CALCIUM GLYCEROPHOSPHATE ATTENUATES HYPOXIA AND CYTOKINE INDUCED INCREASES IN EPITHELIAL PERMEABILITY IN THE CACO-2 MODEL OF INTESTINAL TRANSPORT.

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Calcium glycerophosphate and intestinal epithelial permeability.

Abstract:

Ischemia/reperfusion (I/R) injury is a broad area of medical significance, and includes gut ischemia consequent to such diverse causes as endurance sports activity and congestive heart failure. Few studies have focused on assessment of the direct effects of ischemia on intestinal epithelial integrity. Furthermore, clinical efforts at mitigating the effect of hypoperfusion on gut permeability have focused on restoring gut vascular function. Here we report that, in the Caco-2 cell model of transepithelial transport, calcium glycerophosphate (CGP), an inhibitor of intestinal alkaline phosphatase F3, has a significant effect to preserve transepithelial electrical resistance and to attenuate increases in mannitol flux rates during hypoxia or cytokine stimulation. The effect was observable even at concentrations as low as 1μM. As celiac disease is also marked by a loss of gut epithelial integrity, the effect of CGP to attenuate the effect of the α-gliadin peptide 31-55 was also examined. In this instance, CGP exerted little effect of preservation of TEER, but significantly attenuated peptide induced increase in mannitol flux. In conclusion, it appears that CGP treatment might synergise with other therapies to preserve gut epithelial integrity.

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Introduction:

Ischemia/reperfusion (I/R) injury is a broad area of medical significance. Stroke and coronary artery disease (CAD) are the most obvious examples of I/R injury. However, the fundamental problem is much broader, and includes gut ischemia consequent to splanchnic hypoperfusion. Chronic gut ischemia is not so dramatic as stroke or CAD, but may be more insidious. Splanchnic hypoperfusion is a consequence of endurance sports activity [Peters *et al*., 1999; Van Wijck *e al*., 2012a], and a significant element in the pathophysiology of congestive heart failure [Sandek *et al*, 2010; Celik *et al*, 2010;Sandek *et al*, 2007; Krüger *et al*, 2007], shock [Arvidsson *et al*, 1991], cardiac surgery [Jakob, 2002] and hemodialysis [Jakob *et al*, 2001]. Although the ultimate causes of hypoperfusion are different in each of these clinical situations, they share similar clinical consequences.

During simulated gut ischemia, there is extensive neutrophil recruitment to the post-capillary venule, a process that is dependent, at least in part, on long-chain fatty acyl CoA synthetase [Blakeman *et al*., 2012; Prior *et al*, 2014], likely acting by inhibiting cell adhesion protein palmitoylation [Sardjono *et al*., 2006; Sim *et al*., 2007]. The subsequent immune system activation increases synthesis of an array of cytokines, including TNFα, ILβ and IFNγ. While these have been studied extensively in various pathologies involving splanchnic hypopoperfusion [Esposito *et al*., 2007; Cavriani *et al*., 2007], few studies have focused on assessment of the direct effects of either hypoxia or cytokine liberation on intestinal epithelial integrity [Xu *et al*., 1999; Qiu *et al*., 2014]. Furthermore, clinical efforts at mitigating the effect of hypoperfusion on gut permeability have focused on restoring gut vascular function.

Gut epithelial integrity is dependent, at least in part, on sphingosine-1-phosphate (S1P) generation [Greenspon *et al*., 2011]. Earlier studies revealed that beta-calcium glycerophosphate (CGP) is an inhibitor of intestinal F3 alkaline phosphatase [Tardivel *et al*., 1988]. As alkaline phosphatase catalyzes the conversion of S1P to sphingosine and inorganic phosphate [Edsall and Spiegel, 1999], here we test the hypothesis that, by inhibiting intestinal alkaline phosphatase, CGP might raise the S1P concentrations, thus helping to preserve intestinal integrity during ischemic insult.

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Methods:

All experiments were conducted on Caco-2 cells, a line of cells derived from a colorectal carcinoma, and obtained from American Type Culture Collection (Rockville MD). Upon prolonged culture, these cells express the characteristics of small intestinal enterocytes [Jumarie and Malo, 1991]. They are widely used as a model of transepithelial transport in the small intestine, and are considered a model for celiac disease [Iacomino *et al*, 2013; Caputo *et al*, 2012; Rauhavirta *et al*, 2013]. Cells were grown on a transwell inserts (6-well, 12-well or 24-well, 0.4 micron pore size, PET Polyester, Corning) a permeable barrier system, in minimal essential media (MEM) supplemented with 20% fetal calf serum. Cells were seeded at a density of 3.9 X $10^4/cm^2$, and media was changed at 2 day intervals. The media volumes in the apical and basolateral chambers were 1.5ml and 1 ml (6-well plates), 0.5 ml and 1 ml (12-well plates), and 0.1 ml and 0l5 ml (24-well plates), respectively.

Measurement of transepithelial electrical resistance: The tightness of the tight junctions in a transporting epithelium was evaluated by measuring the transepithelial electrical resistance (TEER). TEER measures the movement of ions across the cell (transcellular) and between cells (paracellular). In a very tight monolayer, transport is nearly all transcellular, hence the electrical resistance is high; the electrical resistance across the membrane was measured using the EVOM2 epithelial voltohmmeter (World Precision Instruments, Sarasota FL, USA). At 3 to 4 weeks in culture, TEER was in the range of 600-700 Ω/cm^2.

Mannitol flux: The TEER results were complemented by measuring mannitol flux. Cells were seeded into 6 well plates, and [^{14}C] labeled mannitol (10 μM) was added to the apical media; 10μl samples were removed from the basolateral media at intervals throughout the experiment. The rate of mannitol flux was determined by calculating the slope of dpm in the basolateral chamber as a function of time.

E-cadherin expression: E-cadherin is the principle protein of tight junctions. The levels of E-cadherin was measured in cells by western blot analysis (primary antibody from BD biosciences).

