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Full Length Research Article

Activation of Rat Intestinal Alkaline Phosphatase by Taurine May be an Alternative Mechanism of Endotoxemic Injury Protection

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ABSTRACT: Investigation of the effect of taurine on the hydrolysis of para-nitrophenylphosphate (p-NPP) by rat intestinal alkaline phosphatase (ALP), L-phenylalanine inhibition of ALP and the mechanism of ALP activation by taurine as well as its role in endotoxemic injury protection was carried out. Rat intestinal ALP was exposed to taurine, and L-phenylalanine at varying concentrations and periods of time. Substrate concentration-dependent kinetic analysis was carried out at 10 mM concentration of taurine and 5.17mM of p-NPP. The concentration dependent kinetic analysis of L-phenylalanine was also investigated at 60 mM. The partially purified rat intestinal alkaline phosphatase activity was also investigated in the presence of taurine. Their interactive effect on L-phenylalanine inhibition was also analyzed. Investigation of the effect of taurine on rat intestinal ALP hydrolysis of p-NPP revealed that taurine is an activator of intestinal ALP. At 10 mM taurine and 60 mM L-phenylalanine, taurine relieved L-phenylalanine inhibition of rat intestinal ALP. The effect of lipopolysaccharide in the absence and presence of taurine on ALP activity was also carried out *in vivo.* The kinetic analysis of the data from the *in vivo* study revealed that rat intestinal ALP activity is higher ($12x10^{-3}$nmol^{-1}min^{-1}mg protein) in the presence of taurine and LPS when compared with the activity in the presence of LPS ($9x10^{-3}$nmol^{-1}min^{-1}mg protein) or taurine ($8.8x10^{-3}$nmol^{-1}min^{-1}mg protein) alone. From this study, it may be concluded that the activation of rat intestinal ALP by taurine may be one of the mechanisms of endotoxemic injury protection.
Keywords: Intestinal Alkaline Phosphatase, Taurine, Endotoxemic , Lipopolysaccharide

INTRODUCTION

Lipopolysaccharide (LPS), also known as endotoxin, plays a major role in the development and/or aggravation of several multifactoral diseases, including sepsis, atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis (Gardiner *et al*., 1995; Patel *et al*., 1995; Vincent *et al*., 2002). It is a major constituent of the outer leaflet of the Gram-negative bacteria cell membrane and a single bacterial cell contains approximately 3.5 x 106 LPS molecules, which are essential for growth and stability of the bacterium (Rietschel *et al*., 1994). A full length LPS molecule consists of four different parts: a lipid A moiety, an inner core, an outer core, and an O-antigen (Raetz, 1990). The lipid A moiety of LPS is composed of two phosphorylated glucosamine saccharides linking at least six fatty acids. This part is the toxic component of the molecule, since injection of a chemically synthesized lipid A induces effects in vivo similar to an injection with a full length LPS molecule (Galanos *et al*., 1985; Kotani *et al*., 1985).

The two phosphate groups attached to the saccharides are essential for the toxic activity of lipid A. Removal of one phosphate group results in the formation of monophosphoryl lipid A (MPLA), which has a significant reduced bioactivity compared to lipid A (Loppnow *et al*., 1989; Schromm *et al*., 2000; Gangloff *et al*., 2005). Alkaline phosphatase (ALP) functions as a host defense molecule at physiologic pH when endotoxin is used as a substrate, and is present in many cells and organs and abundant in kidney tissue. In several animal studies, exogenous administration of ALP resulted in attenuation of the inflammatory response induced by gram-negative bacteria and lipopolysaccaride (LPS) and reduced mortality (Chen *et al*., 2010). It is suggested that upregulation of endogenous glomerular ALP reflects a local host defense principle against LPS in the kidney, and that ALP can be inactivated by reacting oxygen species and peroxynitrite generated during sepsis (Li and Shah, 2004). Additionally, the expression of iNOs protein levels correlates negatively with the activity of ALP in duodenum of rats exposed to LPS (Munford, 2005). There has been emerging evidence for the functional role of intestinal alkaline phosphatase (IALP) as a detoxifying enzyme for lipopolysaccaride (Goldberg *et al*., 2008). Intestinal alkaline phosphatase activity has been associated with the reduction of inflammatory conditions in the bowel (Goldberg *et al.,* 2008). Exposure of intestinal wall to LPS induces IALP gene expression.

