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ARTICLE

Capsaicin Induced the Upregulation of Transcriptional and Translational Expression of Glycolytic Enzymes Related to Energy Metabolism in Human Intestinal Epithelial Cells

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Capsaicin is the major pungent component in hot chili pepper. It is known that capsaicin induces the increase of energy metabolism through adrenaline activation. The aim of this study was to investigate the mechanism underlying the effect of capsaicin on energy metabolism in human intestinal epithelial cells (Caco-2). To clarify the mechanism, we performed proteomics, real time-PCR, ATP measurement, and MTT in this study. Glycolytic enzymes, namely, phosphoglycerate mutase (PGM) and triosephosphate isomerase (TPI), were overexpressed in capsaicin-treated Caco-2 cells. mRNA expression levels of TPI and PGM were also increased in capsaicin-treated Caco-2 cells. Furthermore, intracellular adenosine triphosphate (ATP) content, which is the end product of glycolysis, was increased. To demonstrate the intracellular ATP production and ATP accumulation, the viability of Caco-2 cells incubated with glucose free medium was measured. The viability of capsaicin pretreated cells was higher than that of untreated cells. All these observations strongly indicate that capsaicin increases energy metabolism in human epithelial cells through the activation of glycolytic enzymes.

KEYWORDS: Capsaicin; TRPV1; Caco-2 cells; energy metabolism; glycolytic enzyme

INTRODUCTION

Capsaicin (8-methyl-*N*-vanillyl-6-nonennamide) is a compound of a number of structurally related moieties characterized by their pungent flavor. Capsaicin has been widely studied because of its importance in spices, food additives and drug (*1*). Studies indicate that capsaicin alters thermogenesis and energy metabolism through sympathetic nervous system activation, which induces the reduction of body weight and suppression of body fat accumulation (*2*, *3*). Also, capsaicin inhibits adipocyte differentiation via activation of AMPK (AMK-activated kinase) (*4*).

Recently, it was reported that capsaicin and capsaicin receptor play an important role in increasing the metabolic rate: the metabolic rates of capsaicin receptor knockout mice and normal mice were measured by oxygen consumption (*5*). The oxygen consumption of normal mice, which have the capsaicin receptor, was increased by injection of capsaicin into intestine, but the oxygen consumption of knockout mice was not.

In the small intestine, goblet cells secrete mucus that forms a coating over the epithelial layer. The main function of the epithelial layer includes absorption of food compounds (*6*). The ingested capsaicin was carried into the intestinal epithelium, which is in contact with a high concentration of food ingredients (*7*, *8*). Considering the relevance of the food components and the effects of the oral route for human exposure, we have investigated the effect of capsaicin on the energy metabolism of the intestine, by using the Caco-2 cell line, a well-known *in vitro* model of intestinal epithelium (*6*). A study on the absorption of capsaicin in rats studied *in vivo* and *in situ* showed that about 80% of the administered capsaicin was absorbed in the intestine (*9*).

Caco-2 cells are derived from a human colorectal carcinoma, with a remarkable morphological and biochemical similarity (tight junction, various enzymes, various transporters) to the human small intestinal columnar epithelium (*10*).

Our previous studies revealed that capsaicin receptor (transient receptor potential vanilloid receptor 1; TRPV1) expression in human intestinal epithelial cells (Caco-2) was increased by capsaicin treatment as a function of time (*11*). We have also reported that the tight junctional permeability of intestinal epithelium was modulated by capsaicin (*12*, *13*).

In this study, the mechanism underlying the effect of capsaicin on energy metabolism of intestinal epithelium was clarified by performing two-dimensional (2D)- polyacrylamide gel electrophoresis (PAGE) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis; real-time PCR was conducted to quantitate mRNA and was performed to measure ATP production. Moreover, we determined the cell viability in medium without glucose to test the hypothesis that the increased ATP by capsaicin contributes to the cell viability of the intestinal epithelium. We reported for the first time that capsaicin can activate energy metabolism in human intestinal epithelial cells.