Sphingosine-1-Phosphate: Calcium glycerophosphate has activity as a phosphatase inhibitor, inviting our hypothesis that CGP elevates S1P concentrations by inhibiting dephosphorylation. S1P concentrations were measured in the media of Caco-2 cells using a commercially available ELISA kit (Echelon Catalog # K-1900).

Baseline values of mannitol flux, TEER and S1P were obtained in unstimulated cells, in the presence and absence of increasing concentrations of CGP.

The effect of hypoxia was measured for TEER and mannitol flux, in the presence and absence of CGP. Cells were grown to confluence on transwells, 0.4 micron pore size. Experiments in hypoxic conditions were conducted in a hypoxia chamber (Coy Laboratory Products, Grass Lake MI, USA), as detailed elsewhere [Yang *et al.*, 2006]. Briefly, confluent cultures were placed in the chamber under a 95% N_2/5% CO_2 atmosphere and fed with serum and glucose free MEM, , and bubbled with 95% N_2/5% CO_2. Under these conditions, the PO_2 in the media is below 25 mm Hg (6%). Normoxic conditions were the standard CO_2 incubator and media containing glucose.

The effect of exogenous cytokines were measured for each parameter, in the presence and absence of CGP. Both hypoxia/ischemia and celiac disease are characterized by cytokine release, which increase monolayer permeability [5]. A mixture of the cytokines TNFα , ILβ and

IFγ (final concentrations: 10 ng/ml; 1 ng/ml; 100 ng/ml, respectively) then TEER, mannitol flux, E-Cadherin expression and media concentrations of S1P were measured.

The effect of α -gliadin 31-55 peptide was measured for each parameter. α-Gliadin is the offending protein initiating the symptoms of celiac disease in susceptible patients. Gastric and pancreatic proteases cleave α-gliadin to peptides, which are likely the direct cause of the enteropathy. The literature [Iacomino *et al*, 2013] suggests that the peptide comprised of α-gliadin amino acids 31-55 is a likely candidate. The peptide (Thermo Scientific, Custom synthesis), and added to the media at 100µg/ml.

Results:

Effect of CGP under basal conditions:

Dpm mannitol movement from the apical to the basolateral fluid was graphed as a function of time (Figure1). The slopes of these lines (mannitol flux) are presented in Table 1. CGP had no effect on apical to basolateral mannitol flux. Since there was no significant difference among the treatment groups, a pooled rate of mannitol flux was calculated for the first 4 hours, and was 20 ± 1 dpm/hour.

The effect of CGP on baseline trans-epithelial electrical resistance (TEER) is shown in Figure 2. The slope of TEER as a function of time was calculated, and is presented in Table 1. Vehicle caused a small but statistically significant loss of TEER over time, an effect that was reversed by CGP in a concentration dependent fashion.

CGP had no effect on 24 hour media sphingosine 1-phosphate concentrations, as shown in Figure 3.

Effect of CGP during hypoxia:

Hypoxia/ischemia is a state of diminished oxygen and nutrient supply such that cellular demands are not met. During hypoxia, intestinal epithelial permeability is increased [Xu *et al*., 1999]; we hypothesized that the permeability might be alleviated by CGP treatment. The hypothesis was tested by placing Caco-2 cells in a hypoxia chamber, as described in METHODS, and measuring both apical to basolateral mannitol flux and TEER.

CGP significantly reduced the effect of hypoxia on apical to basolateral mannitol flux. The effect was both concentration and time dependent, and had disappeared by 5 hours in all treatment groups (Figure 4). At 5 hours, the mean apical to basolateral mannitol flux for all groups was 1380 ± 6 dpm (range 1358 to 1395 dpm). The rate of mannitol flux (dpm/hour) was calculated for the first three hours of hypoxia, and results are shown in Table 2. CGP treatment reduced the rate of mannitol flux at all concentrations tested.

Figure 5 shows the effect of CGP on TEER during hypoxia. Baseline TEER values were >600 $m\Omega/cm^2$ for all groups. As there is some variability in baseline TEER, all data have been expressed as a % of baseline TEER. After 1 hour hypoxia, TEER in control cells was significantly decreased, and continued to drop for the duration of the experiment. CGP preserved TEER in a concentration and time dependent manner such that by 5 hours there was no longer a discernable effect. The rate of decrease in TEER during the first three hours of hypoxia is shown in Table 2. The CGP treatment had a significant effect to reduce the rate of TEER loss at all concentrations tested.

Effect of CGP during cytokine stimulation:

In vivo, I/R injury is mediated, at least in part, by neutrophil recruitment [Esposito *et al*, 2007], with subsequent release of pro-inflammatory cytokines such as TNFα, IFN-γ and IL-β [Anker *et al*., 1998; Celik *et al*, 2010]. This inflammatory response was mimicked by adding a mixture of these cytokines (cytomix composition) to the culture media. As shown in Figure 6, cytokine alone increased mannitol flux. CGP decreased cytokine stimulated mannitol flux in a time and concentration dependent manner. The rate of mannitol flux (dpm/hour) was calculated for the first 4 hours of cytokine exposure, and is presented in Table 3.

The onset of the effect of cytomix on TEER is slower than for mannitol flux. For the sake of clarity, Figure 7 shows only the 8 and 10 hour results. Because there is considerable variability in the absolute TEER at baseline (0 hours), values are expressed as a % of baseline. Cytomix does not begin to reduce TEER until 8 hours exposure (upper panel). At 10 hours, TEER in all CGP treated groups except 1 μM was significantly greater than cytomix alone and not different from control (lower panel). By 24 hours, all CGP effect is lost (data not shown). The rates of TEER decline are presented in Table 3.

The effect of CGP on S1P concentrations in the presence of cytomix is shown in Figure 8. Contrary to expectations, cytomix alone failed to decrease S1P. However, in the presence of cytomix, CGP significantly increased S1P concentrations, an effect that was particularly noted at 4 hours. These data suggest that perhaps the effect of CGP on cytomix induced mannitol permeability is mediated by S1P. However, the data are insufficient to make any definitive conclusions regarding this mechanism.

