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PIPERINE-MEDIATED CHANGES IN THE PERMEABILITY OF RAT INTESTINAL EPITHELIAL CELLS

THE STATUS OF γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY, UPTAKE OF AMINO ACIDS AND LIPID PEROXIDATION

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Abstract—The effect of piperine (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine), (from *Piper nigrum*) on the absorptive function of the intestine was studied. *In vitro* experiments showed that piperine (25–100 μM) significantly stimulated γ-glutamyl transpeptidase (γ-GT, EC 2.3.2.2.) activity, enhanced the uptake of radiolabelled L-leucine, L-isoleucine and L-valine, and increased lipid peroxidation in freshly isolated epithelial cells of rat jejunum. The kinetic behaviour of γ-GT towards substrate and acceptor altered in the presence of piperine. In the presence of benzyl alcohol, an enhanced γ-GT activity due to piperine was maintained. These results suggested that piperine may interact with the lipid environment to produce effects which lead to increased permeability of the intestinal cells.

Piperine (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine), a pungent alkaloid present in various *Piper* species of the Piperaceae family [1, 2], has been shown to possess diverse biochemical [3–5] and pharmacological [6, 7] activities. Various studies have also pointed to its role as a bioavailability enhancer [8–17]. At present the mechanisms responsible for the multifold effects of piperine are not properly understood, in particular its bioavailability-enhancing action. The black and long peppers from which this active alkaloid is derived have long been used in Ayurveda as a therapeutic modality [18] but the rationale behind the inclusion of these substances in the majority of Ayurvedic prescriptions has remained unexplained. However, the reported enhancement of bioavailability by piperine/pepper extracts could be due to the effect of the alkaloid on the absorption process in the intestine [19].

In order to study this aspect we determined the effect of piperine on three interrelated biochemical events: (1) γ-glutamyl transpeptidase (γ-GT, EC 2.3.2.2) activity, (2) amino acid uptake and (3) lipid peroxidation (LPO) in freshly isolated epithelial cells from rat jejunum. The effect of benzyl alcohol (a membrane-fluidizing agent) on γ-GT and solute uptake was also studied. Taken together, the results raise the possibility that the changes brought about by piperine may have an important bearing on epithelial cell permeability.

MATERIALS AND METHODS

Chemicals. Chromatographically pure piperine was prepared from *P. nigrum* as per the procedure standardized in this laboratory [1, 8]. γ-Glutamyl-*p*-nitroanilide, *p*-nitroaniline (pNA) and 1,1,3,3,tetraethoxypropane (malonaldehyde, MDA) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and Triton X-100 from BDH (Poole, U.K.). [^{14}C]Leucine and [^{14}C]isoleucine (198 mCi/mmol), and [^{14}C]valine (165 mCi/mmol) were obtained from Bhabha Atomic Research Centre (Bombay, India). All other chemicals were of high purity reagent grade.

Samples. Healthy Charles–Foster rats (150–175 g) kept under uniform husbandry conditions were used. Food was withdrawn 20 hr before the experiments which commenced at a fixed time of day. Animals were killed by cervical blow. Intestines (15 cm beyond 10 cm of pyloric end) were excised and rinsed thoroughly in ice-cooled saline. Epithelial cells (4–5 mg protein/mL) were obtained by the method as described by Lin and Williams [20]. Cell viability was determined by measuring lactate dehydrogenase (LDH) activity [21] in extracellular fluid after centrifugation of cell samples (50 *g*, 5 min). All incubations were done at 37°. In all the experiments the concentrations mentioned were final for the volumes stated.

Determination of γ-GT. Specific activity of the enzyme was determined by the modified method of Szasz [22] using Tris–HCl buffer (120 mM, pH 8.6) in suitable aliquots of cell sample incubated for different time periods (0–30 min) without or with piperine (6.25–100 μM). In another set of experiments benzyl alcohol (50 mM) and/or Triton X-100 (2.5%) were also added. Appropriate blanks were run in parallel incubations omitting one or more of these exogenous substances. Initial experiments showed that addition of exogenous substances did not interfere with the enzyme assay.

