Energy dependence of restitution in the gastric mucosa

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Cheng, Aaron M., Sarah W. Morrison, David X. Yang, and Susan J. Hagen. Energy dependence of restitution in the gastric mucosa. Am J Physiol Cell Physiol 281: C430–C438, 2001.—Rapid epithelial repair (restitution) after injury is required to maintain barrier function of the gastrointestinal mucosa and skin and is thought to be a highly ATP-dependent process that would be inhibited under hypoxic conditions. However, little is known about the metabolic pathways required for restitution. Thus, this study was undertaken to evaluate, in vitro, the role of oxidative respiration and glycolysis in restitution after injury. To this end, restitution of the bullfrog gastric mucosa was evaluated under the following conditions: 1) blockade of mitochondrial respiration; 2) blockade of glycolysis; or 3) absence of glucose. The extent of mucosal repair after injury was evaluated by electrophysiology and morphology. Cell migration, repolarization, and the formation of tight junctions after injury occurred during blockade of mitochondrial respiration, whereas the recovery of mucosal barrier function did not. In contrast, glycolytic inhibition completely blocked all aspects of restitution by inhibiting the migration of surface epithelial cells. Restitution occurred in tissues incubated with glucose-free solutions, suggesting that cells contain sufficient glucose (glycogen) to drive glycolysis for many hours. Our results demonstrate that the glycolytic pathway is essential for restitution after injury in the bullfrog gastric mucosa and that all but complete repair of barrier function occurs in the absence of mitochondrial respiration.

Rana catesbeiana; cell migration; metabolic inhibition

EPITHELIAL CELLS OF THE GASTROINTESTINAL (GI) tract form an effective barrier between luminal and serosal compartments. Epithelial cells are often injured, resulting in denudation of the surface and an increase in permeability of the mucosa. An important response to injury is rapid epithelial repair (restitution), which serves to reestablish epithelial continuity and barrier function of the mucosa.

Restitution was first described in vitro in the bullfrog gastric mucosa (9, 40), but is now known to be a generalized response to superficial injury along the entire GI tract and skin (7, 13, 14, 25, 33, 47). From extensive morphological and physiological studies in the bullfrog gastric mucosa, restitution has been described as a two-part process (9, 40). First, uninjured surface cells extend lamellapodia and migrate to form a thin confluent sheet of epithelial cells at the apical surface of the mucosa. Next, the monolayer of flattened cells reestablishes tight junction structure (and cell polarity) that acts to restore barrier function (9, 40). Restitution has been shown to be a rapid, nonmitotic process that can take place in vitro within 4 h in the amphibian gastric mucosa (9) and is dependent on bicarbonate, Ca^{2+}, actin filaments, and certain growth factors (9, 32, 41, 48). Restitution is also thought to be highly energy dependent and, therefore, likely to be inhibited under conditions of ATP depletion (42). This suggests that restitution would be impaired when the O_2 concentration is reduced (natural hypoxia) or when the mucosa is exposed to substances that cause depletion of intracellular ATP (chemical hypoxia). No study, to date, addresses the energy requirements for restitution.

Thus the present study examines metabolic pathways that support restitution of the bullfrog gastric mucosa. Our results show that the first requirement for restitution (cell migration after injury) is not dependent on mitochondrial respiration but on glycolysis, because either 2-deoxy-d-glucose (2-DG) or iodoacetic acid completely blocked the migration of surface epithelial cells after injury. In contrast, the second requirement for restitution (reestablishment of mucosal barrier function) requires ATP generated by mitochondrial respiration. Although cells repolarize and tight junctions form in the absence of mitochondrial ATP, the junctions are significantly more permeable to small molecules. We propose that surface epithelial cells produce sufficient ATP to support cell migration, even in the absence of molecular oxygen. This may be due to an intimate relationship between metabolism and the actin cytoskeleton that exists in migrating cells.

