Calcium Glycerophosphate (CGP) Preserves Transepithelial Integrity

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Abstract

We hypothesized that CGP would mitigate the effect of hypoxia, cytokines (CK) and/or α-gliadin peptide 31-55 (α-GP, the gluten peptide fragment of celiac disease) to increase transepithelial permeability. All experiments used Caco-2 monolayers with transepithelial electrical resistance (TEER) of 600-700 Ω/cm^2. All agents were added to the luminal side of the cell monolayer. In normoxic cells, TEER increased at 0.69 ± 0.11% / hour and mannitol flux was 28 ± 4 dpm / hour. In hypoxic cells, TEER decreased 19.4 ± 1.31 % / hour and mannitol flux increased to 162 ± 27 dpm / hour. 10 μM CGP, reduced hypoxia induced TEER loss to 10.6±1.86 % / hour and mannitol flux to 35±dpm / hour ($p<0.001$ compared to normoxia or hypoxia alone). CK stimulated TEER loss was 2.1 ± 0.31% / hour and mannitol flux was 54 ± 1 dpm / hour. 10 μM CGP reduced TEER loss to 1.4 ± 0.28% / hour, and mannitol flux to 13 ±1 dpm / hour ($p<0.001$ compared to control or to hypoxia alone). In separate experiments, α-GP + CK increased mannitol flux from 21±1 to 39 ± 3 dpm / hour; α-GP + CK + CGP (1 mM) group, mannitol flux was 32 ±1 dpm / hour ($p<0.05$ *vs* α-GP + CK). In these same experiments, TEER loss was 2.72 ± 0.497% / hour in the peptide + CK group *vs.* 1.20 ± 0.327% /hour in the α-GP + CK + CGP group ($p<0.05$ *vs* α-GP + CK). E-cadherin protein was 75.8±8.6% of control in the α-GP + CK group, while in the α-GP + CK + CGP treated cells, E-cadherin was 101 ± 2.9% of control ($p<0.05$ *vs* α-GP + CK. In summary, CGP showed a significant time and concentration dependent effect to attenuate increased gut permeability caused by hypoxia, CK, or α-GP an effect that was observable even at 1μM. These factors may make it worthwhile to pursue calcium glycerophosphate as an adjunct to other therapies to prevent loss of gut epithelial integrity in hypoxia or celiac disease.

Introduction

Ischemia/reperfusion (I/R) injury is a broad area of medical significance, and includes gut ischemia consequent to such diverse causes such as endurance sports activity and congestive heart failure. The subsequent immune system activation increases synthesis of an array of cytokines, including TNFα, ILβ and IFNγ. 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. As alkaline phosphatase catalyzes the conversion of S1P to sphingosine and inorganic phosphate, 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.

Materials and Methods

All experiments were conducted on Caco-2 cells, a line of cells that express the characteristics of small intestinal enterocytes. Cells were grown on a transwell inserts 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. The tightness of the tight junctions in a transporting epithelium was evaluated by measuring the transepithelial electrical resistance (TEER) which was in the range of 600-700 Ω/cm^2.

Materials and Methods—Continued

Mannitol Flux: Measuring mannitol flux complemented the TEER results. ^{14}C labeled mannitol at 10 μM concentration was added to the apical media. 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 were measured in cells by western blot analysis .

Sphingosine-1-Phosphate: S1P concentrations were measured in the protein lysate of Caco-2 cells using a commercially available ELISA kit.

Hypoxia: The effect of hypoxia was measured for TEER and mannitol flux, in the presence and absence of CGP. 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.

Cytokines: A mixture of the cytokines TNFα, ILβ and IFNγ (final concentrations: 10 ng/ml; 1 ng/ml; 100 ng/ml, respectively) was added to the apical media then TEER, mannitol flux, E-Cadherin expression and concentrations of S1P were measured.

Gliadin peptides: The α-gliadin 31-55 peptide was custom synthesized and was added to the media at 100μg/ml.