IALP detoxifies LPS by removing its phosphate esters *in vitro*. Inhibitors of alkaline phosphatase activity have been used in shedding more light on the physiological role of the enzyme which is still elusive (Shirazi *et al*., 1981; Cyboron *et al*., 1982).

Phenylalanine is a known inhibitor of alkaline phosphatase (Malomo *et al*., 2003) and it is believed to have some roles in inflammatory response (Campbell *et al*., 2010). The immune system during microbial invasion is generally protective, septic shock develops in a number of patients as a consequence of excessive or poorly regulated immune response to the offending organism (Gram-negative or Gram-positive bacteria, fungi, viruses, or microbial toxins) (Klosterhalfen *et al*., 1992). Many mechanisms are involved in the pathogenesis of septic shock, including the release of cytokines (Ulloa and Tracey, 2005), the activation of neutrophils (Yuo *et al*., 1991), monocytes and microvascular endothelial cells (Gougerot Podicalo *et al*., 1996), as well as the activation of neuroendocrine reflexes (Van den Berghe *et al*.,1998) and plasma protein cascade systems, such as the complement system (Sprung *et al*., 1986), the intrinsic and extrinsic pathways of coagulation and the fibrinolytic system (Corrigan *et al*., 1968).

Although the lipopolysaccharide of the gram negative bacterial wall (endotoxin) and the peptidoglycan of gram-positive bacterial wall are the most extensively studied (Ahmed and Christou, 1996), several other agents, like the formyl-peptide, formylmethionyl- leucyl-phenylalanine (FMLP), also promote the inflammatory response (Becker, 1987). Phenylalanine is a component of the bacterial N-formyl peptides, such as N-formyl-Met-Leu-Phe (fMLF), which is the first identified and most potent chemo attractant for phagocytic leukocytes (Marasco *et al*., 1984). FMLP induces activation of leukocytes, initiates chemotaxis, stimulates cell aggregation, enzyme secretion, arachidonic acid and oxidative metabolism (Tiffany *et al*., 2001), and causes systemic hypotension associated with a decrease in systemic vascular resistance (Jonsson *et al*., 1997).

Alkaline phosphatase on the other hand is a dimeric metalloenzyme that can be activated or inhibited by modifiers. However, taurine has been reported to activate ALP, outside its two important metal ions; Mg^{2+} and Zn^{2+}. Taurine has been reported to be the most abundant sulphonic amino acid found in the small intestine (Huxtable, 1992) and an inhibitor of intestinal translocation (Wang, 1995). It is also known to protect against endotoxemic injury (Wang, 1995). The mechanism of this endotoxemic protection might be linked to taurine interaction with alkaline phosphatase. Thus, this study was carried out to investigate the effect of taurine on the hydrolysis of para-nitrophenyl phosphate (p-NPP) by rat intestinal ALP, L-phenylalanine inhibition of ALP and the mechanism of activation as well as the role of taurine in endotoxemic injury protection. And to investigate whether this phenomenon can be employed in further strengthening the defense against LPS toxicity by ALP's ability to detoxify it through dephosphorylation.

MATERIALS AND METHODS

Reagents, chemicals, and enzyme source

ALP substrate, the sodium salt of para-nitrophenyl phosphate (p-NPP) and lipopolysaccharide were obtained from Sigma Chemical Company, St. Louis, US. Magnesium sulphate, L-phenylalanine, sodium ortho-vanadate, sodium hydroxide, sodium hydrogen carbonate and anhydrous sodium carbonate, taurine were all products of BDH Laboratory Reagents, Poole England. All other chemicals used in this study were of high quality research grade. The tissue-specific ALP used here was obtained from the intestines of two albino rats (*Rattus novergicus*) among those obtained from small Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria.

Preparation of Rat Tissue Homogenate

Fresh intestinal tissue from *Rattus novergicus* was homogenized in 0.25 M sucrose solution at 4°C and the crude homogenate was centrifuged at 4000 rpm for 20 minutes at the same temperature. The supernatant was collected and dispensed in Eppendorf tubes and stored frozen. Fresh aliquots were used each day for the experiments described in this report. It is generally believed that to perform a large number of kinetic measurements under constant conditions it is more important to have large stabilized enzyme preparations than small preparations with a higher activity (Ahlers, 1975). Thus, we did not attempt further purification since the enzyme is unusually stable even in impure crude preparations (Garen and Levinthal, 1960). The key experimental results obtained with the crude ALP extract were validated with homogenous calf intestinal ALP obtained from New England Biolabs.

