Clinical and experimental studies on treatment of acute mesenteric ischemia

Schoots, I.G.

Citation for published version (APA):
Hypoxia/reoxygenation impairs glucose absorption and cAMP-mediated secretion more profoundly than glutamine absorption and Ca\textsuperscript{2+}/PKC-mediated secretion in rat ileum \textit{in vitro}.

Ivo G. Schoots\textsuperscript{1}, Pieter B. Bijlsma\textsuperscript{1}, Geert I. Koffeman\textsuperscript{1}, Thomas M. van Gulik\textsuperscript{1}

Departments of \textsuperscript{1}Surgery and Surgical Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

\textit{Intraluminal fluid sequestration following intestinal ischemia – a better understanding}

Submitted
Abstract

**Background:** Intestinal ischemia and reperfusion may lead to profuse secretion of water and electrolytes. The underlying mechanisms have been related to increased hydrostatic pressure, to denudation of intestinal villi and recently, to adenosine-mediated enhancement of chloride secretion.

**Methods:** We studied the effects of hypoxia and reoxygenation on baseline electrophysiological parameters, on glucose- and glutamine-induced absorption, on secretion induced by carbachol, histamine and forskolin, and on epithelial barrier function to disodium-fluorescein and horseradish peroxidase, in rat ileum mounted in Ussing chambers.

**Results:** We observed that 30 minutes of hypoxia followed by 60 minutes of reoxygenation differentially affected glucose- and glutamine-absorption to respectively 11 % and 42 % of control values. cAMP-mediated secretion induced by forskolin was reduced to 9 % of controls. In contrast, Ca$^{2+}$/PKC-mediated secretion induced by carbachol or histamine was only reduced to 35-48 % of controls. Furthermore, the epithelium was fully capable to maintain its barrier function to small and large permeability probes, even after 90 minutes of hypoxia.

**Conclusion:** We conclude that hypoxia and reoxygenation differentially impairs nutrient absorption, corroborating recent absorption data in *in vivo* models of ischemia, and that it differentially affects secretory capacity in crypts, dependent on the intracellular messenger pathway. The relative persistence of Ca$^{2+}$/PKC-mediated secretion to hypoxia and reoxygenation indicates that secretagogues that activate this pathway play a significant role in the intraluminal fluid sequestration and diarrhea observed after intestinal ischemia and reperfusion.
Chapter 10

Introduction

Intestinal ischemia and reperfusion injury may occur in a variety of pathophysiologic conditions, e.g., hemorrhage, trauma, and sepsis, and can instigate multiple organ failure. Ischemia and reperfusion result in intestinal epithelial dysfunction which can lead to the disruption of the physiological barrier between the intestinal lumen and the internal milieu, and therefore result in increased exposure to pro-inflammatory cytokines and susceptibility to infection.

An early manifestation of epithelial dysfunction due to ischemia/reperfusion is the switch from net intestinal absorption to net secretion leading to intraluminal fluid sequestration and diarrhea. At the microscopical level, the first visible damage consists of detachment of the villus epithelial cells, with the formation of subepithelial blebs. Salt and nutrient uptake, with fluid co-transport, is localized in the villi. This active transport may be decreased after deprivation of oxygen due to depletion of ATP reserves and loss of Na⁺-K⁺-ATPase activity. Under normal physiological conditions active anion secretion and associated water secretion is mainly confined to the intestinal crypts. Immediately after acute ischemia, a profuse secretion of water and electrolytes develops during reperfusion. The underlying mechanisms causing this net secretion are still incompletely understood. It has been attributed to an increase in “filtration flow” into the lumen as a result of increased hydrostatic pressure in the subepithelial tissue, combined with an increased hydraulic conductivity of the intestinal epithelium due to a loss of villus epithelial integrity following ischemia. Robinson and coworkers questioned this hypothesis, because after ischemia the capillary circulation is obstructed, so that movement of fluid across the capillaries of the villus core is rather unlikely. Instead, they proposed that the net secretion results from the observed epithelial cell loss of the villi (villus denudation) after ischemia, leading to an arrest of the absorptive process, while the less damaged crypt epithelium still continues to secrete, thus causing an imbalance between absorption and secretion after ischemia. More recently, it was hypothesized that ischemia may actually stimulate secretion, based on the observation that chemical hypoxia induced adenosine-mediated anion secretion in T84 colon carcinoma cell monolayers. This hypoxia-induced secretory response in cell lines is not observed in in vivo animal models, where secretion is not apparent during the ischemic episode, but manifest after the initiation of reperfusion.

