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Interaction of 5-aminosalicylic acid with nitric oxide on rat aortic strips and human platelets

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Abstract

We have examined the interactions of 5-aminosalicylic acid with nitric oxide (NO). Phenylephrine-precontracted rat aortic strips with intact endothelium were further contracted by 5-aminosalicylic acid (50–200 μM) in a concentration-dependent manner. Removal of endothelium, inhibition of guanylate cyclase by methylene blue, inhibition of NO biosynthesis by N^G-nitro-L-arginine as well as inactivation of NO by oxyhemoglobin abolished the effect of 5-aminosalicylic acid. The antiaggregatory effects of 3-morpholinosydnonimine and rat peritoneal neutrophils, which are due to release of NO, were diminished in a concentration-dependent manner by 5-aminosalicylic acid (50–250 μM). In both experimental models the effects of 5-aminosalicylic acid were significantly reduced by superoxide dismutase in a concentration which alone exhibited no effect. Since NO might act as a cytotoxic and vasodilating mediator, our results suggest that inactivation of NO by 5-aminosalicylic acid could contribute to the therapeutic activity of the drug in inflammatory bowel disease.

Keywords: 5-Aminosalicylic acid; Nitric oxide (NO); Inflammatory bowel disease

1. Introduction

A markedly elevated nitric oxide (NO) production in active inflammatory bowel disease has recently been described in several studies (Middleton et al., 1993; Tran et al., 1993; Boughton-Smith et al., 1993). Plasma concentrations of endotoxin and mucosal concentrations of cytokines which have been shown to induce NO synthase (Moncada et al., 1991) are also known to be enhanced during active inflammatory bowel disease (MacDonald et al., 1990; Gardiner et al., 1991; Balfour Sartor, 1994) and, thus, could contribute to the raised NO production. Since NO not only causes smooth muscle relaxation and vasodilatation, but is also an effector molecule of macrophage cytotoxicity (Moncada et al., 1991), it might be a relevant factor in the pathogenesis of inflammatory bowel disease. The cytotoxicity of immunologically activated macrophages, which synthesize NO from L-arginine (Hibbs et al., 1988), can be prevented by inhibitors of NO biosynthesis (James and Glaven, 1989; Green et al., 1990). Glucocorticoids, the most effective drugs for treating inflammatory bowel disease, inhibit the induction of NO synthase (Radomski et al., 1990). 5-Aminosalicylic acid is also established as an effective drug in inflammatory bowel disease, but it is unknown whether its therapeutic efficacy is mediated by effects on specific mediators or relies on the pluripotency of the drug. 5-Aminosalicylic acid interacts with the biosynthesis or action of a variety of mediators possibly involved in the pathogenesis or pathophysiology of inflammatory bowel disease, such as prostaglandins, leukotrienes, cytokines and platelet-activating factor. In order to investigate whether 5-aminosalicylic acid affects the NO system, we have examined the actions of the drug on precontracted rat aortic strips (Furchgott and Zawadzki, 1980) and on inhibition of platelet aggregation by the NO donor, 3-morpholinosydnonimine (SIN-1), or rat peritoneal neutrophils elicited after glycogen injection which are known to produce NO (Rimele et al., 1988). We have selected these cells for our study because they

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contain an inducible, calcium-independent NO synthase. The induction of this enzyme is inhibited by dexamethasone (McCall et al., 1991).

2. Materials and methods

2.1. Effects on rat aortic strips

Spirally cut strips of rat thoracic aorta with intact endothelium were mounted in 5 ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11. The solution was continuously gassed with 95% O_2/5% CO_2 at 37°C. The preload applied to the strips was 0.7 g. Contractions and relaxations were recorded using an isotonic lever transducer (Hugo Sachs, Hugstetten, Germany) and displayed on a Watanabe multipen recorder. The tissues were allowed to equilibrate for 60 min with buffer changes every 10–15 min. A first set of experiments analysed then effects of 5-aminosalicylic acid on rat aortic strips which had not been precontracted. Then the strips were precontracted by phenylephrine (100 nM) and the functional preservation of endothelium was tested by the induction of relaxations with carbachol (0.1–1.0 μM). When phenylephrine-induced contractions had reached a plateau, 5-aminosalicylic acid (final concentration 50–200 μM) was added to the organ bath. Some of these experiments were performed in the presence of indomethacin (5 μM) or superoxide dismutase (0.05 U/ml). Experiments were repeated in the presence of methylene blue (10 μM), N^G-nitro-L-arginine (100 μM) or oxyhemoglobin (2 μM) or after mechanical removal of the endothelium. In additional experiments, precontracted aortic strips were relaxed by SIN-1 (1 nM), carbachol (300 nM) or isoproterenol (1–30 nM) in the absence and in the presence of 5-aminosalicylic acid (100 μM). The results were expressed as percent of the contraction induced by 100 nM phenylephrine (100%).

