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Salicylic acid modulates oxidative stress and glutathione peroxidase activity in the rat colon ☆

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Abstract

Oxidative stress is a characteristic of cancerous colon tissue and inflammatory bowel diseases that increase colon cancer risk. Epidemiological evidence supports a protective effect of plant-derived compounds. Aspirin is also protective against colon cancer. The mechanism of action is unclear although salicylic acid, the main metabolite of aspirin, has been shown to decrease the synthesis of pro-inflammatory and potentially neo-plastic prostaglandins. Salicylic acid is found in significant quantities in a plant-based diet. However, in plants salicylic acid is also reported to modulate the expression of numerous enzymes with antioxidant activity. The aim of this study was to assess whether salicylic acid can modulate pro-cancerous biological pathways in the colon. Oxidative stress, prostaglandins and cytosolic glutathione peroxidase (cyGPX) were analysed in proximal, transverse and distal colon from a rat model of diet-induced oxidative stress. Elevated plasma pyruvate kinase activity (1293 ± 206 U/ml) and increased indices of lipid peroxidation in colon (proximal 6.4 ± 0.84 nM MDA/mg protein; transverse 6.9 ± 0.97 nM MDA/mg protein; distal 5.2 ± 0.62 nM MDA/mg protein) from rats fed a Vitamin E deficient diet were significantly decreased on supplementation with salicylic acid (plasma pyruvate 546 ± 43 U/ml; salicylic acid proximal 3.6 ± 0.39 nM MDA/mg protein; transverse 4.5 ± 0.61 nM MDA/mg protein; distal 4.4 ± 0.27 nM MDA/mg protein). Reductions in oxidative stress and prostaglandin production on supplementation with salicylic acid were associated with an elevation in glutathione peroxidase activity (Vitamin E deficient proximal 0.056 ± 0.013 U/mg protein; transverse 0.073 ± 0.008 U/mg protein; distal 0.088 ± 0.010 U/mg protein; Vitamin E deficient with salicylic acid proximal 0.17 ± 0.01 U/mg protein; transverse 0.23 ± 0.016 U/mg protein; distal 0.16 ± 0.020 U/mg protein). Gpx1 and Gpx2 gene transcripts were not elevated in association with increased activity of the soluble glutathione peroxidase activity. Glutathione peroxidases are key antioxidant enzymes, catalysing the decomposition of potentially toxic lipid peroxides. Gpx activity and regulation of Gpx gene transcription has been shown previously to be complex with activity not necessarily mirrored by a corresponding elevation in gene transcription. By supplementing the diet of Vitamin E deficient rats with salicylic acid (1 g/kg diet), this study assessed effects of salicylic acid on cytosolic glutathione peroxidase activity in the colon. The ability of salicylic acid to modulate antioxidant enzymes in colon tissue may be an important mechanism in inhibiting colon cancer development.

Keywords: Glutathione peroxidase; Colon cancer; Vitamin E; Prostaglandins

1. Introduction

In the past, salicylic acid, prepared from plant extracts has been used to provide pain relief and treat inflammatory conditions and fevers. Salicylic acid functions in plants as a hormonal mediator of the systemic acquired resistance responses to pathogen attack, environmental stress and oxidative stress [1–3]. Consequently, salicylic acid occurs in a wide range of fruits, vegetables, herbs and spices of dietary relevance. Aspirin is a synthetic analogue structurally related to the salicylate family of compounds, salicylic acid being its principal metabolite. After absorption from the stomach and small intestine, aspirin is rapidly hydrolysed to salicylic acid (2-hydroxybenzoic acid) in the

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liver and blood where it is tightly bound to plasma proteins and distributed to all tissues in the body. Consequently, some of the anti-inflammatory and anti-carcinogenic effects of aspirin are potentially due to salicylic acid. Since salicylic acid is common to both aspirin and fruits and vegetables, and both are known to protect against colon pathologies, including cancer, it is possible that this phenolic acid may be responsible for some of the beneficial effects of a plant-based diet on colon health [4–6]. Swain et al. [7] estimate potential intakes of between 10 and 200 mg/day.

