British Journal of Pharmacology (1999) 128, 1268–1274 © 1999 Stockton Press All rights reserved 0007–1188/99 $15.00
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The involvement of protein kinase C in nitric oxide-induced damage to rat isolated colonic mucosal cells

***,[1]B.L. Tepperman, [1]Q. Chang & [1]B.D. Soper**

[1]Department of Physiology, University of Western Ontario, London Ontario, Canada, N6A 5C1

1 The role of protein kinase C (PKC) in colonic cellular injury in response to high concentrations of nitric oxide (NO) released from the donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP) was investigated.

2 Addition of SNAP (0.1 – 1000 μM) to the cellular suspension resulted in a dose-dependent increase in the extent of damage to isolated colonic mucosal cells as assessed by Trypan blue dye uptake and release of the lysosomal enzyme, N-acetyl-β-glucosaminidase. SNAP treatment also resulted in an increase in cellular total PKC activity. These increases were reduced or eliminated by pretreatment of the cells with the PKC antagonists staurosporine or GF 109203X or the NO scavenger, phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO).

3 PKC-α, PKC-δ, PKC-ε and PKC-ζ were detected in colonic cellular lysates by immunoblotting. However, only PKC-ε protein was increased in response to SNAP treatment. Furthermore, SNAP treatment resulted in activation of PKC-ε by causing translocation of the enzyme from the cytosolic to membrane fraction of the cell. This effect was eliminated if cells were preincubated with the NO scavenger, PTIO.

4 The extent of cellular damage in response to addition of SNAP to the incubation medium was enhanced by coincubation with the PKC activator, phorbol 12-myristate 13-acetate (PMA; 1 and 10 μM).

5 PKC activity and the extent of cell damage in response to SNAP were reduced by preincubation of the cells with the peroxyl scavenger, ebselen (0.01 – 10 μM).

6 These data suggest that the PKC-ε isoform of the enzyme mediates NO-induced damage to colonic mucosal cells. This response may occur, at least in part, due to peroxynitrite formation.

Keywords: Colon; protein kinase C; nitric oxide; cell damage; peroxynitrite; ebselen; PKC isoform; PTIO; phorbol ester

Abbreviations: cGMP, cyclic GMP; DFB, Dulbecco's phosphate buffer; ECL, enhanced chemiluminescence; GFX, GF 109203X; HRP, horseradish peroxidase; NO, nitric oxide; PKC, protein kinase c; PMA, phorbol 12-myristate 13 acetate; PMSF, phenylmethylsulphonyl fluoride, PMSF; PTIO, phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3 oxide; SNAP, S-nitroso-N-acetyl-DL-penicillamine

Introduction

Recent evidence has suggested that generation of high levels of NO can lead to induction of cell death by the processes of necrosis and apoptosis in a number of cell types including adenocarcinoma cells, smooth muscle cells and colonic epithelial cells (Nishio & Watanabe, 1997; O'Connor & Moncada, 1991; Tepperman *et al*., 1994). The mechanism by which this occurs remains undefined although NO is known to interact with various target molecules such as oxygen, thiol groups and metals within the prosthetic groups of various enzymes resulting in activation or inactivation, production of peroxynitrite and lipid peroxidation as well as *via* the potentiation of oxygen radicals. Indeed, oxidant scavengers can ameliorate the cytotoxic actions of NO donors. Furthermore, NO donors have been shown to exacerbate the cytotoxic effect of hydrogen peroxide in gastric mucosal cells (Hata *et al*., 1996).

NO might cause cell injury through the production of cyclic GMP (cGMP). Inhibition of guanylate cyclase activity have been shown to reduce the extent of NO-mediated intestinal epithelial cell damage (Tepperman *et al*., 1998). However, the results of that study also indicated that inhibition of guanylate cyclase activity did not appear to account for the complete cytotoxic actions of NO. Alternatively NO may act through different mechanisms and toxicity may be regulated by the activity of the signal transduction system involving protein kinase C. The evidence for the involvement of such a pathway includes findings that NO can activate PKC activity in a number of cell types (Loweth *et al*., 1997; Maiese *et al*., 1993; Nishio & Watanabe, 1997; Sharma *et al*., 1995). Furthermore, PKC activity has been shown to be elevated in a variety of cell types in response to inflammatory challenges and increases in PKC activity have been associated with cellular injury or death (Brawn *et al*., 1995; Jones *et al*., 1997; Phelps *et al*., 1995). Within the gastrointestinal tract, PKC activity is increased in inflamed segments of the colonic mucosa (Sakanoue *et al*., 1992) and PKC activators such as phorbol ester can reduce the integrity of colonic epithelial cells (Brown *et al*., 1999).

Evidence also suggests that PKC may be one of the pathways activated within cells in response to oxidative stress and that this may be linked to oxidative-mediated cell damage. *In vitro* studies have shown that hydrogen peroxide treatment, which results in the induction of epithelial cellular damage and lipid peroxidation, also increases PKC activity (Brawn *et al*., 1995). This oxidant-induced damage to these cells can be reversed by pretreatment with a PKC inhibitor.

*Author for correspondence: Department of Physiology, Medical Sciences Building, Room M226, University of Western Ontario, London Ontario, Canada, N6A 5C1;
E-mail: btepper@physiology.uwo.ca

Therefore, in the present study, we have examined the role of PKC in the mediation of NO-induced cell injury to rat isolated colonic epithelial cells. We have also investigated the role of NO-induced production of reactive oxidants in this response as well as the specific isoform(s) of PKC which most likely mediate the injurious effects of NO on colonic cells.

