B) for 1 h followed by LPS exposure for 2 h. Cytochalasin B was present throughout the experimental study.

2.10. IL-6 assay

Cells at a density of 10^6/well were plated in 24-well plates supplemented with DMEM and 1% FCS. Different concentrations of LPS prepared in DMEM-1% FCS were added to the cultures. Depending on the

reaction conditions, the plates were incubated for 2 h at 37°C/5% CO_2. After the indicated time interval, supernatant samples were collected, centrifuged and stored at −80°C until use. In certain experiments, cells were preincubated with 2.5 μg/ml cytochalasin B. ELISA kits for cytokine the IL-6, from commercial source (R&D System, Minneapolis, MN) were used according to the manufacturer's instructions.

2.11. Statistical analysis

Results are expressed as mean values of experiments repeated several times in triplicate ± S.D. The F- and G-actin pool are normalized against the total protein concentration of the respective experiment. A Paired Student's *t*-test was used to analyze the data and a *P*-value less than 0.05 was considered statistically significant. In some cases, S.D. values were too low to appear in the graph as error bars.

3. Results

3.1. Effect of LPS on the cytoskeletal organization in HSILPF

3.1.1. Actin

F-actin labeled HSILPF monolayers exposed to LPS (10 μg/ml) or medium alone for 30 min, 2, 4, 6, and 8 h were examined by fluorescence microscopy (Fig. 1). All control monolayers contained continuous transcytoplasmic actin filaments (Fig. 1a). After 30 min of incubation of the cells with LPS, actin filaments appeared as irregular spikes with more orthogonal filaments in contrast to straight fibers in cells not treated with LPS (control cells). By 2, 4, 6 and 8 h, short spikes disappeared and actin filaments accumulated in the cell periphery (Fig. 1b–f).

3.1.2. Vimentin

Vimentin in the control cells was exhibited as a fine network from nuclear lamina to the cell periph-

Table 1
Time-dependent reorganization of cytoskeletal structures in lamina propria fibroblasts by LPS[a]

Cytoskeletal filaments	Time of LPS exposure				
	30 min	2 h	4 h	6 h	8 h
Actin[b]	Appearance of orthogonal spikes (10 ± 3.5)	Marginal accumulation (6 ± 2.5)	Marginal accumulation (10 ± 2.5)	Marginal accumulation (15 ± 1.5)	Marginal accumulation (50 ± 5.5)
Vimentin[c]	Fine network pattern	Accumulation and bundling (10 ± 2.5)	Increased bundling (20 ± 2.5)	Diffuse vimentin staining and extensive bundling (30 ± 5.5)	Diffuse vimentin staining and extensive bundling (80 ± 5.7)
Vinculin[d]	Loss of vinculin plaques (2 ± 0.05)	(5 ± 1.5)[e]	(10 ± 2.1)[e]	(25 ± 2.5)[e]	(40 ± 5.5)[e]
Tubulin		–[f]	–[f]	–[f]	–[f]

[a]Cells were plated on glass coverslips and treated with LPS (10 μg/ml) and fixed at 30 min, 2, 4, 6 and 8 h. Cells were processed for indirect immunofluorescence with antibody to actin, vimentin, vinculin and tubulin and FITC-conjugated anti-mouse IgG.
[b]The cells were observed under the fluorescence microscope and randomly 50 fields were counted for the total cells and presence of cells with orthogonal actin fibers and peripheral actin arrangement and expressed as the mean percentage of cells ± S.D. of the specific pattern observed at different periods (values in brackets).
[c]Fifty fields were counted for the presence of total cells and the cells with bundling of filaments and expressed as the mean percentage of cells ± S.D. of the specific pattern (values in the brackets).
[d]Fifty fields were counted for the total cells and the cells exhibiting peripheral arrangement of vinculin plaques and expressed as the mean percentage of cells ± S.D. of the specific pattern (values in brackets).
[e]Complete margination of vinculin along the cell periphery.
[f]No change observed.