2.2. Effects on platelet aggregation

Washed human platelets were prepared as described by Radomski and Moncada (1983). Rat peritoneal neutrophils were elicited in male Wistar rats (250–350 g) by i.p. injection of 15 ml of 0.6% oyster glycogen solution (Palmer et al., 1980). The rats were killed 4 h later and cells were harvested by peritoneal lavage and centrifugation of the lavage fluid ($300 \times g$, 4°C, 10 min). Platelets as well as rat peritoneal neutrophils were resuspended in calcium-free Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $MgCl_2$ 1.1, glucose 5.6. Aliquots (1 ml) of washed platelet suspensions (2.5×10^8 cells/ml) were incubated in a Born aggregometer under continuous stirring after addition of calcium ions (final concentration 1 mM). SIN-1 (final concentration 0.5–4.0 μM), rat peritoneal neutrophils ($0.8–4.0 \times 10^5$ cells/ml) or iloprost (final concentration 0.5–3.0 nM) was added 1 min before induction of platelet aggregation by threshold concentrations (20–30 mU/ml) of thrombin. Further experiments were performed in the presence of superoxide dismutase (60 U/ml) or indomethacin (5 μM) added 30 s before rat peritoneal neutrophils. 5-Aminosalicylic acid (final concentration 50–250 μM) was added 30 s after thrombin. The decrease of optical density of the platelet suspension was recorded for 4 min. The results were expressed as percent inhibition of aggregation induced by thrombin.

2.3. Statistics

Means ± S.E.M. were calculated. Statistical analysis was performed by means of Student's t-test and analysis of variance combined with the Newman-Keuls test, respectively.

2.4. Materials

3-Morpholinosydnonimine (SIN-1) was a gift from Cassella-Riedel Pharma, Frankfurt/Main, Germany. Bovine thrombin was obtained from Behring-Werke, Marburg, Germany. Superoxide dismutase was from Serva, Heidelberg, Germany. Iloprost was kindly provided by Schering, Berlin, Germany. All other reagents were purchased from Sigma Chemicals Co., St. Louis, MO, USA. Oxyhemoglobin was purified from bovine hemoglobin type I as described by Martin et al. (1985).

3. Results

3.1. Effects on rat aortic strips

While rat aortic strips which had not been precontracted were unaffected by the addition of 5-aminosalicylic acid, phenylephrine-precontracted rat aortic strips with intact endothelium were further contracted by the addition of 5-aminosalicylic acid in a concentration-dependent manner (Fig. 1) by 27 ± 3% (50 μM; $n = 15$), 43 ± 4% (100 μM; $n = 20$) and 54 ± 9% (200 μM; $n = 13$), respectively. Removal of endothelium, inhibition of guanylate cyclase by methylene blue or inhibition of NO biosynthesis by N^G-nitro-L-arginine as well as inactivation of NO by oxyhemoglobin abolished the effects of 5-aminosalicylic acid (Fig. 1). SIN-1 (1 nM)-or carbachol (300 nM)-induced relaxations, which are mediated by NO, were significantly reduced by 5-aminosalicylic acid (100 μM) from 33 ± 6% to

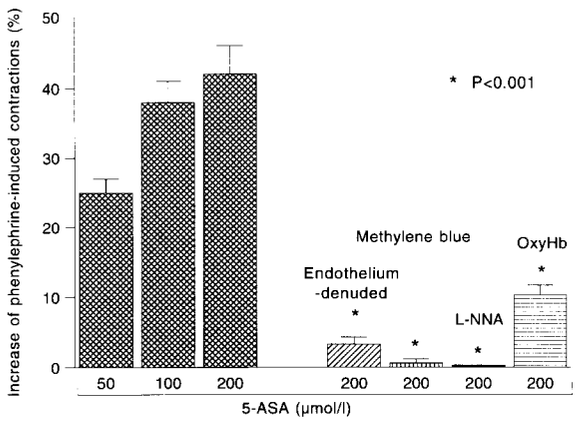

Fig. 1. 5-Aminosalicylic acid (5-ASA) induces concentration-dependent contractions of phenylephrine (100 nM)-precontracted rat aortic strips. Removal of endothelium, methylene blue (10 μM), N^G-nitro-L-arginine (L-NNA; 100 μM) and oxyhemoglobin (oxy Hb; 2 μM) increase basal tone and simultaneously abolish the 5-aminosalicylic acid-induced contractions ($n = 6$–20/group).