Methods

Isolation of colonic epithelial cells

Non-fasted male Sprague Dawley rats (250 – 300 g) were killed by cervical dislocation and epithelial cells were isolated from the colonic mucosa as described by Butler *et al.* (1988). Briefly, the colon was excised, everted and rinsed in ice-cold saline and distended with Dulbecco's phosphate buffer (DFB; pH 7.2; GIBCO, Burlington Ontario, Canada). The colon was incubated for 60 min at 37°C in 0.2% trypsin (type 1A, Sigma, St Louis, U.S.A.) in 50 ml DFB in a water bath that shook at 50 oscillations per min. Epithelial cells were then removed by scraping with a glass slide and were suspended in 5 ml of DFB containing 0.5% bovine serum albumin and 0.5 mM dithiothreitol. Cells were washed twice with DFB by centrifugation at $2000 \times g$ for 2 min, resuspended and dispersed using a Potter-Elverjheim mortar with a Teflon pestle to reduce the number of cell aggregates and crypts. The dispersed cells were filtered through 100 μm polypropylene mesh. The cells were centrifuged again and resuspended in a buffer containing 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol and (in mg ml^{-1}) 0.01 soybean trypsin inhibitor, 0.01 leupeptin and 0.002 aprotinin (pH 7.4).

Treatments

Cells harvested from the rat colon were incubated (total incubation volume, 1 ml) with the NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP, 1 – 1000 μM; Sigma, St. Louis, U.S.A). The cells were incubated in the presence of SNAP for 20 min at 37°C under 95% O_2, 5% CO_2. All agents were added to the cells in volumes of 10 μl or less. In some experiments, SNAP was incubated in the absence of cells at 37°C for 1 h. This procedure caused the inactivation of the SNAP by the release of NO. After this time, the inactivated SNAP was added to the cell suspension. Control cells were incubated with the vehicle for SNAP (100% ethanol, 10 μl). In some experiments, cells were co-incubated with SNAP and one of the following: the protein kinase C inhibitors, staurosporine (10 μM, Biomol, Plymouth Meeting, PA, U.S.A.), bisindolymaleimide I (GF 109302X; 10 μM, Biomol), the nitric oxide scavenger phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO, 1 mM; Sigma) or the peroxynitrite scavenger 2-phenyl-1,2-benzisoselenazol-3-(2H)-one (ebselen, 0.01 – 10 μM, Alexis Biochemicals, San Diego, CA, U.S.A.). In some experiments, SNAP in the concentration range of 1 – 1000 μM was co-incubated with phorbol 12-myristate 13-acetate (PMA, 1 and 10 μM, Precision Biochemicals, Vancouver, Canada).

Determination of cell viability

Trypan blue dye uptake More than 90% of the cells harvested from each colonic segment were identified as epithelial cells by light microscopy. In all experiments, an aliquot of cells was examined for viability as determined by Trypan blue dye uptake (0.5% Trypan blue in phosphate-buffered saline) which has previously been shown to be a reliable index of gastrointestinal epithelial cell injury (Tepperman *et al*., 1991). Cells were counted in a random manner by a naïve observer using a hemocytometer and the number of non-viable cells was determined by light microscopy ($\times 200$ magnification) by counting those cells that took up the dye and a proportion of the total number of cells within the field.

N-Acetyl-β-glucosaminidase The appearance of this lysosomal enzyme in the supernatant was taken to indicate cell lysis. Enzyme activity was determined by incubating 100 μl of supernatant with 100 μl of 0.1 M citrate-phosphate buffer (pH 4.5) containing 1.5 mg ml^{-1} p-nitrophenyl-N-acetyl-β-glucosaminide for 2 h at 37°C. The reaction was terminated with 100 μl of 1 M NaOH-glycine and the absorbance was measured at 405 nm. A unit of enzyme activity was defined as that activity which liberates 1 μmol of p-nitrophenol from the substrate per h at 37°C.

To determine the total enzyme content, the cells in an aliquot (1 ml) of the incubation suspension were lysed by rapid freezing on dry ice followed by re-warming to 37°C (three times). The cell debris was removed by centrifugation ($10,000 \times g$, 1 min) and the maximal enzyme release into the supernatant was determined as described above. Enzyme release was expressed as a percentage of the total enzyme content.

Measurement of protein kinase C activity

Cells were centrifuged at $2000 \times g$ for 10 min (4°C) and were then resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing EDTA (10 mM), phenylmethylsulphonyl fluoride (PMSF; 50 μg ml^{-1}), benzamide (10 mM), soybean trypsin inhibitor (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}), aprotinin (10 μg ml^{-1}), β-mercaptoethanol (0.3% w v^{-1}) and okadaic acid (10 nM). The cells were lysed by sonification for 10 s. A 25 μl aliquot of the sonicate was removed for determination of PKC activity using a commercially available kit (Amersham) which measures the transfer of [γ-^{32}P]-ATP to a peptide specific for PKC. Results are expressed as pmol min^{-1} 10^6 cells^{-1}.

Measurement of PKC protein content

Materials Affinity-purified rabbit polyclonal anti-PKC-α, anti-PKC-δ, anti-PKC-ε and anti-PKC-ζ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The secondary antibody was a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) purchased from Amersham. Rainbow electrophoresis molecular weight marker, the enhanced chemiluminescence (ECL) kit, Hybond ECL nitrocellulose membrane and Hyperfilm ECL were also purchased from Amersham.

Preparation of cytosolic and particulate fractions Cell samples were resuspended in buffer and sonicated for 15 s on ice. The buffer consisted of 50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 1 mM EGTA, 25 μg ml^{-1} each of aprotinin, leupeptin and pepstatin, 1 μg ml^{-1} soybean trypsin inhibitor, 50 μg ml^{-1} PMSF and 10 mM β-mercaptoethanol. The samples were centrifuged at $100,000 \times g$ for 60 min. The supernatant was taken as the cytosolic fraction. The pellet was resuspened in the buffer described above to which was added 10% Triton X-100 and centrifuged at $25,000 \times g$ for 20 min. The protein concentration of each sample was subsequently determined.

