# Nitric oxide inhibits enterocyte mitochondrial phospholipase D

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**Abstract Mitochondrial damage is one of the prominent features of cell death in oxidative stress and related pathological conditions. Alteration in membrane lipid composition may be responsible for the mitochondrial damage. In this study, we have shown that intestinal mitochondria contain an active phospholipase D (PLD) which is activated by oxidants, $Ca^{2+}$ or polyamines and this results in degradation of phosphatidylethanolamine (PE) and formation of phosphatidic acid (PA). This PLD activity is inhibited by nitric oxide (NO) which prevents the lipid alteration in mitochondria when exposed to these agents. This can be reversed by the NO scavenger, haemoglobin. This suggests that alteration of mitochondrial membrane lipid composition by activation of PLD in certain pathological condition such as oxidative stress may be prevented by the simultaneous presence of nitric oxide.**



*Key words:* Phospholipase D; Enterocyte mitochondria; Nitric oxide; Phosphatidic acid; Phosphatidylethanolamine

## 1. Introduction

The free radical, nitric oxide has emerged as an important signal and effector molecule in mammalian physiology. It has been implicated in various normal and pathological conditions, including neurotransmission, vasodilation and inflammation [1–3], but can also be cytotoxic, particularly at elevated concentrations [4,5]. The major recognized target of NO action is a heme protein, the soluble form of guanylate cyclase, whose activity is increased by binding of NO to the heme moiety of the enzyme [1–3]. Cellular targets include key enzymes in respiration, glycolysis, regulation of iron metabolism, and DNA repair [6–9].

With its radical nature, $NO^{\bullet}$ can rapidly react with oxygen and its derivatives to form other potentially toxic radicals. For example, peroxynitrite ($ONOO^{\bullet-}$) is formed in aqueous solutions by an interaction between superoxide anion ($O_2^{\bullet-}$) and $NO^{\bullet}$. Peroxynitrite decays, once protonated, to the very reactive hydroxyl radical ($OH^{\bullet}$) and nitrogen dioxide radical $NO_2^{\bullet}$ [10]. Respiring cells endogenously produce oxygen radicals such as $O_2^{\bullet-}$ and $H_2O_2$ that can react with $NO^{\bullet}$ inside the cell. One of the sources of these intrinsic radicals is the mitochondrial electron transport chain in which 2–3% of the oxygen consumed is leaked in the form of free radicals [11]. Recently we have shown that intestinal mitochondria contain a PLD activity which can be activated by oxidants [12], $Ca^{2+}$ [13] or polyamines [14] resulting in the formation of phosphatidic acid. This PLD preferentially hydrolyses endogenous phosphatidylethanolamine and changes in the mitochondrial lipid composition brought about by PLD activation may have a role in $Ca^{2+}$ cycling that is seen during oxidative stress. Since NO is known to have both protective and damaging effects on cell, the present study looks at the alteration in mitochondrial lipids induced by oxidants, $Ca^{2+}$ or polyamines and the effect of nitric oxide on these lipid changes.

## 2. Materials and methods

### 2.1. Materials

Various lipid standards, fluorescamine, HEPES, BSA, spermine, xanthine, xanthine oxidase, menadione, haemoglobin, glutathione (GSH) and sodium nitroprusside were all obtained from Sigma Chemical Co. All other chemicals used were of analytical grade. Mitochondria were prepared from isolated enterocytes as described [15] and suspended in a solution of 250 mM sucrose, 5 mM HEPES pH 7.4 at a protein concentration of 8–10 mg/ml and used within 2–3 hours.

### 2.2. Synthesis of S-nitrosoglutathione

*S*-Nitrosoglutathione was prepared according to the method of Arnelle [16]. Briefly, GSNO was synthesized by mixing an equimolar concentration of glutathione (100 mM in 250 mM HCl), 0.1 mM EDTA (sodium salt), and 100 mM $NaNO_2$ in water at 25°C for 6 min. GSNO solution was prepared fresh daily, pH adjusted to 7.4, and the concentration was confirmed by absorption spectroscopy as described [17]. This stock solution was diluted to a final concentration of 1 mM in the reactions described below.