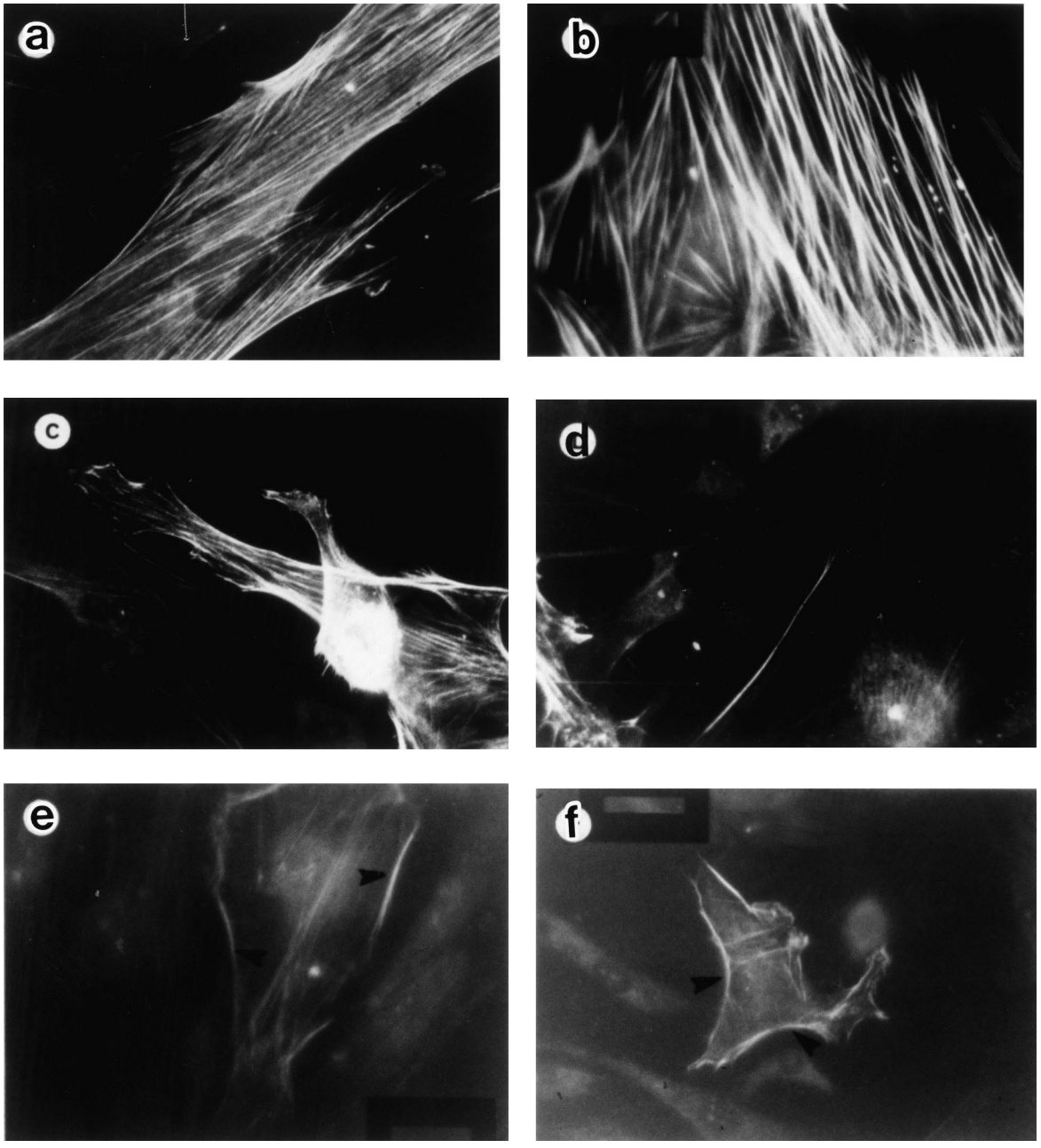

Fig. 1. Effect of LPS on the organization of actin. Fluorescent micrographs of cells labeled with anti-actin and FITC conjugated. (a) Medium control: actin in this control cell is organized in elongated bundles. (b) Thirty minutes after LPS exposure: the formation of small orthogonal actin fibers can be seen. (c) Two hours after LPS exposure: the arrow indicates margination of the actin filament along the cell periphery. (d). Four hours after LPS exposure. (e) Six hours after LPS exposure. (f) Eight hours after LPS exposure exhibiting complete margination of actin along the cell periphery. Magnification: ×63.

ery (Fig. 2a). Treatment with LPS for 30 min did not induce any noticeable change in the vimentin pattern (Fig. 2b). By 2 h of incubation of HSILPF with LPS, accumulation and bundling of vimentin filaments were observed (Fig. 2c). At 4, 6 and 8 h, LPS-treated cells exhibited extensive bundling with loss in the fine network structure (Fig. 2d–f). Along with the bundling of vimentin, the fine network as observed in control cells was changed to a diffused network in LPS-treated cells.

