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Effect of nordihydroguaiaretic acid on glucose absorption, metabolism and $(Na^+ + K^+)$-ATPase activity in rat jejunum

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Abstract—A regulatory role of endogenously synthesized eicosanoids on the absorption, transmural transport and metabolism of glucose in perfused, isolated loops of jejunum *in vitro* was investigated using the lipoxygenase/cyclooxygenase inhibitor, nordihydroguaiaretic acid (NDGA). NDGA diminished glucose absorption over the range 100–500 μM: maximal inhibition at 500 μM NDGA was 52 ± 9 and 64 ± 9% (mean ± SE, $P < 0.001$) for jejuna from fed rats and rats maintained on glucose water for 48 hr, respectively. In each instance, transmural transport was effectively abolished. The vectorial disposition of lactate release was also changed such that the ratio of luminal to serosal production was increased from 0.19 ± 0.02 to 1.72 ± 0.12 ($P < 0.001$) in fed rats, indicating inhibition of the Na^+ pump. NDGA inhibited $(Na^+ + K^+)$-ATPase activity in whole mucosal homogenates with a concentration dependence similar to that observed for glucose absorption. However, NDGA also inhibited Mg^{2+}-ATPase activity in whole homogenates and purified rabbit skeletal muscle phosphofructokinase under the same conditions. The results are discussed in terms of the dissipation of the transmembrane Na^+ gradient via direct inhibition of the $(Na^+ + K^+)$-ATPase by NDGA. Inhibition of the ATPase precludes the use of NDGA as a suitable drug with which to investigate the role of endogenously synthesized eicosanoids in the regulation of intestinal function.

Eicosanoids have been implicated in both physiological and pathological processes in the small intestine; for example, ion secretion [1], sugar absorption [2] and inflammatory bowel disease [3]. One approach to investigate the role of eicosanoids in such processes has been to inhibit their endogenous or hormone-stimulated synthesis from arachidonic acid using non-steroidal anti-inflammatory drugs. These act through inhibition of either cyclooxygenase and hence prostanoid production or lipoxygenases and, therefore, leukotriene production. In this way, the use of nordihydroguaiaretic acid (NDGA*), an inhibitor of lipoxygenase at low concentrations and also cyclooxygenase at higher concentrations, has implicated eicosanoids in the regulation of Cl^- secretion.

Luminal perfusion or arterial infusion with high concentrations of prostaglandin E_2 (PGE_2) is reported to inhibit glucose absorption in the small intestine of rat [2, 4] and mouse [5], and to inhibit α-methyl-D-glucoside uptake in enterocytes isolated from mouse jejunum incubated with PGE_2 [6]. The present study investigates whether endogenous lipoxygenase products may also influence glucose absorption in isolated loops of rat small intestine.

Materials and Methods

Animals. Female Wistar rats (220–250 g) were fed *ad lib.* on a standard chow (Bantin & Kingman Ltd, Hull, U.K.) with free access to water. For some experiments, the chow diet was removed and rats were then maintained on glucose (0.5% w/v) in the drinking water for 48 hr.

Measurement of intestinal absorption and metabolism. Glucose absorption, transmural transport and metabolism were studied in isolated jejunal loops *in vitro* using a preparation modified from Fisher and Parsons and co-workers [7–9], and described previously [5, 10]. Briefly, the abdomen of an anaethetised (Sagatal: sodium pentobarbitone: 0.2 mL/100 g body weight for a fed rat and 0.12 mL/100 g body weight for a glucose-fed rat) female Wistar rat was opened by midline incision. A 20 cm segment of jejunum distal to the Ligament of Treitz was located and flushed with modified Krebs–Henseleit buffer at 37° (containing in mM: NaCl, 118; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, $7H_2O$, 1.2; $CaCl_2.2H_2O$, 1.25; KCl, 4.7; gassed with a 95% O_2:5% CO_2 mixture to pH 7.4). This jejunal segment was cannulated, connected up to the luminal perfusion circuit and the perfusion commenced immediately. Only then was the loop removed from the associated mesentery and from the body of the rat, such that at no stage was the jejunum subjected to anoxia. The perfused jejunal loop was rinsed briefly in modified Krebs–Henseleit buffer to remove excess blood and placed in the serosal chamber. The jejunum is free from the effects of blood flow and neurotransmission, although it will retain the enteric nervous network in the subepithelium.

