Research Article

Bisphenol S (BPS) Alters Xanthine Oxidoreductase Activity to Elevate Oxidative Stress Mediated Protein Carbonylation in Small Intestine of Rat *ex vivo*

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ABSTRACT

Bisphenol S (BPS) is a replacement bisphenol used instead of bisphenol A (BPA) to manufacture polycarbonate plastics, epoxy resins, and thermal papers utilized in storage and packaging of foodstuffs, beverages and drinking water. Consequently, leaching of BPS from the food contact materials into the comestibles, followed by absorption of BPS-tainted comestibles by small intestine becomes the predominant source of BPS contamination to humankind. Though, BPS is known to elevate oxidative stress, its effects on xanthine oxidoreductase (XOR) system mediated oxidative induction of proteins remains unexplored. Therefore, the present study aims to evaluate the effect of BPS on oxidation of proteins by involving XOR system in small intestine of male albino rats ex vivo. BPS exposure has been found to significantly ($P < 0.001$) increase protein carbonylation; alter the activities of XOR system by augmenting the activities of cytosolic and mitochondrial xanthine oxidase (XO) and cytosolic xanthine dehydrogenase; and diminish the concentrations of cytosolic and mitochondrial nitrite in a dose dependent manner compared to the control group. These results indicate that BPS elevates protein carbonylation and diminishes nitrite concentration probably by XOR system mediated oxidative stress generation in mammalian small intestine.

Keywords: Bisphenol S, Small intestine, Mitochondria, Protein carbonylation, Xanthine oxidase, Xanthine dehydrogenase, Xanthine oxidoreductase.

INTRODUCTION

Bisphenol S (BPS) is one of the replacement bisphenol compounds gradually taking over the place of bisphenol A (BPA) since the implementation of a set of restrictions on the latter compound due to the revelation of its potent endocrine disrupting capabilities[1-4]. In the manufacturing of polycarbonate based plastics, polyvinyl chloride (PVC) plastics, epoxy resins and a variety of thermal papers, BPS is considered to be one of the major substitutes of BPA[5-7]. Utilization of BPS might also occur as an ingredient in polyethylene terephthalate (PET), in order to induce thermal rigidity in the polymer ester, widely used to manufacture soft drink and water bottles[8].

Among the aforementioned sources, polycarbonate based plastics, epoxy resin based inner coats in metal containers, thermal papers and thermal labels come in direct contact with the foods, beverages and drinking water packaged and stored within. Leaching of BPS from those BPS-containing contact sites into the stored comestibles, followed by oral ingestion of BPS-tainted comestibles, leading to their absorption through small intestine is the predominant source of BPS contamination to human population[9-15].

Though, BPS has been introduced and is supposed to be served as a safe BPA-alternative, a number of its characteristics including its affinity for estrogen receptors, dermal penetrability, oral bioavailability and biological half-life exceed the levels of those parameters for BPA[16-18]. Moreover, another adverse feature of BPS is its ability to induce oxidative stress through reactive oxygen species (ROS) generation[19-25]. Furthermore, in our previous study, BPS has been found to alter activities of antioxidant enzymes and the status of biomarkers of oxidative stress in duodenum of rat[26]. Nevertheless, the specific sources leading to oxidative stress induction by BPS remains quite elusive. Xanthine oxidoreductase (XOR) system, present in high quantities in small intestine, serves as a well known ROS generating source in mammals[27]. On the contrary, oral ingestion followed by absorption through small intestine being the predominant source of BPS contamination, undoubtedly exposes the organ to the risk of BPS exposure[9]. However, the effect of BPS on XOR system mediated oxidative stress induction is not reported in literature. Therefore, the aim of the present study is to examine the effect of BPS on XOR system in small intestine of male albino rats *ex vivo*.

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals were used in this study. BPS (Purity 98%, CAS No. 80-09-1) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, USA, and Merck Life Science Private Limited, Mumbai, India, respectively.