Kinetic measurements were carried out in cell

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† Abbreviations: piperine, 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperadine; γ-GT, γ-glutamyl transpeptidase; LPO, lipid peroxidation; MDA, 1,1,3,3, tetraethoxypropane (malonaldehyde); LDH, lactate dehydrogenase; pNA, *p*-nitroaniline; GSH, glutathione.

samples incubated for 10 min in the absence or presence of piperine (50 μM). The saturation curves for the γ-GT activity dependent on acceptor (glycylglycine) were drawn by keeping the concentration of donor substrate (γ-glutamyl-*p*-nitroanilide) constant at 4.0 mM against five concentrations 10, 20, 30, 40 and 80 mM of glycylglycine. Similarly, γ-GT activity dependent on donor substrate was determined at a fixed acceptor concentration (80 mM) by varying the donor substrate (1, 2, 3, 4, and 5 mM). From the initial velocity data V_{max} and K_m were calculated.

Uptake of amino acids. Suitable aliquots of epithelial cell preparations were preincubated for 10 min without or with piperine (50 μM). In another set of identical incubations benzyl alcohol (50 μM) was used. To these samples 1.0 mM of radioactive solute was added and incubations allowed to proceed. After a total incubation time of 12 or 20 min aliquots were drawn successively into ice-cooled buffer and centrifuged to terminate uptake. Each pellet was washed and solubilized in 1.0 M NaOH at 65° for 2 hr and finally neutralized with 0.5 mL of 1.0 M HCl. Radioactivity was counted in LKB 1214 Rackbeta LSC.

Measurement of LPO. Suitable aliquots from cell preparations were incubated for different time periods (0–30 min) without or with piperine (25–100 μM). At the end of the incubations the amount of MDA was determined by the thiobarbituric acid assay [23].

Protein. Protein was determined by the method of Lowry *et al.* [24].

Statistics. ANOVA followed by Dunnett's test, Student's *t*-test, correlation and regression analysis were applied to the data.

RESULTS

Viability of cells

LDH leakage was determined in all the cell preparations (Fig. 1). The results show an increase in response to increasing concentrations of piperine at different time intervals ($P < 0.05$) but no significant difference was found between different incubation times (5–30 min) for any given concentration. The effect of benzyl alcohol and Triton X-100 on LDH leakage is shown in Table 1.

Effect of piperine on γ-GT

Piperine was found to stimulate γ-GT activity in the epithelial cells (Table 2). Statistically significant changes began after 10 min of incubation with 50 and 100 μM piperine. At later times (15–30 min) this effect was noted for all the treatments (12.5–100 μM). Piperine at 50 μM and an incubation time of 10 min produced the maximum effect (1.3-fold enhancement of γ-GT activity) and under these conditions the kinetic profile of γ-GT was determined (Figs 2 and 3, Table 3). The donor substrate-dependent plots showed intersecting lines: V_{max} values for control and piperine-treated were 625 ± 21 and 500 ± 23 nmol/mg protein/min ($P < 0.01$), respectively, and the K_m values were 8.33 ± 0.35 and 5.55 ± 0.22 mM ($P < 0.001$), respectively. The acceptor-dependent plots showed parallel curves; V_{max} for control and piperine-treated groups were 333 ± 13 and 526 ± 21 nmol/mg protein/min ($P < 0.01$), respectively, and the K_m values were 50 ± 3 and 83 ± 7 mM ($P < 0.001$), respectively.

The effect of piperine (50 μM) on the enzyme activity in the presence of benzyl alcohol is summarized in Table 1. The addition of benzyl alcohol significantly decreased γ-GT activity in untreated ($P < 0.001$) as well as piperine-treated samples ($P < 0.01$) but the level of significance differed and the value in piperine-treated samples was significantly high as compared to the control sample ($P < 0.001$). Triton X-100 showed an inhibitory effect ($P < 0.001$). In Triton X-100-treated samples, benzyl alcohol produced a further decrease ($P < 0.001$) in the absence of piperine but no significant effect in the presence of piperine. The value in the piperine-treated preparation differed significantly ($P < 0.001$) in comparison to corresponding untreated perparations. LDH leakage due to benzyl alcohol and Triton X-100 was 15 and 30%, respectively, and was not affected by the presence of piperine.