The luminal perfusate (50 mL) and serosal medium (40 mL) both consisted of modified Krebs–Henseleit buffer containing 5 mM D-glucose at 37°. These media were well-oxygenated in their respective reservoirs by the use of gas-lifts (95% O_2, 5% CO_2). The luminal perfusate was recirculated at a rate of 25 mL/min [8] and was segmented with gas at a rate of 3 mL/min to minimize the effect of unstirred layers [9]. Equimolar concentrations of glucose on the two sides of the intestine ensured that active transport was being studied. Samples (0.1 mL) were taken every 5 min for 45 min from both sides of the jejunum for the analysis of glucose and lactate by fluorescence spectrometry [11]. The procedure did not appear to result in any deterioration in the integrity of the jejunal mucosa as assessed by standard histological fixation and staining methods (unpublished work) and in agreement with Plumb *et al.* [12]. Fisher and Parsons [7] showed that the isolated loop preparation is in fact viable for 2 hr. After the perfusion period, the intestinal loop was cut from the cannulae, drained and dried to constant weight by heating overnight in an oven at 110°.

The synthesis of endogenous lipoxygenase products was inhibited by the use of NDGA at doses of 100, 250 and 500 μM. Since depletion of endogenous products occurs over a period of 15–20 min [1, 13], the jejunum was preperfused for 30 min with glucose (5 mM) and in the presence of NDGA (0–500 μM) in both the luminal perfusate and serosal medium. Both solutions were then renewed and the jejunum was perfused for 45 min.

In the isolated loop preparation, glucose is absorbed from the lumen across the brush border membrane; a part of it is then utilized by the tissue and the remainder is transported intact across the basolateral membrane into

* Abbreviations: NDGA, nordihydroguaiaretic acid; PGE_2, prostaglandin E_2; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetra acetic acid.

the serosal medium. In this system, therefore, absorption is represented by the disappearance of glucose from the lumen, transmural transport by the glucose appearing on the serosa and tissue utilization of the absorbed glucose by their difference under steady-state conditions [5]. Total lactate production is given by the sum of the steady-state rates of appearance in both compartments: the proportion of glucose converted to lactate is given by half the total lactate production expressed as a percentage of the rate of glucose utilization, the remainder being an indication of the glucose oxidation. All rates measured were corrected for water transport ("initial − final" serosal fluid volume) and sampling volumes, and expressed in μmol/hr/g dry weight.

Determination of enzyme activities. Total $(Mg^{2+} + Na^+ + K^+)$-ATPase activity was determined in whole mucosal homogenates by measurement of the rate of release of inorganic phosphate (P_i) [10]. Scrapings of jejunal mucosa were homogenized in 50 vol. (mL/g) of 100 mM Tris–HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl and 10 mM KCl, using a Polytron homogenizer at setting 8 for 30 sec. The sample was preincubated for 30 min with 0.075% Triton X-100 to expose all the ATPase sites [14] and the given concentration of NDGA; the homogenate was then assayed for $(Mg^{2+} + Na^+ + K^+)$-ATPase activity by the addition of 40–80 μL of homogenate to 1 mL of preincubation medium containing 3 mM ATP. After 30 min at 37°, the reaction was terminated by the addition of 30% trichloroacetic acid and the free P_i liberated was assayed by the reduction of molybdate [15].