Glutathione peroxidases play a role in regulation of COX-2 and formation of prostaglandins via the arachidonic acid cascade [8]. This is significant since the protective effects of salicylic acid and some non-steroidal anti-inflammatory drugs have also been linked to COX-2 dependent regulation of inflammatory processes and arachidonic acid metabolism in colon tissue [9,10]. However, the mechanism(s) for the beneficial effects are not fully understood and may not be entirely dependent on COX-2 targets [11]. Glutathione peroxidases are a family of enzymes that mediate the elimination of hydro-peroxides in the gut [12,13]. Oxidative stress in the colonic environment, induced by Vitamin E deficiency leads to a decrease in glutathione peroxidase activity (unpublished observations). Glutathione peroxidase activity has also been linked to protection from colon cancer [14,15]. The importance of the gut specific glutathione peroxidase, GPX-2, is emphasised as it is one of the few selenoprotein mRNA transcripts that is elevated in selenium deficiency [16,17]. Controversy exists as to whether the gut Gpx1 and Gpx2 enzymes are functionally redundant or whether they may be complementary. Although different sites of gene expression were observed in the small intestine, suggesting complementary functions, the lack of a phenotype in unchallagened homozygous Gpx1-KO and Gpx2-KO mice supported redundancy [18]. The lack of detailed study of Gpx1 and Gpx2 expression and activity in the colon, with investigations focusing on the small intestine necessitate cautious interpretation of investigation in the colon. Elucidation of the physiological roles of these isozymes will be aided by detailed mapping of Gpx1 and Gpx2 protein and corresponding gene transcripts in the colon.

Indices of lipid peroxidation are increased in association with many colon disorders such as ulcerative colitis, inflammatory bowel syndrome and colon cancer [19]. Low Vitamin E status was shown to generate analogous increased oxidative stress in colon tissue with associated perturbations in prostaglandin metabolism and glutathione peroxidase activity (unpublished observations). Based on these observations this study was conducted using the rat model of diet induced oxidative stress to assess modulation of these parameters in the colonic environment by the dietary anti-inflammatory compound salicylic acid.

2. Materials and methods

2.1. Animals and diets

Weanling male rats of the Rowett Hooded Lister strain ($n = 8$) were offered a semisynthetic diet as described previously [20] for 12 weeks ad libitum. The diet was prepared in-house and was composed of sucrose, 71.8 g; lard, 3.5 g; Vitamin E depleted cod liver oil, 1.5 g; amino acid mixture, 18.0 g; and a vitamin/mineral/trace-metal mixture, 5.2 g) [20]. This was either sufficient in Vitamin E, 1 g Vitamin E/kg diet (as dα-tocopherol acetate, Sigma) (+E diet), deficient in vitamin E, less than 0.5 mg Vitamin E/kg (−E diet), or deficient in Vitamin E with less than 0.5 mg Vitamin E, but supplemented with 1 g salicylic acid/kg (−E + SA diet). Salicylic acid (Sigma, UK) was mixed in powdered form with the diet. The diet formulations were stored frozen (−20 °C) prior to use. Body weights were recorded daily over the course of the experiment. After 12 weeks on the diet the rats were anaesthetised with isofluorane and blood removed by cardiac puncture into a pre-heparinised syringe (Becton Dickinson, Oxford, UK). Plasma was obtained by centrifugation ($2500 \times g$, 10 min, 4 °C). Livers were perfused in situ with chilled 0.15 M KCl. Plasma and excised liver were immediately snap frozen in liquid nitrogen and stored at −70 °C. Colons were excised from the caecum at the ileo-caecal valve and rinsed with ice cold PBS to remove contents. Colon length measurements were recorded and proximal (ascending), transverse and distal (descending) segments were snap frozen separately.