Effect of piperine on uptake of amino acids

The uptake of all three amino acids increased significantly in an identical manner at peak time (2 min) in cells which were preincubated with piperine ($P < 0.001$) (Table 4). At equilibrium (10 min or after) the uptake decreased considerably ($P < 0.001$) both in control and piperine-treated preparations which showed similar values. Benzyl alcohol significantly ($P < 0.001$) reduced the uptake of amino acids by the cells (Table 5). In samples preincubated with piperine (50 μM) the uptake remained inhibited in the presence of benzyl alcohol.

Effect of piperine on LPO

Increased LPO in response to the incubation of cells with piperine was observed. The increase in MDA level was significant ($P < 0.01$) with 25–100 μM piperine after 5 min of incubation and reached a maximum by 30 min. No further elevation occurred after this time.

Association between LPO and enzyme activities

A positive correlation (assessed by correlation index r^2) between MDA level (Table 6) and γ-GT activity (Table 2) was found which was statistically significant ($P < 0.001$) at each concentration–time point. Between MDA level (Table 6) and LDH activity (Fig. 1) a positive correlation existed but critical values of the correlation coefficient (r) declined in a significant manner with increasing concentration–time points (data not shown).

DISCUSSION

γ-GT is a membrane bound enzyme localized on the external surface of the cell membrane which catalyses the initial reaction in the γ-glutamyl cycle by transferring the γ-glutamyl moiety of the natural substrate glutathione (GSH) to an amino acid acceptor. Although the amino acid transport involves multiple transporters, the role of the γ-glutamyl cycle is unequivocal. The intestinal epithelial cells

Table 1. Effect of benzyl alcohol/Triton X-100 on enzyme activity in epithelial cells

Epithelial cell sample	γ-GT (nmol pNA released/mg protein/min) − Piperine	γ-GT (nmol pNA released/mg protein/min) + Piperine (50 μM)
Control (no addition)	180.0 ± 4.7	231.0 ± 6.8‖
+ Benzyl alcohol (50.0 mM)	143.0 ± 4.7 (15)*	197.0 ± 5.9 (15)†‖
+ Triton X-100 (2.5%)	140.0 ± 3.3 (30)*	169.0 ± 5.1 (30)*
+ Benzyl alcohol + Triton X-100	111.0 ± 3.9 (30)‡	162.0 ± 3.2 (30)†§

Values are means ± SEM from five separate experiments. Values in parentheses, % LDH leakage. Total volume of incubation medium was 250 μL comprising 100 μL sample, 10 μL piperine solution in dimethyl sulfoxide or 10 μL dimethyl sulfoxide, and 140–150 μL buffer. For further details refer to Materials and Methods.

* $P < 0.001$; † $P < 0.01$ as compared to control. ‡ $P < 0.001$ and § not significant compared to sample with Triton X-100 only: ‖ $P < 0.001$ compared to corresponding values in the first column (Student's *t*-test).