Experiments on the time course of the inhibition of enzyme activity by NDGA showed that inhibition of P_i formation was complete within the preincubation period. $(Na^+ + K^+)$-ATPase activity was determined as the activity inhibited by 1 mM ouabain. ATPase activity was expressed in μmol P_i/hr/mg protein.

The ability of NDGA to inhibit purified rabbit skeletal muscle phosphofructokinase was also investigated. Phosphofructokinase (0.1 mg/mL in 50 mM Tris–PO_4 buffer, pH 8.0) was preincubated at 25° with NDGA (0–500 μM). The activity was then determined at pH 8.0 by measurement of the rate of formation of fructose 1,6-bisphosphate (μmol/min/mg) [16].

NDGA and phosphofructokinase were obtained from the Sigma Chemical Co. (Poole, U.K.).

Statistical analysis. Results are given as the mean ± 1 SE for six observations. Significance was assessed by Student's *t*-test and is given as $P < 0.05$, <0.01, <0.001 (see Tables).

Results

NDGA inhibited glucose absorption in the intestine from fed rats over the concentration range 100–500 μM as shown in Table 1. Maximal inhibition at the highest dose was 52 ± 9% of the control rate (466 ± 18 to 223 ± 13 μmol/hr/g dry weight). This was accompanied by an inhibition of approximately 40% of the rates of both glucose utilization and total lactate production. The percentage absorbed glucose converted to lactate was unchanged. Similar results were observed for the intestine from rats maintained on glucose water for 48 hr (Table 2), except that there was little, if any, effect of NDGA on the rate of glucose utilization. However, in the absence of NDGA, the rate of utilization in the jejuna from rats maintained on glucose water was 41% lower than from fed rats (namely 206 ± 22 compared to 352 ± 5 μmol/hr/g dry weight, respectively).

NDGA also altered the vectorial disposition of lactate. In the absence of NDGA, the ratio of luminal to serosal lactate release was 0.19 ± 0.02, whereas at 500 μM NDGA this was 1.72 ± 0.12 for the jejuna from fed rats (Table 1). Similar changes in disposition were observed for the jejuna from rats maintained on glucose water (Table 2). We have reported previously [10] that the inhibition of intestinal

Table 1. Dose-dependent effects of NDGA on luminally perfused jejunal loops *in vitro* isolated from fed rats

NDGA (μM)	Rates (μmol/hr/g dry weight)						Absorbed glucose converted to lactate (%)	Luminal/serosal lactate release ratio
	Absorption	Glucose Transmural	Utilization	Lactate production Luminal	Serosal	Total		
0	466 ± 18	113 ± 17	352 ± 5	77 ± 6	397 ± 26	474 ± 26	67 ± 2	0.19 ± 0.01
100	393 ± 35	186 ± 44	207 ± 18‡	52 ± 8*	200 ± 27‡	254 ± 32‡	61 ± 6	0.26 ± 0.04
250	295 ± 17‡	7 ± 17†	288 ± 22*	134 ± 7‡	170 ± 9‡	304 ± 8†	53 ± 3	0.79 ± 0.10†
500	223 ± 13‡	14 ± 12†	209 ± 25‡	183 ± 7‡	107 ± 7‡	290 ± 9†	69 ± 5	1.71 ± 0.09‡

Loops were perfused with 5 mM glucose in Krebs–Henseleit bicarbonate buffer on both luminal and serosal sides, preincubated and perfused in the absence and presence of NDGA at the stated concentrations, as described in Materials and Methods.

The data presented are the rates of glucose luminal disappearance (absorption), glucose serosal appearance (transmural) and utilization of the absorbed glucose (absorption − transmural transport), and total lactate production. Also given is the % absorbed glucose converted to lactate and the vectorial disposition of the luminal and serosal lactate release.