Another manifestation of epithelial dysfunction following ischemia is the increase of epithelial permeability. This has also been attributed to the desquamation of villus intestinal cells and the resulting villus denudation as observed in in vivo studies. Alternatively, the loss of intestinal barrier function may be caused by increased tight junctional permeability, which can be induced by chemical hypoxia in T84 colon carcinoma cell monolayers. The intestinal permeability to small (e.g., Cr-EDTA) and large (Bovine Serum Albumin) probe molecules after ischemia and reperfusion has been widely investigated in vivo, and intestinal permeability increments in various animal models are detectable after ischemic episodes of 20 to 60 minutes. In contrast, T84 and Caco-2BBe intestinal monolayers are extremely resistant to true hypoxia, and in the latter cell line the earliest permeability increases to small probe molecules like fluorescein sulfonic acid appeared after 12 hours of true hypoxia, and such increments were still indetectable for fluorescein-isothiocyanate dextran 70 (MW 70 kD) after 48 hours of hypoxia. On the other hand, interpretation of particularly macromolecular
permeability measurements from blood-to-lumen or lumen-to-blood as shown in vivo animal studies is complicated, as also the vascular endothelial barrier is restrictive to macromolecules and may be affected by ischemia.

Considering the complex interplay between vascular, subepithelial and epithelial factors in in vivo models of ischemia and reperfusion, and taking into account that compared to in vivo studies, monolayers of intestinal cell-lines show a notably different response to hypoxia, it is of interest to study the effects of hypoxia and reoxygenation in in vitro small intestinal preparations. We determined baseline electrophysiological parameters, glucose absorption, glutamine absorption, cAMP-mediated secretion induced by forskolin, Ca\(^{2+}\)/PKC-mediated secretion induced by carbachol or histamine, and epithelial barrier function using disodium-fluorescein and horseradish peroxidase as permeability probes, during varying periods of hypoxia and reoxygenation in rat ileum mounted in Ussing chambers.

**Material & Methods**

**Animals.** All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Academic Medical Centre and the University of Amsterdam. Adult, male Wistar rats (Charles Rivers, Broekman Instituut BV, Someren, the Netherlands), weighing 300-350 g, were fed standard rat chow (Hope Farms, Woerden, the Netherlands) and water ad libitum. Rats were acclimatized to laboratory conditions for at least 7 days prior to experiments. Animals were housed in stainless-steel cages at a constant temperature (22°C) and subjected to a regimen of 12:12 hours light-dark cycle regimen.

**Experimental procedure and electrophysiological measurements.** Rats were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) via intramuscular injection. Laparotomy was performed by midline incision. A segment of distal ileum was isolated 3 to 5 cm from the coecum, and the lumen was rinsed with Ringer’s solution (37°C) to remove intestinal contents. After ligating the blood supply, the ileal segment was rapidly excised and placed in ice-cold Ringer’s solution. Animals were then killed by intracardial injection of sodium-pentobarbital. The isolated segments were stripped of muscle layers and mounted in Ussing-chambers within 10 minutes after excision. Inclusion of Peyers patches was avoided and silicone grease was used to minimize edge damage. The exposed serosal tissue surface area was 50 mm\(^2\). Mucosal and serosal compartments contained 10 ml of Ringer’s solution, which was thermostated to 37°C, carbogenated (humidified 95% O\(_2\), 5% CO\(_2\); pH 7.3) and circulated by gas-lifting. The serosal solution also contained 2.0 mM L-Glutamine. Transepithelial potential difference was measured with Ag/AgCl-electrodes. Transepithelial resistance (R\(_t\)) was determined every 30 seconds from voltage deflections induced by 10 \(\mu\)A bipolar current pulses through platinum wires. Electrodes and platinum wires were connected to the perfusion solution via Ringer-agar bridges. The equivalent short circuit current (I\(_{sc}\)) and R\(_t\) were calculated according to Ohm’s law. Continuous monitoring of the transepithelial potential difference was performed by a customized computer program using Lab View (National Instruments, USA). Electrophysiological parameters were monitored during the entire experimental time course.