Animals

Sprague-Dawley strain of adult male albino rats, weighing around 120-140 grams was used to conduct this study. Animals were maintained in the animal house at 25-27°C temperature with approximately 12h light-dark cycle. They were provided with the access to standard laboratory diet

and water *ad libitum*. Recommended guidelines of Institutional Animal Ethics Committee were followed for performing experimentations. Animal sacrifice was performed by cervical dislocation after keeping them at fasting condition for overnight.

Tissue collection

Small intestine of the sacrificed animals was isolated after opening the abdominal cavity followed by separation of the muscularis layer to obtain visceral smooth muscle (VSM) cells used for either homogenate preparation or mitochondria isolation for performing the experiments[28].

Homogenate preparation

The VSM homogenate (10% w/v) was prepared utilizing a homogenizer (Remi Elektrotechnik Limited, Vasai, India) at cold condition using 50mM phosphate buffer (pH 7.4) as the homogenization medium except for the protein carbonyl assay where 0.9% NaCl was used and during isolation of mitochondria where mitochondrial isolation buffer (pH 7.8) was used.

Mitochondria isolation

Mitochondria were isolated from VSM of small intestine by applying differential centrifugation technique[29].

Preparation of different doses of BPS

DMSO was used as the vehicle to dissolve BPS. Four different doses of BPS dissolved in 10% DMSO solution, viz., 100µM, 200µM, 400µM and 800µM were used in this study. Dose selection was performed on the basis of our previous study[26].

Group division

A total of six groups including the control group, the vehicle control group and four BPS-exposure groups, were established in this study. The control group was devoid of BPS or DMSO exposure whereas the vehicle control group was administered with 10% DMSO solution. BPS-exposure groups were administered with 100µM, 200µM, 400µM and 800µM doses of BPS in 10% DMSO solution and were named as BPS100, BPS200, BPS400 and BPS800 groups respectively.

Incubation of homogenate and mitochondria

The samples of VSM homogenate and mitochondria were separately divided into six groups in equal proportions and were incubated for 1h at 37°C in phosphate buffer (50mM, pH 7.4) after adding BPS or DMSO according to the desired concentrations as described in 'group division' section.

Measurement of protein carbonyl content

Protein carbonyl content was measured by following the standard method[30,31]. 3.2ml of 10mM 2,4-dinitrophenylhydrazine (DNPH) solution dissolved in 2(N) HCl was added into 0.8ml incubated VSM homogenate and were kept in dark for 1 hour at room temperature. Then 4ml of 20% cold tri-chloro acetic acid (TCA) was added and the mixtures were kept at ice cold condition for 10 minutes prior to centrifugation at 3000rpm for 5 minutes. This step was again repeated with 10% TCA solution. The excess DNPH was removed by thoroughly washing the pellets multiple times in ethanol-ethyl acetate (1:1 v/v) solution. Into the washed pellets, 1.6ml of 6M guanidine hydrochloride, dissolved in 50mM potassium phosphate buffer (pH 2.5), was added, mixed thoroughly and kept at 37°C for 10 minutes. The preparations were finally centrifuged at 3000rpm for 10 minutes to collect the supernatant for measurement of absorbance at 370nm using a UV-VIS spectrophotometer (BIO-RAD, USA) against the sample blank tube, where 3.2ml of 2(N) HCl was added instead of DNPH solution. To compensate for the loss of protein present in the pellets during each step of washing, protein content in the supernatants was estimated by measuring absorbance at 280nm using a UV-VIS spectrophotometer and was determined from bovine serum albumin (BSA) standard curve prepared from measuring absorbance of known concentrations of BSA at 280nm.