MATERIALS AND METHODS

Cell Culture. Caco-2 cells (passage 35−45) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO)

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supplemented with 10% fetal calf serum (Sigma), 1% penicillin−streptomycin (Sigma), and 1% nonessential amino acids (Cosmo Bio Co. Ltd., Tokyo, Japan) and incubated in an atmosphere of 5% CO_2 at 37 °C. The cells were passaged at a split ratio of 4−8 every 3 or 4 days. To culture for the subsequent extraction of protein and total RNA, cells were seeded onto Petri plates at a density of 1×10^6 cells per well, and for protein extraction, capsaicin (Sigma) was added (final concentration: 100 μM). For protein extraction and RNA extraction, Caco-2 cells were incubated for 2−3 weeks, to induce cell differentiation, and then treated with capsaicin for 24 h.

Two-Dimensional Gel Electrophoresis. Proteins were extracted by cell lysis in 4% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 7 M urea, 2 M thiourea, 25 mM spermine base (Sigma), 1 M ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 5 mM 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), followed by centrifugation at 100000*g* for 20 min. The supernatant protein concentration was determined using a 2-D Quant kit (GE Healthcare, USA). Proteins from capsaicin-treated or untreated cells were resuspended in 350 μL of 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) immobilized pH gradient (IPG) buffer (GE Healthcare), and 10 mM DTT. Solubilized proteins were electrophoresed in the first dimension using a commercial flatbed electrophoresis system (Multiphor II, GE Healthcare) and 18 cm IPG dry strips (GE Healthcare) with a pH 3−10 linear range. The IPG strip was rehydrated for approximately 14 h at room temperature, and the proteins were electrophoresed in gradient mode using an EPS 3501 XL power supply (GE Healthcare) under the following conditions: 150 V, 1 mA for 30 min; 300 V, 1 mA for 30 min; 500 V, 1 mA for 1 min; 3500 V, 1 mA for 1.5 h; 3500 V, 1 mA for 7.8 h. After isoelectric focusing, the IPG strips were reequilibrated for 20 min in 2% (w/v) sodium dodecyl sulfate (SDS), 6 M urea, 30% (v/v) glycerol, 0.05 M Tris-HCl (pH 8.8), and 2% (w/v) DTT and for 20 min in 2% (w/v) SDS, 6 M urea, 30% (v/v) glycerol, 0.05 M Tris-HCl (pH 8.8), and 5% (w/v) iodoacetamide. The strip was placed on a gradient SDS−PAGE gel (12−14% (w/v) polyacrylamide) and run at 1000 V, 20 mA for 45 min and at 1000 V, 40 mA for 160 min (*14*, *15*). The proteins were visualized by Coomassie Brilliant Blue staining (using PhastGel Blue R-350, GE Healthcare) for spot analysis. The spot analysis software, ImageMaster 2D Platinum (Ver. 5.0; GE Healthcare), allows magnification of specific fields of view and assists in the visual comparison of proteins between control and experimental gels.

Mass Spectrometry. Protein spots of interest were excised from the gel, washed and digested in-gel with trypsin (sequencing grade, Boehringer Mannheim). All MALDI-TOF mass spectra were acquired using a Voyager-DE STR mass spectrometer (Applied Biosystems). The matrix solution was prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid (Sigma) in 1 mL of 50% acetonitrile and 0.1% trifluoroacetic acid in deionized water. The peptide sequence tags obtained were used to identify the proteins by searching databases using BLAST (http://www.ncbi.nlm.nih.gov:80/blast) or Prospector MS-Tag software (http://prospector.ucsf.edu/).