Transepithelial permeability, α-gliadin, and CGP:

Although celiac disease is not caused by gut ischemia, α-gliadin stimulates cytokine release [Larson *et al*, 2014]. The α-gliadin peptide fragment 31-55 is the offending peptide initiating the inflammatory symptoms of celiac disease in susceptible patients [Iacomino *et al*., 2013]. The effect of the peptide fragment on mannitol flux and TEER were measured in the presence and absence of cytokine mix so as to mimic that inflammation. The results of these experiments are shown in Figure 9. At 1 hour, there were no differences among the experimental groups. At 2 hours, mannitol flux was significantly elevated in the peptide-cytomix treated cells as compared to control. When 1 mM CGP was included with the peptide-cytomix, the mannitol flux at 2 hours was not significantly different from control. At 4 hours, mannitol flux in both the peptide and peptide + cytomix groups was significantly greater than control. CGP (1 mM) significantly reduced both the peptide-induced and peptide + cytomix induced increases in mannitol flux. The rate of mannitol flux for each experimental group is shown in Table 4.

The effect of CGP on α-gliadin peptide induced reduction in transepithelial resistance (TEER) is shown in figure 9. In contrast to the mannitol experiments, these data indicate that there is only a very small effect of CGP on TEER. Notably, though, the rate of TEER loss in the peptide + cytomix group was 0.0272 ± 0.00497% per hour. In the presence of 1 mM CGP, this rate was significantly less, 0.0120 ± 0.00327 % per hour ($p=0.019$). At the end of the TEER experiments, the media was assayed for E-cadherin, a major tight junction protein. Contrary to expectations, E-cadherin was 75.7±8.6% of control ($p=0.0479$) in the peptide-cytokine treated cells. However, in the peptide-cytokine CGP treated cells, E-cadherin levels were 101±2.9% of control ($p=0.048$ *vs*. peptide-cytokine alone).

Discussion:

To our knowledge, prior studies of gut ischemia/reperfusion injury have focused on whole-animal or intact tissue studies. The present study demonstrates the direct effect of hypoxia/ischemia and cytokine stimulation on a model of intestinal epithelial transport, in the absence of the underlying muscularis and without the complicating factor of blood flow. Furthermore, the study shows that a pharmacologic agent, calcium glycerophosphate, attenuates the I/R induced increase in gut permeability.

Under baseline conditions, CGP has, at most, only a small effect on transepithelial permeability. Furthermore, under baseline conditions, CGP does not appear to have any significant effect on S1P concentrations. In contrast, calcium glycerophosphate has a significant time and concentration dependent effect to attenuate increased gut permeability caused by hypoxia, cytokine stimulation, and α-gliadin peptide 31-55.

It was somewhat surprising that the cytokine effect on mannitol flux was observed at an early time point, suggesting that the cytokine effect on mannitol flux may not require new protein synthesis. Rather, the time course over which CGP increases S1P in cytokine-stimulated cells suggests that the effect of CGP on mannitol flux may be linked to the increased S1P levels. However, the present data are insufficient to permit any firm conclusions.

Gut hypoperfusion is a ubiquitous complication of CHF [Sandek *et al*, 2007; Jakob 2002], and contributes to poor nutritional status and CHF-induced cachexia [Sandek *et al*, 2011; Celik *et al*, 2010; Sandek *et al*, 2010]. In 2010, there were more than 1,000,000 hospital discharges for CHF, up from about 400,000 in 1980, and the number continues to rise. The incidence of CHF is about 1% of the population over 65, with a 5 year survival rate of about 50%. The most recent estimate of CHF prevalence is more than 5.1 million persons, a number that is expected to rise to more than 8 million by 2030. In 2010, heart failure was the direct or contributing cause of about 280,000 deaths in the U.S [Go *et al.*, 2014]. To date, treatment of CHF associated gut ischemia has focused on improving the gut perfusion rate, itself dependent entirely on improved overall cardiac performance [Anker *et al*, 2003]. We have been unable to find evidence of therapies aimed directly at improving gut epithelial integrity. One could reasonably anticipate that modest improvements in cardiac performance (about all that can currently be achieved) would synergize with improved gut integrity, leading to an overall improvement in the status of the CHF patient.

Aside from CHF, gut ischemia has been identified as a complication of endurance exercise. In the U.S., nearly 10 million persons run or jog 110 or more days per year [http://endurancesportswire.com/state-of-the-sport-part-ii-running-industry-report-from-running-usa/] and an additional 3.3 million ride a bicycle 110 or more days per year [http://www.gluskintownleygroup.com/downloads/The US Bicycle Market - A Trend Overview Report.pdf]. A study of long-distance runners and cyclists found that the prevalence of exercise related lower GI symptoms was 71% for runners and 64% for cyclists [Peters *et al*., 1999]. The same study revealed that 5% of runners and 6% of cyclists experienced symptoms severe enough to require medication. These symptoms have been linked to splanchnic hypoperfusion [Reviewed by van Wijck *et al.*, 2012a] and are exacerbated by concomitant administration of non-steroidal anti-inflammatory drugs such as ibuprofen [van Wijck *et al*, 2012b]. More recently, it has been suggested that ingestion of L-citrulline might be useful in mitigating the symptoms of exercise induced gut ischemia, most likely by increasing the availability of arginine to vascular endothelial nitric oxide synthase [van Wijck *et al*., 2014]. Like CHF, the proposed treatment rests on improving gut blood flow rather than by directly improving gut epithelial integrity.

Celiac disease is a debilitating condition, with limited treatment options. These include corticosteroids and monoclonal antibodies aimed at specific mediators of inflammation as well as

severely restricted diets. The corticosteroids have significant and potentially damaging side effects, as do the monoclonal antibodies. The latter have the further disadvantage of requiring parenteral administration. Dietary therapies have limited efficacy, where the ubiquitous presence of hidden gluten makes its effective elimination nearly impossible. These factors may make it worthwhile to pursue CGP as an adjunct to celiac dietary therapy, as CGP might limit the negative impact of hidden dietary gluten.