Measurement of the activities of xanthine oxidoreductase system

Incubated VSM homogenates of six different groups were separately centrifuged at 2000rpm for 10mins in cold, followed by collection of the supernatant that was again centrifuged at 10,000rpm for 40mins in cold to collect the final supernatant for estimating cytosolic xanthine oxidoreductase (XOR) activity. Xanthine oxidase (XO) activity was measured spectrophotometrically at 295nm and expressed as mUnits/min/mg of protein[32]. A final volume of 1.0ml assay mixture contained 50mM phosphate buffer (pH 7.8) containing 0.1mM xanthine and suitable quantity of incubated mitochondria or supernatant obtained from incubated VSM homogenate as the source of enzyme for determining the mitochondrial and cytosolic XO activities respectively. Xanthine dehydrogenase (XDH) activity was measured spectrophotometrically at 340nm through NAD^+ reduction and expressed as mUnits/min/mg of protein[33]. A final volume of 1.0ml assay mixture contained 50mM phosphate buffer (pH 7.5) containing 0.3mM xanthine, 0.75mM NAD^+, 0.05mM sodium azide and suitable quantity of supernatant obtained from incubated VSM homogenate as the source of enzyme. XO+XDH activity was calculated as the cumulative activities of XO and XDH. XO/XDH and XO/XO+XDH ratios were calculated through dividing the activity of XO by XDH and by XO+XDH respectively.

Determination of nitrite concentration

Nitrite concentration was determined spectrophotometrically at 548nm by following the standard method[34,35]. 0.1ml of Griess reagent containing 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl-ethylenediamine dihydrochloride in 1:1 ratio was added into 0.3ml of incubated sample containing VSM homogenate or mitochondria followed by addition of 2.6ml of deionized water and were kept in dark for 30mins

at room temperature. Dilution of samples were performed to get nitrite concentration in the range of 5-20µM. Absorbance of the mixture was measured spectrophotometrically at 548nm against the blank. Nitrite concentration was determined from the standard curve prepared from measuring absorbance of known concentrations of nitrite generated from aqueous solution of sodium nitrite at 548nm.

Determination of protein content

Protein contents of VSM homogenate and mitochondrial samples were estimated by following the standard method[36].

Statistical analysis

Statistical analyses were performed on Microcal Origin (version 7.0 for Windows) software. Data were represented as mean ± standard error of the mean (SEM) after performing Tukey's test of one way analysis of variances (ANOVA) to analyze significance difference between mean values of different groups.

RESULTS

Protein carbonylation

BPS exposure has been found to significantly ($P < 0.001$) increase cytosolic protein carbonyl content in a dose dependent manner in VSM of small intestine of rat *ex vivo* in BPS exposure groups compared to the control and vehicle control group (Figure 1).

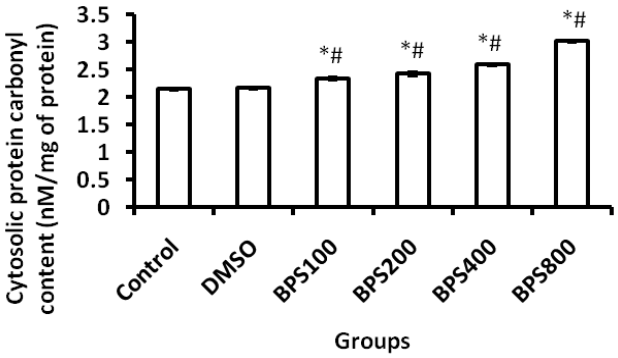

Figure 1: Graphical representation of changes in cytosolic protein carbonyl content in VSM of small intestine of rat is shown. Control represents the control group, not exposed to BPS and DMSO; DMSO represents the vehicle control group exposed to 10% DMSO solution; BPS100, BPS200, BPS400 and BPS800 represent the BPS exposure groups exposed to respectively 100µM, 200µM, 400µM and 800µM doses of BPS dissolved in 10% DMSO solution. Results are expressed as mean ± standard error of the mean (n=6); at the significance level *P < 0.001 vs. control and #P < 0.001 vs. vehicle control using ANOVA.

Activities of xanthine oxidoreductase system

Exposure to BPS has been found to significantly ($P < 0.001$) increase the activities of cytosolic and mitochondrial XO, cytosolic XDH, cytosolic XO+XDH; along with the cytosolic XO/XDH ratio and cytosolic XO/(XO+XDH) ratio in a dose dependent manner in VSM of small intestine of rat *ex vivo* in BPS exposure groups in comparison to the control and vehicle control group (Figure 2).