Immunoblotting of cellular samples Each sample of 10 – 15 μg protein was boiled for 10 min in an equal volume of SDS sample buffer (125 mM, pH 6.8, 20% glycerol and 10%

mercaptoethanol) before subjecting to 10% SDS–PAGE gel. After electrophoresis, the gel was soaked for 30 min in transfer buffer and electroblotted onto nitrocellulose membranes using Mini Trans-blot. The membranes were blocked for 1 h with 10% nonfat dry milk in phosphate-buffered saline (80 mM Na_2PO_4, 20 mM NaH_2PO_4, 10 mM NaCl and 0.05% Tween-20 (pH 7.5). The blots were then incubated for 3 h with specific PKC-α antibody (1 : 1000). PKC-δ antibody (1 : 800), PKC-ε antibody (1 : 800) or PKC-ζ antibody (1 : 800) at room temperature. Following washes with PBS (three times for 10 min), a 1 : 5000 dilution of HRP-linked secondary antibody was added for 2 h at room temperature. The ECL kit was used to visualize the immunoreactive bands according to the manufacturer's protocol. The density of the immunoreactive bands on the autoradiogram was quantified by measurement of the absolute integrated optical density (IOD) which estimates the volume of the band in the lane profile as calculated by Image Master VDS software (Pharmacia Biotech).

Statistical calculations

The statistical significance of the differences within groups was determined using analysis of variance and Duncan's multiple range test. Statistical differences between groups treated with different agents was determined by the *t*-test for paired data. $P < 0.05$ was the accepted level of significance. Data are expressed as means ± s.e.mean with *n* being the number of cell preparations, each from a different rat.

Results

Increasing concentrations of SNAP in the incubation medium (1–1000 μM) resulted in an increase in the extent of cell injury as assessed by both Trypan blue dye uptake and N-acetyl β glucosaminidase release into the medium (Figure 1A,B). Inactivation of the SNAP by preincubation for 1 h at 37°C resulted in a loss of the molecule's ability to injure the epithelial cells as assessed by either dye uptake (Figure 1A) or lysosomal enzyme release (Figure 1B). Furthermore, addition of SNAP to the incubation medium resulted in a dose-dependent increase in PKC activity within the colonic epithelial cells (Figure 2). Significant increases in PKC activity were observed at SNAP concentrations of 10 μM and greater. Inactivation of SNAP (1000 μM) resulted in a loss of the PKC activation previously observed in this study.

Figure 3 demonstrates the effects of SNAP on PKC isoform protein levels in colonic epithelial cells. Proteins for PKC-α, PKC-δ, PKC-ε and PKC-ζ were each demonstrated in unstimulated cells. Addition of SNAP resulted in a significant increase in the protein for PKC-ε only. Although immunoblot analysis revealed that, in untreated cells, the protein levels for the other PKC isoforms were expressed in greater abundance than that observed for PKC-ε, these isoforms were not significantly affected by NO donor treatment (Figure 3A,B).

PKC-ε protein levels in cytosolic and membrane fractions of cellular lysates are shown in Figure 4. A significant increase in PKC-ε was observed in the membrane fraction in response to SNAP treatment. Addition of the NO scavenger, PTIO, significantly reduced the PKC-ε protein expression in the membrane fraction.

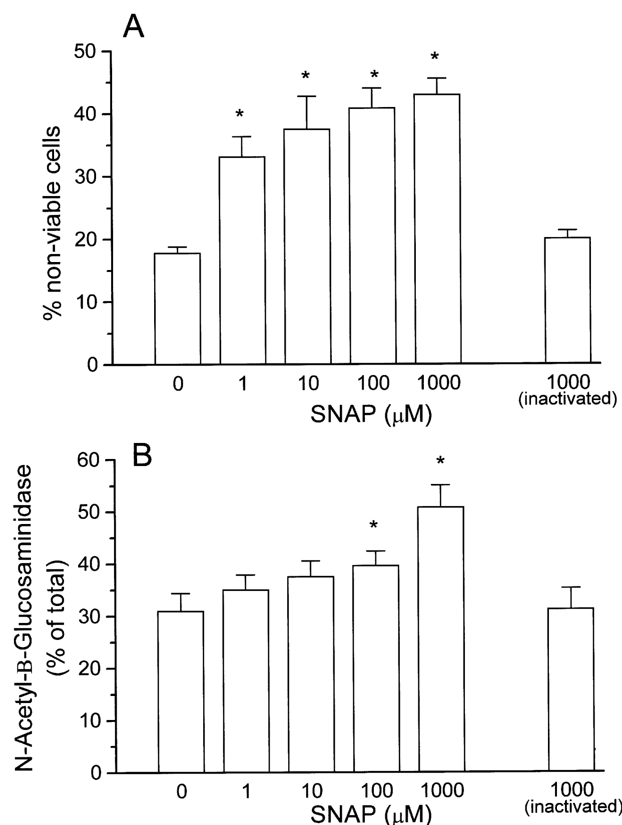

Figure 1 The effect of increasing concentrations of SNAP (1–1000 μM) in the incubation medium on the extent of colonic cellular injury as assessed by (A) Trypan blue dye uptake and (B) N-acetyl-β-glucosaminidase release. In some experiments, SNAP (1000 μM) was incubated at 37°C for 1 h to release NO prior to commencing the experiment. The inactivated form of SNAP was used to assess cellular integrity. Values are displayed as means ± s.e.mean of 5–7 independent experiments. *Indicates significant increases over untreated cells as determined by Duncan's multiple range test.