Determination of ALP-Catalysed Hydrolysis of P-NPP
Alkaline phosphatase activity was measured by the rate of hydrolysis of appropriate concentrations of p-nitrophenylphosphate (pNPP) at 25°C in 0.1 M $Na_2CO_3/NaHCO_3$ buffer, pH 10.1 as previously described (Ahlers, 1975). Enzyme activity is expressed as the mMol of p-nitrophenol released per minute. Reaction mixtures containing the enzyme source and buffers in the presence of the appropriate concentrations of the modifiers (magnesium, taurine, vanadate, and L-phenylalanine) were kept at 25 °C for 10 minutes. In all assays, 100 µl of the crude enzyme preparation equivalent to 0.5 mg total protein were added to catalyse the hydrolysis of p-NPP. Reactions were initiated by the addition of the appropriate concentration of the substrate, p-NPP. Incubation was allowed for 10 minutes before stopping the reaction by the addition of 0.35 M NaOH. The absorbance was read at 400 nm against a blank of the buffered substrate on a Spectronic-21 UV Vis spectrophotometer and the corresponding activities recorded. All measurements of reaction rate were performed in triplicate. Protein concentration was determined using Biuret method (Gornall *et al*., 1949).

In Vivo Effect of LPS and/ or Taurine on ALP Activity
To investigate the effect of LPS on ALP activity, 1ml of LPS (0.5mg/ml) was administered to a set of rat intraperitoneally (group 1) and the effect on the rats was observed for 20 minutes after which the rats were sacrificed through cervical dislocation. The small intestines were collected and the kinetics of ALP investigated. The p-NPP concentration used was 5.17mM. The K_m and V_{max} values were consequently estimated. To investigate the effect of LPS and taurine on ALP activity, 1ml of taurine (4.8mg/ml) was first administered to the rats orally and the rats were left for 20 minutes after which 1ml LPS (0.5mg/ml) was administered to each rat intraperitoneally. The effect on the rats was observed for few minutes after which the rats were sacrificed through cervical dislocation. The liver and intestine were collected and the kinetics of ALP activity investigated. The p-NPP concentration used was 5.17mM. The K_m and V_{max} values were consequently estimated.

RESULTS

Activation of ALP by Mg^{2+} and taurine

An analysis of the roles of Mg^{2+} ions and taurine in the activation of ALP was carried out by investigating the kinetics of p-NPP hydrolysis in the presence of 0 and 40 mM concentrations of the metal ion (Figure 1A) and 0 and 10 mM concentrations of taurine (Figure 2B). In experiments to determine kinetic constants, activity was monitored by measuring the absorbance change at varying substrate concentrations (0.2–1.8 mM p-NPP) in the presence of varying ligand concentrations. The effects of Mg^{2+} and taurine on the Km for pNPP and V_{max} values were obtained from Lineweaver-Burk plots (Figures 1B and 2B; Table 1). Within the range of substrate concentration examined, the hydrolysis of pNPP by ALP followed saturation kinetics (Figures 1 and 2). Kinetic analysis showed that both Mg^{2+} and taurine activate ALP catalyzed p-NPP by increasing the maximum reaction rate (V_{max}) as shown in Table 1. The activation of alkaline phosphatase by Mg^{2+} and taurine was also accompanied by a change in the Michaelis constant (Km).

Inhibition of rat intestinal ALP by L-phenylalanine

The Michaelis–Menten's curve and the double reciprocal plot for the hydrolysis of p-NPP over a range of concentrations by alkaline phosphatase in the presence of 60 mM L-phenylalanine are shown in Figures 3A and 3B. The values of kinetic parameters, V_{max} and Km are given in Table 1. There was an evidence of inhibition of ALP activity when exposed to 60 mM L-phenylalanine as indicated by sharp drop in the V_{max}.