Values are means ± SE, N = 4–12. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Table 2. Effect of NDGA on glucose absorption, transmural transport and utilization in the intestine from rats maintained on glucose (0.5% w/v) in the drinking water for 48 hr

NDGA (μM)	Rates (μmol/hr/g dry weight)						Absorbed glucose converted to lactate (%)	Luminal/serosal lactate release ratio
	Absorption	Glucose Transmural	Utilization	Lactate production Luminal	Serosal	Total		
0	539 ± 19	333 ± 17	206 ± 22	42 ± 5	167 ± 14	210 ± 18	51 ± 5	0.25 ± 0.03
100	340 ± 65‡	229 ± 61*	111 ± 12*	40 ± 8	115 ± 4*	156 ± 10	70 ± 4	0.35 ± 0.04
250	259 ± 21‡	55 ± 17†	204 ± 28	124 ± 11‡	132 ± 8	256 ± 17	87 ± 9†	0.94 ± 0.07†
500	196 ± 26‡	48 ± 23†	148 ± 18	240 ± 26‡	141 ± 21	380 ± 46‡	128 ± 16‡	1.70 ± 0.22‡

See Table 1 for details and footnote symbols.
Values are means ± SE, N = 5–9.

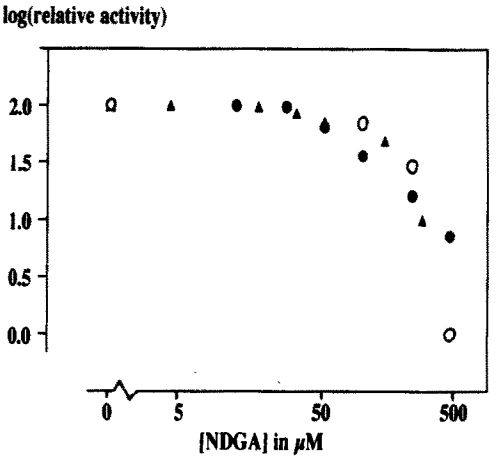

Fig. 1. The inhibition of $(Mg^{2+} + Na^+ + K^+)$-ATPase (●), muscle phosphofructokinase (○) and glucose absorption (▲) by NDGA. Relative activities are given as % initial enzyme activity and % maximal change in glucose absorption. Each point represents the mean of three to eight values, but error bars (<10% SE) are omitted for clarity. Exponential fits yielded $y = 91.4*e^{(-0.0055563x)}$ $R = 0.97$: $K_i = 125\ \mu M$ (●); $y = 108.2*e^{(-0.0074132x)}$ $R = 0.98$: $K_i = 94\ \mu M$ (○); $y = 105.3*e^{(0.0049382x)}$ $R = 0.99$: $K_i = 140\ \mu M$ (▲).

glucose absorption by vanadate is also accompanied by a change in disposition of lactate, which is caused by inhibition of the $(Na^+ + K^+)$-ATPase. The effect of NDGA on $(Na^+ + K^+)$-ATPase activity in mucosal homogenates was, therefore, investigated. Initial experiments in the absence of NDGA and in the presence of 1 mM ouabain showed that $(Na^+ + K^+)$-ATPase activity accounted for 61 ± 5% of the total $(Mg^{2+} + Na^+ + K^+)$-ATPase activity. With intestine from fed rats, NDGA significantly inhibited $(Mg^{2+} + Na^+ + K^+)$-ATPase activity at concentrations greater than 20 μM and almost completely at 500 μM (Fig. 1). The fact that $(Mg^{2+} + Na^+ + K^+)$-ATPase and $(Na^+ + K^+)$-ATPase activities were both inhibited and that the curve for their combined inhibition was essentially monophasic suggested that inhibition was caused by direct inhibition of the enzymes by NDGA rather than by changes in the concentrations of lipoxygenase products. In agreement with this idea, NDGA inhibited purified phosphofructokinase from rabbit skeletal muscle over the same concentration range (Fig. 1).

