ORIGINAL ARTICLE

Lipopolysaccharide- and Proinflammatory Cytokine-Induced Energy Production in Intestinal and Colonic Epithelial Cell Lines

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Fukushima K, Sasaki I, Takahashi K, Naito H, Matsuno S. Lipopolysaccharide- and proinflammatory cytokine-induced energy production in intestinal and colonic epithelial cell lines. Scand J Gastroenterol 1999;34:291–296.

Background: Although epithelial cells in ulcerative colitis may be metabolically deficient, it remains unknown whether epithelial cells modulate energy metabolism in inflamed mucosa. The purpose of the present study is to investigate whether inflammatory mediators such as lipopolysaccharide (LPS), interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) alter energy metabolism in epithelial cells. **Methods:** Adenosine 5′-triphosphate (ATP) levels in HT29 cells cultured with LPS, IL-1β, IL-6, or TNF-α were measured with high-performance liquid chromatography, using a reversed-phase chromatography column. Cellular and mitochondrial (antimycin A-sensitive) respiration rates were determined polarographically, using a Clark-type oxygen electrode. **Results:** When the cells were cultured with LPS, IL-6, and TNF-α but not IL-1β, ATP levels increased significantly at 6 h, followed by a decrease at 24 h. Enhancement of oxygen consumption, which was completely blocked by antimycin A, was also shown at 3 h by the exposure to these substrates. **Conclusion:** LPS and proinflammatory cytokines induced cellular ATP generated by mitochondrial phosphorylation. An active energy production in epithelial cells on the exposure to inflammatory mediators may be critical for escape from chronic mucosal inflammation.

Key words: Cytokine; energy; epithelial cells; mitochondrial phosphorylation; ulcerative colitis

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Although ulcerative colitis (UC) is a disease of unknown etiology, many studies have been carried out to find possible abnormalities in epithelial cells. These include the investigation of surface molecules (1, 2), the synthesis of active chemicals, and the secretion of immunoregulatory and proinflammatory cytokines (3–7). The energy metabolism in epithelial cells has been also investigated, leading to the relatively new proposal that epithelial cells in UC may be metabolically deficient because of the decreased utilization of energy substrates, short-chain fatty acids (8). This hypothesis for UC pathogenesis appears to be proved by the fact that short-chain fatty acid enemas are effectively used for treatment of some UC patients (9–12). However, even if impaired energy metabolism is critical for UC pathogenesis, there is little direct evidence that epithelial cells modulate energy metabolism in inflamed mucosa. It remains unknown whether epithelial cells increase or decrease energy production when they are exposed to inflammatory mediators such as lipopolysaccharide (LPS) and proinflammatory cytokines. To address this issue, HT29, a human colonic cancer cell line, and IEC6, a rat crypt-derived non-transformed intestinal epithelial cell line, were stimulated by these mediators, and then cellular adenosine 5′-triphosphate (ATP) concentration and mitochondrial oxygen consumption were evaluated.

The present study showed that LPS, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) but not IL-1β enhanced the intracellular energy substrates that were generated by mitochondrial respiration in a time-dependent fashion.

Materials and Methods

Cell culture

HT29 and IEC6 cells were routinely cultured with McCoy's 5A or Dulbecco's modified Eagle's medium containing penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% or 5% fetal calf serum, respectively. The cells were grown in 90-mm petri dishes or six-well plates at 37 °C in a 5% CO_2 atmosphere. When the cells reached confluence, they were exposed to *Escherichia coli* 026-derived LPS (Wako Co., Osaka, Japan), human IL-1β (Genzyme, Cambridge, Mass., USA), IL-6 (Peprotech Inc., Rocky Hill, N.J., USA), or TNF-α (Peprotech Inc.) and cultured for 24 h at most. Preliminary studies showed that no significant difference in number of cells was observed among the above cultures.