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Treatment	**Rate of TEER Change % per hour**	**Rate of Mannitol Flux dpm per hour**
Control	0.69 ± 0.11†	28 ± 4
Vehicle	-0.24 ± 0.32*	19 ± 4
1 mM CGP	0.80 ± 0.14†	31 ± 4
100 μM CGP	0.28 ± 0.13	19 ± 3
10 μM CGP	-.09 ± 0.37	21 ± 5
1 μM CGP	0.48 ± 0.23*	31 ± 6

Table 1. Baseline changes in TEER and mannitol flux from time 0 to 4 hours. TEER was significantly lower in vehicle treated cells, an effect that was reversed by CGP in a concentration dependent fashion. *=$p<0.05$ compared to control; †=$p<0.05$ compared to vehicle. Each value represents the results of three independent experiments.

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Treatment	**Rate of TEER Loss % per hour**	**Rate of Mannitol Flux dpm per hour**
Hypoxia	19.4 ± 1.31	162 ± 27
1 mM CGP	6.79 ± 1.12*	19 ± 4*
100 μM CGP	9.67 ± 1.42*	39 ± 3*
10 μM CGP	10.61 ± 1.86*	35 ± 3*
1 μM CGP	12.2 ± 1.56*	49 ± 7*

Table 2. The effect of hypoxia on the rate of TEER loss and mannitol flux during the first three hours of hypoxia. CGP treatment reduced both parameters in a dose-dependent fashion. *=$p<0.001$ compared to hypoxia alone. Each value represents the results of three independent experiments.

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Treatment	**Rate of TEER Loss % per hour**	**Rate of Mannitol Flux dpm per hour**
Control	0.064 ± 0.38*	28 ± 3*
Cytokine	2.1 ± 0.31	54 ± 1
Cytokine + 1 mM CGP	0.76 ± 0.24*	4 ± 1*
Cytokine + 100 μM CGP	0.87 ± 0.32*	10 ± 1*
Cytokine + 10 μM CGP	1.4 ± 0.28*	13 ± 1*
Cytokine + 1 μM CGP	2.4 ± 0.34	16 ± 2*

Table 3. The rate of TEER loss and mannitol flux during the first four hours (mannitol flux) or 10 hours (TEER) following cytokine stimulation. CGP treatment reduced both parameters in a dose-dependent fashion. *=$p<0.001$ compared to cytokine alone. Each value represents the results of three independent experiments.

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Treatment	Mannitol Flux dpm/hr	% of control
Control	21 ± 1	100%
Peptide	36 ± 1*	171%
Peptide + 1mM CGP	28 ± 1*	133%
Peptide + Cytomix	39 ± 3*	186%
Peptide + CGP + Cytomix	32 ± 1*	152%

Table 4. The effect of CGP on α-gliadin peptide 31-55 induced mannitol flux. * = $p < 0.01$ *vs.* control; each value represents the mean of 3 independent experiments.

Calcium glycerophosphate and intestinal epithelial permeability.

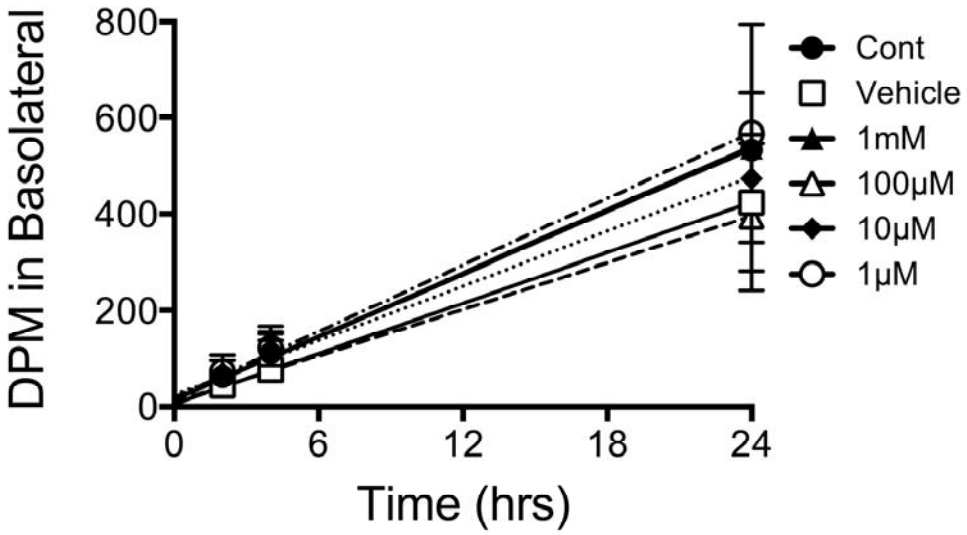

Figure 1. Baseline mannitol flux was not influenced by CGP at any concentration tested, as determined by 2-way analysis of variance. Data represent the mean of three independent experiments.

Calcium glycerophosphate and intestinal epithelial permeability.