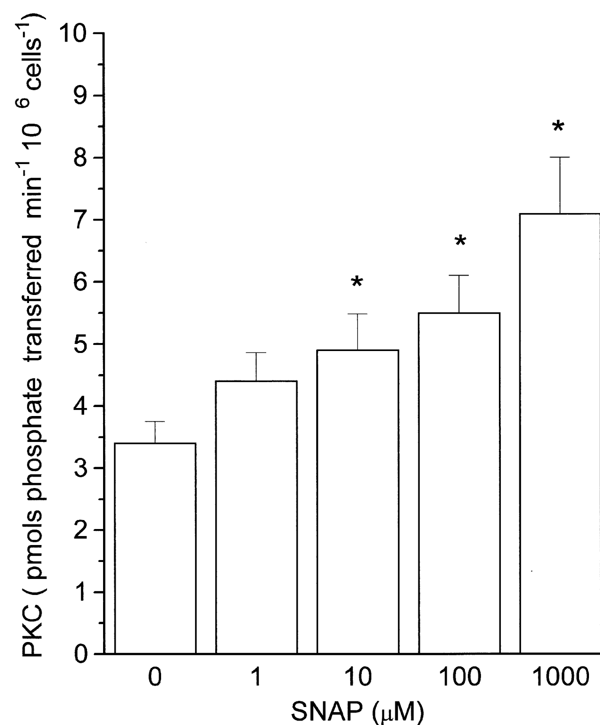

Figure 2 The effect of SNAP (1–1000 μM) on mean ± s.e.mean cellular PKC activity in six independent experiments. *Indicate significant increases compared to untreated cells by Duncan's multiple range test.

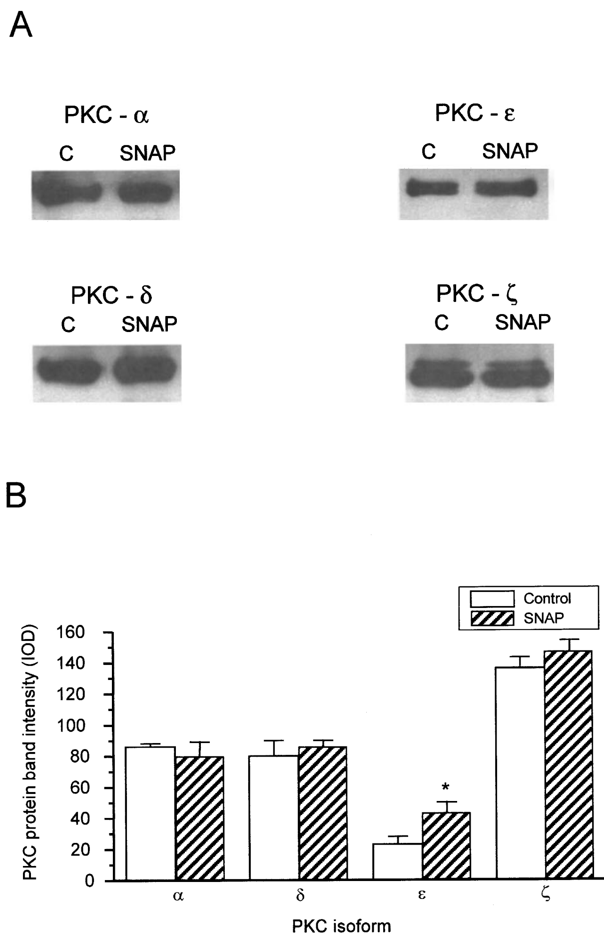

Figure 3 The effect of SNAP (100 μM) on PKC isoforms in lysates of colonic epithelial cells. (A) Displays of representative Western immunoblots of samples from untreated and SNAP-treated cells. (B) Displays the mean $\pm$ s.e.mean protein band intensity for each isoform from six independent experiments. *Indicates a significant increase compared to control cells as determined by the *t*-test for paired data.

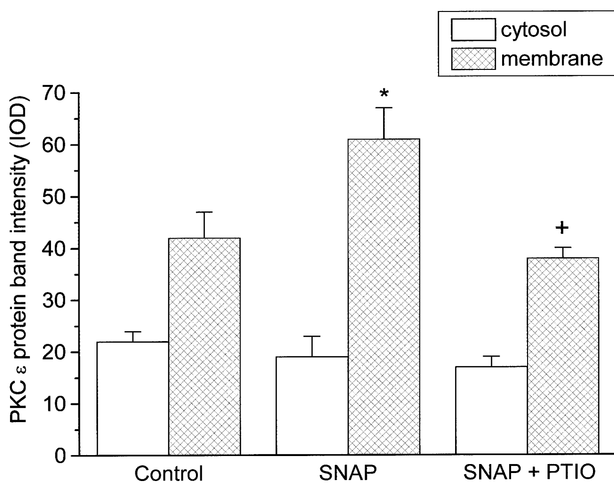

Figure 4 The mean $\pm$ s.e.mean protein band intensity for PKC-ε in the cytosolic and membrane fractions of colonic cells treated with SNAP (100 μM) alone or SNAP in combination with the NO scavenger, PTIO (1 mM). Values are derived from five independent experiments and represent PKC-ε protein band intensity determined in the cytosolic and membrane particulate fractions of lysates of colonic epithelial cells. *Indicates significant increases in the protein band intensity in the membrane fraction in comparison to the membrane function of untreated cells whereas + indicates significant decreases in the band intensity in comparison to the response to SNAP alone as determined by Duncan's multiple range test.