### 2.3. Phospholipase D assay

Mitochondrial PLD was assayed using endogenous phospholipids as substrate. Mitochondria (approximately 1 mg protein) in 250 mM sucrose, 5 mM HEPES pH 7.4 in a total volume of 1 ml were incubated with the following compounds separately at 37°C for 30 min. PLD activity was stimulated by 100 μM $Ca^{2+}$ or 0.5 mM spermine or 1 mM xanthine+100 munits xanthine oxidase or 50 mM menadione (all final concentration). To check the NO effect, experiments were started by exposing the mitochondria to sodium nitroprusside (1 mM) or *S*-nitrosoglutathione (1 mM) as NO donor. In those experiments where $Ca^{2+}$ or spermine were added, 0.3 mM haemoglobin was included in the incubations as a scavenger of NO. At the end of incubation, total lipids were extracted by Bligh and Dyer's method [18] and PA content analyzed by TLC. Extracted lipids were spotted on silica gel G plates impregnated with 0.5 M oxalic acid and separated using the solvent system, chloroform:methanol:conc. HCl (85:13:0.5 v/v) [19]. PA corresponding to standard was identified by iodine exposure, eluted and quantitated by phosphate estimation after acid digestion [20]. Our earlier studies using intestinal mitochondrial PLD have shown that this enzyme does not catalyze transphosphatidylation in the presence of alcohol [12–14] which is similar to a recent report of a yeast PLD unable to catalyze transphosphatidylation [21].

### 2.4. Lipid analysis

Neutral lipids were separated on silica gel G plates using the solvent system hexane:diethyl ether:acetic acid (80:20:1 v/v). Spots corresponding to standard were identified by iodine exposure and eluted. Cholesterol [22], diglycerides and triglycerides [23] were estimated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on 5% EGSS-X column. Heptadecanoic acid was used as internal standard. Individual phospholipids

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*Abbreviations:* PLD, phospholipase D (EC 3.1.4.4); NO, nitric oxide; PA, phosphatidic acid; PE, phosphatidylethanolamine; $O_2^{\bullet-}$, superoxide anion; $ONOO^{\bullet-}$, peroxynitrite; Hb, haemoglobin; SNP, sodium nitroprusside; GSNO, *S*-nitrosoglutathione


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were separated on silica gel H plates using the solvent system chloroform:methanol:acetic acid:water (25:14:4:2 v/v) [24] and quantitated by phosphate estimation after acid hydrolysis. Individual aminophospholipids were also quantitated after derivatization with fluorescamine and separation on silica gel H plates impregnated with 3% magnesium acetate using the solvent system chloroform:methanol:$NH_4OH$:water (60:40:5:2 v/v) [25]. Eluted individual spots were quantitated using Shimadzu SF 5000 spectrofluorometer with excitation at 395 nm and emission at 468 nm.

*2.5. Protein estimation*

Protein was estimated by the method of Lowry et al. [26] using bovine serum albumin as standard.

*2.6. Statistical analysis*

Three separate experiments were carried out and results are presented giving the mean ± S.E.M. Mann Whitney U test was done to compare the changes.

## 3. Results

Exposure of the intestinal mitochondria to oxidants, $Ca^{2+}$ or spermine induced alteration in the phospholipid composition and among the phospholipids only PE level was decreased (Fig. 1). There was no change in the level of other phospholipids including lysophospholipids (data not shown). This decrease in the PE level induced by these agents was abolished by the simultaneous presence of NO donor, SNP (Fig. 1). Phosphatidic acid, the product of PLD action on phospholipids was measured after exposure to these agents. As shown in Fig. 2, oxidants, $Ca^{2+}$ or spermine stimulated the formation of PA which was completely inhibited by NO. Sodium nitroprusside (SNP) which was used as a donor of NO was tested at 1 or 5 mM and both concentrations showed similar results and hence for all experiments, 1 mM SNP was used. A nitroprusside molecule contains five cyanide ions and one nitric oxide surrounding a central iron atom $[Fe(CN)_5NO]^{2-}$ and to rule out the possibility of a cyanide effect on the response, mitochondria were pretreated with haemoglobin before addition of SNP. This reversed the inhibitory effect of SNP as shown in Fig. 3. To further confirm, another NO donor, *S*-nitrosoglutathione was used in presence and absence of NO scavenger haemoglobin and the effect of $Ca^{2+}$ and spermine on intestinal PLD activity was measured. As shown in Fig. 4, GSNO inhibited the PA formation which was reversed by haemoglobin. Incubation of mitochondria with oxidants, $Ca^{2+}$ or spermine did not alter the composition of neutral lipids (Table 1).