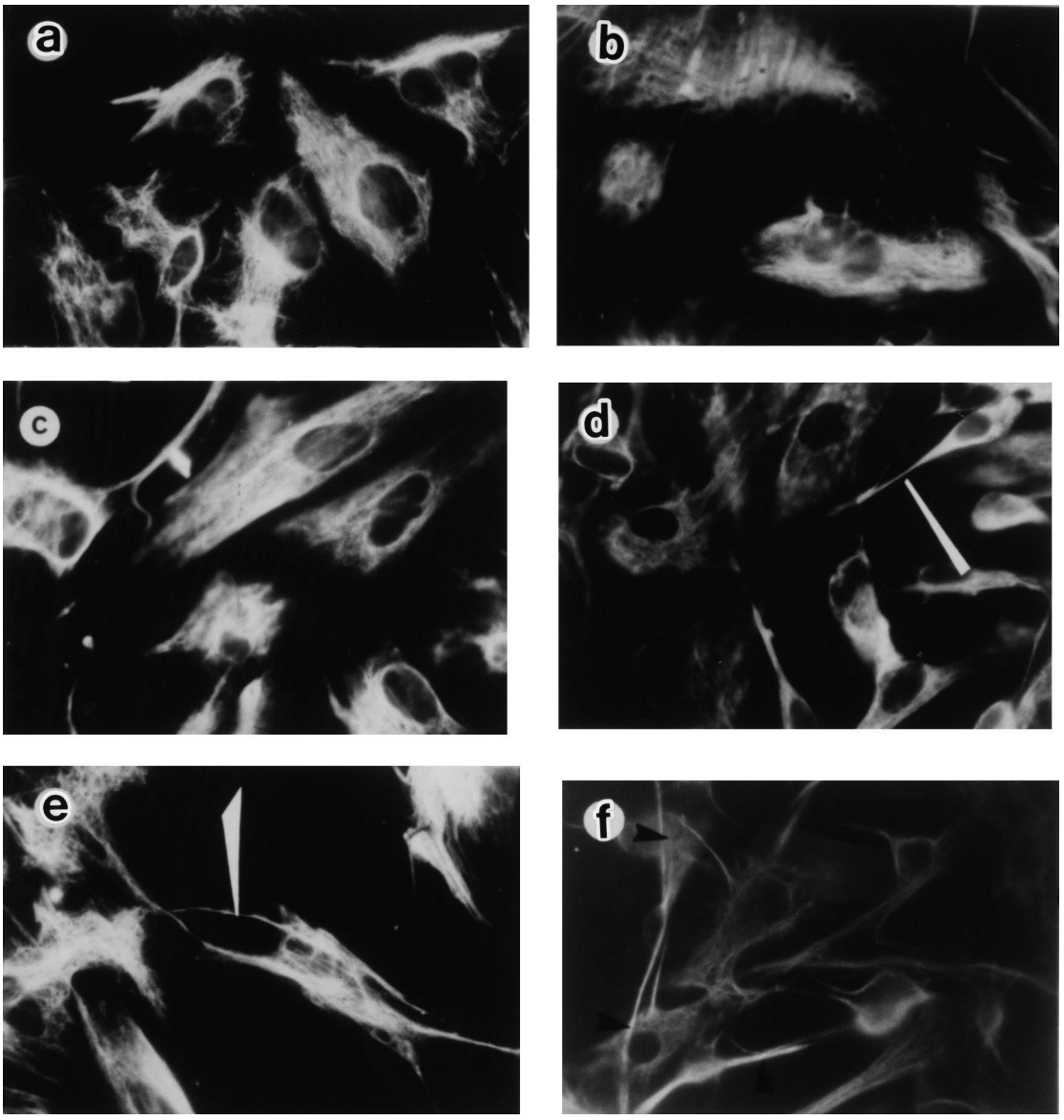

Fig. 2. Effect of LPS on vimentin arrangement. Fluorescent micrographs of cells labeled with anti-vimentin and FITC conjugated anti-mouse IgG. (a) Medium control: vimentin appeared as a fine network distributed from the nuclear lamina to the periphery of the cells. (b) Thirty minutes after LPS exposure: the vimentin pattern is the same as that observed in the control cells. (c) Two hours after LPS exposure: bundling of the filament can be seen. (d) Four hours after LPS exposure: the fine network changed into diffused vimentin and extensive bundling (arrow). (e) Six hours after LPS exposure. (f) Eight hours after LPS exposure. Arrows in e and f indicate the fine bundling in the LPS-treated lamina propria fibroblasts. Magnification: ×63.

3.1.3. Vinculin

Vinculin, an actin binding protein was distributed as plaques in the cell body as well as cell periphery in the control cells (Fig. 3a). Incubation of cells with LPS for 30 min resulted in loss of vinculin plaques from the cell body (Fig. 3b). By 2 h, there was marginal accumulation of the vinculin bordered along the cell periphery (Fig. 3c). No further change in the vinculin pattern was observed at 4, 6 and 8 h as compared to 2 h (Fig. 3d–f).