**Secretory and absorptive function.** The viability of the ileal epithelium to react to a secretory stimulus during the time course of the experiments was judged by the measurements of carbachol-induced I\(_{sc}\)-changes \((10^{-5} \text{ M, added serosaly})\), determined at 30, 60, 90 or 120 minutes in four different control tissues per animal. The viability to react to an absorptive stimulus was judged in the same tissues by determination of I\(_{sc}\)-changes, induced by mucosal addition of 20 mM D-glucose at 10 minutes after carbachol administration (20 mM of mannitol was added concomittantly to the serosal side to maintain iso-osmolality).
**Barrier function.** After mounting the tissues, the permeability probes disodium-fluorescein (Na$_2$Fl) and horseradish peroxidase (HRP) were added mucosaly to a final concentration of 10$^{-5}$ M. Serosal samples of 500 µL were taken every 30 min for two hours and were replaced by an equal volume of Ringer’s solution. Permeability of the ileal epithelium was determined by the serosal appearance of Na$_2$Fl and HRP. Na$_2$Fl was detected in a fluorescence-reader (Cytoflour®, Series 4000, PerSeptive Biosystems, Inc., Framingham, MA, USA) at excitation and emission wavelengths of 485/20 and 530/25 nm, respectively. HRP was measured enzymatically. Samples of 100µL were mixed in a 96 wells plate with 100µL phosphate buffer (0.1 M, pH 6.0) containing 0.003% H$_2$O$_2$ and 0.009% ortho-dianisidine dihydrochloride (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). After 15 minutes the reaction was halted by adding a 50 µL 2.0 M H$_2$SO$_4$ solution. The HRP concentration-dependent optical absorption rate was determined with a spectrophotometer at 450-490 nm wavelength (Thermo Max, Molecular Devices Co., Sunnyvale, CA, USA).

**Hypoxia and reoxygenation.** The effects of hypoxia and reoxygenation on nutrient and electrolyte transport were studied after 30 min equilibrium time. Hypoxia was induced by ending carbogenation and perfusing both compartments with 95% N$_2$ and 5% CO$_2$. As a result oxygen pressure decreased from 540-560 mmHg to 45-50 mmHg within 2-3 minutes. Reoxygenation was achieved by returning to carbogen perfusion. After 30 min of hypoxia and 60 min of reoxygenation of tissues the I$_{sc}$-changes induced by glucose, glutamine, carbachol, histamine and forskolin were measured. Baseline electrophysiological parameters were monitored during the entire time course of the experiments. The barrier function was studied in control tissues during 120 minutes, and in the hypoxia-reoxygenation experiments mentioned above, using the permeability probes Na$_2$FL and HRP. The epithelial barrier function was furthermore studied in a second series of experiments, in which hypoxia was induced not only for 30 minutes, but also for 60 and 90 minutes. In these experiments, hypoxia was induced at t = 0 minutes. The equilibrium time was omitted to ensure a 90, 60 or 30 minute reoxygenation period, respectively.

**Histology.** Following the experiments, the ileal tissues were fixed in 10% formaldehyde, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) for histological examination. Test solution composition, chemicals. Krebs-Ringer’s solution contained 117.5 mM NaCl, 5.7 mM KCl, 25.0 mM NaHCO$_3$, 1.2 mM MgSO$_4$, 1.2 mM NaH$_2$PO$_4$, and 2.5 mM CaCl$_2$. All chemicals, with HRP being type II were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands), except L-Glutamine (BioWittaker, Verviers, Belgium) and disodium-fluorescein (Molecular Probes, Leiden, The Netherlands). Forskolin and indomethacin were dissolved in ethanol, and final concentrations of ethanol in the serosal compartment of the Ussing chambers were 0.1 %. This concentration was without detectable effect on all measured parameters.

**Statistical analysis.** Results reported represent mean values ± standard error of the mean (SEM). Statistical analysis was performed by the Mann-Whitney U test. A value of $P < 0.05$ (two-tailed) was considered statistically significant.