MATERIALS AND METHODS

Restitution. Animals used for this study were maintained in accordance with the guidelines of the Committee on Animals at the Beth Israel Deaconess Medical Center and those prepared by the Committee on Care and Use of Laboratory Animals by the National Research Council. Bullfrogs (Rana catesbeiana), caught in the wild, were purchased from either West Jersey (Wenonah, NJ) or Charles D. Sullivan (Nashville, TN) and kept at room temperature in large water tanks until use. Stomachs were removed from pithed frogs, and the fundic mucosa was stripped from underlying external muscle layers and submucosa to bare the muscularis mucosa as

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described previously (18). Stripped mucosae were divided into two halves, and each was mounted between two Lucite halves of an Ussing-type chamber with an exposed mucosal area of 0.636 cm². Mucosal surfaces were bathed with a solution containing (in mM) 102.4 Na⁺, 4.0 K⁺, 0.8 Mg²⁺, 91.4 Cl⁻, 2.0 Ca²⁺, 10.1 SO₄²⁻, and 19.3 mannitol and continuously gassed with 100% O₂. Mucosal solutions were unbuffered and kept at pH 4.0 with a pH-stat device (Radiometer America, Cleveland, OH). Serosal surfaces were bathed and kept at pH 7.4 and were continuously gassed with 95% O₂-5% CO₂.

For studies of restitution, tissues were used in the “resting” state with no added stimulant or inhibitor of acid secretion. Tissues were injured by exposure of the luminal surface to 1 M NaCl for 10 min as described in detail by Svanes et al. (40). After injury, both nutrient and luminal solutions were replaced. One tissue was used as a control, and the other tissue was subjected to one of the following conditions: 1) potassium cyanide (KCN; 2 mM) was added to nutrient and luminal solutions to block mitochondrial respiration; KCN inhibits the reoxidation of cytochrome a₉ by molecular oxygen. In control tissues, an additional 2 mM potassium chloride (KCl) was added to nutrient and luminal solutions; 2) sodium azide (3 mM; replacing 3 of the 83.3 mM NaCl) was added to the nutrient solution to inhibit mitochondrial respiration. Sodium azide inhibits the activity of respiratory chain complex IV (cytochrome c oxidase); 3) 2-DOG (10 mM), replacing 10 mM glucose in the nutrient solution, was used to inhibit glycolysis; 2-DOG enters cells like glucose but is not metabolized; 4) 2-DOG (10 mM) and sodium pyruvate (20 mM) replaced 10 mM glucose and 20 of the 83.3 mM NaCl in the nutrient solution. As pyruvate is the end product of glycolysis that is blocked by 2-DOG, added pyruvate enters the citric acid cycle and generates ATP via mitochondrial respiration; 5) iodoacetic acid (300 μM) was added to nutrient and luminal solutions; iodoacetic acid is converted to iodoacetate at neutral pH and inhibits glycolysis and the hexose monophosphate shunt by inhibiting glyceraldehyde-3-phosphate dehydrogenase; 6) glucose-free solutions where 10 mM mannitol replaced 10 mM glucose in the nutrient solution; this condition was to verify whether blocking glucose uptake into cells affected restitution; and 7) glucose-free solution, as above, for 4 and 20 h before injury; this condition was used (in an attempt) to glycogen-deplete tissues before injury. Tissues were monitored under the above conditions for 4 h to evaluate recovery of transmucosal epithelial resistance (TER) after injury. TER was calculated from Ohm’s law using measurements of potential difference that were monitored continuously by KCl-saturated agar bridges connected via two calomel electrodes to a DVC-1000 voltage/current clamp (World Precision Instruments, Sarasota, FL).

Mannitol flux studies. The flux of [³H]mannitol from luminal to serosal solutions after injury was used to determine and how blockade of mitochondrial respiration or glycolysis affects mucosal permeability after injury. [³H]mannitol (50 μCi and 15–30 Cl/mmol; NEN, Boston, MA) was added to the luminal solution (containing 19.3 mM of cold mannitol) immediately after injury. Aliquots (0.25 ml) were taken every 30 min from the nutrient solution, replacing this aliquot with an equal volume of unlabeled nutrient buffer. Samples were diluted with 3 ml of scintillation fluid (Atomlight; NEN), and the amount of [³H] in each sample was determined in a liquid scintillation counter (Packard Instruments, Meriden, CT). Mucosal-to-serosal flux was calculated by standard techniques.