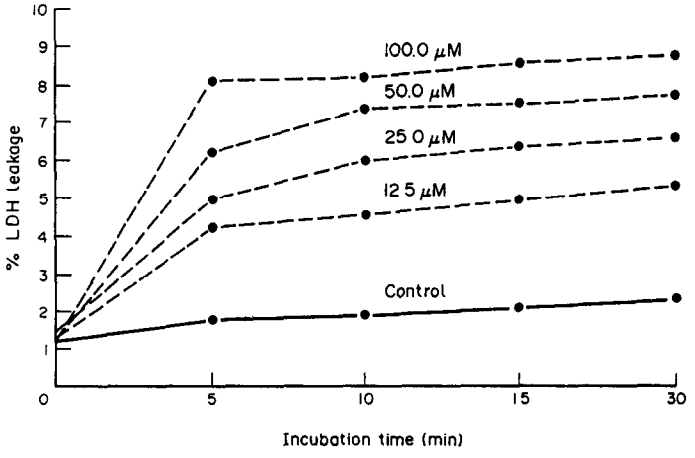

Fig. 1. LDH leakage in epithelial cell preparations. Cells were incubated as described in Table 1. LDH activity was expressed as percentage of the amount of enzyme activity originally present in the extracellular fluid collected before incubation of cell samples. Lines show one representative experiment out of three performed in duplicate. For details refer to Materials and Methods.

Table 2. Effect of piperine on γ-GT activity in epithelial cells

Piperine (μM)	Specific activity (nmol pNA released/mg protein/min) after incubation for different periods of time (min) 0	5	10	15	30
0.0	179.5 ± 3.9	179.6 ± 3.0	175.6 ± 4.0	178.5 ± 3.0	175.0 ± 3.1
6.25	180.0 ± 2.9	188.4 ± 3.5	189.8 ± 4.0	187.9 ± 4.1	185.0 ± 3.5
12.5	181.0 ± 4.1	189.8 ± 3.5	190.8 ± 4.2	219.5 ± 4.2	218.0 ± 4.0
25.0	180.0 ± 3.0	190.0 ± 3.9*	195.0 ± 2.7*	224.0 ± 4.5*	222.0 ± 3.9*
50.0	182.0 ± 4.2	195.3 ± 4.2*	237.8 ± 5.2*	237.5 ± 4.9*	232.5 ± 3.7*
100.0	181.3 ± 5.0	200.8 ± 4.6*	204.3 ± 3.7*	210.3 ± 3.2*	213.0 ± 3.5*

Values are means ± SEM of five separate experiments. Incubations were run as described in Table 1. For further details refer to Materials and Methods.

ANOVA F 2.55, $P < 0.1$.

* $P < 0.01$, Dunnett's test.

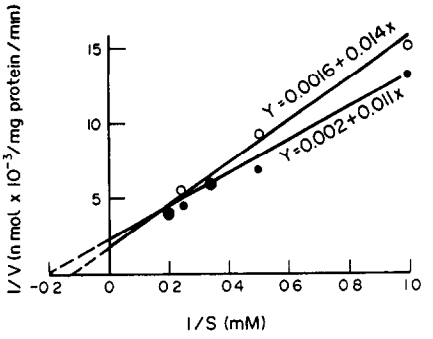

Fig. 2. Double-reciprocal plot showing dependence of γ-GT activity on donor substrate (γ-glutamyl-*p*-nitroanilide) in the absence (○) and presence (●) of piperine (50.0 μM). Cells were incubated as described in Table 1 for 10 min. Glycylglycine concentration was held constant at 80.0 mM. For further details refer to Materials and Methods and Results.

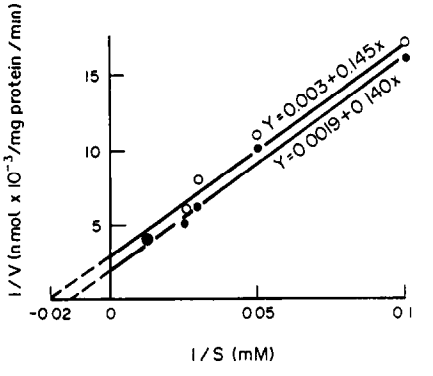

Fig. 3. Double-reciprocal plot showing dependence of γ-GT activity on the acceptor (glycylglycine) in the absence (○) and presence (●) of piperine (50.0 μM). Cells were incubated as described in Table 1 for 10 min. The concentration of γ-glutamyl-*p*-nitroanilide was held constant at 4.0 mM. For further details refer to Materials and Methods and Results.

which are involved in the transport of solutes are a location of high γ-GT activity [25, 26].