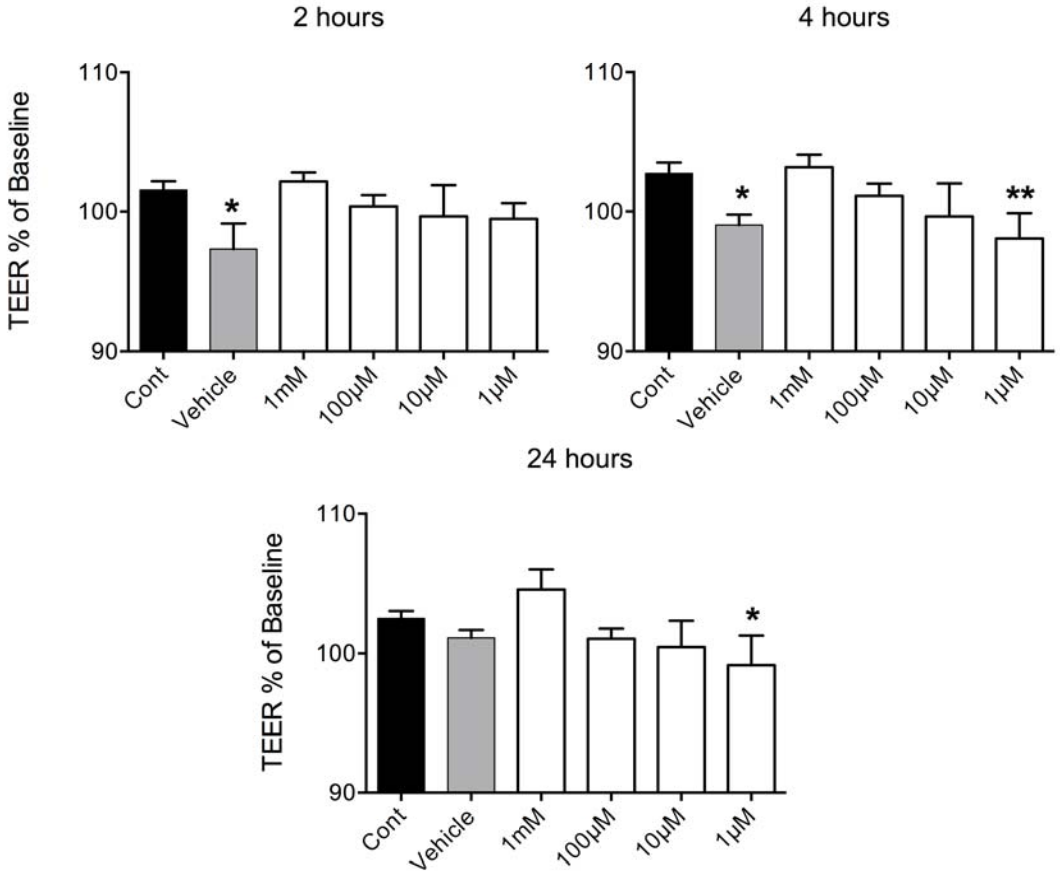

Figure 2. The vehicle reduced TEER slightly, but significantly ($p<0.05$ compared to control) at two and 4 hours. In the presence of 10 μM to 1 mM CGP, TEER was not different from control at any time point. Data were analyzed by two-way analysis of variance followed by Dunnett's multiple comparison test.

Calcium glycerophosphate and intestinal epithelial permeability.

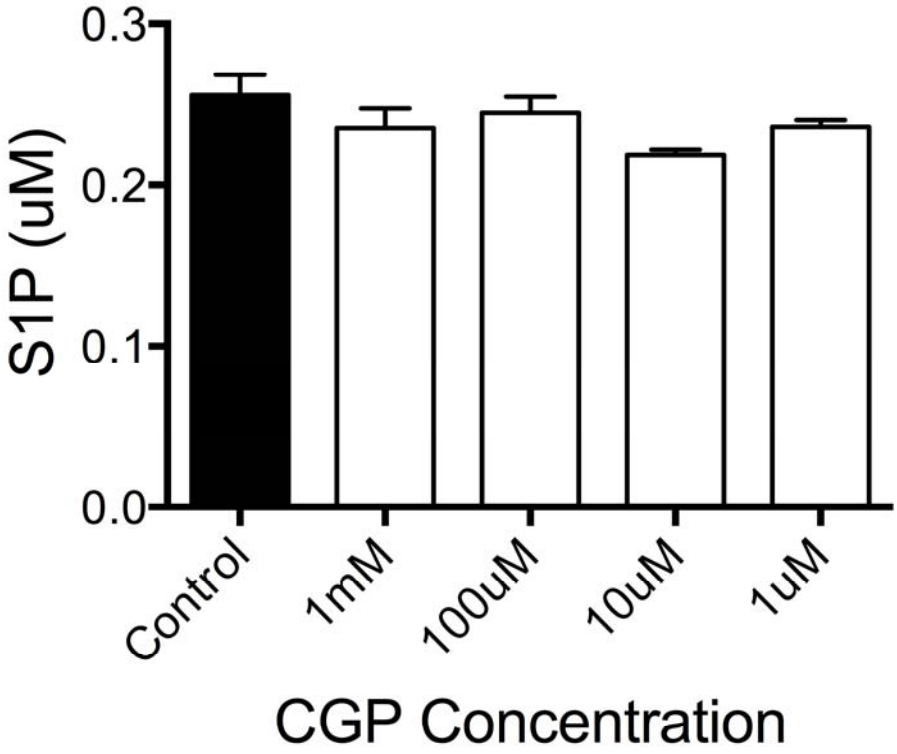

Figure 3. CGP had no significant effect on media S1P concentrations. The mean results of 3 independent experiments are shown; data were analyzed by one-way analysis of variance followed by Dunnett's test for multiple comparisons.

Calcium glycerophosphate and intestinal epithelial permeability.