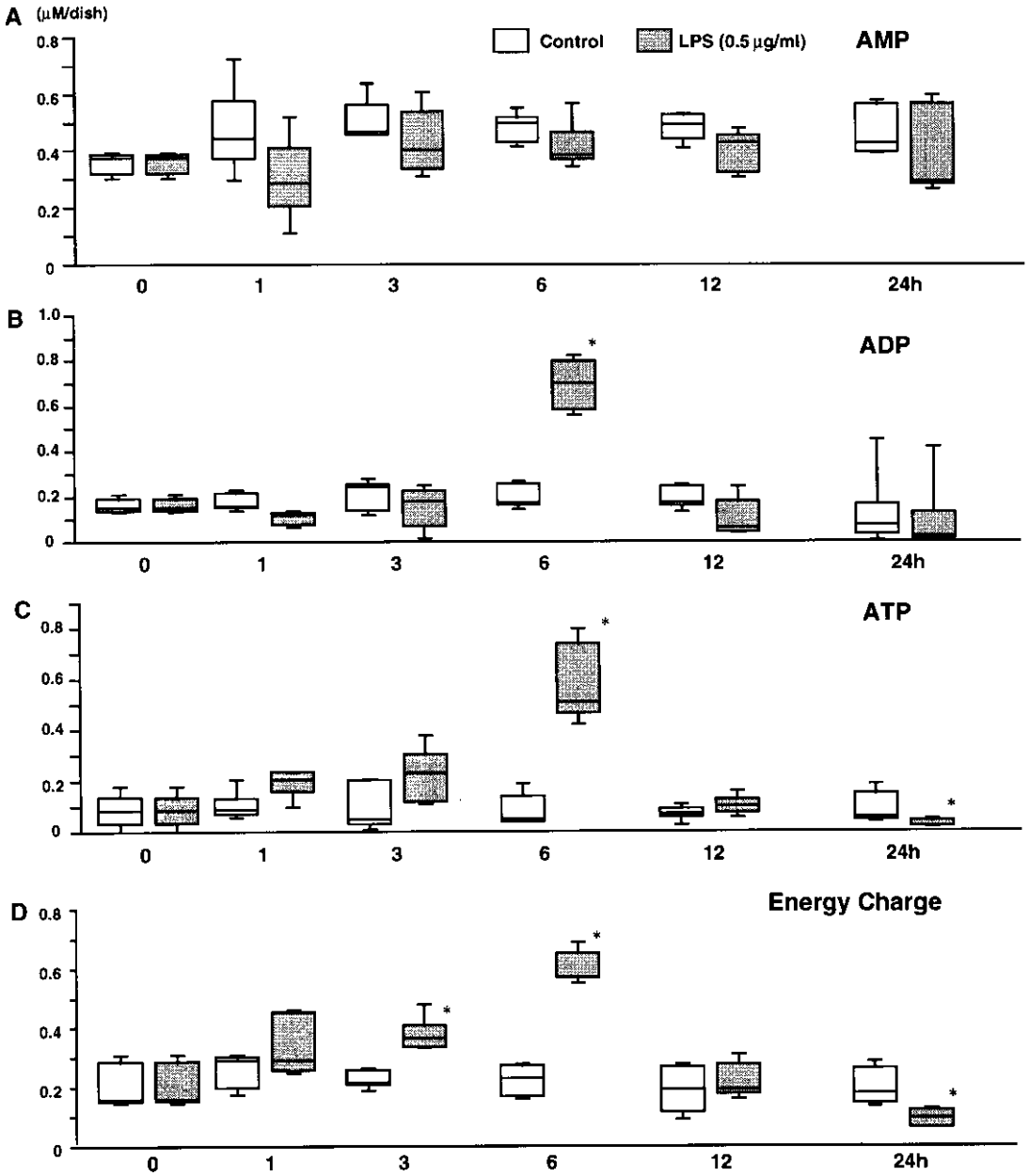

Fig. 1. The time course of adenosine monophosphate (AMP) (A), adenosine diphosphate (ADP) (B), and adenosine triphosphate (ATP) concentration (C) and energy charge (D) in HT29 cells cultured with or without lipopolysaccharide (LPS) (0.5 μg/ml) ($n = 5$). Energy charge (0.5 ADP + ATP/AMP + ADP + ATP) was calculated on the basis of nucleotide concentrations. Asterisks indicate significant difference with and without LPS.

Extraction of nucleotide

The cells were washed with phosphate-buffered saline. Nucleotides were immediately extracted with 0.9 *N* $HClO_4$ solution, followed by neutralization with cold 3.75 M K_2CO_3 solution, as described elsewhere (13). The final supernatants were collected and stocked in a −80 °C freezer until measurement.