3.1.4. Tubulin

Tubulin exhibited a nested filamentous network in control cells as well as in cells treated with LPS. In contrast to other cytoskeletal proteins, the tubulin organization did not undergo any change in response

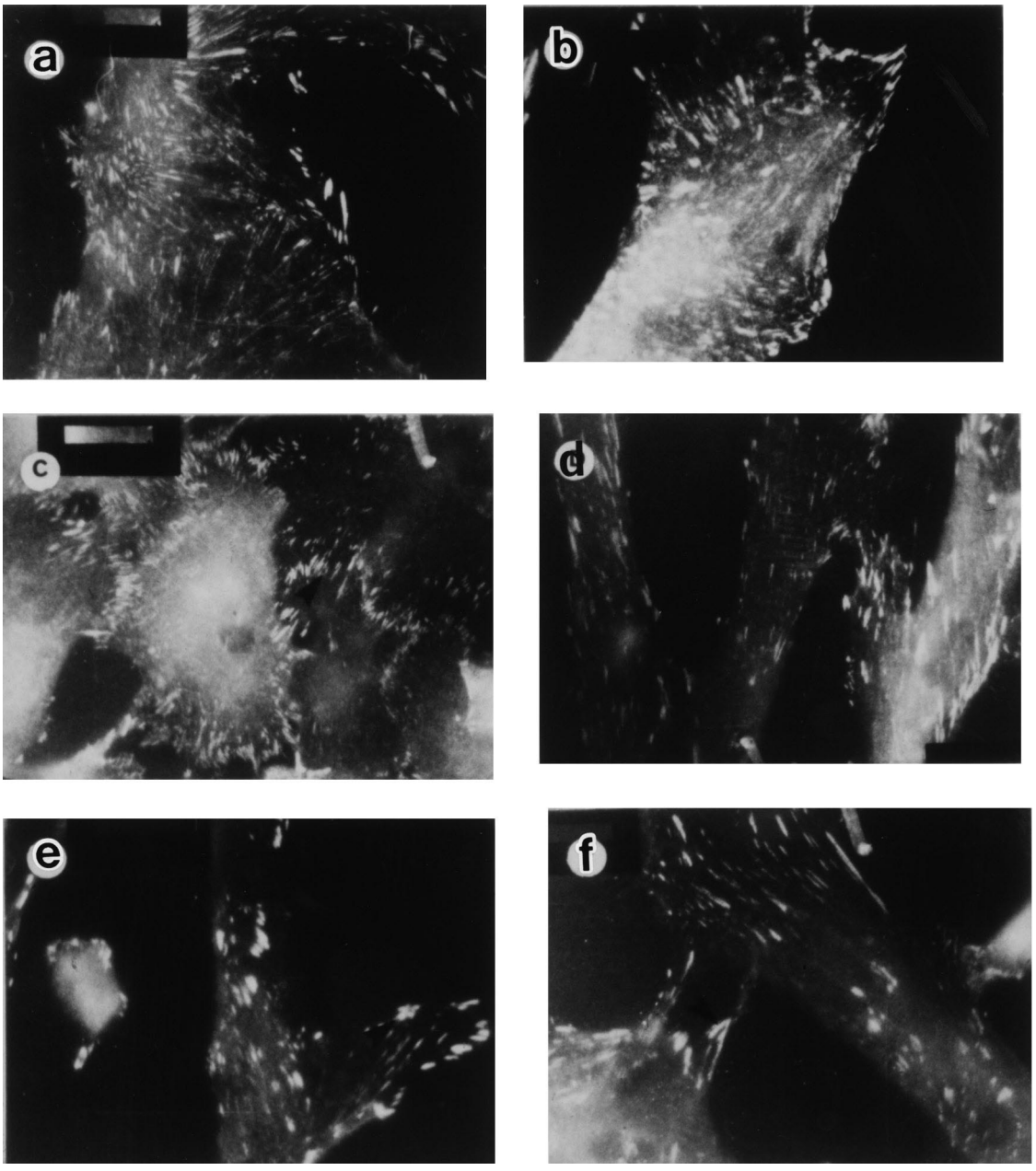

Fig. 3. Effect of LPS on vinculin arrangement. Fluorescent micrographs of cells labeled with anti-vinculin and FITC conjugated anti-mouse IgG. (a) Medium control: the vinculin plaques distributed both in the cell body as well as cell periphery can be seen. (b) Thirty minutes after LPS exposure: vinculin plaques rearranging to the cell periphery. (c) Two hours after LPS exposure: the arrangement of the vinculin along the cell periphery can be seen. (d) Four hours after LPS exposure. (e) Six hours after LPS exposure. (f) Eight hours after LSP exposure. The vinculin plaques in the cell periphery can be seen in e and f. Magnification: ×63.

to LPS during the time period used for he study (data not shown).