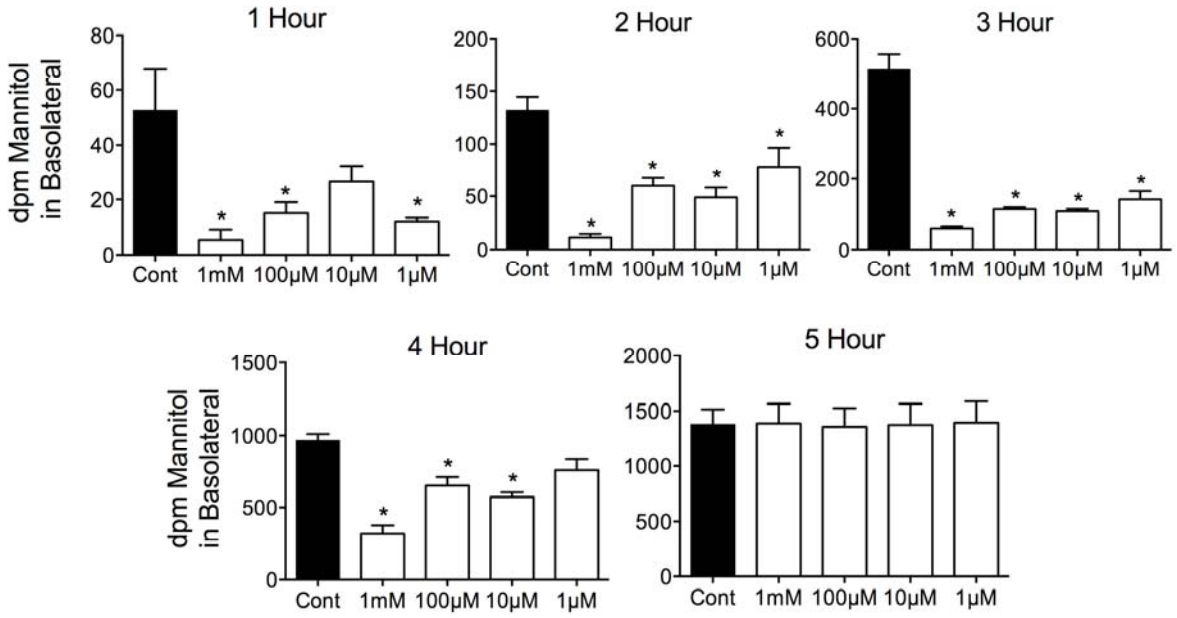

Figure 4. Mannitol flux during hypoxia increased significantly for all groups. CGP attenuated the rate of mannitol flux in a concentration dependent fashion. Slopes of these plots are given in Table 2.

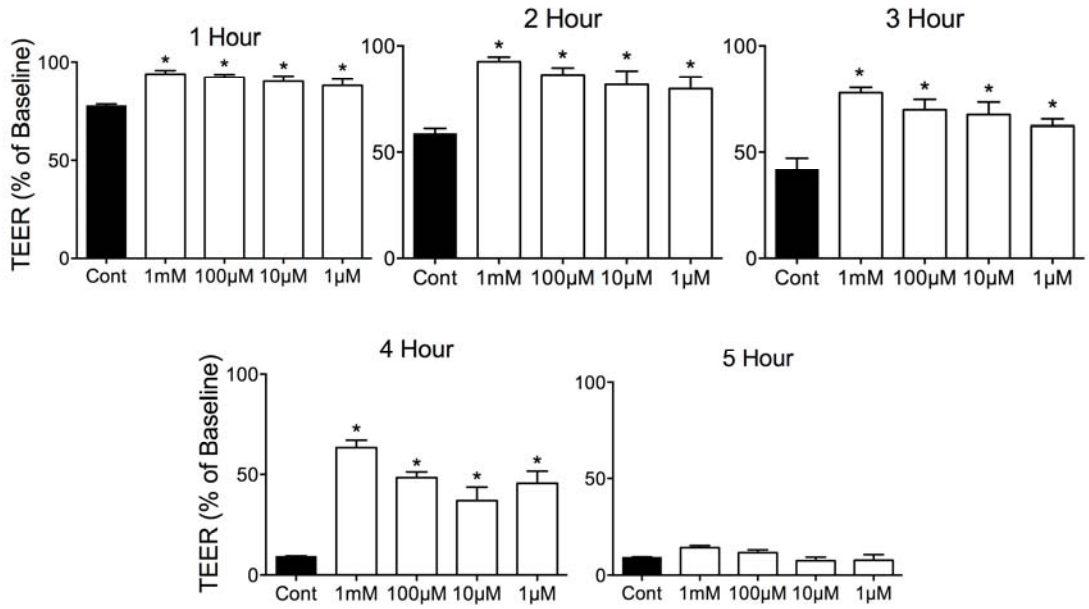

Figure 5. TEER decreased significantly during hypoxia for all groups. However, the rate of decrease was attenuated by CGP in a concentration dependent manner. The slopes of these plots are shown in Table 2. Each concnetration represents data from three independent experiments. Data were analyzed by two-way analysis of variance, followed by Tukey's .multiple comparisons test. * = $p < 0.05$ compared to control.

Calcium glycerophosphate and intestinal epithelial permeability.