Morphology. Tissues were fixed for light and electron microscopy for 10 min at room temperature in the chamber and then overnight at 4°C in 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Tissues were postfixed with 1% OsO₄ in 0.1 M cacodylate buffer, stained en bloc with aqueous 2% uranyl acetate, dehydrated through graded alcohols and propylene oxide, and then embedded in LX112 resin.
Thick sections (0.5 mm), stained with toluidine blue for light microscopy, were used to evaluate restitution in each experiment. All samples were coded, and morphological evaluation was conducted without foreknowledge of their source by four investigators. Each tissue was evaluated from 0 to 5, as follows. A score of 0 indicated that the apical surface of the mucosa was denuded without cell migration from the gastric pits (no restitution). A score of 1 indicated that <25% of the apical surface was covered with flattened epithelial cells. A score of 2 indicated that 25–50% of the apical surface was covered with flattened epithelial cells. A score of 3 indicated that 50–75% of the apical surface was covered with flattened epithelial cells. A score of 4 indicated that 100% of the apical surface was covered by cuboidal or columnar, rather than flattened, epithelial cells. After analysis by each investigator, the code was broken, and the results were analyzed as described below.

**RESULTS**

Injury and repair in control gastric mucosa. Exposure of frog gastric mucosa to 1 M NaCl (luminal) for 10 min induced an immediate decrease in TER (Fig. 1A). After the hypertonic salt was replaced with buffer, TER recovered gradually to ~88% of preinjury levels.
within 4 h (Fig. 1A). The flux of mannitol in control tissues (Fig. 1B) was 0.341 pmol·h⁻¹·cm⁻² for the first 90 min after injury (initial flux), but was reduced 3.8-fold to 0.09 pmol·h⁻¹·cm⁻² by 120 to 240 min after injury (final flux). Morphological analysis of control tissues 4 h after injury showed that 100% of the denuded surface was populated with surface epithelial cells that were cuboidal or columnar in shape (Fig. 2A). The mean histological score of control tissues was 4.59 ± 0.06 (Table 1).

Effects of metabolic blockade with KCN or sodium azide. We recently showed that 2 mM KCN completely inhibits stimulated acid secretion in the frog gastric mucosa and significantly reduces the concentration of intracellular ATP in isolated gastric glands (17). Sodium azide (1–5 mM) has a similar effect on acid secretion in the frog gastric mucosa (21). Because our goal was to use a concentration of metabolic inhibitor that blocks the generation of ATP and cell function, we chose 2 mM KCN and 3 mM sodium azide for our studies.

Tissues were incubated for 30 min with KCN or sodium azide before injury. TER increased significantly in these tissues before injury (Fig. 1A), which was due to inhibition of spontaneous acid secretion. Exposure to 1 M NaCl for 10 min induced an immediate decrease in TER (Fig. 1A). After the hypertonic salt was replaced with buffer containing KCN or azide, there was a modest recovery of TER to 50% of that in control tissues (Fig. 1A). Because KCN is volatile and produces a toxic gas, mannitol flux experiments were done only in the presence of sodium azide. Mannitol flux was 0.387 pmol·h⁻¹·cm⁻² for the first 90 min after injury but decreased to 0.256 pmol·h⁻¹·cm⁻² by 120 to 240 min after injury; the final rate of mannitol flux was 2.86-fold greater than in control tissues (Fig. 1B). Morphology of tissues incubated with KCN (not shown) or sodium azide (Fig. 2B) was nearly identical and showed that 100% of the apical surface of the gastric mucosa was covered with a flattened layer of surface epithelial cells. The mean histological score of tissues treated with KCN was 3.91 ± 0.26 and with sodium azide was 3.96 ± 0.16 (Table 1). Surface epithelial cells from tissues treated with KCN (not shown) or sodium azide (Fig. 3) were polarized, and tight junctions were present between cells.