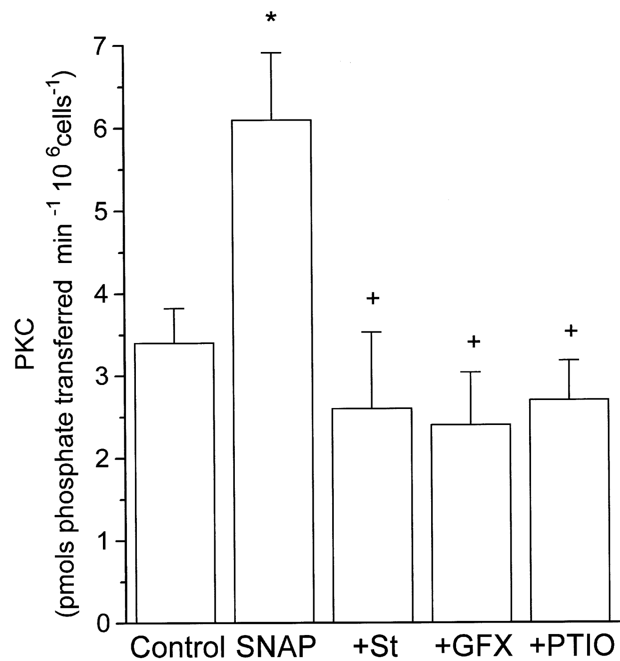

Figure 5 The effect of the PKC antagonists, staurosporine (St) or GF 109203X (GFX) (10 μM) on the mean $\pm$ s.e.mean cellular PKC activity in response to addition of SNAP (1000 μM) to the incubation medium. *Indicates significant increases over control whereas + indicates significant decreases when compared to SNAP alone as determined by Duncan's multiple range test.

PKC activity was increased by SNAP treatment (1000 μM) and this increase was reduced by addition of the PKC antagonists, staurosporine or GF 109203 X to the incubation medium (Figure 5). Furthermore, cellular PKC activity in response to SNAP treatment was reduced by addition of the NO scavenger, PTIO to the cell suspension (Figure 5).

The increase in cell injury as assessed by either Trypan blue dye uptake (Figure 6A) or release into the medium of the lysosomal enzyme, N-acetyl-β-glucosaminidase (Figure 6B), was increased in response to SNAP treatment (1000 μM). This injury was significantly reduced when staurosporine, GF 109203X or PTIO were added to the cellular incubation medium.

Addition of the PKC activator, phorbol 12-myristate 13-acetate (PMA; 1 and 10 μM) to the incubation medium augmented the extent of cellular injury in response to SNAP treatment (1 – 1000 μM). The lower concentration of PMA (1 μM) significantly increased the extent of cell damage in response to the lowest concentrations of SNAP (1 and 10 μM) whereas the high concentration of PMA (10 μM) significantly increased cell injury at all concentrations of SNAP used in the present study (Figure 7).

Incubation of cells with the peroxyl scavenger, ebselen (0.1 – 10 μM) resulted in a dose-dependent reduction in the extent of cell damage in response to 1 mM SNAP (Figure 8A). Treatment with 1 or 10 μM doses of the scavenger resulted in cellular injury which was not significantly different than that observed in untreated cells. Similarly ebselen resulted in a dose-dependent reduction in the cellular PKC activity in response to SNAP treatment (Figure 8B).

Discussion

In the present study, the nitric oxide donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP) resulted in a dose-dependent

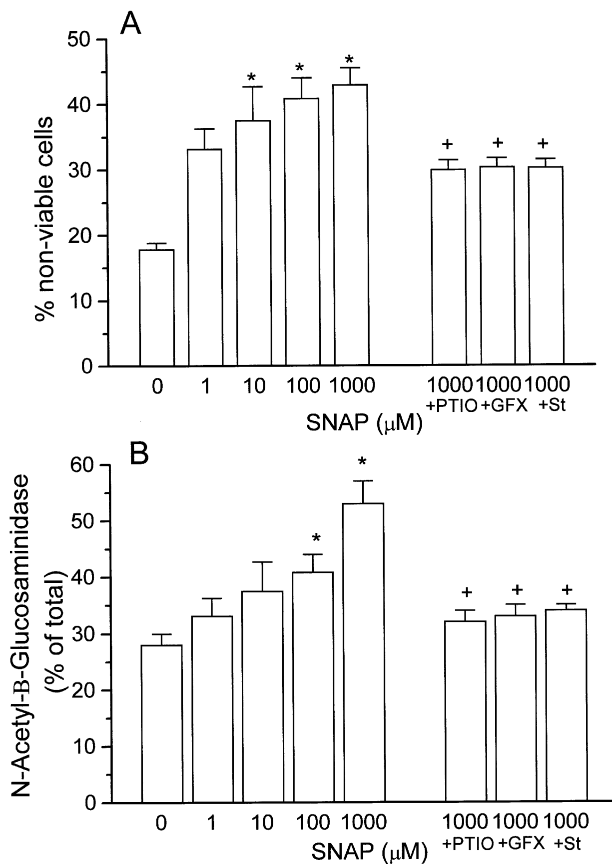

Figure 6 The effect of the NO scavenger, PTIO (1 mM) and the PKC antagonists, staurosporine (St; 10 μM) and GF 109203X (GFX; 10 μM) on the mean $\pm$ s.e.mean extent of cellular injury as assessed by (A) Trypan blue dye uptake and (B) N-acetyl-β-glucosaminidase release in six independent experiments. *Indicates significant increases in the extent of cell damage in response to SNAP whereas + indicates significant reductions in cellular injury when compared to the response to 1000 μM SNAP alone as determined by Duncan's multiple range test.