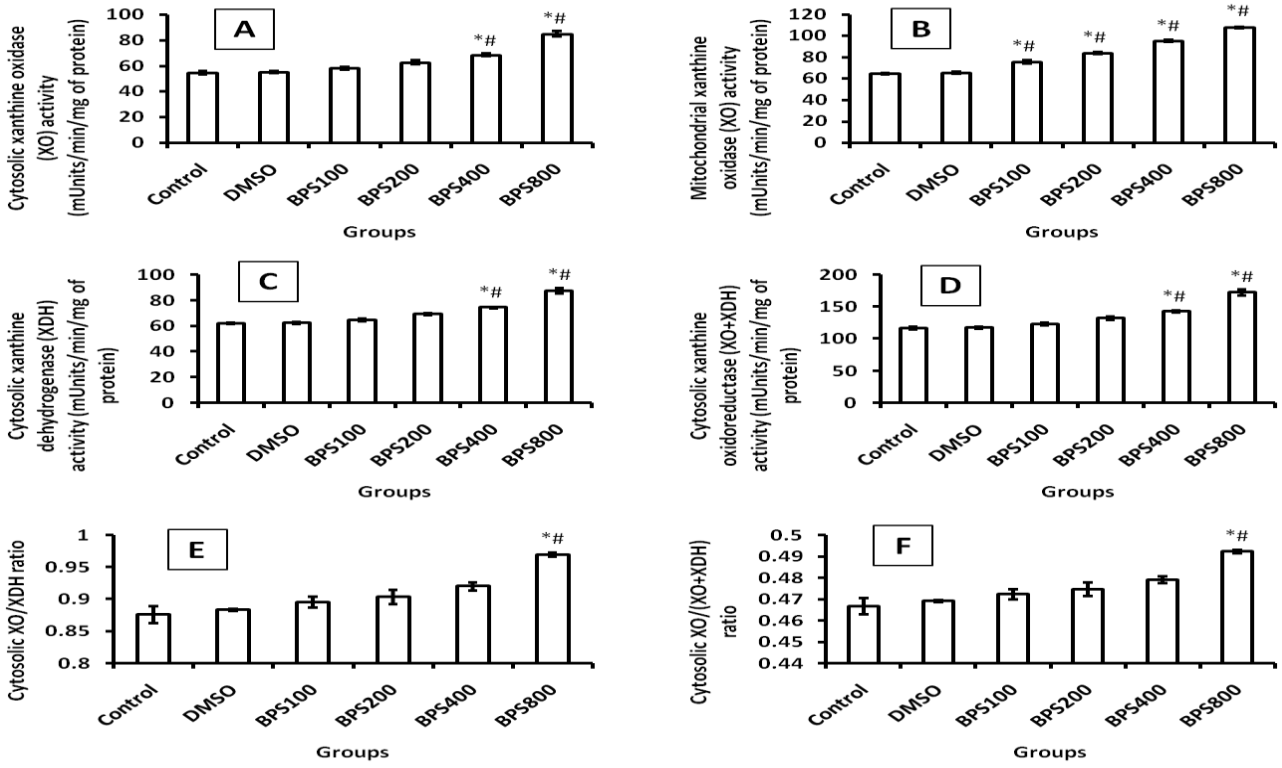

Figure 2: Graphical representations of changes in the activities of xanthine oxidoreductase system in VSM of small intestine of rat. Activity levels of cytosolic xanthine oxidase (XO) (A), mitochondrial xanthine oxidase (XO) (B), cytosolic xanthine

dehydrogenase (XDH) (C), cytosolic xanthine oxidoreductase (XO+XDH) (D); and ratios of cytosolic XO/XDH (E) and cytosolic XO/(XO+XDH) (F) are shown. Control represents the control group, not exposed to BPS and DMSO; DMSO represents the vehicle control group exposed to 10% DMSO solution; BPS100, BPS200, BPS400 and BPS800 represent the BPS exposure groups exposed to respectively 100µM, 200µM, 400µM and 800µM doses of BPS dissolved in 10% DMSO solution. Results are expressed as mean ± standard error of the mean (n=6); at the significance level *P < 0.001 vs. control and #P < 0.001 vs. vehicle control using ANOVA.