Effects of blockade of glycolysis with 2-DOG or iodoacetic acid. Tissues were incubated for 30 min with 2-DOG or iodoacetic acid before injury. These glycolytic inhibitors did not affect the rate of spontaneous acid secretion within the first 30-min period (they reduced acid secretion to 0 within 2 h) and thus did not significantly increase TER before injury (Fig. 1A). Exposure to 1 M NaCl for 10 min induced an immediate decrease...
in TER (Fig. 1A). After the hypertonic salt was replaced with buffer that contained either 2-DOG or iodoacetic acid, there was virtually no recovery of TER (Fig. 1A). In addition, initial and final rates of mannitol flux in tissues treated with 2-DOG (not shown) or iodoacetic acid (Fig. 1B) were not significantly different and were approximately sevenfold greater than the final rate of flux in control tissues. The cumulative mannitol flux was 0.543 pmol·h⁻¹·cm⁻² in tissues treated with 2-DOG and 0.57 pmol·h⁻¹·cm⁻² in tissues treated with iodoacetic acid. Morphological analysis of tissues treated with 2-DOG showed that only some migration of cells occurred from the gastric pit (Fig. 4A). In tissues treated with 2-DOG, <25% of the denuded basement membrane was covered with flattened epithelial cells, and the mean histological score was 0.75 ± 0.15 (Table 1). In contrast, tissues treated with iodoacetic acid showed no migration of surface epithelial cells from gastric pits or along the denuded basement membrane at the surface of the gastric mucosa (Fig. 4B). The mean histological score for tissues treated with iodoacetic acid was 0 ± 0 (Table 1).

**Added pyruvate facilitates cell migration after injury in tissues treated with 2-DOG.** Pyruvate is the end product of glycolysis and is not formed when glycolysis is blocked with 2-DOG or iodoacetic acid. This inhibits the ability of cells to generate ATP because both aerobic respiration and anaerobic glycolysis depend on the production of pyruvate. 2-DOG with added pyruvate should increase the generation of ATP and improve the rate of cell migration after injury.

Tissues were incubated for 30 min with 2-DOG and sodium pyruvate (2-DOG/pyruvate) before injury. Treatment of tissues with these substances had no affect on acid secretion and thus did not cause a significant increase in TER before injury (Fig. 1A). Exposure to 1 M NaCl for 10 min induced an immediate decrease in TER. After the hypertonic salt was replaced with buffer containing 2-DOG/pyruvate, there was virtually no recovery of TER (Fig. 1A). In addition, initial and final rates of mannitol flux were not significantly different (Fig. 1B), similar to that from tissues incubated with iodoacetic acid or 2-DOG alone. The cumulative mannitol flux in tissues incubated with
2-DOG/pyruvate was 0.615 pmol·h⁻¹·cm⁻². The morphology of tissues treated with 2-DOG/pyruvate (not shown) was nearly identical to that of tissues treated with 2-DOG (Fig. 4A). Surface epithelial cells were found to migrate out of the gastric pit, but the apical surface of the gastric mucosa remained largely denuded. However, the mean histological score of tissues treated with 2-DOG/pyruvate was 2.13 ± 0.20 (Table 1), which demonstrates that a greater amount of the apical surface was covered with flattened epithelial cells.

Restitution occurs in glucose-free solutions. Tissues were incubated for 30 min in glucose-free solution before injury. This treatment had no affect on acid secretion and did not significantly increase TER before injury (Fig. 5A). Exposure to 1 M NaCl for 10 min induced an immediate decrease in TER (Fig. 5A). After the hypertonic salt was replaced with buffer devoid of glucose, there was complete recovery of TER within 4 h (Fig. 5A). Furthermore, tissues incubated in glucose-free solutions for 4 or 20 h (before injury) also showed complete recovery after injury (Fig. 5B). The initial rate (30 to 120 min after injury) of mannitol flux in glucose-free solutions was 0.49 pmol·h⁻¹·cm⁻², which is 1.4-fold greater than in control tissues (Fig. 5C). This increase in mannitol flux may reflect a significantly slower rate of recovery of TER in glucose-free solutions after injury (Fig. 5A). However, the final rate (180 to 240 min after injury) of mannitol flux was 0.106 pmol·h⁻¹·cm⁻², which is not significantly different from that of control tissues. Morphology of tissues treated with glucose-free solutions showed complete recovery after injury (Fig. 6). Surface epithelial cells were cuboidal or columnar and covered 100% of the surface of the gastric mucosa (Fig. 6). The mean histological score for tissues treated with glucose-free solutions was 4.21 ± 0.08 (Table 1).