**Results**

All investigated tissues demonstrated a serosa-positive transepithelial potential difference after mounting tissues in the Ussing chambers. After 30 min of equilibrium time, the I$_{sc}$ of control experiments decreased from 60.0±7.0 to 32.5±4.0 µA/cm$^2$ (n = 16) during the experimental time course (Figure 1A). R$_t$ decreased from 42.5±1.9 to 22.1±1.7 Ω.cm$^2$ at t = 120 min (Figure 1B). Thereafter, both the I$_{sc}$ and R$_t$ stabilized in control tissues monitored for 150 min. Serosally applied to control tissues at 30 min, tetrodotoxin (TTX, 10$^{-6}$ M), atropine (10$^{-5}$ M) or indomethacin (10$^{-5}$ M) had no detectable effect on spontaneous I$_{sc}$ or R$_t$ (all tested at n = 6, data not shown).
Effects of hypoxia and reoxygenation on short circuit current (I_{sc}) and transepithelial resistance (R_{t}). During 30 min of hypoxia the I_{sc} declined sharply from 65.8±7.5 to 2.8±2.8 μA/cm² (n = 6) within 10 min of hypoxia (Figure 1A), whereas R_{t} did not change compared to control epithelia (Figure 1B). Within minutes, the I_{sc} showed a rapid rise during reoxygenation. A peak value of I_{sc} was invariably observed, immediately following reoxygenation, after which I_{sc} levels increased further to control values during reoxygenation.

**Figure 1 A, B.** Short circuit current (I_{sc}) (Figure 1A) and trans-epithelial resistance (R_{t}) (Figure 1B) in rat ileum control tissues (---; n = 16) and in tissues exposed to hypoxia from t = 30 min to t = 60 min (----; n = 6). During hypoxia the I_{sc} declined significantly from 65.8±7.5 to 2.8±2.8 μA/cm² within 10 min of hypoxia (* P < 0.05). A rapid increase of I_{sc} was observed immediately after reoxygenation. This increase of I_{sc} was followed by a limited decline, after which I_{sc} levels regained initial control values. R_{t} remained unchanged throughout hypoxia and reoxygenation. Values represent means ± SEM.

Effects of hypoxia and reoxygenation on nutrient-induced absorption and carbachol-induced secretion. Changes in the I_{sc} (ΔI_{sc}), induced by serosal addition of 10^{-5} M carbachol and by mucosal administration of 20 mM D-glucose remained constant throughout the time course of control experiments, with no significant differences between additions at 30, 60, 90 or 120 minutes (n = 5 - 6, Table 1).

Changes in I_{sc}, induced by carbachol or by D-glucose after 30 min hypoxia and 60 min of reoxygenation (Figure 2A), showed significant reductions compared to I_{sc}-changes in control tissues. Carbachol-induced I_{sc}-changes decreased 2-fold, from 72.9±17.4 to 33.8±11.4 μA/cm² (n = 6, P = 0.041), and D-glucose-induced I_{sc}-changes decreased 10-fold, from 17.6±2.7 to 1.8±0.8 μA/cm² (n = 6, P = 0.002). The decrease of I_{sc}-changes after D-glucose stimulation to 11±6 % of matched controls (=100 %) was significantly different from the decrease of I_{sc}-changes after carbachol stimulation to 47±8 % of matched controls (P = 0.015).

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Table 1. $I_{sc}$-changes induced by addition of carbachol and glucose at 30, 60, 90 or 120 minutes after tissue mounting.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Glucose $\Delta I_{sc}$</th>
<th>Carbachol $\Delta I_{sc}$</th>
<th>Number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>20.7 ± 3.7</td>
<td>87.7 ± 2.5</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>23.5 ± 6.4</td>
<td>79.8 ± 17.7</td>
<td>5</td>
</tr>
<tr>
<td>90</td>
<td>17.0 ± 2.1</td>
<td>83.7 ± 10.2</td>
<td>6</td>
</tr>
<tr>
<td>120</td>
<td>17.6 ± 2.7</td>
<td>72.9 ± 17.4</td>
<td>6</td>
</tr>
</tbody>
</table>

$I_{sc}$-changes ($\Delta I_{sc}$) induced by serosal addition of $10^{-5}$ M carbachol at 30, 60, 90 or 120 minutes after tissue mounting, and $I_{sc}$-changes, induced by mucosal addition of 20 mM D-glucose at 10 minutes after carbachol administration. No significant differences in $I_{sc}$-changes were observed between different timepoints of addition of either carbachol or D-glucose.