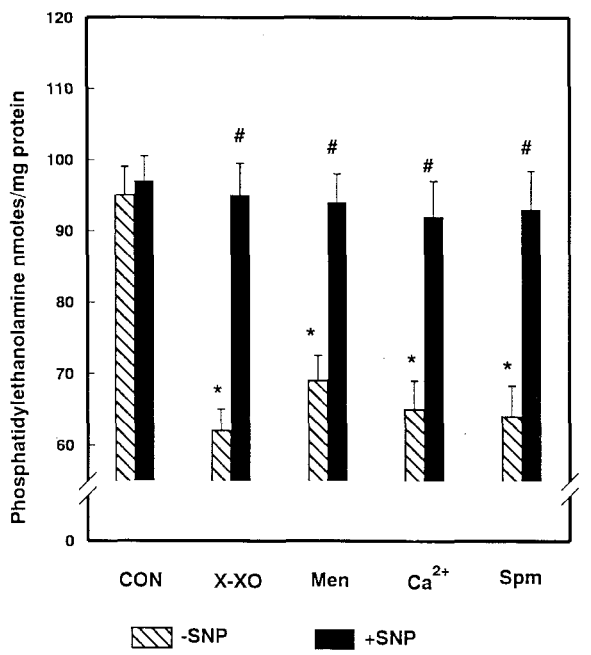


Fig. 1. Effect of NO on intestinal mitochondrial PLD activity induced by oxidants, spermine and $Ca^{2+}$ as judged by phosphatidylethanolamine degradation. Experimental conditions are described in the text. Each value represents mean ± S.E.M. of three separate estimations. $*p < 0.05$ in treated samples compared to control. $p$, 0.05 in treated samples compared to NO incubated samples.

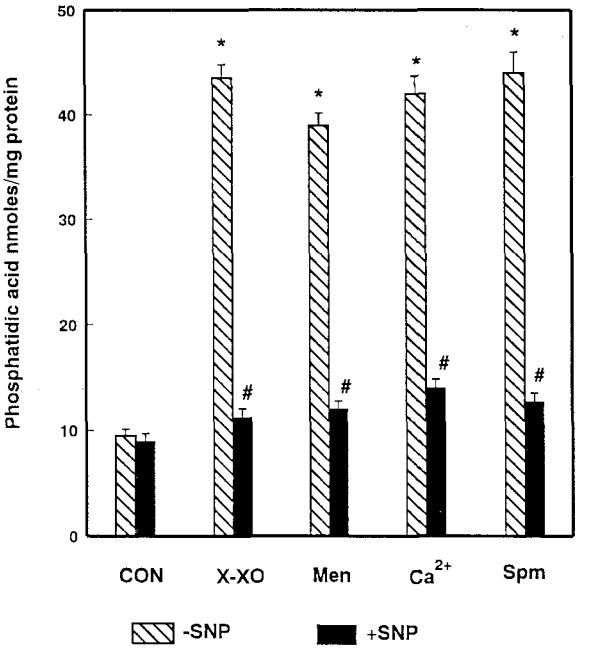


Fig. 2. Effect of NO on intestinal mitochondrial PLD activity induced by oxidants, spermine and $Ca^{2+}$ as assessed by phosphatidic acid formation. Experimental conditions are described in the text. Each value represents mean ± S.E.M. of three separate estimations. $*p < 0.05$ in treated samples compared to control. $p$, 0.05 in treated samples compared to NO incubated samples.