$9 \pm 3\%$ ($P < 0.01$; $n = 6$) and from $20 \pm 6\%$ to $7 \pm 2\%$ ($P < 0.05$; $n = 6$), respectively, while relaxations elicited by isoproterenol were not significantly affected by 5-aminosalicylic acid. Isoproterenol (1, 10 and 30 nM) relaxed rat aortic strips (with intact endothelium) by $11 \pm 3\%$, $50 \pm 7\%$ and $85 \pm 5\%$, respectively, in the absence and by $8 \pm 1\%$, $43 \pm 5\%$ and $86 \pm 4\%$, respectively, in the presence of 5-aminosalicylic acid (100 μM; $n = 6$ for each isoproterenol concentration). In several experiments, the effects of 5-aminosalicylic acid were determined in the presence of a concentration (0.05 U/ml) of superoxide dismutase which alone reduced the tone of the precontracted aortic strips by less than 3%. In these experiments the contractions induced by 5-aminosalicylic acid (100 μM) were signifi-

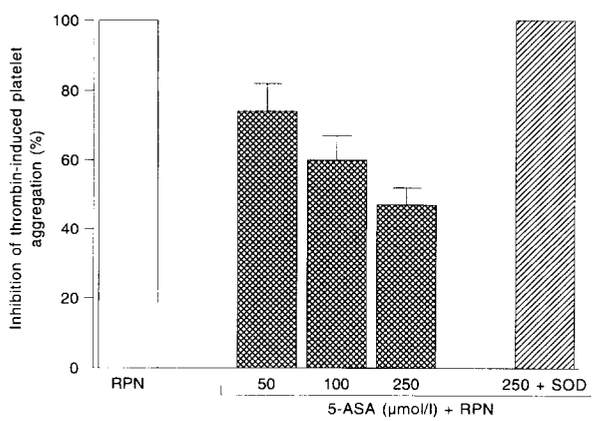

Fig. 2. 5-Aminosalicylic acid (5-ASA) antagonizes in a concentration-dependent manner the inhibition of platelet aggregation by rat peritoneal neutrophils (RPN; 0.8–4.0×10^5 cells/ml; $n = 6$–17/group). The antagonism of the anti-aggregatory effect of rat peritoneal neutrophils by 5-aminosalicylic acid (250 μM) is lost in the presence of superoxide dismutase (SOD; 60 U/ml; $n = 5$).

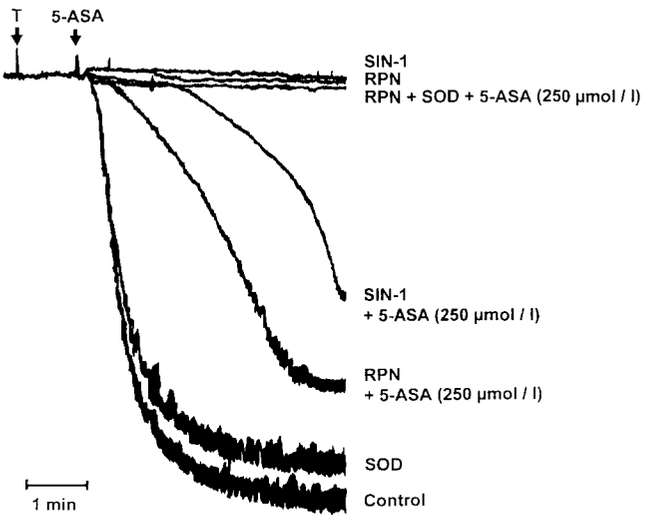

Fig. 3. Effects of 5-aminosalicylic acid (5-ASA) on inhibition of thrombin (T)-induced platelet aggregation by rat peritoneal neutrophils (RPN; 0.8×10^5 cells/ml) or SIN-1 (0.5 μM). The antagonism of the anti-aggregatory effect is lost in the presence of superoxide dismutase (SOD; 60 U/ml): traces representative of ≥ 5 experiments.

cantly ($P < 0.01$; $n = 6$) smaller ($23 \pm 5\%$) than those obtained in the absence of superoxide dismutase ($45 \pm 8\%$). Indomethacin (5 μM) did not affect the contractions induced by 5-aminosalicylic acid.

3.2. Effects on platelet aggregation

The antiaggregatory effects of concentrations of rat peritoneal neutrophils (0.8–4.0×10^5 cells/ml) which caused just complete inhibition (100%) of platelet aggregation were diminished in a concentration-dependent manner by 5-aminosalicylic acid to $74 \pm 8\%$ (50 μM; $n = 6$), $60 \pm 7\%$ (100 μM; $n = 15$) and $47 \pm 5\%$ (250 μM; $n = 17$), respectively (Fig. 2). Indomethacin