The present results show that piperine caused an enhancement of γ-GT in intestinal epithelial cells. The data suggest that this stimulatory effect might be due to an altered kinetic behaviour of the enzyme which was evident by a change in the V_{max}/K_m values. The apparent affinity of the substrate towards enzyme seemed to increase in the presence of piperine. However, in the case of acceptor both V_{max} and K_m were enhanced. This pattern is consistent with a bisubstrate (ping-pong) mechanism. Therefore, it is likely that the availability of enzyme intermediate ("modified enzyme") or the protein turn-over number was enhanced in the presence of piperine, thereby altering the overall rate-determining step. A covalent enzyme intermediate, although not experimentally demonstrated, has been suggested to be involved in the mechanism of action of γ-GT [27]. Assuming this to be the case, the altered kinetic pattern of the enzyme towards the endogenous substrates could be caused either by the influence of this lipophilic compound on the conformation of the enzyme or on the lipid environment within the membrane.

Hydrophobic drug interaction in the vicinity of the membrane bound enzyme and also transporter protein is not unexpected and may result in the altered catalytic activity of various enzymes [28]. The modulation of membrane protein function by its lipid environment is a well known phenomenon and has been demonstrated amply with fluidizing agents. Benzyl alcohol is an agent which selectively fluidizes the outer cell membrane [29]. The fluidity determines the freedom of motion of membrane molecules within the lipid bilayer [30] and any modification of this motion is reported to affect numerous enzyme activities [29] and transport systems [31].

The results presented here show that in the presence of benzyl alcohol γ-GT is inhibited. In normal cells, benzyl alcohol augmented this inhibition in the presence of Triton X-100, possibly due to an additive effect of these two compounds. On the other hand, in the piperine-treated cells, the stimulation of γ-GT was maintained because of the presence of this lipophilic substance of exogenous origin in the membrane. This stimulatory effect of piperine was still observable when cells were treated

Table 3. Analysis of Lineweaver–Burk plots

Assay	Kinetic parameter	Control	Piperine-treated
Donor substrate dependence	V_{max}	625.0 ± 21.0	500.0 ± 23.0*
	K_m	8.33 ± 0.35	5.55 ± 0.22†
Acceptor dependence	V_{max}	333.0 ± 13.0	526.0 ± 21.0†
	K_m	50.0 ± 03.3	83.3 ± 07.0*

See Figs 2 and 3 for plots.
Results are expressed in mM (K_m) and nmol pNA/mg protein/min (V_{max}) ± SEM.
* $P < 0.01$; † $P < 0.001$ (Student's *t*-test).

Table 4. Uptake of amino acids in epithelial cells

[^{14}C]Amino acids	Uptake (pmol/mg protein) Peak Control	Peak Piperine-treated*	Equilibrium Control†	Equilibrium Piperine-treated†‡
L-Leucine	543 ± 17	680 ± 12	201 ± 11	217 ± 19
L-Isoleucine	510 ± 20	695 ± 18	240 ± 17	249 ± 13
L-Valine	490 ± 15	610 ± 13	211 ± 13	220 ± 11

Values are means ± SEM from five separate experiments. Samples (100 μL) were preincubated for 10 min at 37° without or with piperine (50 μM) in Krebs–Hanseleit buffer and loaded with 1.0 mM of radioactive solutes. The incubations were allowed to proceed. Peak uptake occurred at 12 min and equilibrium uptake was measured after 20 min of total incubation period.

* $P < 0.001$ compared to control values at peak time; † $P < 0.001$ compared to corresponding values at peak time; ‡ not significant compared to control values at equilibrium (Student's *t*-test).

Table 5. Effect of benzyl alcohol on amino acid uptake in epithelial cells

[^{14}C]Amino acid	Uptake (pmol/mg protein) Control (no addition)	+ Benzyl alcohol*	+ Piperine + benzyl alcohol*
L-Leucine	570 ± 19	290 ± 13	301 ± 13
L-Isoleucine	500 ± 17	295 ± 19	320 ± 19
L-Valine	530 ± 17	280 ± 17	330 ± 17

Uptake values (measured at peak time) are means ± SEM from five separate experiments. Incubations were carried out as described in Table 4 in the presence of piperine (50 μM) and/or benzyl alcohol (50 mM).