## 4. Discussion

Oxidative stress is associated with mitochondrial damage and altered cellular $Ca^{2+}$ homeostasis. This process may be a consequence of impairment of $Ca^{2+}$ sequestration by the endoplasmic reticulum (ER) and mitochondria which results in inability of the cell to maintain its cytosolic free $Ca^{2+}$ concentration within the physiological range [27]. During oxidative stress, $Ca^{2+}$ overload may result in alteration of cell function and ultimately in cell death. Oxidative stress is associated with non-specific opening of a membrane pore in mitochondria which leads to leakage of many compounds including $Ca^{2+}$. This results in mitochondrial swelling, altered energy production, damage to mitochondria and finally cell death. During ischemia, mitochondria tries to augment the formation of ATP by increased $Ca^{2+}$ uptake [28].

Supporting data are available in the literature for both a protective and a cytotoxic role for NO in biological systems. Nitric oxide has been shown to inhibit mitochondrial function of activated macrophages [29,30]. Mitochondria respiration

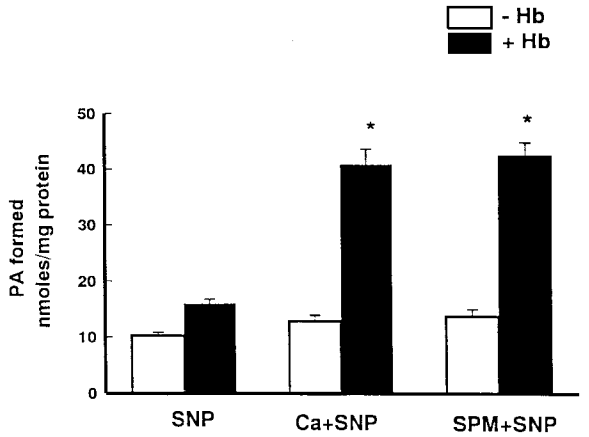


Fig. 3. Effect of haemoglobin (Hb) on sodium nitroprusside (SNP) mediated PLD inhibition. Mitochondria were incubated with 100 μM $Ca^{2+}$ or 0.5 mM spermine (SPM) with 1 mM SNP in absence and presence of 0.3 mM haemoglobin. After 30 min incubation, PA was analyzed. Each value represents mean ± S.E.M. of three separate estimations. *$p < 0.05$ in Hb treated samples as compared to SNP incubated sample alone.

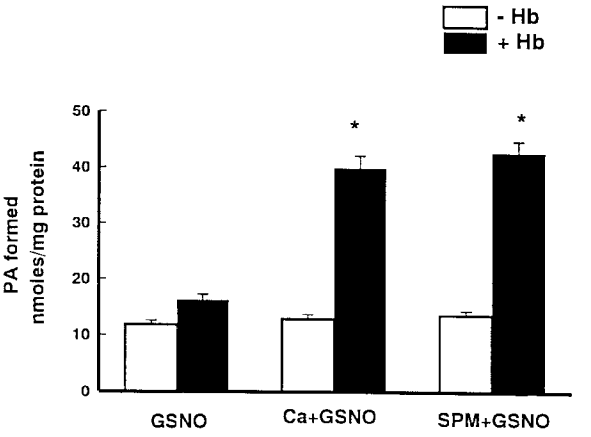


Fig. 4. Effect of *S*-nitrosoglutathione (GSNO) on enterocyte mitochondrial phospholipase D activity and the reversibility of GSNO mediated PLD inhibition by haemoglobin. Mitochondria were incubated with 1 mM GSNO in absence and presence of 0.3 mM haemoglobin. Each value represents mean ± S.E.M. of three separate estimations. *$p < 0.05$ in Hb treated samples as compared to GSNO incubated sample alone.

was inhibited by NO resulting in reduced ATP synthesis in a concentration dependent manner without affecting the activity of energy transducing enzymes and the inhibition was significantly stronger at physiologically low intracellular oxygen tension [31]. NO has been shown to inhibit SH- and iron-containing proteins, and electron transport system [32,33]. Peroxynitrite which is formed by the reaction of NO with $O_2^-$ may produce oxidant injury itself or through the formation of a hydroxyl-like radical [34]. Examples of ONOO-induced cell injury include oxidation of cellular thiols, thus depleting cellular GSH and cysteine and exposing cellular macromolecules to damage from reactive oxygen species [35], inactivation of MnSOD [36] and inactivation of succinate dehydrogenase and fumarate reductase in *Trypanosoma cruzi*, thus producing cytotoxicity [37]. NO also increases cytosolic concentration of free calcium ion by denergizing mitochondria and kills freshly isolated hepatocytes [38,39]. Mitochondrially located NO synthase might be involved in the regulation of oxidative phosphorylation, because of the ability of NO to bind to cytochrome oxidase and inhibit electron transport [40].