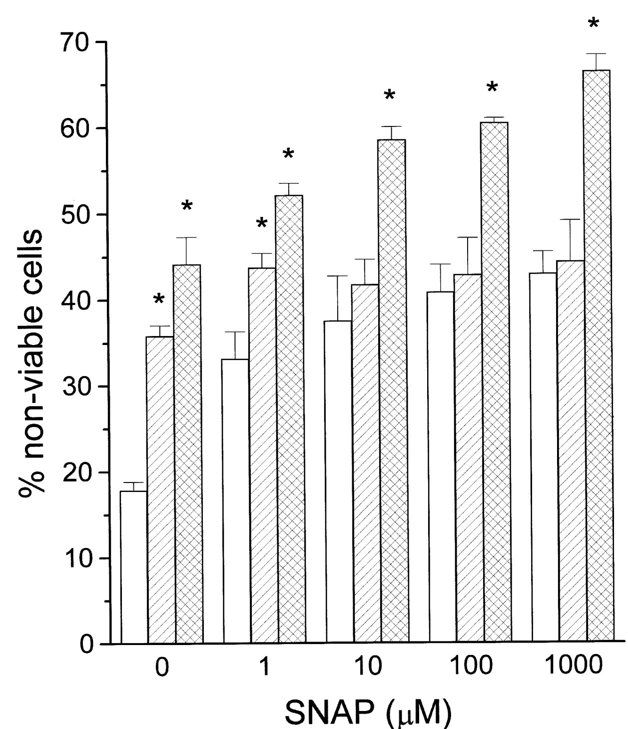

Figure 7 The effect of co-incubation of colonic epithelial cells with SNAP (1 – 1000 μM) and the PKC activator phorbol 12-myristate 13-acetate (PMA; 1 and 10 μM) on the mean change in cellular integrity as assessed by Trypan blue dye uptake in 5 – 7 independent experiments. *Indicates significant increases over the respective SNAP control group as determined by Duncan's multiple range test.

increase in the extent of cellular injury in isolated colonic mucosal cell. This phenomenon has been observed previously in isolated cells from the gastrointestinal mucosa as well as endothelial cells, pancreatic B cells, smooth muscle cells and neurons (Loweth *et al*., 1997; Maiese *et al*., 1993; Nishio & Watanabe, 1997; O'Connor & Moncada, 1991; Palmer *et al*., 1992; Tepperman *et al*., 1993; 1994). The present study also indicates that SNAP treatment of these cells resulted in an increase in total PKC activity of these cells. Similar increases in PKC activity in response to NO donors have also been shown in a variety of cell types (Maiese *et al*., 1993; Nishio & Watanabe, 1997). Furthermore, this increase in PKC activity appears to be causally related to the increase in cellular injury. This is confirmed by the demonstration that the cytotoxic effects of SNAP could be reduced by inactivation of the compound and overcome by addition, not only of NO and peroxyl scavengers such as PTIO, but also by PKC antagonists such as staurosporine and GF 109203X. Furthermore, the damaging actions of SNAP could be augmented by incubating cells with the PKC activator, phorbol myristate acetate. This enhancement of cell death in response to a NO challenge and PKC activation *via* phorbol ester treatment has been demonstrated previously in other cell types (Jun *et al*., 1997).

The role of PKC in cytotoxicity has been complicated by contradictory reports. PKC activation has been shown to result in cell death in a variety of cell types including colonic mucosal cells (Brown *et al*., 1999; Jones *et al*., 1997; Ye *et al*., 1993). Furthermore inhibition of PKC activity have been shown to prevent or reduce cellular injury in response to a number of challenges (Brown *et al*., 1999; Shaposhnikova *et al*., 1994). However, in contrast to these findings, PKC activation has also been associated with a reduction in lymphocyte damage and to prevent radiation-induced apoptosis *in vitro* (McConkey *et al*., 1992; Tomei *et al*., 1988). Furthermore, inhibition of PKC has been shown to induce cell death in human myeloid leukemic cells and down-regulation of PKC was found to suppress the induction of apoptosis in human prostatic carcinoma cells (Freemerman *et al*., 1996; Rusnak & Lazo, 1996). It is conceivable that the conflicting observations regarding the apparent role of protein kinase C in the regulation of cellular integrity may reflect cell type-specific responses to triggering agents (Jarvis *et al*., 1994; Lucas & Sanchez-Margalet, 1995).

The presence of multiple PKC isozymes have previously been demonstrated in the colonic epithelium of the rat (Jiang *et al*., 1995). In other cell types including hepatocytes and promyeloid cells, cell injury was associated with differential expression of some isozymes of PKC (Jones *et al*., 1994; Pongracz *et al*., 1996). In the present study, while the proteins of a number of PKC isoforms were detected by immunoblotting, only PKC-ε was increased in response to addition of SNAP to the incubation medium. Nishio & Watanabe (1997) have similarly observed a selective increase in PKC-ε in smooth muscle cells in response to challenge with SNAP and PKC-ε activation has been shown to be selectively mediated by NO in heart tissue (Ping *et al*., 1999). Furthermore, we have demonstrated that the addition of the NO donor resulted in the translocation of the enzyme from cytosol to membrane.

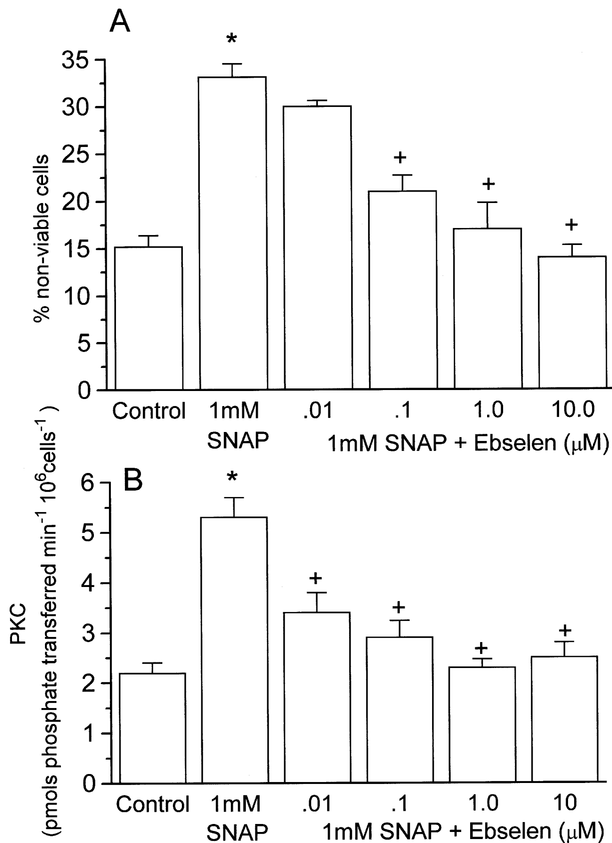

Figure 8 The effect of the peroxyl scavenger ebselen (0.01 – 10 μM) on the extent of (A) cell damage as assessed by Trypan blue dye uptake and (B) cellular PKC activity in response to addition of SNAP (1 mM) to the incubation medium. Values represent the means ± s.e.mean of six independent experiments. *Indicate significant increases in response to SNAP in comparison to untreated cells. + Indicates significant decreases from the response to SNAP alone as determined by Duncan's multiple range test.