After 30 min hypoxia and 60 min of reoxygenation, changes in $I_{sc}$, induced by L-glutamine or by carbachol (Figure 2B) also showed significant reductions compared to $I_{sc}$-changes in control tissues. Carbachol-induced $I_{sc}$-changes decreased 3-fold, from 51.7±8.5 to 15.6±2.3 $\mu$A/cm$^2$ ($n = 7$, $P = 0.002$), and L-glutamine-induced $I_{sc}$-changes also decreased 3-fold, from 22.6±4.5 to 7.3±1.3 $\mu$A/cm$^2$ ($n = 7$, $P = 0.002$). The decrease of $I_{sc}$-changes after L-glutamine stimulation to 42±10 % of matched controls (=100 %) was comparable to the decrease of $I_{sc}$-changes after carbachol stimulation to 35±7 % of matched controls ($P = 0.63$).

Figure 2 A. Changes in short circuit current ($\Delta I_{sc}$) in rat ileum ($n = 6$), induced by 20 mM D-glucose or by $10^{-5}$ M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), but to a different extent, respectively 11±6 % and 47±8 % of matched controls ($P = 0.015$). Values represent means ± SEM. Inserts: typical recording of control responses to D-glucose and to carbachol.

Figure 2 B. Changes in short circuit current ($\Delta I_{sc}$) in rat ileum ($n = 7$), induced by 20 mM L-glutamine or by $10^{-5}$ M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), to a comparable extent, respectively 42±10 % and 35±7 % of matched controls ($P = 0.63$). Values represent means ± SEM. Inserts: typical recording of control responses to L-glutamine and to carbachol.

Effects of hypoxia and reoxygenation on forskolin- and histamine-induced secretion compared to carbachol-induced secretion. Control values of $10^{-5}$M forskolin-induced secretion at $t = 120$ minutes were 49.0±14.7 $\mu$A/cm$^2$ (Figure 3A). Control values of $10^{-5}$M carbachol-induced secretion, added 10 minutes later, were 93.8±21.6 $\mu$A/cm$^2$ (Figure 3A),
not significantly different from control values in Figure 2A and 2B, indicating that prior addition of forskolin did not decrease $I_{sc}$-responses to carbachol. After 30 min hypoxia and 60 min of reoxygenation changes in $I_{sc}$, induced by forskolin or by carbachol (Figure 3A), showed significant reductions compared to $I_{sc}$-changes in control tissues. Forskolin-induced $I_{sc}$-changes decreased 10-fold to $3.0\pm1.2 \mu A/cm^2$ ($n = 6$, $P = 0.002$), and carbachol-induced $I_{sc}$-changes decreased 2-fold to $38.4\pm6.1 \mu A/cm^2$ ($n = 6$, $P = 0.026$). The decrease of $I_{sc}$-changes after forskolin addition to $9\pm3\%$ of matched controls ($=100\%$) was significantly different from the decrease of $I_{sc}$-changes after carbachol administration to $49\pm11\%$ of matched controls ($P = 0.004$).

Control values of $10^{-4}$M histamine-induced secretion at $t = 120$ minutes were $56.4\pm9.3 \mu A/cm^2$ (Figure 3B). Control values of $10^{-5}$M carbachol-induced secretion, added 10 minutes later, were $70.5\pm9.1 \mu A/cm^2$ (Figure 3B), not significantly different from control values in Figure 2A and 2B, indicating that prior addition of histamine also did not decrease $I_{sc}$-responses to carbachol. After 30 min hypoxia and 60 min of reoxygenation changes in $I_{sc}$, induced by histamine or by carbachol (Figure 3B) showed significant reductions compared to $I_{sc}$-changes in control tissues. Histamine-induced $I_{sc}$-changes decreased 2-fold to $26.0\pm9.2 \mu A/cm^2$ ($n = 9$, $P = 0.024$), and carbachol-induced $I_{sc}$-changes decreased once more 2-fold to $32.4\pm9.3 \mu A/cm^2$ ($n = 9$, $P = 0.014$). The decrease of $I_{sc}$-changes after histamine addition to $44\pm14\%$ of matched controls ($=100\%$) was comparable to the decrease of $I_{sc}$-changes after carbachol administration to $47\pm15\%$ of matched controls ($P = 1.0$).

**Figure 3 A.** Changes in short circuit current ($\Delta I_{sc}$) in rat ileum ($n = 6$), induced by $10^{-5}$M forskolin or by $10^{-5}$M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), but to a different extent, respectively $9\pm3\%$ and $49\pm11\%$ of matched controls ($P = 0.004$). Values represent means $\pm$ SEM. Inserts: typical recording of control responses to forskolin and to carbachol.