Interaction of Taurine with Mg^{2+} and Phenylalanine on Rat Intestinal Alkaline Phosphatase

Results presented in Figures 1 and 2 show that both Mg^{2+} and taurine are required for ALP activation. Hence, we sought to determine if any synergistic interactions exist between both ligands in the activation of ALP, and to determine the optimal concentrations of both ligands for full activity. The effects of Mg^{2+} and taurine on ALP activity were investigated by determining the rate of hydrolysis of 5.17 mM pNPP in the presence of 10 mM taurine and 40 mM Mg^{2+}. The results of this experiment are shown in Figure 4 and the pattern indicates a synergistic interaction between the two ligands as indicated by increase in the V_{max}. The values of kinetic parameters, V_{max} and Km are given in Table 1.

The effect of interaction of 10 mM taurine and 60 mM L-phenylalanine on rat intestinal alkaline phosphatase hydrolysis of p-NPP was also investigated and the result obtained is shown in Figure 5. The result shows that taurine modulates L-phenylalanine inhibition of rat intestinal ALP activity as indicated by increase in the V_{max} (Table 1).

Interaction of Lipopolysaccharide (LPS), Taurine and a Combination of LPS and Taurine with Intestinal ALP Activity

The Michaelis-Menten's curve and double reciprocal plot for effect of taurine, LPS and interaction of taurine and LPS on the hydrolysis of p-NPP over a range of concentration (0.207 – 1.861) by alkaline phosphatase is shown in Figures 5 and 6 respectively. The values of kinetic parameters K_m and V_{max} are given in Table 2.

The kinetic analysis of the data revealed that rat intestinal ALP activity is highest in the presence of taurine and LPS combination when compared with the activity in the presence of LPS alone and taurine. The data showed only slight difference in the activity of ALP in the presence of LPS and taurine as depicted by the values of V_{max} (Table 15). The presence of taurine and LPS affects the binding affinity (K_m) of ALP for p-NPP. A low affinity is recorded in the presence of LPS when compared with the affinity in the presence of a combination of LPS and taurine alone. The highest affinity is observed in the presence of taurine only. This is deduced from low K_m value (in the case of taurine only) and slightly different values of K_m (in the presence of LPS and a combination of LPS and taurine).

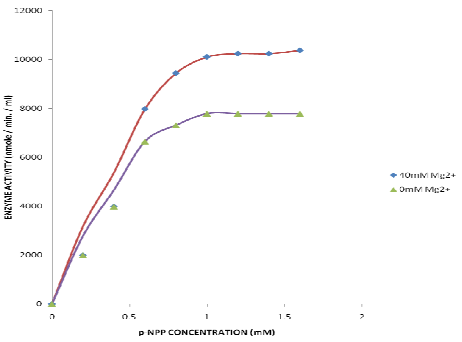

Figure 1A: Kinetic analysis of the effects of Mg^{2+} on ALP-catalyzed hydrolysis of p-NPP.

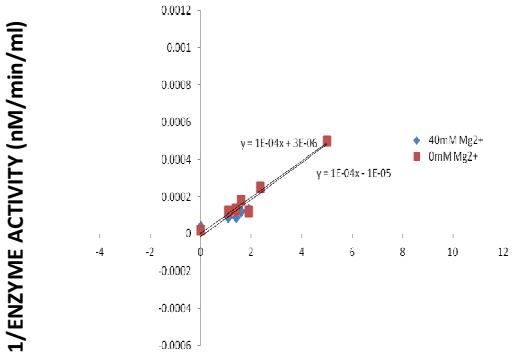

Figure 1B: Double reciprocal plot of the effects of Mg^{2+} on ALP-catalyzed hydrolysis of p-NPP.

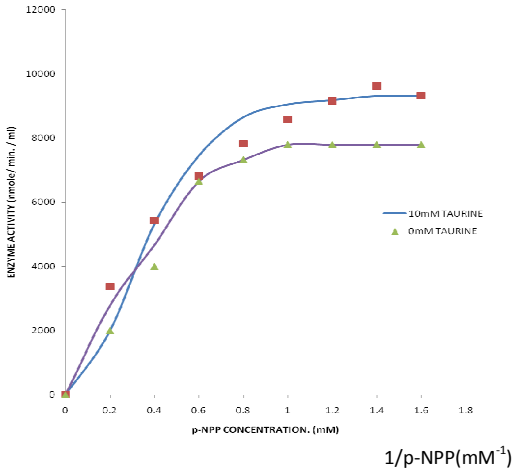

Figure 2A: Kinetic analysis of the effects of taurine on ALP-catalyzed hydrolysis of p-NPP.