Measurement of nucleotide levels

Adenosine monophosphate (AMP), adenosine diphosphate (ADP), and ATP were measured with high-performance liquid chromatography, using a reversed-phase chromatography column (Kanto Chemical Co., Tokyo, Japan). Data were initially obtained as a nucleotide concentration per dish or well. Changes in the degree of nucleotide concentration (percentage change) were calculated considering the nucleotide concentration obtained in the absence of stimulants as 100%. Energy charge (0.5ADP + ATP/AMP + ADP + ATP) was also calculated as proposed by Atkinson (14).

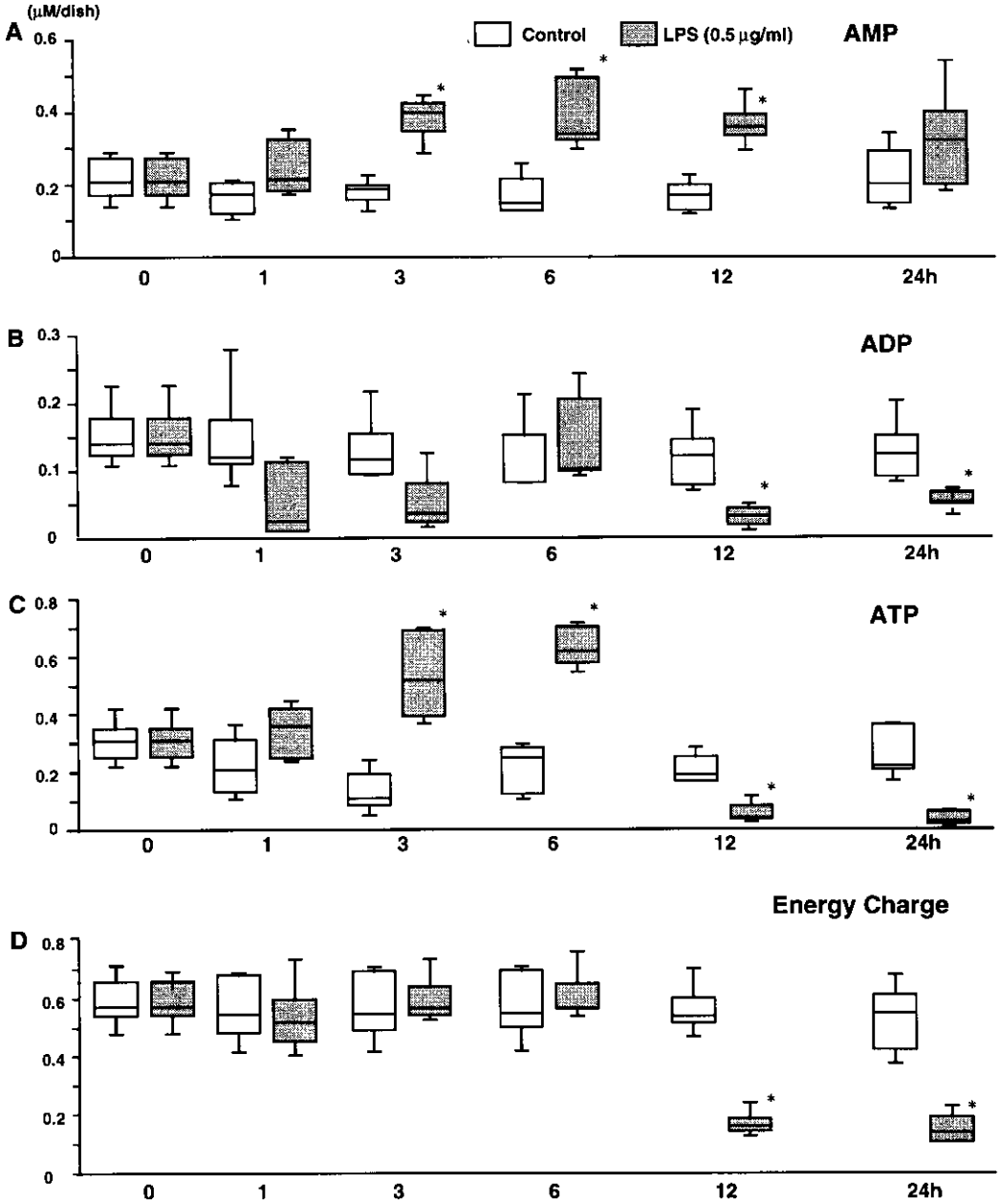

Fig. 2. The time course of adenosine monophosphate (AMP) (A), adenosine diphosphate (ADP) (B), and adenosine triphosphate (ATP) concentration (C) and energy charge (D) in IEC6 cells cultured with or without lipopolysaccharide (LPS) (0.5 μg/ml) ($n = 5$). Energy charge (0.5 ADP + ATP/AMP + ADP + ATP) was calculated on the basis of nucleotide concentrations. Asterisks indicate significant difference with and without LPS.