Quantitative Real-Time PCR. After incubating seeded plates for 24 h, total RNA was purified using the ISOGEN kit (Nippon Gene Co. Ltd., Japan). Total RNA was quantified by measurement at 260 nm with a UV spectrophotometer and was also measured at 280 nm to assess purity. Only RNA with a 260/280 ratio higher than 1.8 was used for the real-time PCR. Template cDNA was obtained from total RNA using the SuperScript reverse transcriptase system (Invitrogen). Briefly, RNA was denatured at 65 °C for 5 min and incubated with 1 mL oligo$(dT)_{12-15}$ primers and chilled at 4 °C. After adding SuperScript II reverse transcriptase (200 units) the reaction mix was incubated at 42 °C for 60 min, then 10 min at 70 °C (*16*). For the quantification of mRNA, nested primers were designed using Primer3 input software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/primer3_www.cgi). Quantitative PCR reactions were performed in a MiniOpticon instrument (Bio-Rad, USA) and carried out as recommended for iQ SYBR Green supermix (Bio-Rad). Briefly, the RT mix (2 mL) was used as template for the real-time PCR mix containing 0.5 mM forward (5′-CTTCACCATGGACATCTACGCCAA-3′) and reverse (5′-CTGGTGATGGACTGGATGTAATCG-3′) nested primers (2 mL each) and 2× SYBR Green supermix (10 μL). The amplification conditions were: 3 min at 95 °C, 10 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C for 34 cycles. At the end of the reaction, a melting curve analysis was carried out to check for the presence of primer-dimers.

Measurement of Intracellular ATP Content. ATP was assessed by firefly bioluminescence using the luminescence luciferase assay kit (TOYO Ink, Tokyo, Japan) (*17*). To determine the increase of intracellular ATP content due to capsaicin, treatment Caco-2 cells (2×10^3 cells/well) were pretreated with or without capsazepine (10 μM) for 1 h. The non-glucose medium contains 10% fetal calf serum, 1% penicillin−streptomycin, and 1% nonessential amino acids. Capsazepine is an antagonist of capsaicin receptor (TRVP1). After capsaicin (100 μM) treatment, cells were lysed with 100 μL of lysis buffer (Toyo ink) and placed directly into the chamber of a luminometer (Powerscan HT; Dainippon Pharmaceutical, Osaka, Japan). Light emission was recorded after addition of 100 μL of luciferin−luciferase solution (Toyo ink). When ATP is the limiting component in a luciferase reaction, the intensity of light emitted is proportional to the concentration of ATP in the cytosolic extracts.

Determination of Cell Viability in Medium without Glucose. To investigate the effect of increased ATP due to capsaicin treatment, by capsaicin on the cell viability of intestinal epithelium, Caco-2 cells (2×10^3 cells/well) were cultured in 96-well plates at 37 °C for 24 h with medium including capsaicin (100 μM) or not. Then the medium of the capsaicin-treated cells was changed to the non-glucose medium without capsaicin for 12 h, 24 h and 48 h. On the other hand, the medium of non-capsaicin-treated cells was changed to the non-glucose medium with or without capsaicin for 12 h, 24 h and 48 h. This was followed by the addition of 10 μL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) (Dojin Laboratories, Kumamoto, Japan) working solution. After 6 h of incubation, 10% sodium dodecyl sulfate (SDS, Wako Pure Phamaceutical Co., Ltd., Japan) was added to dissolve the formazan and the plates were again incubated for 12 h. The amount of formazan produced by the cells was determined at 470 nm wavelength. Cell viabilities are presented relative to those of the nontreated cells.

Statistical Analysis. All experiments were conducted in quadruplicate ($n = 4$), and the results were expressed as mean ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test (Stat-100, BIOSOFT, U.K.) to determine significant differences among the means. Statistical significance was defined as $p < 0.05$ compared with control values.