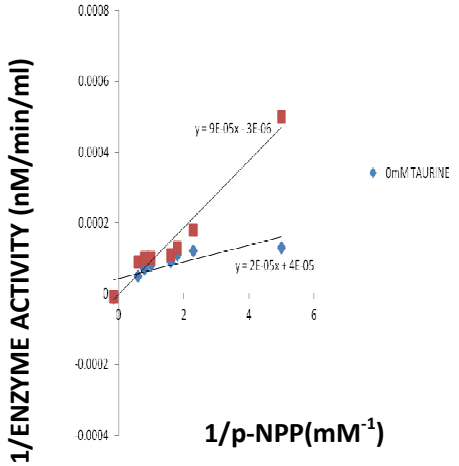

Figure 2B: Double reciprocal plot the effects of taurine on ALP-catalyzed hydrolysis of p-NPP.

Reaction mixture containing 0.1M $Na_2CO_3/NaHCO_3$ buffer (pH 10.1) and 10 µl crude ALP (0.5 mg total protein) was pre-incubated at 25 °C both in the absence of Mg^{2+} and in the presence of 40 mM Mg^{2+} for 10 minutes. Reaction was initiated by the addition of 0.2-1.8 mM p-NPP and incubation was carried out for 10 minutes before stopping the reaction by the addition of 1.0 ml 0.35 M KOH. Enzyme activity is expressed as the mMol of para-nitrophenol released per minute.

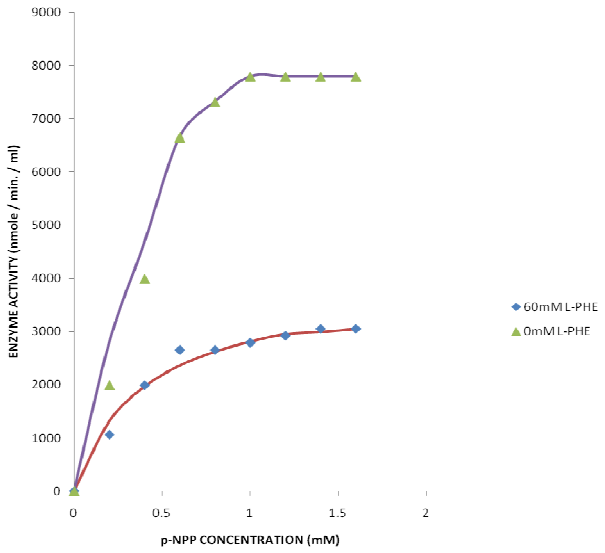

Figure 3A: Kinetic analysis of the effects of L-phenylalnine on ALP-catalyzed hydrolysis of p-NPP.

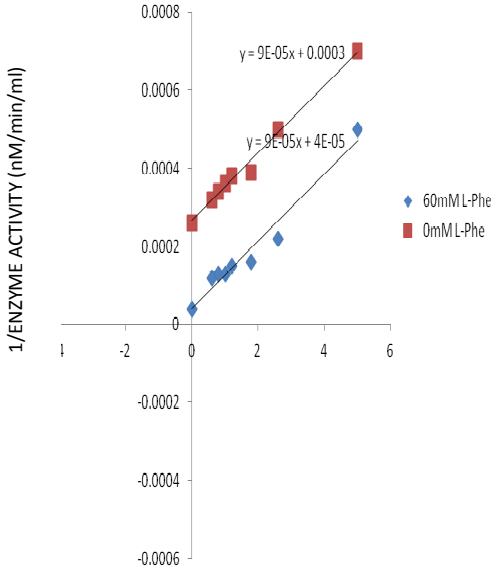

Figure 3B: Double reciprocal plot of the effects of L-phenylalanine on ALP-catalyzed hydrolysis of p-NPP.