DISCUSSION

Mitochondria generate ATP via aerobic respiration, which can be blocked with chemicals, such as KCN or azide (chemical hypoxia), or by reducing the concentration of O₂ (natural hypoxia). The present study shows that blockade of mitochondrial respiration (chemical hypoxia) has two different effects on restitution in the bullfrog gastric mucosa. First, chemical hypoxia does not impede the migration, repolarization, or formation of tight junctions in surface epithelial cells after injury. Because cell migration after injury is not dependent on respiration to generate ATP, aerobic glycolysis (or some other pathway) must be activated. Second, chemical hypoxia significantly impairs the recovery of barrier function after injury even though tight junctions form at the apical surface of surface epithelial cells.
These results suggest that complete assembly of the tight junction and recovery of mucosal barrier function after injury is dependent on ATP generated by mitochondrial respiration. Why cell migration and recovery of barrier function after injury require different energy sources is not known.

The results presented here suggest that all aspects of restitution are, however, dependent on an intact glycolytic pathway. These results are in contrast to the energy requirements for gastric acid secretion, a process known to be highly ATP dependent (1, 2, 4, 10, 11, 19, 21, 34–37). Whereas gastric acid secretion in the frog mucosa is rapidly inhibited (nearly completely) with 1–5 mM sodium azide (21) or 2 mM KCN (17), we show here that the same concentration of azide or KCN has little effect on cell migration, repolarization, or the formation of tight junctions after injury. In contrast, incubation with the glycolytic inhibitors 2-DOG or iodoacetic acid completely inhibits restitution by blocking cell migration. Although the frog mucosa is an excellent model in which to study mucosal repair after injury, the metabolic requirements for mucosal restitution may differ in larger wounds, in other species, and in other tissue types.

The metabolic requirements for cell migration after injury, presented here, are consistent with those in which cell migration, in general, was studied in either explant cultures of skin epithelial cells or in primary cultures of human keratinocytes during chemical or natural hypoxia (15, 24, 47). In the oral mucosa (antemolar plate), migration of skin epithelial cells from an explant was evaluated by the formation of a “tapered tongue” along the cut edge of the tissue (15). The length of the tapered tongue was compared during blockade of mitochondrial respiration with cyanide, antimycin A, or hypoxia, or by blockade of glycolysis with sodium fluoride, 2-DOG, or iodoacetic acid. It was found that although the length of the tapered tongue did not change in the presence of glycolytic inhibitors, it increased significantly in length in the presence of mitochondrial inhibitors (15). This later observation was verified in cultured human keratinocytes. Under hypoxic conditions, the migration of keratinocytes (neonatal foreskin) was accelerated, both in nonconfluent migrating cells and in wounded, confluent monolayers (31). It was found that during hypoxia, keratinocytes exhibited increased expression and redistribution of ezrin/radixin/moesin proteins, decreased secretion of laminin 5 (a protein known to inhibit the migration of keratinocytes), and enhanced expression of type IV collagenase, all of which were thought to facilitate cell migration (31). In the cornea, cell migration was evaluated in explant cultures as “sliding” of the epithelium after injury. Because glycolytic inhibitors (parahydroxymercuronibenzolate and iodoacetic acid) blocked sliding and a respiratory inhibitor (cyanide) did not, it was concluded that sliding of the corneal epithelium requires energy from a source that may be glucose or glycogen (24).