RESULTS

Proteomics Analysis of the Capsaicin-Treated Caco-2 Cells. The proteins overexpressed following treatment with capsaicin (100 μM) in Caco-2 cells were identified by proteomics technologies (**Figure 1A**). From our previous results, 100 μM capsaicin concentration can induce various bioactivity of Caco-2 cells including tight junction regulation and capsaicin receptor response (*11*−*13*). Proteins from capsaicin-treated cells and control cells were separated on immobilized pH gradient strips followed by SDS−PAGE (**Figure 1B**). Stained gels were analyzed with image analysis software. We focused on the spots that were overexpressed by more than 170%. The two protein spots that showed a significant change—spot 1 was upregulated 210% and spot 2 was upregulated 170% in intensity compared to that of controls—had been excised and analyzed by MALDI-TOF mass spectrometry, which gave sufficient confirmation of protein identity for both spots. The results of database searches showed that the protein sequences most closely corresponded to that of human phosphoglycerate mutase (PGM; spot 1) and human triosephosphate isomerase (TPI; spot 2), with matching peptides covering 67% of human PGM (171/254 amino acids) and 56% of human TPI (140/249 amino acids). PGM is an enzyme that catalyzes the internal transfer of a phosphate group from C-3 to C-2 which results in the conversion of 3-phosphoglycerate to 2-phosphoglycerate through a 2, 3-bisphosphoglycerate intermediate. TPI is an enzyme that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. TPI plays an important role in glycolysis and is essential for efficient energy production.

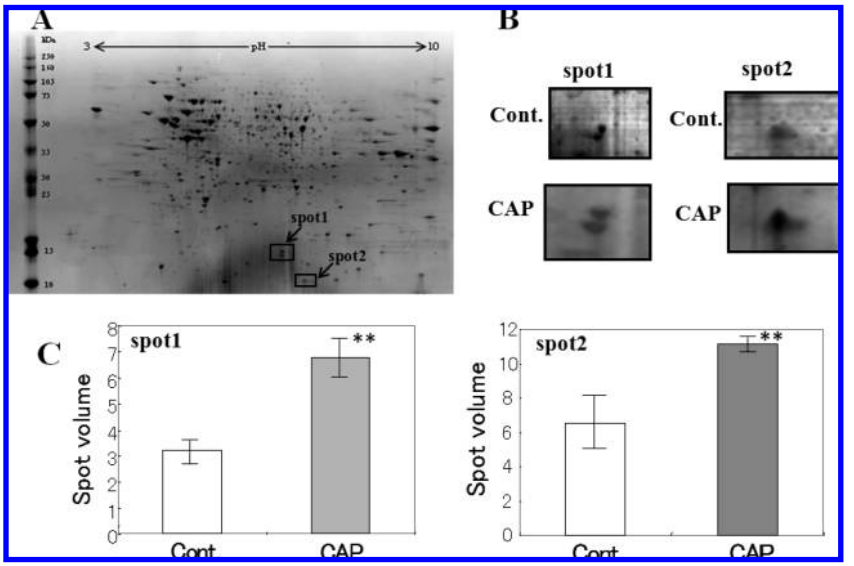

Figure 1. Two-dimensional gel electrophoresis of capsaicin-treated Caco-2 cells (**A**), the magnified images of the boxed regions (spot 1 and spot 2) (**B**) and spot volume of spot 1 and spot 2 (**C**). Caco-2 cells were treated with 100 μM capsaicin for 24 h. The 2-DE gel was stained with coomassie brilliant blue. Spot volume was measured by ImageMaster 2D Platinum software. These spots were identified as phosphoglycerate mutase (PGM; spot 1) and triosephosphate isomerase (TPI; spot 2) by MALDI-TOF mass spectrometry. Each bar represents the mean ± SD (n = 3). $^{**}p < 0.01$ vs control.

Effects of Capsaicin on the mRNA Expression Levels of PGM and TPI. The mRNA expression levels of PGM and TPI proteins were evaluated by quantitative real-time PCR (**Figure 2**) using GAPDH as a control gene. The mRNA expression levels of PGM and TPI proteins were highly increased by 100 μM capsaicin treatment on Caco-2 cells. In fact, the mRNA expression levels of PGM and TPI were upregulated by 280% and 130% respectively. These results have the same tendency as the result of 2-DE.