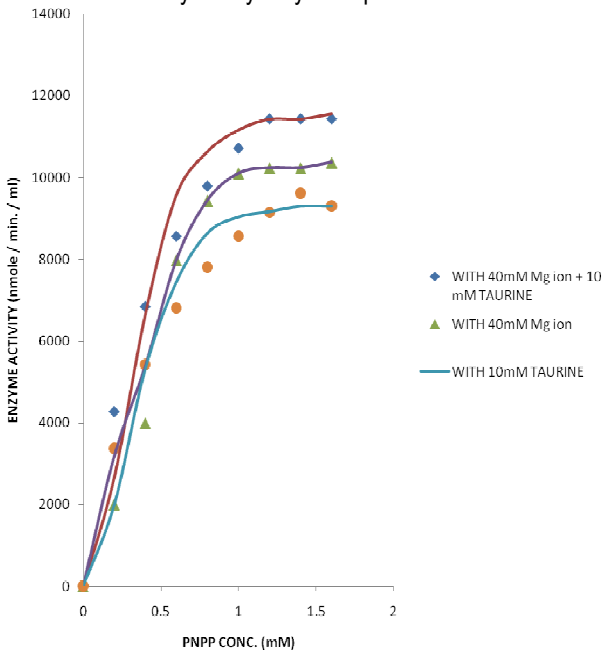

Figure 4: Synergistic activation of ALP-catalyzed hydrolysis of p-NPP by Mg^{2+} and taurine.

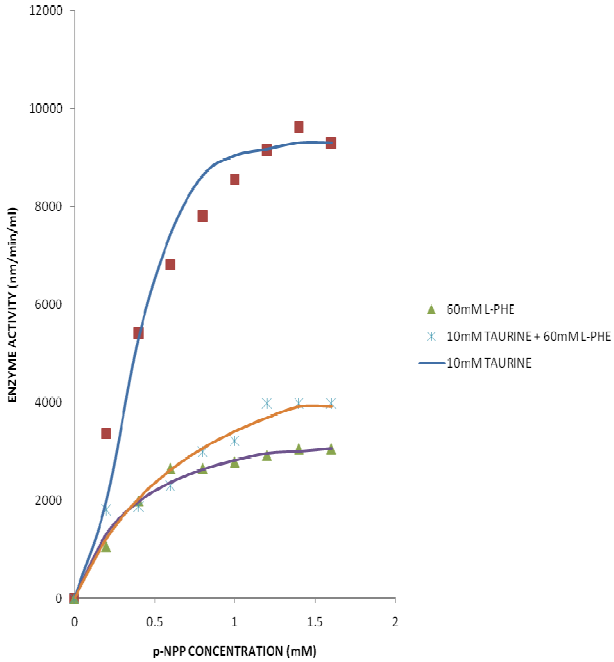

Figure 5: Kinetic analysis of the modulatory effect of taurine on L-phenylalanine inhibition of rat intestinal ALP activity.

Reaction mixture containing 0.1M Na_2CO_3/$NaHCO_3$ buffer (pH 10.1) and 10 µl crude ALP (0.5 mg total protein) was pre-incubated at 25 ^oC both in the absence of L-Phenylalanine and in the presence of 60 mM L-Phenylalanine for 10 minutes. Reaction was initiated by the addition of 0.2-1.8 mM p-NPP and incubation was carried out for 10 minutes before stopping the reaction by the addition of 1.0 ml 0.35 M KOH. Enzyme activity is expressed as the mMol of para-nitrophenol released per minute

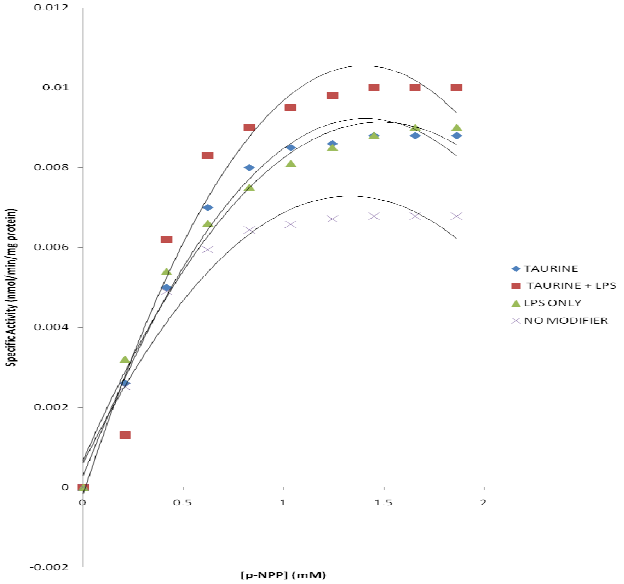

Figure 6A: Michaelis-Menten curve of intestinal ALP activity following intra-peritoneal administration of rat with LPS, taurine, and taurine + LPS.