**Figure 3 B.** Changes in short circuit current ($\Delta I_{sc}$) in rat ileum ($n = 9$), induced by $10^{-4}$M histamine or by $10^{-5}$M carbachol after 30 min hypoxia and 60 min reoxygenation (hatched bars), were significantly (* $P < 0.05$) decreased compared to control values (open bars), to a comparable extent, respectively $44\pm14\%$ and $47\pm15\%$ of matched controls ($P = 1.0$). Values represent means $\pm$ SEM. Inserts: typical recording of control responses to histamine and to carbachol.

**Effects of hypoxia and reoxygenation on barrier function.** Na$_2$Fl-fluxes reached steady-state values after 60 to 90 min (Figure 4A). The permeability of rat ileal epithelium for Na$_2$Fl did not change when subjected to 30 min of hypoxia and reoxygenation,
irrespective of whether the hypoxia was introduced after a 30 min equilibrium period (with 60 min reoxygenation time, data not shown), or at the onset of the experiment at $t = 0$ min (without an equilibrium period, with 90 min reoxygenation time) (Figure 4A). Even after prolonged periods of hypoxia, 60 and 90 min, no change in $\text{Na}_2\text{Fl}$ permeability was observed compared to controls ($n = 6 - 10$, see legend Figure 4). HRP-flux reached steady-state values at 90 min in control experiments (Figure 4B). Also the HRP-fluxes at the three different periods of hypoxia and reoxygenation were comparable to control values.

![Figure 4A and B](image)

**Figure 4 A, B.** Mucosal to serosal flux of $\text{Na}_2\text{Fl}$ (Figure 5A) and HRP (Figure 5B) in rat ileum *in vitro* in control tissues (open bars; $n = 10$), or exposed to 30 min (forwards hatched bars; $n = 6$), 60 min (backwards hatched bars; $n = 6$) or 90 min (crossed bars; $n = 6$) of hypoxia, which were introduced at $t = 0$ min. No significant differences were observed between hypoxia-exposed tissues as compared to controls. Values represent means ± SEM.

**Effects of hypoxia and reoxygenation on morphology.** Histological appearance of the tissues at the end of the hypoxia-reoxygenation experiments as determined by light microscopy was unchanged compared to control tissues. In both control and hypoxia-reoxygenated tissues a shortening of the villi was observed, with some cell sloughing at the tips of the villi, as well as widened intercellular spaces between and occasionally under villus epithelial cells, corresponding to previously reported data on morphology of oxygenated rat ileum mounted in Ussing chambers at a 120 min time course.

**Discussion**

This *in vitro* study demonstrates the effects of a graded level of hypoxia, (with oxygen pressures of 45 - 50 mm Hg), and reoxygenation on baseline electrophysiological parameters, induced nutrient and electrolyte transport and barrier function in rat ileal epithelium. We observed three notable findings: a) A differential effect of hypoxia and reoxygenation upon the absorptive capacity of the small intestinal epithelium. Sodium-coupled glucose transport showed a substantial reduction after hypoxia and reoxygenation to 11% of control values, whereas sodium-coupled glutamine transport was less affected, to 42% of controls. b) A differential effect of hypoxia and reoxygenation upon the two main secretory pathways of the small intestinal epithelium was demonstrated: The cAMP-mediated anion secretion, as induced by forskolin, decreased to 9% of control values, comparable to the reduction in glucose transport. In contrast, the $\text{Ca}^{2+}$/PKC-
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mediated secretory capacity, reflected by cholinergic or histaminergic stimulation of anion secretion, decreased to only 35 - 49 % of controls, comparable to the reduction of glutamine uptake. c) A lack of effect of hypoxia and reoxygenation was observed on the small intestinal barrier function to substances with molecular weights ranging from 376 D to 40 kD, even after prolonged episodes of graded hypoxia lasting up to 90 minutes.

A differential influence of hypoxia and reoxygenation on the absorptive and the secretory functions of the small intestine may give rise to a pro-secretory imbalance, leading to intraluminal fluid sequestration and diarrhea. This hypothesis, already put forward by Robinson and coworkers in the late seventies 7-10, was based upon in vivo studies of ischemia and reperfusion in canine small intestine. Following ischemia and reperfusion glucose and water absorption were completely abolished, while electrolyte and water secretion persisted. They explained this by the observation of extensive villus denudation during ischemia and reperfusion.