Discussion

The inhibition of glucose absorption (expressed as % maximal change) shows a very similar dependence on NDGA concentration to that for the inhibition of $(Na^+ + K^+)$-ATPase activity and can, therefore, be explained in terms of the Na^+ gradient hypothesis. In other words, the diminution in glucose absorption is caused by dissipation of the associated Na^+ gradient which drives Na^+/glucose cotransport across the brush border membrane [17]. Cl^- secretion is inhibited by NDGA over the same concentration range and is also presumably caused by inhibition of Na^+ pump activity [1].

In view of the NDGA-induced inhibition of $(Na^+ + K^+)$-ATPase, a reduction in glucose utilization is also expected in the presence of NDGA. Indeed, this is the case for the intestine from fed rats. However, in the intestine from fed

mice, glucose utilization is increased by NDGA [5] and so it appears that NDGA exerts effects other than those solely on $(Na^+ + K^+)$-ATPase. In both chow-fed and glucose-fed rats, the rate of glucose absorption at the highest dose of NDGA matched the rate of glucose utilization. Such an observation is consistent with the idea that the residual absorption, which occurs when the Na^+ pump and hence the active component of absorption is completely inhibited, is caused by metabolic solute drag and emphasizes once again the importance of the inter-relationship between transport and metabolic processes.

The effect of NDGA on lactate production in the perfused rat jejuna appeared to vary between the fed and starved states only with respect to the source of glucose converted to lactate. In both cases, treatment with NDGA resulted in an alteration in the vectorial disposition of lactate release in favour of the luminal side. A reduction in total lactate produced in the jejuna of fed rats reflected the NDGA-induced inhibition of metabolism of the absorbed glucose. In this case, the proportion of glucose converted to lactate remained unchanged, while the rate of lactate released onto the luminal side of the jejunum increased to an excess of that released onto the serosal side. In glucose-fed rats, however, metabolism was inhibited to a lesser degree, but total lactate production increased considerably and above that which could be provided solely from the absorbed glucose, and possibly arose via glycogenolysis. Nevertheless, this is not observed in the fed state, while the increase in luminal over serosal lactate release was identical. An explanation of a NDGA-induced reduction in the Na^+ gradient via $(Na^+ + K^+)$-ATPase inhibition and subsequent diminishment in the capability of both lactate reabsorption and glucose absorption from the lumen is plausible.

In a previous investigation [10], we showed that vanadate causes a marked alteration in the vectorial disposition of lactate in small intestine. This effect is caused by inhibition of the $(Na^+ + K^+)$-ATPase; inhibition of oxidative phosphorylation is unlikely to play any part since vanadate does not form a covalent transition state analogue intermediate with F_1-ATPase. The sensitivity of the vectorial disposition of lactate to NDGA is consistent with the inhibition of $(Na^+ + K^+)$-ATPase and thus provides a useful diagnostic indication of the site of drug action.

Although a dose dependency of the inhibitory effect of NDGA on eicosanoid synthesis was not performed in this study, the inhibition of $(Na^+ + K^+)$-ATPase occurs at concentrations of NDGA which inhibit both lipoxygenases and cyclooxygenase. Nevertheless, it is clear that the inhibition does not involve changes in the concentrations of eicosanoids, since mucosal Mg^{2+}-ATPase and purified phosphofructokinase are inhibited *in vitro* over the same concentration range. In accordance, Dempster and Kellett [6] found that PGE_2 did not affect $(Na^+ + K^+)$-ATPase activity in homogenates of mouse jejunum. In addition, it has been reported [18] that purified myosin ATPase is also inhibited by NDGA and the data presented appear quantitatively indistinguishable from the $(Mg^{2+} + Na^+ + K^+)$-ATPase data given in Fig. 1. Confirmation of the possibility that NDGA inhibits ATP-requiring enzymes in general requires further investigation.

Since NDGA effects glucose absorption by direct inhibition of the Na^+ pump, it is not a suitable drug with which to investigate the role of endogenously synthesized eicosanoids in intestinal function.

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