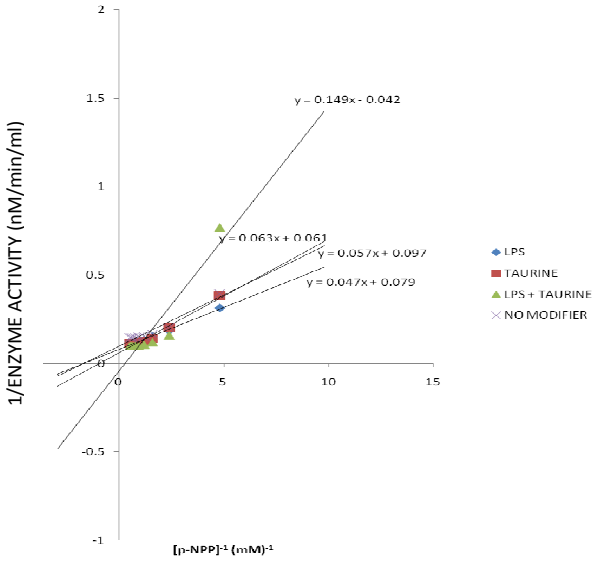

Figure 6B: Double-reciprocal plot showing effect of taurine, LPS and a combination of both on the rate of intestinal ALP-catalyzed hydrolysis of p-NPP.

Reaction mixture containing 0.1M Na_2CO_3/$NaHCO_3$ buffer (pH 10.1) and 10 µl crude ALP (0.5 mg total protein) was pre-incubated at 25 °C both in the absence of L-Phenylalanine and in the presence of 60 mM L-Phenylalanine for 10 minutes. Reaction was initiated by the addition of 0.2-1.8 mM p-NPP and incubation was carried out for 10 minutes before stopping the reaction by the addition of 1.0 ml 0.35 M KOH. Enzyme activity is expressed as the mMol of para-nitrophenol released per minute

Table 1: Kinetics Parameters of Rat Intestinal ALP in the Presence of Different Modifiers at 25 °C

Modifers	V_{max} (nmole/min/ml) x10^3	K_m (mM)
No modifiers	7.80±0.18	0.29±0.01
Mg^{2+}	17.72±0.92	0.40±0.03
Taurine	13.95±0.18	0.49±0.05
L-Phe	3.94±0.18	0.60±0.20
Taurine + Mg^{2+}	19.63±0.05	0.25±0.15
Taurine + L-Phe	6.85±0.05	1.93±0.04

The values represent V_{max} and Km of different modifiers of rat intestinal ALP and the standard deviation (n=3).

Table 2: Kinetic Parameters of intestinal ALP after intraperitoneal administration of lipopolysaccharides (LPS) and taurine at 25 °C

	V_{max} x10^{-3} (nmol/min/mg protein)	K_m x10^{-2} (mM)
No modifier	6.79	27.8
LPS only	9.0	38.0
Taurine	8.8	23.00
LPS + Taurine	12.0	22.0

The values represent V_{max} and Km of different modifiers of rat intestinal ALP and the standard deviation (n=3).

DISCUSSION

The activation of ALP by magnesium as observed is in conformity with earlier studies (Ahlers, 1974; Arise *et al*., 2005). Magnesium may have occupied the structural site on ALP that led to a subsequent conformational change from a less active form of ALP to a more activated form of the enzyme (i.e. activated ALP–Mg^{2+} Complex). The activated ALP-Mg^{2+} complex has a low Km which shows that it has a high binding affinity for the substrate. The activated ALP- taurine complex has a low Km which shows that it has a high binding affinity for the substrate. Taurine may have occupied the structural site on ALP that led to a subsequent conformational change from a less active form of ALP to a more activated form of the enzyme (i.e. Activated ALP–Taurine Complex). Researchers have demonstrated taurine can protect tissues from neutrophil-induced reperfusion injury and oxidative stress because the respiratory burst activity of neutrophils is also significantly reduced in the presence of taurine (Raschke *et al*., 1995). Also, exposure to bacterial endotoxins has been suggested as one factor which can augment the magnitude of individual responses to xenobiotics (Roth *et al*., 1997). Circulating endotoxins of intestinal origin have been found to create a positive feedback on endotoxin translocation from the

gut, stimulating increases in serum endotoxin levels. In experimental animals, taurine was found to significantly inhibit intestinal translocation and to protect the animals from endotoxemic injury (Roth *et al*., 1997). Therefore, it is possible that taurine might be able to modify factors underlying susceptibility to toxic substances since it has been reported that taurine's protective effect is mediated by its antioxidative properties (Raschke *et al*., 1995).