The present study demonstrates that differential influences of hypoxia and reoxygenation on the absorptive and the secretory functions of the small intestine are already regulated at the level of intracellular messenger pathways, instead of villus denudation alone. Hypoxia and reoxygenation in our in vitro model did not lead to extensive villus denudation. A considerable transport capacity of glutamine was still measured after hypoxia and reoxygenation. Furthermore, the intestinal specimen maintained their macromolecular barrier function and structure following hypoxia and reoxygenation as evidenced by permeability and morphological examination.

We observed that the villus enterocytes were still capable to maintain a substantial part of their absorptive capacity for glutamine after a relatively short period of graded hypoxia, while in contrast glucose absorption was almost abolished. This differential effect of hypoxia upon glutamine- versus glucose absorption qualitatively resembles recent findings by Kles et al 27,28 in Ussing chamber studies of rat small intestine, after in situ luminal perfusion of jejunal loops during one hour of mesenteric ischemia. They reported a 70 to 90 % reduction in glucose transport, while glutamine transport was fully preserved, and subsequently showed that the ischemia-induced reduction in glucose absorptive capacity was caused by trafficking of functional SGLT-1 protein from the brush border membrane to intracellular pools. Also their model of ischemia did not result in marked villus denudation 27, which may be related to a protective effect of luminal perfusion during ischemia 32. Thus our in vitro model of hypoxia qualitatively appears to reflect an in vivo animal model of ischemia with a relatively mild degree of epithelial damage.

Besides this differential effect on nutrient absorption, we furthermore observed differential effects of hypoxia and reoxygenation upon the cAMP-mediated and the Ca²⁺/PKC-mediated secretory pathways. It is unlikely that the 10-fold reduction in forskolin-induced, cAMP-mediated secretion after hypoxia and reoxygenation is simply due to ATP depletion in crypt enterocytes, because carbachol addition after forskolin to the same tissues still showed a marked secretory response. The relatively small, 2- to 3-fold reduction of Ca²⁺/PKC-mediated anion secretion after hypoxia and reoxygenation was observed both after cholinergic stimulation and after histaminergic stimulation of this intracellular messenger pathway. In particular the relative persistence of histamine-induced anion secretion after hypoxia may be relevant to the secretory response of the small intestine after ischemia and reperfusion in vivo: 1) Barret and coworkers have
shown that in contrast to cholinergic stimulation, histaminergic stimulation appears to be far less effective in generating negative second messengers, such as inositol 3,4,5,6, tetrakisphosphate. This can block the ability of the epithelium to respond to a second Ca²⁺/PKC-dependent agonist. This implies that histamine-dependent activation of epithelial chloride secretion may be permissive for ongoing responsiveness, whereas cholinergic stimulation is not. 2) Plasma levels of histamine showed little change during ischemia in rabbit small intestine, but promptly increased during reperfusion, thus parallel to the appearance of secretion. Moreover, pretreatment of rats with the histamine-degrading enzyme diamine-oxidase largely prevented the intraluminal fluid accumulation induced by small intestinal ischemia-reperfusion. The released histamine may originate from mucosal and mesenteric mast cells, as both types of mast cells degranulate after ischemia-reperfusion.

The high spontaneous $I_{sc}$ in the rat ileum in our in vitro study (Figure 1A) could not be attenuated by serosally applied TTX, atropine or indomethacin, indicating that it was not caused by ongoing neural activity, a high cholinergic tone or by prostaglandin release. After the induction of hypoxia, an immediate decrease of the spontaneous $I_{sc}$ was observed, and a rapid recovery was determined after reoxygenation. These phenomena were previously observed in an elegant study by Munck, in which rat jejunum was exposed to five minutes of unilateral or bilateral hypoxia in Ussing chambers. He demonstrated that about 80% of the spontaneous $I_{sc}$ was caused by chloride secretion and the remaining 20% was due to sodium uptake. Both serosal and bilateral hypoxia caused a similar drop in $I_{sc}$ as seen in our present experiments, followed by rapid recovery after reoxygenation. Comparable observations have been reported in rat colon. Chloride secretion is driven by the ongoing activity of basolateral Na⁺-K⁺-ATPase in combination with basolateral Na⁺-K⁺-2Cl⁻ co-transport, basolateral K⁺ conductance and the Cl⁻ conductance in apical cell membranes. The high spontaneous $I_{sc}$ in rat small intestine in vitro indicates a high activity of basolateral Na⁺-K⁺-ATPase, and during hypoxia intracellular ATP stores may thus rapidly deplete, leading to the fast reduction of spontaneous chloride secretion. In contrast, in T84 colon crypt cell line monolayers, chemical depletion of ATP stores led to a transient, adenosine-mediated activation of chloride secretion, which disappeared when ATP-levels reached 5% of control values. Moreover, chemical hypoxia decreased the $R_t$ of T84 colon cell monolayers from 1500 Ω.cm² to approximately 700 Ω.cm², while in our study in rat ileum no effect of hypoxia upon $R_t$ could be detected.