Results

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

Figure 1. **Baseline changes** in Trans Epithelial Electrical Resistance (TEER) and Mannitol Flux from time 0 to 4 hours. TEER was significantly lower in vehicle (0.1% citric acid) 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.

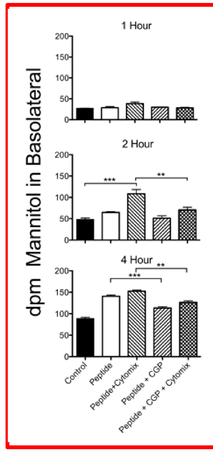
Treatment (Hypoxia)	Rate of TEER Loss% per hour	Rate of Mannitol Flux dpm per hour
Control	19.4 ± 1.31	162 ± 27
1 mM CGP	6.79 ± 1.12*	19 ± 4*
100 μM CGP	9.67 ± 1.42*	39 ± 3*
10 μM CGP	10.61 ± 1.86*	35 ± 3*
1 μM CGP	12.2 ± 1.56*	49 ± 7*

Figure 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

Results—Continued

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 +1mM CGP	0.76 ± 0.24*	4 ± 1*
Cytokine +100 μM CGP	0.87 ± 0.32*	10 ± 1*
Cytokine + 10μM CGP	1.4 ± 0.28*	13 ± 1*
Cytokine + 1μM CGP	2.4 ± 0.34	16 ± 2*

Figure 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.



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%

Figure 4. The table represents the effect of Calcium Glyerophosphate (CGP) on α-gliadin peptide 31-55 induced Mannitol Flux. * = $p < 0.01$ *vs*. control. The graph represents the effect of 1mM Calcium Glycerophosphate (CGP) on α-gliadin peptide fragment 31-55 induced Mannitol Flux

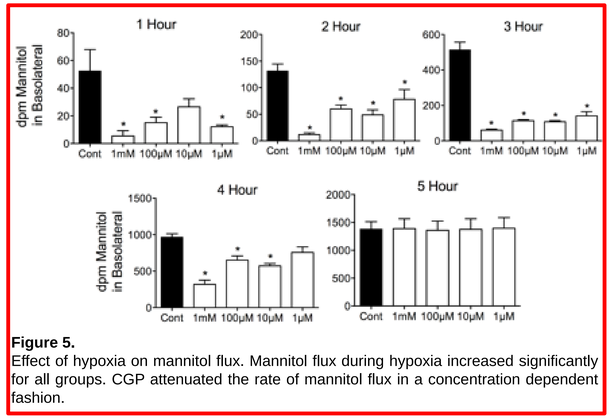


Figure 5. Effect of hypoxia on mannitol flux. Mannitol flux during hypoxia increased significantly for all groups. CGP attenuated the rate of mannitol flux in a concentration dependent fashion.

Results—Continued

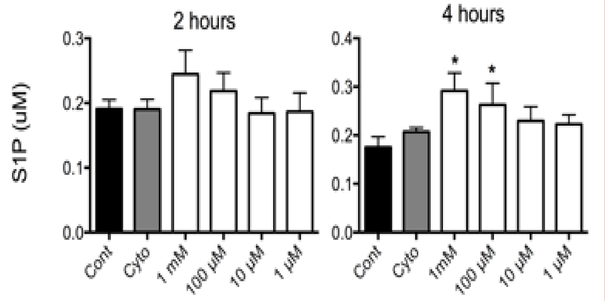


Figure 6: The effect of Calcium Glycerophosphate (CGP) on Sphingosine 1 Phosphate (S1P) concentrations in the presence of cytomix.

Summary/Conclusions

- Calcium Glycerophosphate (CGP) is shown to have only a small effect on transepithelial permeability in normal conditions.
- In contrast, CGP has a significant time and concentration dependent effect to attenuate increased gut permeability caused by hypoxia, cytokine stimulation, and α-gliadin peptide 31-55.
- CGP is also found to increase Sphingosine 1 Phosphate (S1P) in cytokine-stimulated cells suggesting 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.
- Additional research on its mechanism of action is required to further strengthen our hypothesis. So far there have not been any data reflecting this aspect of this action of calcium glycerophosphate.

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