Measurement of oxygen consumption

Cellular and mitochondrial respiration rates were determined polarographically, using a Clark-type oxygen electrode (model UC 12; Central Kagaku Co., Tokyo) as described elsewhere (15). Each culture medium was collected and then centrifuged to remove cell debris. The cells were also harvested after treatment with trypsin (0.25%) and ethylenediaminetetraacetic acid (0.02%). The cells were carefully resuspended with the collected medium to minimize possible metabolic changes and errors associated with formation of cell clumping in preparations. Then, viability and number of cells were evaluated. An aliquot of the cell suspension was added to the reaction vessel such that a final concentration of $3.2–5.4 \times 10^6$ cells/ml was obtained. Cells were maintained in suspension with a magnetic stir bar. The reaction vessel was capped, and then cellular respiration was determined by the rate of O_2 consumption and monitored on a strip chart recorder (15). To investigate the mitochondrial contribution

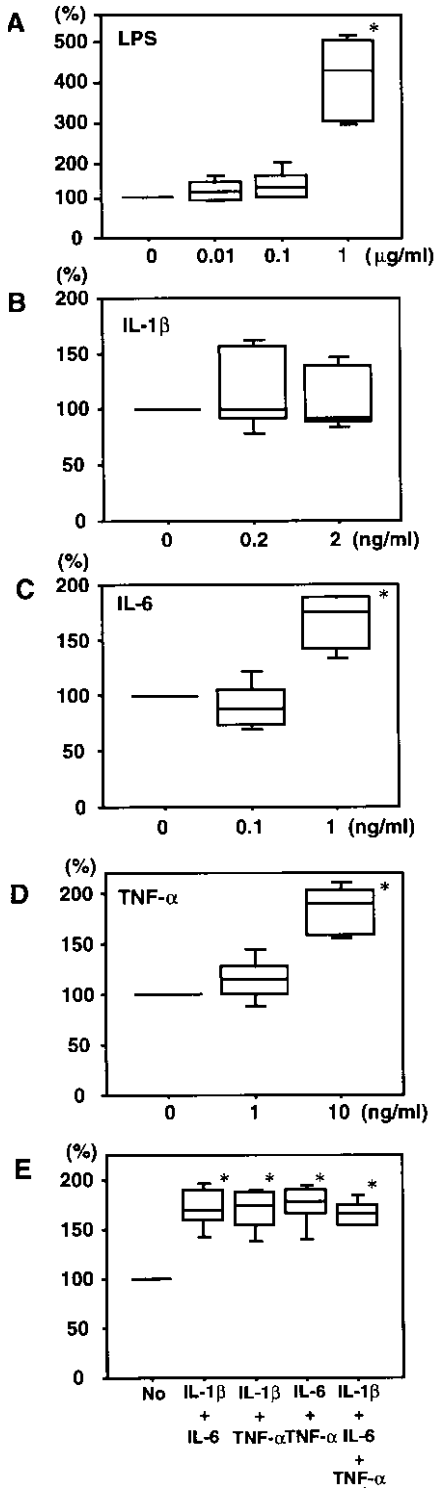

Fig. 3. Adenosine triphosphate (ATP) concentration in HT29 cells cultured with or without lipopolysaccharide (LPS) (A), interleukin-1β (IL-1β) (B), IL-6 (C), and tumor necrosis factor-α (TNF-α) (D) or combination of IL-1β (2 ng/ml), IL-6 (1 ng/ml), and TNF-α (10 ng/ml) (E) for 6 h ($n = 5$). Asterisks indicate significant difference from without stimulants.

to total cellular respiration, antimycin A (Wako, Tokyo, Japan), which is a potent inhibitor of electron flow from the cytochrome bc1 complex to cytochrome c, was added to the reaction vessel at a final concentration of 200 ng/ml (16). Data were expressed as oxygen consumption (mg/l) per 10^6 cells per minute.

