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Protective effect of a novel antioxidant non-steroidal anti-inflammatory agent (compound IA) on intestinal viability after acute mesenteric ischemia and reperfusion

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Abstract

Reactive oxygen species play an important role in the basic pathophysiology of ischemia–reperfusion injury. We investigated whether the administration of a novel non-steroidal anti-inflammatory compound with antioxidant properties, the compound [5-(2-amino-ethylamino)-1-phenyl-2-pentanone] (compound IA), has a beneficial effect on the repair process of the intestinal mucosa after transient mesenteric ischemia in a randomized blind trial. Six groups of rats were subjected to a model of 60 min of intestinal ischemia that was produced by occluding the superior mesenteric artery. At the end of ischemia, compound IA was administered intravenously and the clamp was removed allowing reperfusion. At 60 min after reperfusion, animals were sacrificed and a 10 cm section of terminal ileum was resected. The outcome was evaluated by histopathologic assessment, measurement of polymorphonuclear leukocytes and the extent of lipid peroxidation measuring the small intestine tissue malondialdehyde. After 1 h of reperfusion, the mucosal damage was less in IA-treated rats compared with the control group. Moreover, the number of polymorphonuclear leukocytes in intestinal mucosa was significantly lower in IA group. Compound IA resulted in a statistically significant reduction of the concentration of small intestine tissue malondialdehyde, compared to those of controls. Administration of compound IA decreased the mucosal damage in rats that were subjected to 60 min of ischemia followed by 60 min of reperfusion. The mechanism of compound IA action is considered to be mediated via its potent antioxidant, free radical scavenging activities and inhibition of polymorphonuclear leukocytes infiltration.

Keywords: Antioxidant; Non-steroidal anti-inflammatory agent; Acute mesenteric ischemia; Intestinal viability; Ischemia–reperfusion

1. Introduction

Despite effective surgical treatment, the mortality rate for patients with acute occlusive mesenteric ischemia remains high because of delays in diagnosis and definitive treatment (Schoenberg and Beger, 1988; Nilsson et al., 1994). In an attempt to improve survival after acute mesenteric ischemia, a number of experimental studies have been conducted in order to test several pharmacological agents that might attenuate reperfusion injury of the intestinal mucosa, in conjunction with surgical revascularization (Cicalese et al., 1996; Park et al., 1994; Duranton et al., 1998; Mustafa et al., 1995; Ward et al., 2000). However, in few of these studies, the agent was administered following ischemia and before reperfusion, simulating the likely use of a protective agent in the clinical setting.

Reactive oxygen species are known to play an important role in the basic pathophysiology of ischemia–reperfusion injury (Collard and Gelman, 2001). Reperfusion of ischemic tissues results in the formation of toxic reactive oxygen species, such as superoxide anions, hydroxyl radicals,

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hydrogen peroxide and peroxynitrite. Reactive oxygen species are potent oxidizing agents, the damage of cellular membranes by lipid peroxidation being a major consequence (Toyokuni, 1999).

The compound IA [5-(2-amino-ethylamino)-1-phenyl-2-pentanone] was designed and synthesized as an anti-inflammatory agent with basic character, thus expected to cause less side effects than the commonly used acidic nonsteroidal anti-inflammatory drugs (NSAIDs). It has been shown to inhibit the non-enzymatic peroxidation of rat liver microsomal lipids, almost completely at a concentration of 25 μM. This compound is also a very potent scavenger of hydroxyl radicals ($HO^{\cdot}$) and interacts with the stable 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), a property which expresses the reducing activity of the compound and its ability to scavenge N-centered radicals. This compound also possesses significant anti-inflammatory activity, reducing the carrageenan-induced rat paw edema (Andreadou et al., 1997).

Thus, we found it of interest to study the effect of the above agent in an imitated clinical setting of transient intestinal ischemia and reperfusion.

2. Materials and methods

2.1. Materials

The synthesis of compound IA has been previously described in detail (Andreadou et al., 1997). The chemical structure of compound IA is shown in Fig. 1.

Thirty-six (36) adult male Wistar rats weighing 250–300 g were obtained from Pasteur Institute, Athens. Rats were acclimatized to our laboratory conditions for 1 week prior to use in experiments. They were housed individually in stainless steel cages at a constant temperature (29 °C) and a 12-h day/ night cycle. Rats ate commercial rat chow and had water ad libidum. All experimental procedures described below were approved by Animal Care Committee according to the European Union Act and the Greek Law 160, A-64, May 1991.

2.2. Methods

2.2.1. Experimental protocol

Animals were divided into six groups consisting of six rats each: (1) control–sham-operated; (2) control–ischemia; (3) control–ischemia/reperfusion; (4) IA–sham-operated; (5) IA–ischemia; (6) IA–ischemia/reperfusion. Rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (16 mg/kg) administered intramuscularly. A jugular venous cannula was inserted for fluid and drug administration.