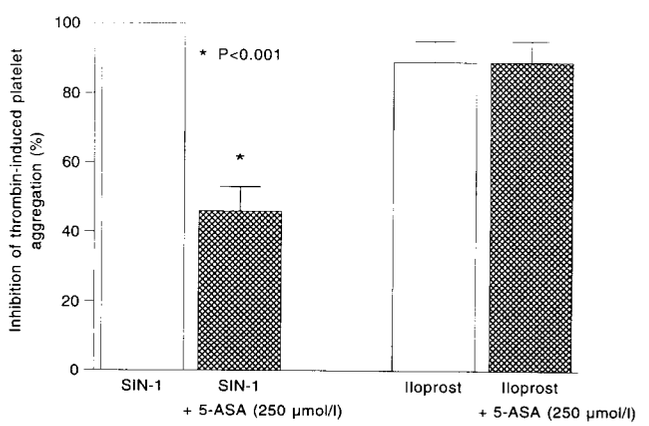

Fig. 4. 5-Aminosalicylic acid (5-ASA) antagonizes SIN-1 (0.5–4.0 μM; $n = 9$)-, but not iloprost (0.5–3.0 nM; $n = 7$)-induced inhibition of thrombin (T)-induced platelet aggregation.

(5 μM) did not influence the antiaggregatory effect of rat peritoneal neutrophils and its modification by 5-aminosalicylic acid. SIN-1 (0.5–4.0 μM)-induced complete inhibition of platelet aggregation was reduced to a comparable extent (46 ± 7%) by 5-aminosalicylic acid (250 μM; $n = 9$), while iloprost (0.5–3.0 nM)-induced inhibition of platelet aggregation was not affected ($n = 7$; Fig. 3 and Fig. 4). In the presence of superoxide dismutase (60 U/ml), 5-aminosalicylic acid did not reduce the inhibition of platelet aggregation by rat peritoneal neutrophils (Fig. 2 and Fig. 3). This concentration of superoxide dismutase alone had no significant effect on inhibition of platelet aggregation by rat peritoneal neutrophils in the absence of 5-aminosalicylic acid.

4. Discussion

Effects of 5-aminosalicylic acid on the tone of rat aortic strips were observed only under conditions of continuous NO generation and were abolished when NO production was suppressed. The results suggest that the contractile effect of 5-aminosalicylic acid as well as the reduction of NO-dependent SIN-1-and carbachol-induced relaxations in the presence of 5-aminosalicylic acid result from inactivation of NO. Likewise, inhibition of platelet aggregation by rat peritoneal neutrophils, which is attributed to NO generation (Rimele et al., 1988), was concentration dependently attenuated by 5-aminosalicylic acid. In contrast, relaxation of rat aortic strips by isoproterenol was unaffected by 5-aminosalicylic acid as was cyclic AMP-dependent inhibition of platelet aggregation by iloprost. The antagonistic action of 5-aminosalicylic acid on vascular smooth muscle relaxation and inhibition of platelet aggregation by NO was markedly diminished in the presence of superoxide dismutase, suggesting that the action of 5-aminosalicylic acid could be related to the generation of oxygen radicals which destroy NO. In concentrations that fully reversed the attenuation by 5-aminosalicylic acid, superoxide dismutase had no significant effect on rat peritoneal neutrophils in the absence of 5-aminosalicylic acid. Thus, scavenging of oxygen radicals generated by rat peritoneal neutrophils cannot explain the complete inhibition of the 5-aminosalicylic acid effect. 5-Aminosalicylic acid in the concentrations used does not inhibit NO synthase (Pallapies et al., 1994). With rat aortic strips, qualitatively identical results were obtained with 4-aminosalicylic acid in concentrations of 1–20 mM (Pallapies et al., 1994). The lack of effect of indomethacin suggests that prostaglandins do not play a major role in the mode of action of 5-aminosalicylic acid in the experimental systems used in this study.

Our results show that NO is inactivated by concentrations of 5-aminosalicylic acid which may be obtained at inflammatory sites of the bowel after administration of the drug to patients with chronic inflammatory bowel disease and which have been shown to be of therapeutic benefit in these patients (Lauritsen et al., 1984; Allgayer, 1992). NO release by intestinal tissue in patients with inflammatory bowel disease is mainly due to the inducible form of the NO synthase and should thus be comparable to generation of NO by rat peritoneal neutrophils after glycogen challenge, which is also catalyzed by an inducible NO synthase. The inactivation of NO by 5-aminosalicylic acid described in this study, as well as the inhibitory effect of 5-aminosalicylic acid on NO synthase-dependent *N*-nitrosation reactions (Grisham and Miles, 1994), might be specially relevant in ulcerative colitis associated with a markedly elevated NO synthase activity in colonic mucosa (Boughton-Smith et al., 1993).

Experimentally induced chronic ileitis has already been shown to be ameliorated by inhibition of NO synthase (Miller et al., 1993). Proinflammatory actions of increased NO production in inflammatory bowel disease could be diminished not only by inhibition of NO biosynthesis but also by inactivation of NO. Our results suggest that efficient local inactivation of the cytotoxic mediator NO might contribute to the therapeutic efficacy of 5-aminosalicylic acid in inflammatory bowel disease.

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