The differences in $I_{sc}$ response to hypoxia between the T84 colon cell monolayers and rat ileum may be related to the very low spontaneous $I_{sc}$ (2 μA/cm²) of the T84 colon cell monolayers, or may be caused by differences between chemical hypoxia and true hypoxia, to which T84 monolayers are extremely resistant. This may also explain the differences in effects of hypoxia upon the $R_t$ of the T84 monolayers and the $R_t$ of rat ileal tissues. Moreover, there are large differences in the $R_t$ in both tissue preparations and in their morphology. In unstripped rat small intestine, 80% of the $R_t$ is formed by the subepithelial layer ($R_s$). In stripped tissue, the $R_s$ still forms about 65% of $R_t$ and 15% of the $R_t$ is caused by the lateral intercellular spaces. Thus, in a stripped small intestinal tissue only 20% of $R_t$ is located in the tight junctions. In comparison, in the T84 colon cell monolayers this value is close to 100%, because one may assume that in an intestinal monolayer with a $R_t$ of 1500 Ω.cm² the relative contribution of the lateral intercellular
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spaces to $R_t$ will be much smaller than 15 %. Therefore, in contrast to the $R_t$ of T84 monolayers, the $R_t$ of rat ileal tissue may be a poor indicator of its barrier function, which may be measured more appropriately by quantification of epithelial permeability to small and/or large probe molecules.

In our in vitro study, neither hypoxia, even after 90 min, nor reoxygenation influenced the ileal permeability to disodium-fluorescein or to horseradish peroxidase. This observation is somewhat puzzling, considering the numerous reports from in vivo animal models, in which intestinal ischemia and reperfusion resulted in decreased intestinal barrier function. The most likely explanation is that the increased intestinal epithelial permeability after ischemia and reperfusion in vivo is not caused by epithelial hypoxia per se, but is a secondary effect induced by agents of vascular endothelial and/or submucosal origin, which may initially be activated or released by ischemia and reperfusion. Kubes and coworkers have provided evidence in support of this interpretation in in vivo animal models of ischemia and reperfusion. They used the blood-to-lumen clearance of $^{51}$Cr-EDTA (MW 243 D), which rapidly equilibrates completely between plasma and interstitium, to assess intestinal permeability in feline ileal loops exposed to ischemia and reperfusion. The increased intestinal permeability, induced by ischemia and reperfusion, was reduced by the inhibition of granulocyte recruitment, by constitutive endothelial nitric oxide production and by nitric oxide donors, and it was enhanced via mast cell activation using nitric oxide synthesis inhibitors. Moreover, ischemia and reperfusion did not induce an increase in intestinal permeability to $^{51}$Cr-EDTA in mast cell-deficient mice, in contrast to ischemia and reperfusion in wild-type mice, and mast cell stabilizers completely prevented the increased mucosal permeability to $^{51}$Cr-EDTA after ischemia and reperfusion in rat small intestine in vivo. These findings are consistent with the concept that microvascular dysfunction with increased vascular albumin leak and associated mast cell degranulation are initial events in ischemia and reperfusion. This may be a prerequisite for the impairment of intestinal barrier function that occurs in ischemia and reperfusion in vivo.

In conclusion, we have shown that hypoxia/reoxygenation in rat small intestine in vitro has a differential effect on glutamine- and glucose-absorption, confirming recent reports of differential nutrient absorption in in vivo models of intestinal ischemia. Furthermore the present study is the first report, to our knowledge, that indicates that hypoxia/reoxygenation affects the cAMP-mediated secretory capacity of enterocytes to a much stronger extent than the Ca$^{2+}$/PKC-mediated secretory capacity, which may be relevant for the understanding of the mechanisms involved in the development of intraluminal fluid sequestration and diarrhea after intestinal ischemia.
References

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