Statistical analysis

Mann-Whitney's U test was used to compare data, using StatView software (Abacus Concepts Inc., Berkeley, Calif., USA). Data are expressed medians and percentiles. $P < 0.01$ was the criterion of statistical significance.

Results

Nucleotide levels and energy charge

When HT29 cells were cultured with LPS (0.5 μg/ml), ATP and ADP levels were dramatically enhanced at 6 h, followed by a significant decrease of ADP and ATP at 24 h (Fig. 1A, B, C). The energy charge also increased at 6 h, displaying a twofold increase (Fig. 1D). When IEC6 cells were examined, a significant increase of AMP level was observed at 3, 6, and 12 h (Fig. 2A). The ATP level was also significantly enhanced at 6 h (Fig. 2C). Both ADP and ATP levels decreased at 12 and 24 h (Fig. 2B, C). In contrast to that of HT29 cells, a decrease and not an increase of the energy charge was observed at 12 and 24 h in IEC6 cells (Fig. 2D).

Because the increase of the ATP level was most evident at 6 h, the effect of LPS and proinflammatory cytokines on ATP was investigated at this time point. An enhancement of ATP was shown when LPS (1 μg/ml), IL-6 (1 ng/ml), and TNF-α (10 ng/ml) but not IL-1β were used (Fig. 3A, B, C, D). No synergistic effect was observed when using any combination of cytokines (Fig. 3E).

Oxygen consumption

Oxygen consumption increased rapidly on addition of LPS (0.5 μg/ml), reached the maximum level after 3 h, and showed more than 50% increase at 12 h (Fig. 4A). On the other hand, the control oxygen consumption was stable up to 12 h (data not shown). Proinflammatory cytokines also enhanced oxygen consumption up to 140%–200% (Fig. 4B). Addition of antimycin A rapidly and completely blocked both LPS- and proinflammatory cytokine-induced oxygen consumption, indicating that the oxygen consumption was primarily dependent on mitochondrial respiration (Fig. 5). The cells' viability was confirmed as nearly 100% by trypan-blue exclusion right after the addition of antimycin A.

Discussion

The present study showed that the ATP concentration in epithelial cells increased rapidly due to the exposure to LPS, IL-6, and TNF-α but not IL-1β. The enhancement of oxygen consumption, which was completely blocked by antimycin A, was also observed under these conditions. We therefore conclude that the enhanced ATP level depended on acceler-

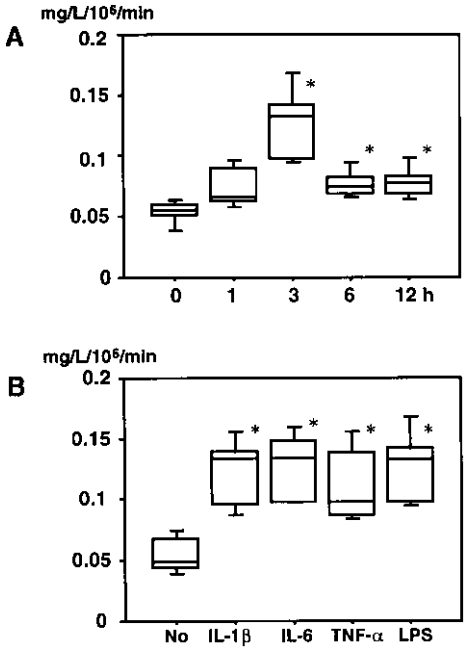

Fig. 4A. Time course of oxygen consumption in HT29 cells cultured in the presence or absence of lipopolysaccharide (LPS) (0.5 μg/ml) ($n = 5$). 4B. Oxygen consumption in HT29 cells cultured with interleukin 1β (IL-1β) (2 ng/ml), IL-6 (1 ng/ml), tumor necrosis factor-α (TNF-α) (10 ng/ml), and LPS (0.5 μg/ml) at 3 h ($n = 5$). Asterisks indicate significant difference from without stimulants.