Effects of Capsaicin on Intracellular ATP Production. Based on the results of proteomics analysis, capsaicin induced the increase of glycolytic enzyme expression. To investigate the upregulated glycolytic enzymes' effects on energy generation, levels of ATP, which is the end product of glycolysis, were evaluated. ATP is a multifunctional nucleotide that is most important as a "molecular currency" of intracellular energy transfer. In this role, ATP transports chemical energy within cells for metabolism. Intracellular ATP accumulation of capsaicin-treated Caco-2 cells was measured by a luciferase reaction method (**Figure 3**). In capsaicin-treated Caco-2 cells, luminescence was 156% higher than in untreated cells. However, this phenomenon disappeared when the cells were pretreated with capsazepine, which is the antagonist of capsaicin receptor (TRPV1). Capsazepine alone did not influence the intracellular ATP level of nontreated Caco-2 cells. The implications of this result are that the production of intracellular ATP on capsaicin-treated Caco-2 cells was related to capsaicin receptor.

Effects of Capsaicin on Cell Viability in the Condition of Non-Glucose Medium. To determine the effect of intracellular ATP accumulation on capsaicin-treated Caco-2 cells, cell viability was investigated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with cells cultured in the non-glucose medium. To assess the effect of intracellular ATP accumulation on cell viability, Caco-2 cells in non-glucose medium were pretreated with capsaicin or without capsaicin. Then the medium of capsaicin-treated cells was changed to the non-glucose medium without capsaicin. On the other hand, the medium of untreated cells was changed to the non-glucose medium with or without capsaicin. The cell viability of Caco-2 cells pretreated with capsaicin (without glucose with pre-CAP) was higher than that of those that were not pretreated with capsaicin (without glucose and without glucose with CAP) (**Figure 4**). Although capsaicin pretreatment induced cell viability endurance when incubated in non-glucose medium, capsaicin treatment induced a quick decrease in cell viability when grown in non-glucose medium.

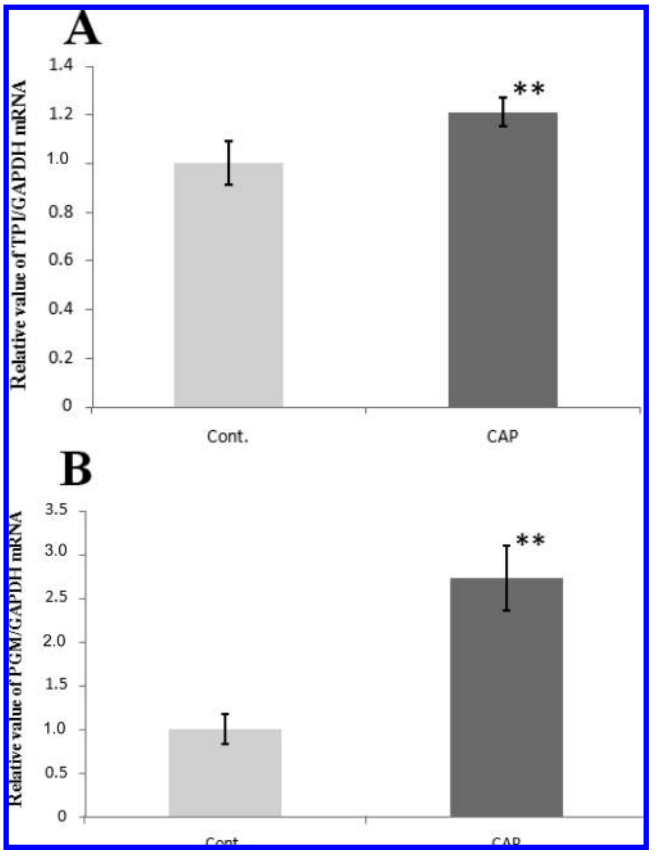

Figure 2. Effect of capsaicin on the expressions of TPI and PGM mRNAs by Caco-2 cells. GAPDH was used as a housekeeping gene. The mRNA expression of TPI and PGM were normalized by GAPDH mRNA expression. Caco-2 cells were treated with 100 μM capsaicin for 24 h. Each bar represents the mean ± SD (n = 3). $^{**}p < 0.01$ vs control.