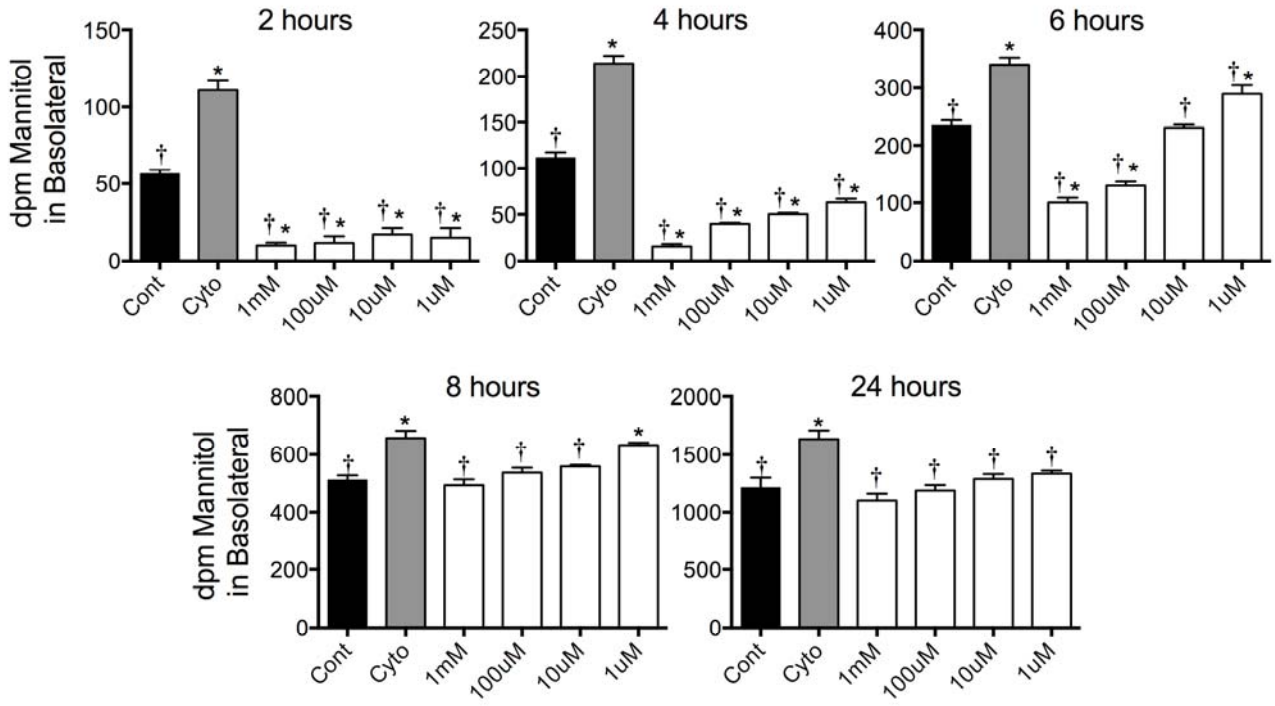

Figure 6. Cytokine treatment stimulated transepithelial mannitol flux, an effect that was attenuated by CGP in a concentration dependent fashion. The rates of mannitol flux are given in Table 3. Each treatment represents the mean of three independent experiments. Data were analyzed by two-way analysis of variance, followed by Tukey's test for multiple comparisons. * = $p<0.05$ compared to control; † = $p< 0.05$ compared to cytokine alone.

Calcium glycerophosphate and intestinal epithelial permeability.

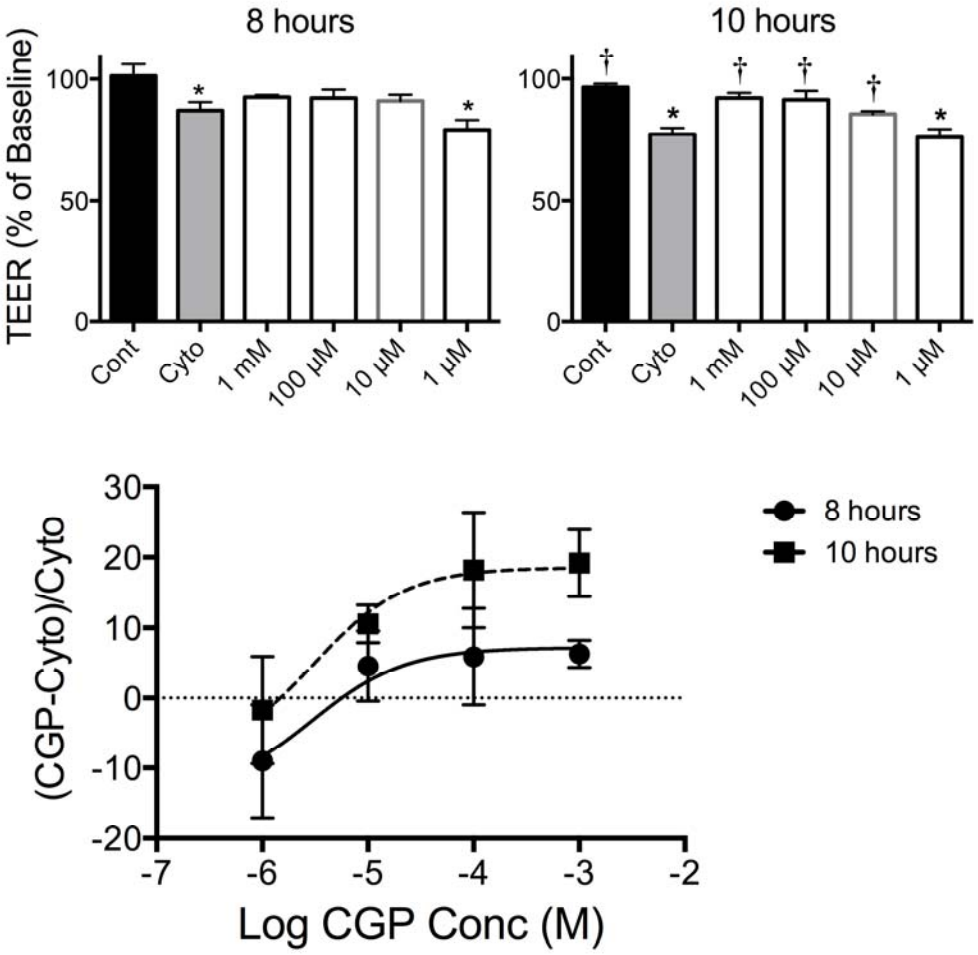

Figure 7. The effect of CGP on transepithlial electrical resistance (TEER) during cytomix treatment. Data were analyzed by two way analysis of variance, followed by Tukey's test for multiple comparisions. Each treatment represents the mean of three independent experiments. * = $p < 0.05$ compared to control; † = $p < 0.05$ compared to cytomix alone.

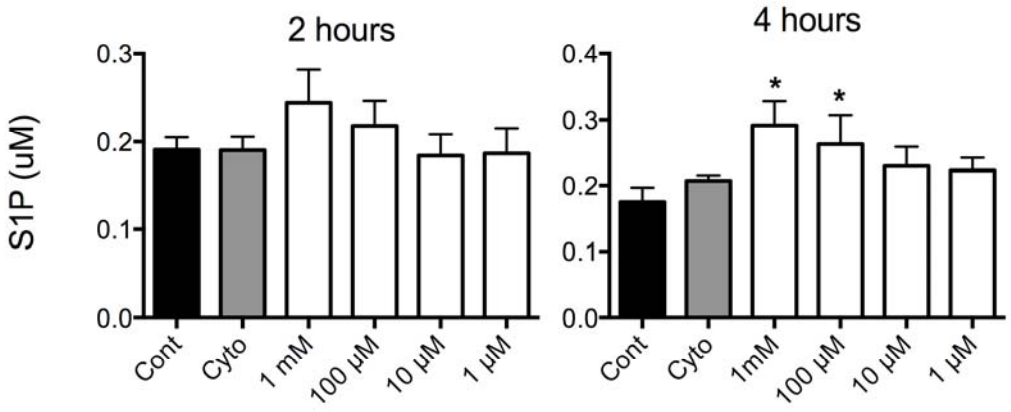

Figure 8. The effect of CGP on S1P concentrations in the presence of cytomix. Each treatment represents the mean of three independent experiments. Data were analyzed by two way analysis of variance, followed by Tukey's test for multiple comparisons. *=$p<0.05$ compared to control.