As summarized in Table 1, 10% of the cells were exhibiting orthogonal actin fibers after 30 min of LPS exposure. By 2 h the small actin spikes disappeared and 6% of the cells were seen with peripherally accumulated actin. The percentage of cells with actin accumulation increased with time. However, no more than 50% of the cells were exhibiting peripheral arrangement of actin at 8 h. The percentage of cells showing bundling of the vimentin filaments increased in a time-dependent manner with 80% of the cells

showing bundling after 8 h of LPS exposure. The loss of vinculin plaques from the cell body and rearrangement in the cell periphery in response to LPS reached 40% by 8 h.

3.2. The F-actin pool was increased in HSILPF in response to LPS

The effect of LPS on the F-actin content in HSILPF (expressed as fluorescent unit/mg total protein) was studied (Fig. 4). Incubation of cells different concentration of LPS (0–10 μg/ml) significantly increased the F-actin pool in HSILPF. At concentrations of 0.001, 0.01 and 0.1 μg/ml LPS, a moderate increase in the F-actin pool with respect to the control was observed, with a sharp increase in F-actin pool at 1 and 10 μg/ml LPS. Possibly, at high concentrations of 1 and 10 μg/ml the F-actin becomes stable and could no longer be extracted with Triton X-100, thereby exhibiting a sharp rise. However, at much higher concentration (100 mg/ml), no further increase was observed.

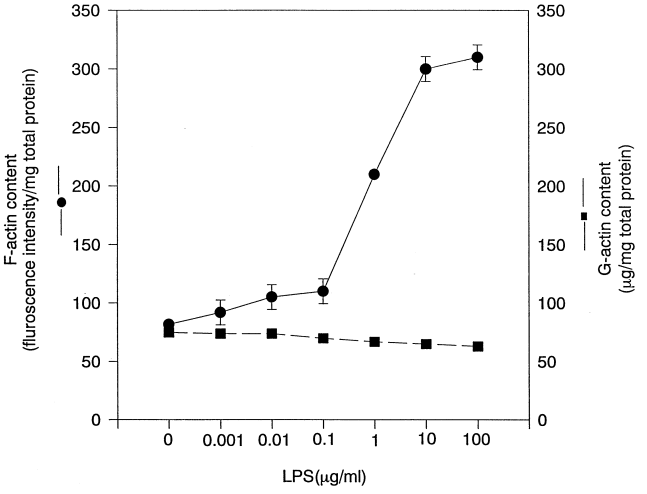

Fig. 4. Concentration-dependent effect of LPS on the F- and G-actin pool in HSILPF. For F-actin, HSILPF were exposed to various concentrations of LPS (0–100 μg/ml) for 2 h, fixed, permeabilized and assayed for F-actin as described in Section 2. F-actin (●) content is expressed as fluorescent unit/mg total protein mean ± S.D. of five separate experiments performed in triplicate. For G-actin measurements, cells were treated with various concentrations of LPS for 2 h and extracted as described in Section 2 and the supernatant tested for G-actin content by DNAse I inhibition assay. Bovine skeletal muscle actin was used as standard. G-actin content (■) is expressed as μg/mg total protein mean ± S.D. of five separate experiments performed in triplicate.

3.3. G-actin pool in HSILPF in response to LPS remained unchanged

The effect of LPS on G-actin pool (expressed as μg/mg total protein) was studied (Fig. 4). There was no significant difference between the G-actin pool of media or LPS-treated HSILPF after an incubation period of 2 h.