2.2. Measurement of indices of oxidative stress

Vitamin E concentrations in plasma, liver and colon ($n = 8$) were determined by reverse phase HPLC with fluorimetric detection [21]. Methodology was validated by participation in the US National Institute of Standards Quality Assurance Scheme for fat-soluble vitamins (Lab 145, Ranking category 1 in 2003). Products of lipid peroxidation were estimated in tissue homogenates as thiobarbituric acid reactive substances (TBARS) using HPLC and fluorimetric detection as previously described [22]. Plasma pyruvate kinase activity, a marker of cell membrane damage, was determined by test kit (Boehringer Mannheim, Sussex, UK).

2.3. Measurement of prostaglandin E_2 derivatives

A Prostaglandin E metabolite EIA kit (Cayman Chemical Company) was used to assay PGE_2 metabolites according to the manufacturer's instructions. Colon segments ($n = 3$) were homogenised in Tris-buffer (20 mM Tris–HCl, pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1 mM Na_3VO_4, leupeptin (4 μg/ml), toluene sulphonyl fluoride (60 μg/ml) and triton X-100 (0.1%, v/v). Homogenates

were derivatised immediately according to the manufacturer's instructions using the carbonate buffer supplied with the kit. The stable derivatives of PGE_2 metabolites formed were then quantified by EIA.

2.4. Glutathione peroxidase activity

Glutathione peroxidase activity in colon segments ($n = 8$) was determined using a glutathione reductase coupled assay with 0.25 mM H_2O_2 and 5 mM glutathione as substrates [23]. A unit of cyGPX is defined as that which oxidises 1 μmole of NADPH per min.

2.5. RNA extraction

RNA was extracted from 1 cm colon segments adjacent to that used for micro-anatomical analysis using an RNeasy Midi Kit (Qiagen, Crawley, UK). All of the extracted RNA samples were subjected to analysis using the Agilent Bioanalyser (Agilent Technologies, Bracknell, UK).

2.6. Semi-quantitative PCR of Gpx1 and Gpx2

First strand cDNA was synthesised from total RNA (0.5 μg) using Superscript II reverse transcriptase (Gibco BRL, Paisley, Scotland) according to the manufacturer's instructions. Semi-quantitative PCR was performed using GAPDH primers (5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′) as an internal reference (95 °C for 1 min, 59 °C for 40 s, 72 °C for 1.5 min). Specific primer pairs for *Gpx1* (5′-aggcaccacgacccgggactacac-3′; 5′-ggacaccggggaccaaatgatg-3′) and *Gpx2* (5′-tcggcctggatggggagaaga-3′; 5′-ttgggatcggtcatgagggagaat-3′) were designed to amplify across intronic regions (95 °C for 40 s, 60 °C for 40 s, 72 °C for 1.5 min). Hot start PCR was performed using 50 pmol primers, 1U Taq (Bioline, UK), 250 μM dNTPs and 1.6 mM $MgCl_2$. PCR amplicons were quantitated on ethidium bromide stained agarose gels and normalised to expression of the housekeeping gene GAPDH using UVIband image analysis software (UVItec, Cambridge, UK).

3. Results

3.1. Measurement of indices of oxidative stress

Consumption of the Vitamin E deficient diet with or without supplementation with salicylic acid for 12 weeks did not affect final weights of the rats compared with those consuming the diet sufficient in Vitamin E (Table 1) and there were no overt signs such as changes in coat condition or significant differences in colon length measurements. However, a marked Vitamin E deficiency was apparent, since concentrations of α-tocopherol in plasma, liver and colon of rats consuming both the Vitamin E deficient or Vitamin E deficient supplemented with salicylic acid diets were undetectable or significantly decreased compared with Vitamin E supplemented controls. Evidence of increased oxidative stress induced by Vitamin E deficiency was confirmed by increased cell membrane damage as indicated by enhanced plasma pyruvate kinase activity and increased indices of lipid peroxidation (TBARS) in plasma, liver (Table 1) and colon (Fig. 1(A)) when compared to Vitamin E sufficient rats. The elevation of plasma pyruvate kinase activity in plasma and increased indices of lipid peroxidation in colon, but not the plasma were significantly decreased on supplementation of Vitamin E deficient rats with salicylic acid (Fig. 1(A) and Table 1).