Though it is known that lipopolysaccharide (LPS) induces apoptosis and inhibits osteoblast differentiation (Guo *et al*., 2014), it has however been reported that pre-treatment with rutin, an anti-inflammatory agent like taurine, inhibited LPS-induced neutrophil infiltration in the lungs, LPS-induced expression of vascular cell adhesion molecule (VCAM)-1 and inducible nitric oxide synthase (iNOS) (Huang *et al*., 2014). Taurine, a non-protein sulfur containing amino acid, is the most abundant free amino acid and has been shown to play several essential roles in the human body. It is widely distributed in very high concentrations in brain, heart, kidney, lens, and reproductive organs (Huxtable, 1992). In these tissues, it functions as an anti-inflammatory substance, neurotransmitter, cell volume regulator, antioxidant, growth promoting factor, etc. At the cellular level, one of its important functions is cytoprotection as demonstrated in this study.

The enzyme activity decreased in the presence of 60 mM L-phenylalanine. This shows that L-phenylalanine inhibit the rat intestinal alkaline phosphatase, which is in conformity with earlier studies on L-phenylalanine (Fishman *et al*.,1962; Arise *et al.,* 2008). There was an evidence of inactivation of ALP exposed to L-phenylalanine. There was reduction of ALP activity by 60% in the presence of 60 mM L-phenylalanine. The inactivation of ALP by L-phenylalanine is also Km dependent as depicted by high Km value of 0.6 mM. This revealed that the binding affinity of ALP for p- NPP gradually reduced in the presence of 60 mM L-phenylalanine. The mechanism of uncompetitive inhibition by L-phenylalanine was linked to its ability to bind to an arginine residue in the active site of the enzyme (Hoylaerts *et al.,* 1992).

Mg^{2+} and taurine are required for ALP activation. When 10 mM taurine and 40 mM Mg^{2+} was combined together, there was a positive synergisitic effect. There was an increase in V_{max} and decrease in Km. This shows a high binding affinity of the enzyme to its substrate. This may be due to the fact that magnesium and taurine may have occupied the structural site on ALP that led to a subsequent conformational change from less active form of ALP to a more activated form of the enzyme (i.e. activated ALP - Mg^{2+} - taurine complex). In the presence of 10 mM taurine, activation of the rat intestinal ALP activity occurred. This may be due to the activatory ability of taurine on L-phenylalanine inhibited rat intestinal ALP. The enzyme activity and V_{max} increased compared to only L-phenylalanine inhibited rat intestinal ALP. This revealed the modulatory ability of taurine on L-phenylalanine inhibition of rat intestinal ALP. Taurine relieved L-phe inhibition of rat intestinal ALP. This may explain some of the roles of ALP physiologically regardless of the inhibitory role of L-phenylalanine, which is believed to have some roles in inflammatory response and a component of FMLP, the most potent chemo attractant for phagocytic leukocytes (Marasco *et al*., 1984). Trachtman *et al*. (1992) proposed that the cytoprotective activity of taurine must be related in part to the scavenging of HOCl. However, the effects of taurine may also include the suppression of neutrophil activity by N-chlorotaurine and in this regard; Trachtman *et al.* (1992) found that a number of other reactive oxygen species and free radicals are produced during the inflammatory response. The activated ALP- taurine complex has a low Km which shows that taurine up-regulated the activity of ALP in the presence of its substrate.

CONCLUSION

This study shows that the hydrolysis of p-NPP and LP by rat intestinal ALP can be stimulated by taurine. The effect of taurine and magnesium on p-NPP hydrolysis as well as LP dephosphorylation by rat intestinal ALP is positively synergistic because of the increased V_{max}. The activation of rat intestinal ALP by taurine may be one of the mechanisms of endotoxemic injury protection. The increased ALP activity mediated by taurine in the presence of L-phenylalanine may be an alternative immunological mechanism against endotoxemic injury.

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