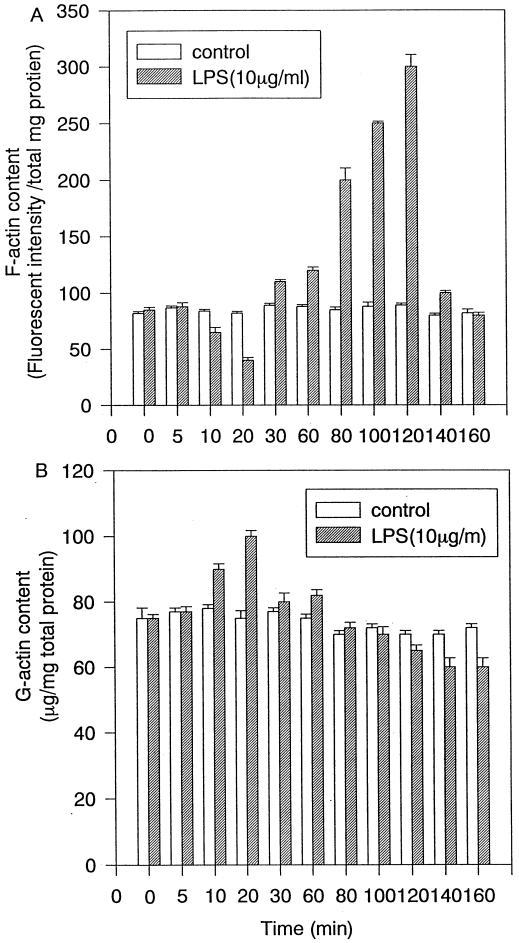

Fig. 5. Time-dependent effect of LPS on the F- and G-actin pool of HSILPF. Cells were treated with LPS (10 μg/ml) for various time periods (0–160 min) and F- and G-actin pools were estimated as described in Section 2. (A) F-actin pool is expressed as fluorescent unit/mg total protein mean ± S.D. of seven separate experiments performed in triplicate. (B) G-actin content is expressed as μg/mg total protein mean ± S.D. of seven separate experiments performed in triplicate.

3.4. Time-dependent variation in the F- and G-actin pool in HSILPF in response to LPS

To determine if there is a specific pattern of the F- and G-actin pool in response to LPS at early time intervals, we measured the F- and G-actin pool over a time period of 0–160 min with an interval of 20 min (Fig. 5).

The F-actin content in control cells was almost constant throughout the time period used for the study. In LPS-treated cells, a decrease in F-actin content was observed at 10–20 min, followed by an increase at 30, 60, 80, 100 and 120 min, with subsequent decrease of F-actin to basal level at 140 and 160 min (Fig. 5A).

The G-actin pool was almost similar in control cells at all time intervals. In LPS-treated cells, the G-actin level increased at 10 and 20 min thereafter the G-actin level decreased slightly (Fig. 5B).

3.5. LPS enhanced synthesis of actin, but not vimentin, vinculin or tubulin

To determine whether LPS exposure altered actin, vimentin, vinculin or tubulin synthesis during the 2 h study, ^{35}S-methionine incorporation into actin, vimentin, vinculin or tubulin was determined and expressed as dpm/mg total protein (Fig. 6). The LPS exposure significantly increased the mean (±S.D.) dpm of actin as compared to the control (15 000 ± 1020 vs. 8000 ± 280). Synthesis of vimentin, vinculin or tubulin remained unchanged.

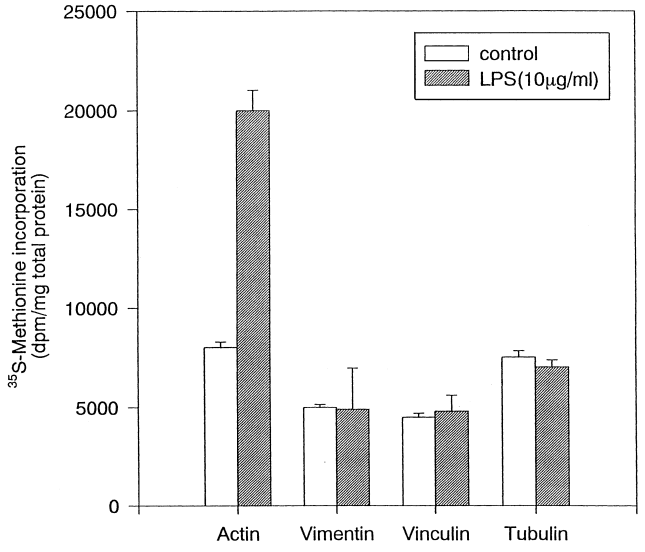

Fig. 6. Effect of LPS on new actin, vimentin, vinculin and tubulin synthesis. ^{35}S-Methionine labeled HSILPF were exposed to LPS for 2 h and immunoprecipated as described in Section 2. ^{35}S-Methionine incorporation into actin, vimentin, vinculin and tubulin immunoprecipitates were counted in a Beckman β-counter and expressed as mean ± S.D. dpm/mg total protein of four individual experiments performed in triplicate

3.6. Cytochalasin B reduced the F-actin pool, increased G-actin pool and inhibited % 'S' phase cells and IL-6 secretion by LPS-treated HSILP

We have previously reported that LPS induced enhanced cell proliferation [5] and synthesis of proinflammatory mediators [6] in HSILPF. To monitor the role of actin in LPS-induced cellular functionality we used Brdu incorporation to study 'S' phase cells and the expression of multifunctional cytokine IL-6, along with F- and G-actin pool.

3.6.1. F- and G-actin pool

Cytochalasin B, an actin-severing compound, completely blocked the increase in LPS induced F-actin in HSILPF and significantly increased the G-actin pool (Fig. 7A,B).

3.6.2. 'S' phase cells

At 2 h, the % 'S' phase cells in LPS-treated HSILPF was 18.5 (±1.5) compared to 3% in control cells (Fig. 7C). Cyto B reduced the 'S' phase cells from 18.5 to 1.8% in LPS-treated HSILPF.