Whether cells do or do not migrate during blockade of aerobic respiration may reflect their origin and/or specific function. For instance, glycolysis is the primary source of energy for the migration of melanoma A2058 cells (5). Neutrophil chemotaxis is inhibited by drugs that inhibit glycolysis (3), and natural hypoxia has no effect on the migration of lymphocytes from peripheral blood, suggesting a dependence on glycolysis (45). In contrast, cell migration in monocytes and macrophages is inhibited during natural hypoxia. It was suggested that a dependence on aerobic metabolism may serve to trap both monocytes and macrophages in hypoxic areas of a tumor (29, 45).

It was argued that the inhibition of cell migration during glycolytic blockade in prostate cancer cells was not due to inhibition of glycolysis per se, but due to a lack of ATP (22). Furthermore, it was suggested that when respiration is inhibited, prostate cancer cells adapt by increasing activity of the glycolytic pathway to maintain cellular levels of ATP and migration. This switching of aerobic-anaerobic metabolism was also described in cardiomyocytes during hypoxia (30). Because it is not possible to block glycolysis without affecting respiration and ATP production, our results do not address whether the activation of glycolysis is
specifically required for cell migration or is a default pathway by which ATP is generated during chemical hypoxia. However, two results from the present study argue in favor of a specific requirement for glycolysis in migrating cells. First, we show that pyruvate added to tissues in glycolytic blockade does not greatly increase the rate of cell migration after injury. Although this condition would (theoretically) restore levels of NADH, acetyl-CoA, and intracellular ATP generated by the TCA cycle and mitochondrial respiration, it has only minimal influence on cell migration after injury. Second, if the sole function of aerobic glycolysis was to generate pyruvate that was then used to generate ATP by respiration (similar to gastric acid secretion, described above), then blockade of respiration would inhibit cell migration, and it does not. These results suggest that glycolysis is, in some way, required for cell migration after injury.

We show here that despite morphological restitution after injury in the presence of KCN or azide, TER of the mucosa is only 50% of control, and mannitol flux remains significantly elevated, even when tight junctions are present at the luminal surface of surface epithelial cells. ATP depletion (due to hypoxia) in cultured epithelial cells is known to cause a decrease in TER and an increase in paracellular permeability (6, 12, 27, 43, 44, 46, 49), even though tight junctions are present and appear normal by electron microscopy (46). Thus our data suggest that impaired recovery of TER and permeability in the gastric mucosa exposed to chemical hypoxia is due to a lack of tight junction integrity. It was recently shown that ATP depletion (due to chemical hypoxia) in Madin-Darby canine kidney cells inhibits Rho signaling and the phosphorylation of tight junction-associated proteins, occludin and ZO-1. Because tyrosine phosphorylation of occludin and ZO-1 is responsible for localization of these proteins to the tight junction (12, 16, 23), barrier function at the tight junction is greatly reduced during ATP depletion. Despite the importance of tight junctions in regulating barrier function of the gastric mucosa, little is known about their structure and regulation in gastric epithelial cells.

Why ATP generated by glycolysis cannot serve to maintain integrity of the tight junction is not known. However, it is possible that once reepithelialization is complete, ATP is not further generated by glycolysis. To support this hypothesis, it was recently proposed that the integration of cell motility and metabolism occurs when actin filaments form at the leading edge of migrating cells. It has been shown that many of the glycolytic enzymes bind to actin filaments (8, 26, 28, 38, 39) and that this binding results in a significant increase in the Michaelis-Menten constant and maximal velocity of each enzyme (8, 20). Thus during cell migration, there may be a maximal rate of ATP generation from glycolysis localized specifically to actin filaments at the leading edge, where energy is used to drive cell migration. Further studies will be required to investigate this interesting possibility.

In conclusion, our data provide new insight into the process of restitution and challenges current thinking concerning the metabolic requirements for cell migration, repolarization of cells, formation of tight junctions, and restoration of barrier function in an intact mucosa after injury. In addition, this work also provides a foundation with which to pursue further studies concerning the relationship between metabolism, the actin cytoskeleton, and cell migration after injury in the GI tract and skin.

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