Activation of PKC is associated with its translocation from the cell soluble to the particulate fraction. Activation of a particular PKC isozyme can be assayed by determining its relative distribution between cell fractions by Western blot analysis. Therefore, the results of the present study suggest that addition of NO to isolated colonic epithelial cells caused the activation of protein kinase C-ε and that the increase in cytotoxic reaction is linked to the increase in this isoform of the enzyme. Yoshida *et al.* (1999) have observed NO-mediated PKC-ε translocation in rat heart during post-ischemic reperfusion. While it is possible that other isoforms of PKC may be involved in the regulation of colonic cellular integrity, these were not observed under the conditions of the present study.

The mechanism of NO-induced cell damage is thought to result from direct cytoxicity of the NO radical. Furthermore, it has been shown that interaction between NO and the superoxide anion can lead to the formation of the highly cytotoxic peroxynitrite radical with subsequent liberation of the hydroxyl radical (Beckman *et al.*, 1990). Oxidants such as peroxynitrite have been shown to increase PKC activity in epidermal cells, endothelial cells, ventricular myocytes and astrocytes (Brawn *et al.*, 1995; Larsson & Cerutti, 1989; Siflinger-Birnboim *et al.*, 1992; Ward & Moffat, 1995). We have investigated the extent of oxidant formation *via* peroxynitrite production on PKC-mediated cell injury by the use of the peroxyl scavenger, ebselen. In the present study, ebselen reduced both the extent of cell injury and PKC activity. Assuming a selective action of ebselen as a peroxyl scavenger, these results suggest that peroxynitrite formation, at least in part, is responsible for the effect of SNAP on the parameters examined in this study. This confirms previous studies in which NO-mediated formation of reactive oxidants produce their biological actions *via* changes in PKC activity (Sheehy *et al.*, 1998).

In conclusion, the present study has demonstrated that NO-induced damage to colonic mucosal cells is mediated *via* an increase in cellular PKC activity and that the primary isoform involved is PKC-ε. Furthermore, the activation of PKC appears to be mediated, to some extent, by peroxynitrite formation.

This work was supported by a grant from the Medical Research Council of Canada (MT-6426).

References

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 1620–1674.

BRAWN, M.K., CHIOU, W.J. & LEACH, K.L. (1995). Oxidant-induced activation of protein kinase C in UC11MG cells. *Free Rad. Res.*, **22,** 23–37.

BROWN, J.F., WAKULICH, C.A., SOPER, B.D. & TEPPERMAN, B.L. (1999). The effect of protein kinase C activation on colonic epithelial cellular injury. *Gastroenterology*, **116,** A674.

BUTLER, R.N., LAWSON, N.H., GOLAND, G.J., JARRETT, I.G. & ROBERTS-THOMASON, I.C. (1988). Proliferative activity in the proximal and distal colon of the rat after fasting and refeeding. *Immunol. Cell. Biol.*, **66,** 193–198.

FREEMERMAN, A.J., TURNER, A.J., FIRRER, M.J., SZABO, E., VALERIE, K. & GRANT, S. (1996). Role of c-jun in human myeloid leukemia cell apoptosis induced by pharmacological inhibitors of protein kinase C. *Mol. Pharmacol.*, **49,** 788–795.

HATA Y., OTA, S., HIRAISHI, H., TERANO, A. & IVEY, K.J. (1996). Nitric oxide enhances cytotoxicity of cultured rabbit gastric mucosal cells induced by hydrogen peroxide. *Biochim. Biophys. Acta*, **1290,** 257–260.

JARVIS, W.D., KOLESNICK, R.N., FORNARI, F.A., TRAYLOR, R.S., GERWITZ, D.A. & GRANT, S. (1994). Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 73–77.

JIANG, Y.H., AUKEMA, H.M., DAVIDSON, L.A., LUPTON, J.R. & CHAPKIN, R.S. (1997). Localization of protein kinase C isozymes in rat colon. *Cell Growth Diff.*, **6,** 1381–1386.

JONES, B.A., RAO, Y.P., STRAVITZ, R.T. & GORES, G.J. (1997). Bile salt-induced apoptosis of hepatocytes involves activation of protein kinase C. *Am. J. Physiol.*, **272,** G1109–G1115.

JUN, C.-D., PARK, S.-J., CHOI, B.-M., KWAK, H.-J., PARK, Y.-C., KIM, M.-S., PARK, R.-K. & CHUNG, H.-T. (1997). Potentiation of the activity of nitric oxide by the protein kinase C activator phorbol ester in human myeloid leukemic HL-60 cells: Association with enhanced fragmentation of mature genomic DNA. *Cell. Immunol.,* **176,** 41–49.

LARSSON, R. & CERUTTI, P. (1989). Translocation and enhancement of phosphotransferase activity of protein kinase C following exposure in mouse epidermal cells to oxidants. *Cancer Res.,* **49,** 5627–5632.

LOWETH, A.C., WILLIAMS, G.T., SCARPELL, J.H.B. & MORGAN, N.G. (1997). Evidence for the involvement of cGMP and protein kinase G in nitric oxide-induced apoptosis in the pancreatic B-cell line, HIT-T15. *FEBS Lett.,* **400,** 285–288.