3.6.3. IL-6 level

LPS induced a high level of IL-6 in HSILPF by 2 h, which was reduced to almost basal level by cytochalasin B (Fig. 7D).

4. Discussion

Cytoskeletal proteins are the major structural backbone of cells and maintain the cellular functionality [13,14]. Reorganization of actin filaments in response to LPS have been reported in various cell types, such as macrophages [9], mesangial cells [10], uroepithelial cells [15] endothelial cells [16] and B-cells [17]. In endothelial cells, LPS distorted the actin architecture thereby affecting the barrier function

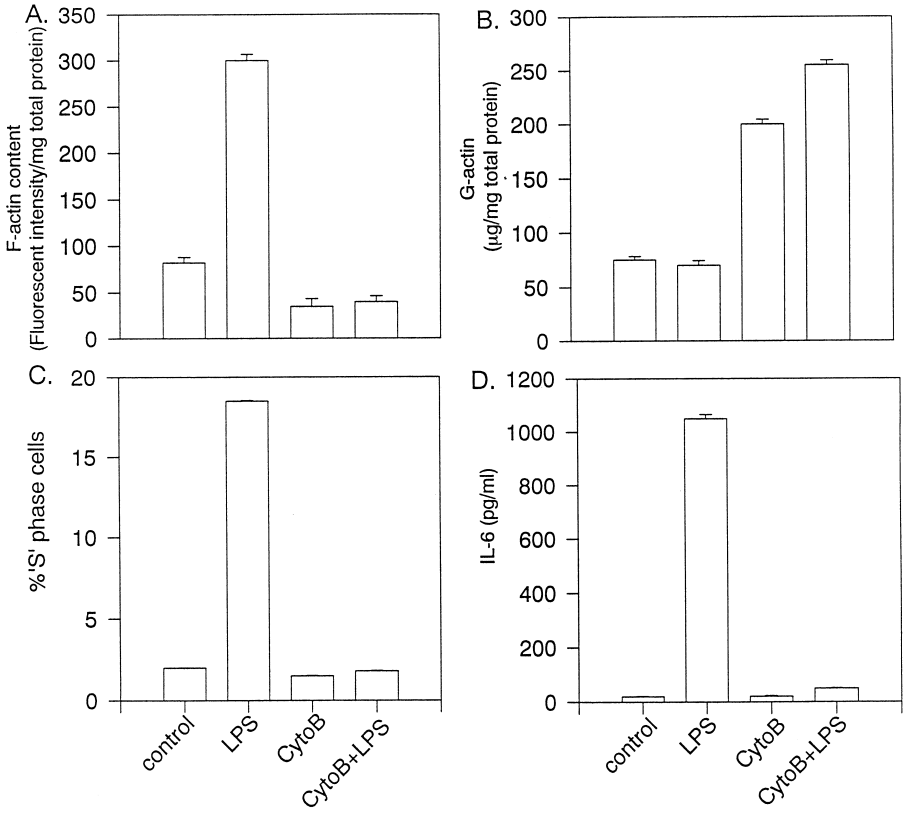

Fig. 7. Effect of cytochalasin B on the LPS induced F- and G-actin pool, % 'S' phase cells and IL-6 secretion. (A,B) Cells were pretreated with cytochalasin B (2.5 µg/ml) for 1 h followed by LPS treatment for 2 h. F- and G-actin contents were estimated as described in Section 2. (A) F-actin pool is expressed as fluorescent unit/mg total protein mean ± S.D. of four separate experiments performed in triplicate. (B) G-actin content is expressed as µg/mg total protein mean ± S.D. of four separate experiments performed in triplicate. (C) % 'S' phase cells. Cells were seeded on glass coverslips and treated as described in Section 2. The coverslips were fixed at 2 h and processed for immunofluorescence staining with anti-Brdu antibody and FITC-conjugated detection antibody. Fifty microscopic fields were counted under Zeiss microscope for total cells and Brdu-positive cells. Results are expressed as mean percentage Brdu-positive cells ± S.D. of three different experiments. (D) Release of IL-6. HSILPF were pretreated with cytochalasin B for 1 h followed by LPS for 2 h. IL-6 in the supernatant was measured as described in Section 2. The results are derived from three individual experiments in triplicate and expressed as the mean values (pg/ml IL-6) ± S.D.

[16]. Altogether, these data suggest strong participation of actin in various LPS-induced alterations in the cell-like barrier function, synthesis of cytokines etc.