Table 1
Indices of Vitamin E status and lipid peroxidation in rats maintained on Vitamin E-sufficient (+E), -deficient with salicylic acid (−E + SA), or -deficient (−E) rations

Parameter	+E	−E + SA	−E
Weight (g)	407 ± 13	402 ± 10	396 ± 15
Plasma pyruvate kinase (U/ml)	69 ± 5[a]	546 ± 43[a]	1293 ± 206
Plasma TBARS (nM MDA/ml)	2.5 ± 0.3[a]	9.6 ± 1.1	10.5 ± 1.4
Plasma dα-tocopherol (μg/ml)	13.5 ± 1.2	BDL	BDL
Liver dα-tocopherol (μg/mg protein)	464 ± 40[a]	3.2 ± 0.4	2.7 ± 0.4
Colon dα-tocopherol (μg/mg protein)			
Proximal	0.119 ± 0.016	BDL	BDL
Transverse	0.077 ± 0.015	BDL	BDL
Distal	0.065 ± 0.006	BDL	BDL

Values are means ± S.E.M., $n = 8$/group. BDL, below detection limits.
[a] $p < 0.001$ compared with −E group.

3.2. Measurement of prostaglandin E_2 derivatives in colon

Vitamin E deficiency and the associated oxidative stress in the colonic environment were associated with an increase in prostaglandin levels measured as PGE_2 derivatives in all colon regions (Fig. 1) when compared to Vitamin E sufficient rats. Supplementation of rats experiencing diet induced oxidative stress with salicylic acid attenuated the increased levels of indices of oxidative stress and the associated elevation of prostaglandin PGE_2 derivatives (Fig. 1). The attenuation was significant in the transverse and distal colon.

3.3. Glutathione peroxidase activity

A reduction in glutathione peroxidase activity was apparent in rats deficient in dietary Vitamin E and this was significant in the proximal and transverse regions of the colon. The elevation in indices of oxidative stress and PGE_2 derivatives caused by dietary Vitamin E deficiency were attenuated with salicylic acid supplementation (Fig. 1(C)). This is associated with an elevation in glutathione peroxidase activity in the colon of Vitamin E deficient rats supplemented with salicylic acid