* $P = 0.001$ compared to control (Student's *t*-test).

Table 6. Effect of piperine on LPO in epithelial cells

Epithelial cell sample	MDA level (pmol/mg protein) after incubation for different periods of time (min) 0	5	10	15	30
Control (no addition)	513 ± 20	540 ± 15	545 ± 19	530 ± 20	500 ± 20
+ Piperine (μM)					
12.5	533 ± 19	540 ± 19	540 ± 17	530 ± 17	540 ± 20
25.0	523 ± 17	610 ± 16*	615 ± 19*	650 ± 19*	680 ± 21*
50.0	529 ± 14	600 ± 18*	700 ± 17*	740 ± 16*	730 ± 22*
100.0	510 ± 13	590 ± 17*	740 ± 18*	800 ± 17*	840 ± 19*

Values are means ± SEM from six experiments. Incubations were set up as described in Table 1 except that piperine was dissolved in ethanol. For other details refer to Materials and Methods.

ANOVA F 3.42, $P < 0.1$.

* $P < 0.01$, Dunnett's test.

with both benzyl alcohol and Triton X-100, suggesting that piperine exerted its effects chiefly by interacting with the fluidized state of the membrane lipids. Since the activity of membrane bound γ-GT depends on a suitable lipid environment, the interaction of piperine with the environment rather than a direct action on the enzyme seemed to stimulate the enzyme activity. This event would also explain the observed enhancement of the uptake of amino acids by piperine-treated cells in order to fulfil the requirement of an activated γ-glutamyl cycle.

In the presence of benzyl alcohol the uptake was low and remained so when piperine was also present. In this situation it was difficult to assess the effect of piperine. One reason may be that benzyl alcohol reduced the intravesicular volume of the cell membrane [32] resulting in cell shrinkage. Nevertheless, it seems logical to infer that piperine did not

act independently of the alterations caused by this fluidizing agent on the physical state of the cell.

Any effect of piperine on γ-GT may also be an important determinant in the utilization and functions of GSH in intestinal epithelial cells, since translocation of thiol groups has been associated with detoxification mechanisms [33]. Perturbation of the GSH pool has been shown to occur as a consequence of chemical reaction of xenobiotics with microsomal oxygenases [33]. A significant influence of piperine on the activity of a variety of cytochrome P450-dependent enzymes in the liver and lungs has been reported earlier [14–17].

The data on LPO revealed a high incidence of MDA accumulation by the cells in the presence of piperine. This event would further increase the polar products thereby affecting the fluidity. There existed a concentration- and time-dependent correlation between the accumulation of MDA and the extent of γ-GT stimulation. The lipid fluidity induced by LPO has been associated with changes in the protein conformation [34]. In addition, the accumulation of lipid peroxides has also been known to alter membrane permeability [35]. MDA levels observed were not cytotoxic as evident by the following two observations; LDH leakage in response to increasing concentrations of piperine was not substantial (< 10%), and was poorly correlated with MDA level at increasing concentration–time points.

In summary, the present results suggest that piperine could alter the structural relationship between lipids and protein (fluidity) to cause stimulation of γ-GT with concomitant rise in solute uptake by the epithelial cells. The enhancement of LPO in epithelial cells could either be the cause or consequence of the action of piperine but is likely to contribute to a piperine-mediated change in cell permeability. Such an alteration in the biochemical milieu of the epithelial cell may have a profound effect on intestinal absorption. In a recent report the effect of piperine on intestinal motility has been demonstrated [36]. The widespread use of piperine/pepper extracts in Ayurvedic formulations may therefore be to augment the absorption process of so many herbal drugs reported in Indian and other folklore medicine [37].

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