Our previous study with HSILPF demonstrated that LPS induced proliferation [5] and enhanced secretion of proinflammatory cytokines in HSILPF [6]. Hence we further investigated the organization of cytoskeletal filaments, actin, vimentin, vinculin and tubulin in HILSPF in response to LPS. The early formation of short actin spikes (by 30 min) probably may be denoting the early response of LPS in HSILPF. Further accumulation of the actin in HILPSF in response to LPS may be depicting the prior actin arrangement required for LPS-increased DNA synthesis and IL-6 production.

Actin accumulation in uroepithelial cells in response to LPS was related to the actin-dependent ICAM distribution [15]. In macrophages, the noticeable change of actin organization began from 10 min and was conspicuous at 30 min, but the change in F- and G-actin pool was obvious within seconds [9]. However, in case of intestinal fibroblasts at a particular time period of 10 and 20 min of LPS exposure decrease in the F-actin and an increase in the G-actin pool was observed (Fig. 5). Probably macrophage being a highly sensitive cells to the extracellular stimuli the response to LPS was much faster than that of

the fibroblasts. Possibly at an early time period after LPS exposure the cells may synthesize new actin or reorganize the pre-existing actin which is easily extractable with Triton X-100, thereby contributing to the increase in the G-actin and decrease in F-actin pool (Fig. 5A,B). However, in endothelial cells the G-actin pool was increased without a concomitant decrease in the F-actin pool [16]. Probably a difference in the pattern of the F- and G-actin pool in endothelial cells and HSILPF can be due to the two different events induced by LPS: disruption of the barrier structure in endothelial cells, a destructive signal and proliferation in HSILPF, a constructive signal.

Possibly the G-actin pool is increased in the cells undergoing cellular injury, whereas the F-actin pool is increased in the cells undergoing proliferation. However, the equilibrium between the F- and G-actin pool and actin polymerization is important for maintaining the cellular function. Actin polymerization is inhibited by cytochalasin B by binding to the barbed end of the actin filament thereby preventing the actin assembly and is used as a useful tool to elucidate the role of actin polymerization in controlling cellular function [18–20]. Prior treatment of cells with cytochalasin B, significantly increased G-actin pool and reduced the F-actin level in LPS-treated HSILPF.

Cytochalasin B hampered both the increase in the % 'S' phase cells as well as IL-6 secretion in HSILPF in response to LPS, thereby suggesting the role of actin in the LPS-induced cellular responses. Vincristine, a tubulin depolymerizing compound which is known to induce structural changes in the vimentin distribution in fibroblasts [21] did not have any effect on the F- and G-actin pool as well as % 'S' phase cells and IL-6 secretion in HILSPF stimulated with LPS (data not shown). These combined data strongly suggest that actin is the major cytoskeletal protein required for cellular signaling and other cytoskeletal protein may interact with actin for the alteration in cell functions induced by LPS in HSILPF. Actin and not vimentin or tubulin have also been suggested to be the major cytoskeletal filament in formation of protrusions in fibroblasts [22]. Actin polymerization was obligatory for epidermal growth factor induced signal transduction in epithelial cells [23]. These reports and our study suggest the involvement of actin in mediating the signal by an effector molecule in the target cell.

To our knowledge, there is no report related to the change in vimentin or vinculin pattern in response to LPS. Vinculin, an actin binding protein rearranged itself to the cell periphery in response to LPS in HSILPF. The disappearance of vinculin plaques from the cell body and arrangement in the cell periphery probably suggests the loss of focal adhesion, thereby preparing the cells to undergo proliferation (Fig. 3a–f). Bundling of the vimentin as observed in response to LPS perhaps may be required for the progression of the cell in the proliferative phase. However, no noticeable change in the tubulin structure was observed in HSILPF in response to LPS. The methionine incorporation study suggested new actin synthesis in HSILPF in response to LPS, thereby suggesting that new actin units may be incorporated by LPS in HSILPF thereby leading to enhanced cell proliferation and IL-6 secretion. Other cytoskeletal filaments did not undergo any change at the synthesis level in response to LPS.

Although the cytoskeleton is an integrated, dynamic structure in which there is a high level of coordination between the components, we found that actin filaments, of all the cytoskeletal filaments are involved in IL-6 secretion and cell proliferation in HSILPF in response to LPS. The phenomenon of actin assembly involves a different set of regulatory proteins [24,25]. The new actin synthesis observed in response to LPS in HSILPF may be controlled by some regulatory proteins and second messenger like diacylglycerol (DAG), inositol triphosphate (IP_3). DAG and IP_3 have been suggested to be involved in the modulation of actin filaments by affecting the PI (phosphoinositide) cycle [22,26]. Probably, LPS may affect the actin organization by interacting with the actin binding proteins like profilin, gelsolin or actinin thereby modulating the PI pathway. Hence, further study is required to elucidate the role of actin regulatory proteins and the role of second messengers in increased actin synthesis and LPS-mediated signaling in lamina propria fibroblasts.

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