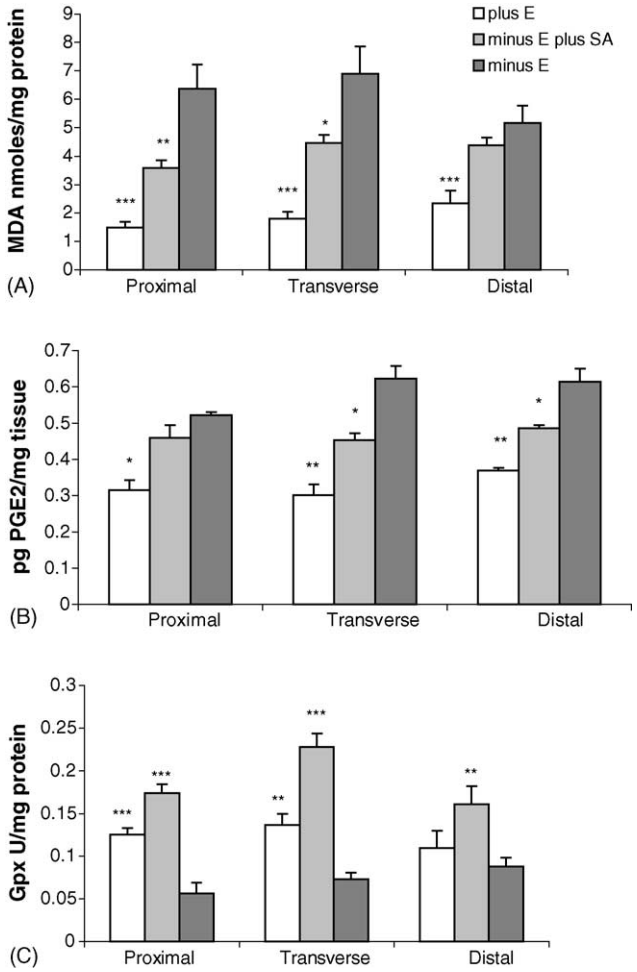

Fig. 1. Indices of (A) lipid peroxidation, (B) PGE2 levels and (C) glutathione peroxidase activity in proximal, transverse and distal colon of rats maintained on Vitamin E-sufficient (plus E), -deficient with salicylic acid (minus E plus salicylic acid), or -deficient (minus E) rations. Results are presented as mean ± S.E. Groups were compared by ANOVA and significant differences relative to diet-induced Vitamin E deficient rats were assessed by post hoc *t*-tests based on the pooled S.E.: * indicates a *p* value of less than 0.05; ** less than 0.01; and *** less than 0.001. A *p* value less than 0.05 are considered significant.

(Fig. 1(C)). The level of glutathione peroxidase activity on supplementation with salicylic acid was greater than that in colons from rats fed adequate levels of the dietary antioxidant, Vitamin E.

3.4. Semi-quantitative PCR of Gpx1 and Gpx2

Semi-quantitative PCR was performed to determine the levels of the cytosolic glutathione peroxidases 1 (*Gpx1*) and 2 (*Gpx2*) since the homogenates used for measurement of total glutathione peroxidase activity contain both soluble glutathione peroxidases (Fig. 2). Semi-quantitative PCR revealed a significant upregulation of *Gpx1* in the distal colon of Vitamin E depleted rats when compared with colon from rats fed sufficient Vitamin E (Fig. 2(C)). There was no significant change in *Gpx1* transcripts in the proximal or transverse colon (Fig. 2(A and B)). No significant change in *Gpx2* transcript levels was observed with Vitamin E deficiency induced oxidative stress. Supplementation of Vitamin E deficient rats with salicylic acid led to a decrease in *Gpx2* transcripts levels in proximal and distal colon (Fig. 2(D and F)). Expression of Gpx2 in transverse and distal colon (Fig. 2(E)) was not significantly altered.

4. Discussion

Vitamin E deficiency resulted in increased oxidative stress in proximal, transverse and distal colon of the rat model with a concomitant increase in pro-inflammtory and carcinogenic biological processes. Evidence of these changes was obtained from measurements of elevated indices of oxidative stress (Fig. 1(A)) and prostaglandin production (Fig. 1(B)). Increased oxidative stress and prostaglandin production were associated with a decrease in glutathione peroxidase activity in rats deficient in Vitamin E (Fig. 1(C)). A reduction in the levels of increased colonic oxidative stress and prostaglandin production was observed when these rats were supplemented with salicylic acid. This may have been a consequence of the associated increase in glutathione peroxidase activity (Fig. 1) on supplementation with the anti-inflammatory phenolic compound, salicylic acid. A similar reduction in oxidative stress as measured by TBARS was not observed in plasma. Notably, Gpx2 is not present in plasma. This would support a role for Gpx2 rather than Gpx1 in the elevated soluble glutathione peroxidase activity in the colon and the associated reduction of lipid peroxidation with salicylic acid supplementation. Conversely, oxidative stress induced by Vitamin E deficiency led to a reduction in glutathione activity. The role of glutathione peroxidase in colon pathologies, such as colon cancer has been the subject of recent investigations [24,25,21]. Lipid peroxidation is associated with abnormal fatty acid metabolism, a factor governing glutathione peroxidase activity and regulation. Changes in colonic glutathione peroxidase activity were not reflected in marked alterations in *Gpx1* and *Gpx2* gene expression. Previous studies in our lab have indicated a complex interplay of glutathione peroxidase activity, fatty acid metabolism and post-transcriptional RNA processing [26]. Hence, there is evidence from previous studies indicating a lack of correlation of glutathione peroxidase activity with gene transcript levels.