LUCAS, M. & SANCHEZ-MARGALET, V. (1995). Protein kinase C involvement in apoptosis. *Gen. Pharmacol.,* **26,** 881–887.

MAIESE, K., BONIECE, I.R., SKURAT, K. & WAGNER, J.A. (1993). Protein kinases modulate the sensitivity of hippocampal neurons to nitric oxide toxicity and anoxia. *J. Neurosci. Res.,* **36,** 77–87.

MCCONKEY, D.J., JONDAL, M. & ORRENIUS S. (1992). Cellular signalling in thymocyte apoptosis. *Semin. Immunol.,* **4,** 371–377.

NISHIO, E. & WATANABE, Y. (1997). Nitric oxide donor-induced apoptosis in smooth muscle cells is modulated by protein kinase C and protein kinase A. *Eur. J. Pharmacol.,* **339,** 245–251.

O'CONNOR, K.J. & MONCADA, S. (1991). Glucocorticoids inhibit the induction of nitric oxide synthase and the related cell damage in adenocarcinoma cells. *Biochim. Biophys. Acta,* **1097,** 227–231.

PALMER, R.M.J., BRIDGE, L., FOXWELL, N.A. & MONCADA, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.,* **105,** 11–12.

PHELPS, D.T., FERRO, T.J., HIGGINS, R.J., SHANKAR, R., PARKER, D.M. & JOHNSON, A. (1995). TNFα induces peroxynitrite-mediated depletion of lung endothelial glutathione via protein kinase C. *Am. J. Physiol.,* **269,** L551–L559.

PING, P., TAKANO, H., ZHANG, J., TANG, X.L., QIU, Y., LI, R.C., BANERGEE, S., DAWN, B., BALAFONOVA, Z. & BOLLI, R. (1999). Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signalling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ. Res.,* **84,** 587–604.

PONGRACZ, J., DEACON, E.M., JOHNSON, G.D., BURNETT, D. & LORD, J.M. (1996). Doppa induces cell death but not differentiation of U937 cells: evidence for the involvement of PKC-beta 1 in the regulation of apoptosis. *Leuk. Res.,* **20,** 319–326.

RUSNAK, J.M. & LAZO, J.S. (1996). Downregulation of protein kinase C suppresses induction of apoptosis in human prostatic carcinoma cells. *Exp. Cell. Res.,* **224,** 189–199.

SAKANOUE, Y., HATADA, T., HORAI, T., SHOJI, Y., KUSUMOKI, M. & UTSUNOMIYA, J. (1992). Protein kinase C activity of colonic mucosa in ulcerative colitis. *Scand. J. Gastroenterol.,* **27,** 275–280.

SHAPOSHNIKOVA, V.V., DOBROVINSKAYA, O.R., EIDUS, L.K. & KORYSTOV, Y.N. (1994). Dependence of thymocyte apoptosis on protein kinase C and phospholipase A_2. *FEBS Lett.,* **348,** 317–319.

SHARMA, K., DANOFF, T.M., DE PIERO, A. & ZIYADEH, F.N. (1995). Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: possible mediation via protein kinase C. *Biochem. Biophys. Res. Commun.,* **207,** 80–88.

SHEEHY, A.M., BURSON, M.A. & BLACK, S.M. (1998). Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: a role for superoxide. *Am. J. Physiol.,* **274,** L833–L841.

SIFLINGER-BIRNBOIM, A., GOLIGORSKY, M.S., DEL VECCHIO, P.J. & MALIK, A.B. (1992). Activation of protein kinase C pathway contributes to hydrogen peroxide-induced increase in epithelial permability. *Lab. Invest.,* **76,** 24–30.

TEPPERMAN, B.L., ABRAHAMSON, T.D. & SOPER, B.D. (1998). The role of cyclic guanylate monophosphate in nitric oxide-injured injury to rat small intestinal epithelial cells. *J. Pharmacol. Exp. Therap.,* **284,** 929–933.

TEPPERMAN, B.L., BROWN, J.F., KOROLKIEWICZ, R. & WHITTLE, B.J.R. (1994). Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: Effect of endotoxin challenge. *J. Pharmacol. Exp. Therap.,* **271,** 1477–1482.

TEPPERMAN, B.L., BROWN, J.F. & WHITTLE, B.J.R. (1993). Nitric oxide synthase induction and intestinal epithelial cell viability in the rat. *Am. J. Physiol.,* **265,** G214–G218.

TEPPERMAN, B.L., TAN, S.Y. & WHITTLE, B.J.R. (1991). Effects of calcium modifying agents on integrity of rabbit isolated gastric mucosal cells. *Am. J. Physiol.,* **261,** G119–G127.

TOMEI, D.L., KANTER, P. & WENNER, C.E. (1988). Inhibition of radiation-produced apoptosis in vitro by tumor promoters. *Biochem. Biophys. Res. Commun.,* **155,** 324–331.

WARD, C.A. & MOFFAT, M.P. (1995). Role of protein kinase C in mediating effects of hydrogen peroxide in guinea pig ventricular myocytes. *J. Mol. Cell. Cardiol.,* **27,** 1089–1097.

YE, X., GEORGOFF, I., FLEISHER, S., COFFMAN, F.D., COHEN, S. & FRESA, K.L. (1993). The mechanism of epipodophyllotoxin-induced thymocyte apoptosis: possible role of a novel calcium-independent protein kinase. *Cell. Immunol.,* **151,** 320–335.

YOSHIDA, K., MIZUKAMI, Y. & KITAKAZE, M. (1999). Nitric oxide mediates protein kinase C isoform translocation in rat heart during postischemic reperfusion. *Biochim. Biophys. Acta,* **1453,** 230–238.

(Received July 1, 1999
Revised August 16, 1999
Accepted August 18, 1999)