NO appears to be capable of both producing and preventing oxidant injury in different in vitro and in vivo systems. The overall outcome of these two effects in any system may depend upon the relative concentrations of individual reactive oxygen species [41]. In response to neutrophil mediated injury, endothelial cells have been shown to release NO in addition to other mediators [42]. Released NO reacts with superoxide to form peroxynitrite, thus acting as a free radical scavenger and blunting oxidant injury [34,35]. In a feline model of myocardial injury NO has been shown to interfere with neutrophil adherence to coronary endothelium and attenuate neutrophil superoxide production [43]. The protective effects of NO in ischemia and reperfusion injury have also been demonstrated in other systems [44,45]. NO has also been shown to inhibit neutrophil ROS production via a direct effect on NADPH oxidase [42] and to function as an antioxidant through the formation of NO-iron adducts, thus reducing the availability of ferrous iron and thereby ROS production [46].

The nitrite production may be a useful index of acute and chronic experimental intestinal injury and nitric oxide may contribute to the functional repair of the epithelial barrier [47]. With respect to intestinal ischemia-reperfusion injury, most of the alterations elicited by this process can be induced by inhibition of NO synthase in normal rats [48,49]. It has also been shown that administration of NO donors provide significant protection against dysfunction associated with ischemia-reperfusion injury [50].

We have recently shown that intestinal mitochondria contain a PLD which can be activated by oxygen free radicals, $Ca^{2+}$ or by polyamines [12–14] and this PLD when stimulated by these agents, specifically hydrolyse endogenous PE resulting in the generation of PA. The present study has shown that the lipid alteration induced by PLD activation by these agents can be prevented by the simultaneous presence of NO which was reversed by haemoglobin as NO scavenger. To our

Table 1
Effect of NO on the intestinal mitochondrial neutral lipids

| | (nmol/mg protein) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | X–XO | | SPM | | $Ca^{2+}$ | |
| | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) |
| Total cholesterol | 139 ± 11.00 | 136 ± 10.00 | 134 ± 10.00 | 132 ± 10.40 | 131 ± 9.40 | 129 ± 9.00 | 133 ± 8.70 | 130 ± 7.90 |
| Triglyceride | 90 ± 6.00 | 87 ± 4.50 | 88 ± 7.80 | 84 ± 4.70 | 87 ± 7.40 | 84 ± 4.90 | 86 ± 5.00 | 84 ± 5.80 |
| Diglyceride | 56 ± 4.50 | 57 ± 4.00 | 57 ± 4.70 | 55 ± 6.00 | 54 ± 6.00 | 58 ± 5.00 | 55 ± 4.70 | 57 ± 6.00 |
| Free fatty acid | 101 ± 11.00 | 103 ± 9.30 | 109 ± 9.00 | 102 ± 9.40 | 107 ± 8.70 | 104 ± 8.50 | 111 ± 8.70 | 102 ± 9.50 |

Xanthine (X) 1 mM and xanthine oxidase (XO) 100 munits, spermine (SPM) 0.5 mM and $Ca^{2+}$ 100 μM were used as final concentration. (+) with 1 mM SNP, (−) without SNP. Each value represents mean ± S.E.M. of three separate estimations.

knowledge, there is no data available on the effect of NO on PLD activity. The effect of NO observed here is not due to scavenging of free radicals since PLD activation by $Ca^{2+}$ or polyamine activated PLD was also inhibited by NO. Lipid alteration of the mitochondrial membrane is likely to alter their function and decrease in PE and increase in PA in mitochondria may have some physiological significance during oxidative stress and on $Ca^{2+}$ loading and these effects may be prevented by NO. This observation supports a possible protective role for NO in enterocyte mitochondrial damage during certain pathological conditions.

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