Nitrite concentration

Cytosolic and mitochondrial nitrite concentrations have been found to be significantly (P < 0.001) decreased following BPS exposure in a dose dependent manner in VSM of small intestine of rat *ex vivo* in BPS exposure groups in comparison to the control and vehicle control group (Figure 3).

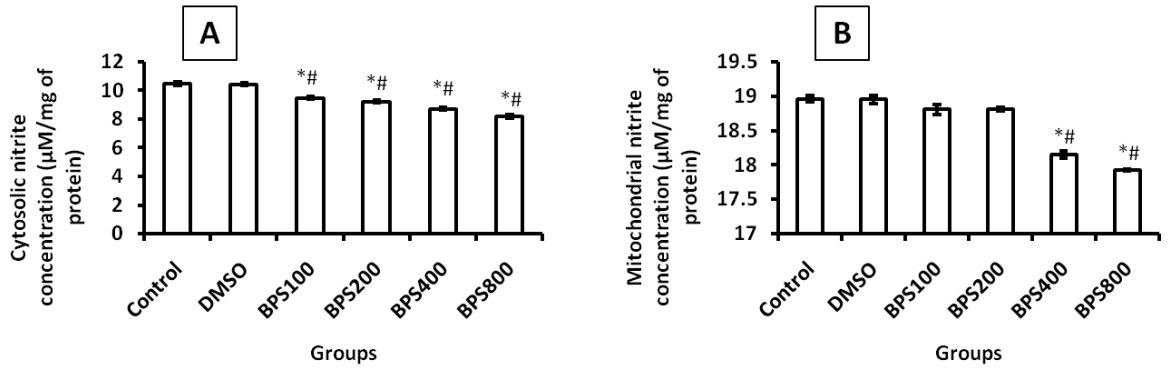

Figure 3: Graphical representations of changes in nitrite concentration in VSM of small intestine of rat. Concentrations of cytosolic (A) and mitochondrial (B) nitrite are shown. Control represents the control group, not exposed to BPS and DMSO; DMSO represents the vehicle control group exposed to 10% DMSO solution; BPS100, BPS200, BPS400 and BPS800 represent the BPS exposure groups exposed to respectively 100µM, 200µM, 400µM and 800µM doses of BPS dissolved in 10% DMSO solution. Results are expressed as mean ± standard error of the mean (n=6); at the significance level *P < 0.001 vs. control and #P < 0.001 vs. vehicle control using ANOVA.

DISCUSSION

Carbonylation of proteins is a non-enzymatic process that occurs due to irreversible oxidative modification to protein molecules[37]. We have observed BPS-induced enhancement in cytosolic protein carbonyl content in this study. This observation corroborates with previous reports of BPS-induced augmentation in protein carbonylation in rat liver, zebrafish brain, human granulosa KGN cells, and HepG2 cells[38,39,20,22]. Increased level of protein carbonylation might hamper inherent functioning of various intracellular and membrane proteins even up to the level of partial or complete inactivation[40]. Through protein carbonylation, reactive carbonyl moieties including aldehydes, ketones and lactams are introduced within a protein that mediates alterations of polypeptide chain conformation[37,41]. Analysis of the mechanism of protein carbonylation suggests that oxidative induction caused by ROS is the principal causative factor. The association between increased ROS formation and enhanced protein carbonylation is so strong that presence of protein carbonyl content is regarded as a devoted biomarker for oxidative stress generation[41]. Oxidation of the side chains of certain amino acids or cleaving of peptide bonds in protein structure by the highly reactive and harmful form of ROS, hydroxyl radical ($HO^{\bullet}$), are the principal sources for yielding reactive carbonyl derivatives[37,41]. Specifically, arginine, lysine, threonine, proline and tryptophan are the major amino acids where carbonylation to their side chain preferentially occurs[41].