There is substantial evidence that both aspirin, and presumably its principal metabolite salicylic acid [5] and diets rich in fruit and vegetables, containing significant levels of salicylic acid [4,6], have colon protective effects. Serum levels of 0.04–2.47, 0.02–0.20 and 0.23–25.40 μmol/l have been measured in subjects consuming vegetarian, non-vegetarian diets and low dose aspirin (75 mg daily), respectively [27]. Since oxidative stress, elevated prostaglandin production and perturbations in glutathione peroxidase activity are associated with many

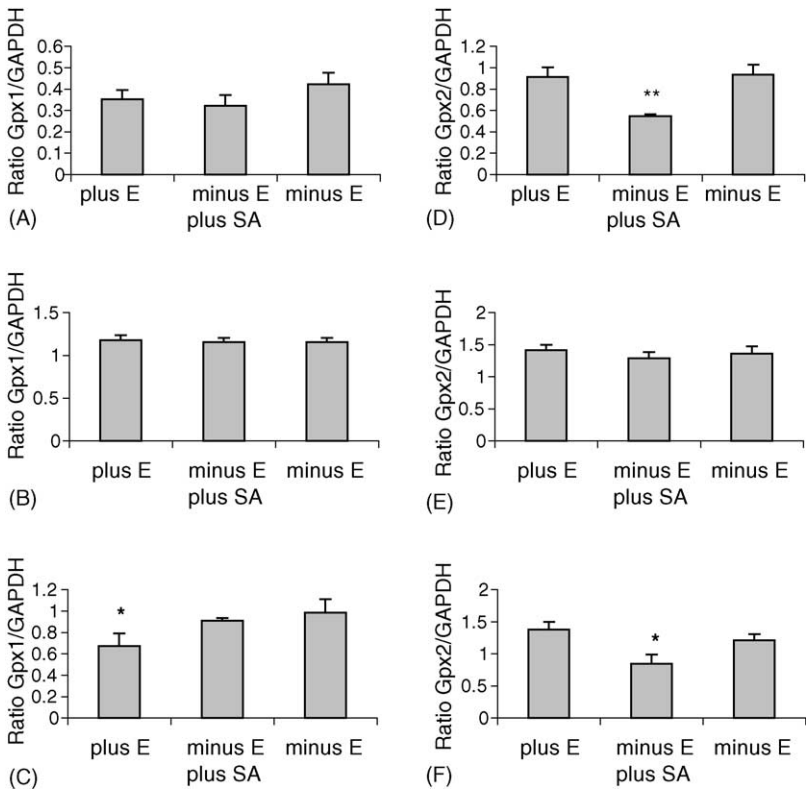

Fig. 2. Semi-quantitative PCR of *Gpx1* and *Gpx2*. Expression normalised to GAPDH for *Gpx1* in proximal (A), transverse (B) and distal (C) and *Gpx2* in proximal (D), transverse (E) and distal (F) colon is shown. The graph is a representative experiment showing the averaged ratio of *Gpx* to GAPDH from five biological replicates ±S.E. Each ratio from the biological replicates was derived from triplicate PCRs. Groups were compared by ANOVA and significant differences were assessed by post hoc *t*-tests based on the pooled S.E.: * indicates a p value of less than 0.05; ** less than 0.01. A p value less than 0.05 are considered significant.

colon pathologies including cancer; the modulation of pro-inflammatory and carcinogenic biological processes by salicylic acid in the colon is highly significant. This study provides evidence of potential mechanisms in vivo for the protective effects of diets rich in salicylates.

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