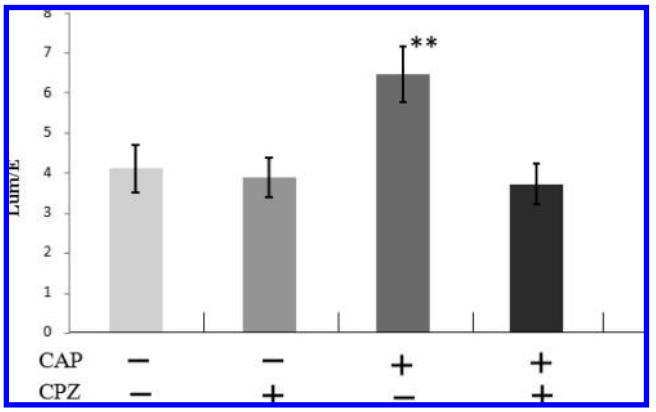

Figure 3. Effect of capsaicin on the intracellular ATP production of Caco-2 cells. Caco-2 cells were treated with 100 μM capsaicin for 24 h and with or without pretreatment with 10 μM capsazepine for 1 h. Intracellular ATP production was increased by capsaicin treatment on Caco-2 cells. Each bar represents the mean ± SD (n = 7). **p < 0.01 vs control.

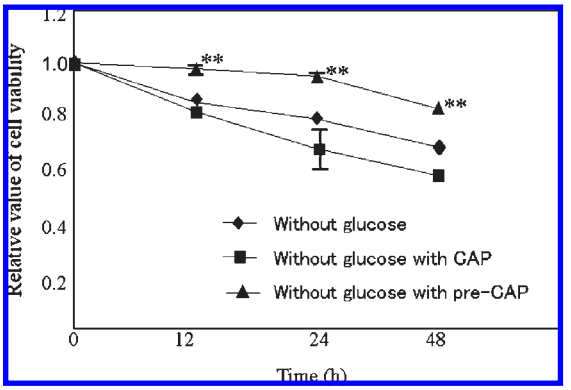

Figure 4. Effect of capsaicin-pretreatment on the cell viability of Caco-2 cells, incubated with or without glucose medium. The cell viability of Caco-2 cells, incubated on non-glucose medium, was measured by using MTT. The cell viability of capsaicin-pretreated Caco-2 cells was higher than that of non-pretreated Caco-2 cells on non-glucose medium. Each bar represents the mean ± SD (n = 9). **p < 0.01 vs without glucose.

DISCUSSION

Capsaicin has various physiological functions such as promotion of hair growth (*18*), induction of apoptosis (*19*−*21*), promotion of intestinal mobility (*22*, *23*), induction of thermal hyperalgesia (*24*, *25*), and suppression of body fat accumulation (*26*). In addition, capsaicin increases thermogenesis by enhancing catecholamine secretion from the adrenal medulla (*5*). It has been reported that the main pathway of metabolism activation by capsaicin was the adrenaline pathway, secreted from the adrenal gland.

We previously reported that capsaicin receptor expressed in Caco-2 cells, which are human intestinal epithelial cells, plays an important role in the regulation of tight junctional permeability by capsaicin (*11*−*14*). From *in vivo* experiment, capsaicin receptor is also involved in the increase in the metabolic rate of adipose tissue following capsaicin administration into mouse intestine (*27*, *28*). This report showed that the intestine organ plays an important role for providing the reaction place with capsaicin in energy metabolite activation. However, the exact mechanism of metabolic activation by capsaicin is still largely unknown. Accordingly, we focused on intestinal epithelial cells and used various procedures for investigating the precise mechanism of metabolic activation by capsaicin in the present study.