Calcium glycerophosphate and intestinal epithelial permeability.

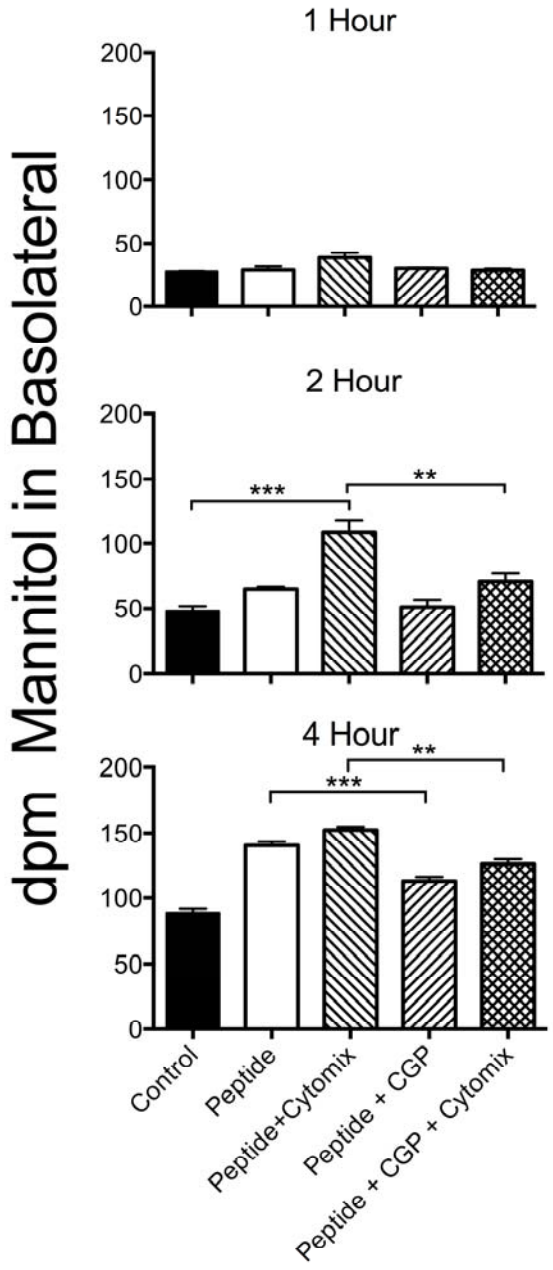

Figure 9. The effect of 1 mM CGP on α-gliadin peptide fragment 31-55 induced mannitol flux. Each treatment represents the mean of three independent experiments. ** = $p< 0.01$; *** = $p<0.001$

Calcium glycerophosphate and intestinal epithelial permeability.

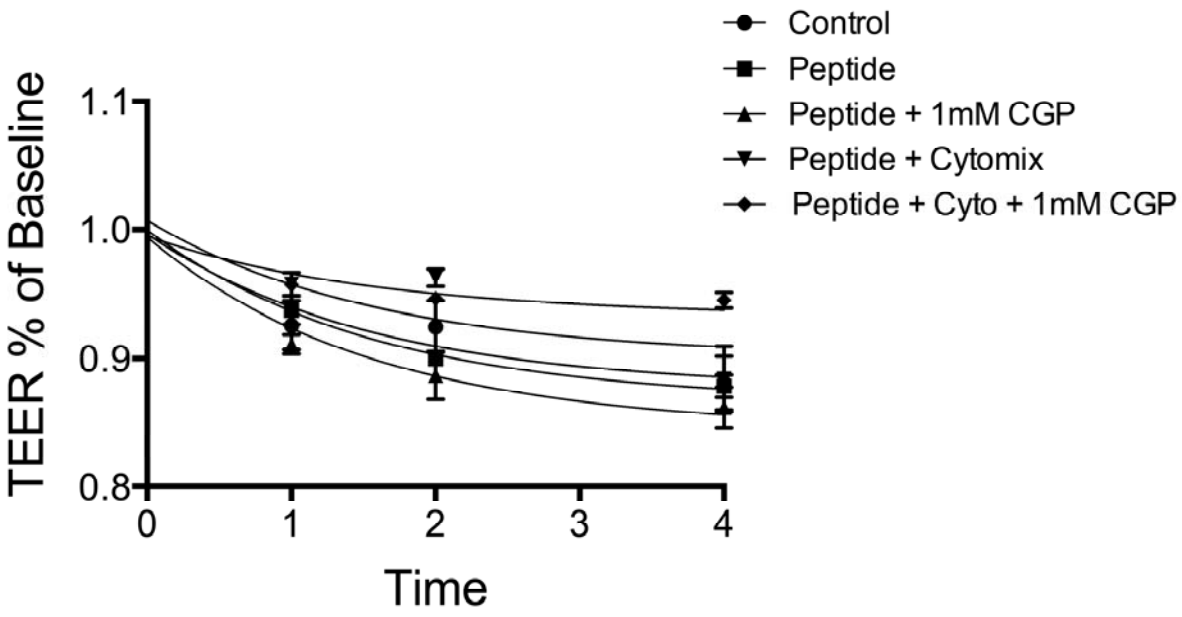

Figure 10. The effect of 1 mM CGP on α-gliadin peptide induced reduction in TEER.