One of the predominant sources of $HO^{\bullet}$ generation exists as an indirect outcome of the activity of XOR system. As presence of this enzyme system in intestine is very prominent, it is considered to notably contribute towards intestinal ROS production[27,42]. This system consists of molybdenum and flavin containing protein structure that shows both the XDH and XO activities performed by reversible inter-conversion between these two forms through oxido-reduction of cysteine residues[43]. Moreover, XDH can also be permanently converted to XO by protease mediated irreversible partial proteolysis[42-46]. Reduction of the cofactor NAD^{+} into NADH is performed solely by the XDH form during uric acid generation from purines[47]. Though, both the forms possess capability for reducing molecular oxygen (O_2) into different forms of ROS, the effectiveness of XO is notably greater than that of XDH in this context[42]. Superoxide anions ($O_2^{\bullet-}$) are produced during generation of uric acid from the oxidation of purines catalyzed by XO activity using O_2 as the cofactor[44,47]. The XO pathway has also been reported to be involved in $O_2^{\bullet-}$ generation in rat skeletal muscle[48]. Reduction by one and two electron transfer to O_2 respectively generates $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) by XO[43,49]. Reduction of O_2 by transfer of three electrons generates $HO^{\bullet}$[49]. Moreover, generated H_2O_2 further participates in formation of additional quantity of $HO^{\bullet}$ through Haber-Weiss reaction in presence of ferric ion (Fe^{3+})[44,45]. The iron storing protein, ferritin, present in mitochondria is capable of releasing Fe^{3+}

that might aid in HO• generation[44]. In addition to the cytosolic compartment, XO is also found in mitochondria[44,50-55]. Mitochondrial XO performs generation of $O_2^{\bullet-}$ and H_2O_2 leading to mitochondrial ROS generation alongside the ROS production by mitochondrial electron transport chain[44,56-58].

BPS has been found to increase XDH and XO activities in cytosolic fraction, as well as to increase mitochondrial XO activity in this study. Furthermore, cytosolic XO+XDH activity, XO/XDH ratio, and XO/XO+XDH ratio have also been elevated following BPS exposure in this study. The increase in XO+XDH activity indicates an overall measure of induction of XOR system. Whereas, increased XO/XDH and XO/XO+XDH ratios demonstrate shift of the XOR system towards XO activity. It is noteworthy to mention that in healthy tissues, XDH is the dominating form of XOR system[44]. In contrary, prevalence of XO activity is associated with oxidative tissue injury[52]. Altogether, these observations strongly support the notion of ROS induction by BPS through involving the XOR system. Hence, it can be speculated that one of the routes of BPS-induced ROS generation occurs through the enhanced XOR activity mediated formation of ROS entities, which itself is triggered by BPS exposure.

Furthermore, XOR also contains nitric oxide (NO) generating domain in its protein structure that leads to its inherent ability to direct NO formation[42]. The formation of NO is mediated through the nitrate reductase and nitrite reductase activities of XOR, reducing nitrate into nitrite and nitrite into NO respectively[43]. We have found that BPS exposure suppressed cytosolic and mitochondrial nitrite concentrations in this study. Consequently, NO generation is also expected to be altered by BPS exposure. This result corroborates with the previous report claiming about the NO suppressing characteristic of BPS[59]. However, in presence of $O_2^{\bullet-}$ the generated NO reacts with the radical instantaneously, even before $O_2^{\bullet-}$ gets the chance to be dismutated by superoxide dismutase[60]. Peroxynitrite ($ONOO^-$), which is a highly reactive pro-oxidant compound reported to induce protein carbonylation, is generated from the reaction of NO with $O_2^{\bullet-}$ at diffusion controlled rate[43,60-62]. Therefore, the ROS arising from XO activity, as well as from other sources, is speculated to contribute towards the BPS exposure-induced decreased NO availability[59].

CONCLUSION

From the results of this study it is concluded that BPS modulates the activities of xanthine oxidoreductase system to generate reactive oxygen species that might be involved in oxidative modification towards proteins to increase protein carbonylation as well as to diminish nitrite concentrations in the visceral smooth muscle of small intestine of rats *ex vivo*. This study reports the effects of BPS on xanthine oxidoreductase activities for the first time as no previous mention regarding this topic is reported in the literature. Future scope for further studies remains to evaluate the detailed mechanism of action of BPS on specific categories of reactive oxygen species generation involving this enzyme system.

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