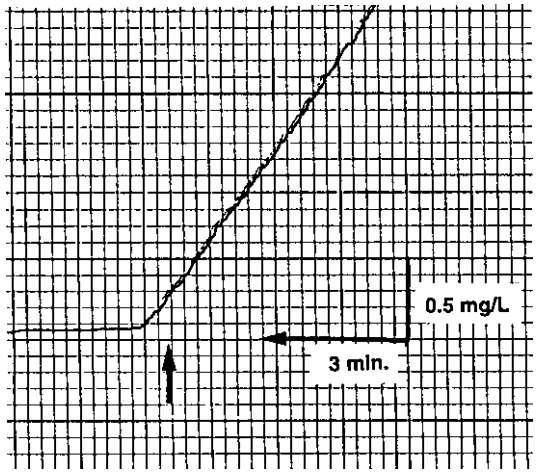

Fig. 5. Antimycin A (200 ng/ml) completely blocked oxygen consumption in HT29 cells cultured with interleukin-6 (IL-6) (1 ng/ml). Similar results were obtained in the presence of lipopolysaccharide (0.5 μg/ml), IL-1β (2 ng/ml), or tumor necrosis factor-α (10 ng/ml). Arrow indicates the administration of antimycin A.

ated mitochondrial oxidative phosphorylation. The consequent decrease of ATP and ADP at 12 h and 24 h in LPS-stimulated HT29 and IEC6 cells may be explained by 'utilization' or 'consumption' of the energy intermediates. The present results indicate that energy status can be actively modulated by these inflammatory modulators, probably to show multiple functional responses (4, 6, 17–21). Although IL-1β failed to enhance the ATP level, this may be due to a different time-course response and/or rapid consumption of ATP. This interpretation is in agreement with the evidence that oxygen consumption was rapidly increased by IL-1β, LPS, and the other cytokines

Another important note is that the energy response to LPS or proinflammatory cytokines may differ qualitatively and/or quantitatively in accordance with the cell origin, the degree of differentiation, and the presence or absence of transformation, as shown by differential AMP and ADP responses to LPS between HT29 and IEC6 cells. Several reports suggest that energy metabolism is partly dependent on differentiation status, which can be modified by culture conditions in vitro. Induction of mitochondrial genes was observed in HT29 cells grown in trehalose-containing medium as compared with those in glucose-containing medium (22). Mitochondrial respiration rate isolated from differentiated HT29 cells is much higher than that from undifferentiated cells (16).

We showed that two epithelial cell lines produced energy intermediates on exposure to LPS, IL-6, and TNF-α, leading to the question whether normal colonic or UC epithelial cells increase energy production in a proinflammatory cytokine-rich mucosal environment. It has been widely accepted that the protein, gene expression, and activity of proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α are increased in experimental and clinical mucosal inflammation (23, 24). Limited energy synthesis in epithelial cells may cause inadequate cellular responses. In practice, it has been shown that IL-1β production was strictly dependent on energy substrate in LPS-stimulated monocytes (25). However, our preliminary experiments using isolated human epithelial cells after dispase digestion failed to show consistent results in energy production, probably owing to inadequate cellular viability (data not shown). An alternative epithelial preparation from mucosal tissues may overcome this methodologic problem. We need an additional consideration for epithelial energy synthesis in inflamed mucosa. We used feasible concentrations of cytokines to stimulate epithelial cells in vitro, in accordance with previous reports measuring these cytokine levels in culture supernatants, tissue, or stool extracts (5, 26–28). In contrast, epithelial cells in mucosa are probably exposed to active chemicals with various concentrations and combinations of LPS, proinflammatory cytokines, and/or immunomodulatory cytokines, including IL-2, IL-4, interferon (INF)-γ and IL-10. We observed similar enhancement of ATP levels on stimulation with IL-1β plus IL-6, IL-1β plus TNF-α, TNF-α plus IL-6, and all of them. However, specific combinations of cytokines may have a pivotal role for modulation of epithelial function. For example, IFN-γ sensitized HT-29 cells to Fas-, TNF-, and endotoxin-mediated cytotoxicity (29).

In conclusion, energy status can be actively modulated by exposure to inflammatory mediators such as LPS, IL-6, and

TNF-α. An active energy production in epithelial cells may play a role in maintaining homeostasis and be critical for the escape from chronic mucosal inflammation. Investigation of dynamic energy metabolism might shed additional light on the relationship between mucosal inflammation and epithelial energy production.

Acknowledgements

We thank Prof. Hiroshi Nagura of Tohoku University, School of Medicine, Dept. of Pathology, for a fruitful discussion. We also thank Ms. Emiko Shibuya for her skillful assistance in the experiments. This work is supported by a grant from the Ministry of Education, Science and Culture of Japan and the Kanae Foundation for the study of new drugs.

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Received 22 October 1998
Accepted 15 January 1999