From this study, it was apparent from results of 2D gel electrophoresis, mass spectrometry, and real-time PCR that the specific glycolytic enzymes TPI and PGM were overexpressed by capsaicin treatment. These results raised the possibility that the activated TPI and PGM enzymes may have enhanced energy metabolism in capsaicin-treated intestinal epithelial cells. The activity of TPI and PGM was not influenced by age or caloric restriction (*29*), while the other glycolytic enzymes were influenced. Furthermore, the activity and expression of these two enzymes, however, are decreased by oxidation and disorders (Alzheimer's disease, hemolytic anemia, erythrocyte destruction, etc.) (*30*, *31*). These observations indicate that the expression and activity of these glycolytic enzymes were upregulated in response to specific signals, such as capsaicin.

Especially, TPI enzyme is essential for energy production, allowing two molecules of glyceraldehyde 3-phosphate to be produced for every glucose molecule, thereby doubling the energy yield. PGM enzyme catalyzes 1,3-bisphosphoglycerate and plays an important role downstream of glycolysis.

Thus, these results demonstrated that glycolytic enzymes, which are closely related to energy metabolism, were upregulated in capsaicin-treated human intestinal epithelial cells.

The intracellular ATP level was determined with a luciferase reaction method. The intracellular ATP production in Caco-2 cells was activated by capsaicin treatment. The activation of glycolytic pathway, which was induced by capsaicin treatment, contributes to intracellular ATP production and accumulation in Caco-2 cells. The increase of intracellular ATP level, as confirmed by the pretreatment of capsazepine, was related with capsaicin receptor.

The intracellular ATP accumulation is important for optimal integrity of the mucosa and has been suggested to play a specific role in the regulation of tight junction function (*32*, *33*). From these reports, it was supposed that the intracellular ATP accumulation contributes to the homeostasis of Caco-2 cells, because tight junction is necessary for Caco-2 cells differentiation. The effect of intracellular ATP accumulation on cell viability was investigated by MTT analysis. The cell viability of capsaicin-pretreated Caco-2 cells was higher than that of untreated Caco-2 cells and capsaicin-treated Caco-2 cells cultured on condition of non-glucose medium.

There is an interesting report on the effects of capsaicin on intestinal metabolism (*34*): capsaicin immediately increased the temperature of the skin for 2 h in rats, suggesting an increase in heat loss. O_2 consumption, an index of heat production, also immediately increased after capsaicin injection, and this increase lasted for > 10 h. The intestinal temperature decreased within 1 h after injection, and this decrease was followed by a long-lasting hyperthermic period. We suggest that the decrease of temperature in this phenomenon was caused by ATP synthesis and the long-lasting hyperthermic period was caused by ATP accumulation.

An increase in thermogenesis is a well-known metabolic function of capsaicin. However, the mechanism of metabolic promotion caused by capsaicin has been mainly studied at the level of activation of adrenal sympathetic nerves (*35*−*37*). Moreover, the report of Kawada et al. (*38*) raises the possibility that the metabolic promotion caused by capsaicin is induced in various pathways.

Based on the results of Kawada et al., we hypothesized that the metabolic activation by capsaicin was caused by the multiple signaling pathway; the pathway concerned with the capsaicin receptor is an attractive candidate for the metabolic activation pathway in intestinal epithelium. We examined the relationship of glycolytic enzymes to the increase in metabolic rate of human intestinal epithelial cells.

We also revealed that TPI and PGM, which are specific glycolytic enzymes responding to capsaicin, play a key role in the increased metabolic rate in intestinal epithelium. These results indicate that the pathway of metabolic activation by capsaicin was concerned with TPI and PGM through ATP generation.

To the best of our knowledge, this is the first study on the mechanism of metabolic activation by capsaicin in intestinal epithelium. From this study, we suggest that capsaicin induced ATP production via the upregulation of glycolytic enzymes, TPI and PGM, in Caco-2 cells.

ABBREVIATIONS USED

ATP, adenosine triphosphate; PGM, phosphoglycerate mutase; TPI, triosephosphate isomerase; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide.

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