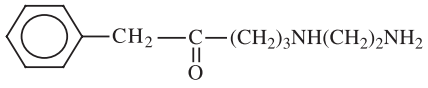

Fig. 1. Chemical structure of compound IA.

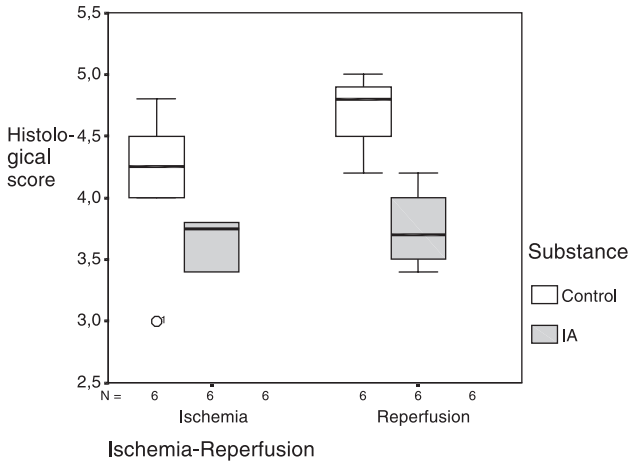

Fig. 2. Histological grade per group when ischemia or ischemia–reperfusion was performed.

The dose for compound IA was 0.3 mmol/kg which is the same with that used for the determination of anti-inflammatory activity (carrageenan experiments). The LD_{50} of IA in rats is 0.86 mmol/kg intraperitoneally (Andreadou et al., 1997). Compound IA was administered intravenously in an acute experimental setting. Control agent was normal saline.

Through a midline abdominal incision using aseptic technique, the superior mesenteric artery was meticulously isolated at its origin, while the accompanying mesenteric vein remained intact. In groups (1) and (4), the tested agent was administered and animals were sacrificed after a 60 min period. In groups (2) and (5), there was occlusion of the superior mesenteric artery by application of a traumatic microsurgical clamp for 60 min. Just before the end of this period, the substance was administered to the respective groups. In groups (3) and (6), a 60 min period of ischemia was performed and administration of the substance took place before the end of ischemia. Then the clamp was removed and a 60 min period of reperfusion followed. At the end of the operations, all animals were exsanguinated, and tissue samples were obtained from small intestine 10 cm proximal to the ileocecal area and subjected to histopathological evaluation. Furthermore, at the end of the experiment, all animals appeared normal both macroscopically and by autopsy.

2.2.2. Histopathologic assessment

Small intestinal tissue specimens were rinsed promptly in cold saline solution and immediately fixed in 10% buffer formalin phosphate. The tissue was embedded in paraffin, sectioned transversely and stained with hematoxylin–eosine. A similarly prepared section was stained with Giemsa stain. Each slide was evaluated in a blind manner by two separate investigators.

Table 1
Results of treatment with compound IA on the extent of tissue damage (mean of grades as defined by Chiu et al., 1970)

Group	Mean ± S.D.
(1) Control–sham-operated	0
(2) Control–ischemia	4.13 ± 0.62
(3) Control–ischemia/reperfusion	4.70 ± 0.30
(4) IA–sham-operated	0
(5) IA–ischemia	3.65 ± 0.20
(6) IA–ischemia/reperfusion	3.75 ± 0.33
	Overall sig.: $P<0.005$

Comparison among groups. Analysis with one-way ANOVA.

Mucosal damage by each slide was graded on a six-tiered scale defined by Chiu et al. (1970), as follows:

Grade 0: Normal mucosa
Grade 1: Development of subepithelial (Gruegenhagen's) spaces near the tip of the villi with capillary congestion.
Grade 2: Extension of subepithelial space with moderate epithelial lifting from the lamina propria.
Grade 3: Significant epithelial lifting along the length of the villi with a few denuded villous tips
Grade 4: Denuded villi with exposed lamina propria and dilated capillaries
Grade 5: Disintegration of lamina propria with hemorrhage and ulceration

Polymorphonuclear leukocytes were counted per high power field of Giemsa stained in 20 separate areas of each slide immediately superior to the muscularis mucosae, and the mean number of polymorphonuclear leukocytes per high power field was determined for each animal.

2.2.3. Determination of tissue malondialdehyde

Determination of the small intestinal tissue malondialdehyde was carried out in order to estimate the extent of lipid peroxidation in the damaged tissues. Samples of the area obtained at the end of each group were frozen at −70 °C until the assay. At the day of analysis, tissue samples were washed in ice-cold NaCl 0.9%, blotted on absorbent paper and weighed. Each sample was then minced in small volume of ice-cold 20 mM Tris–HCl buffer, pH 7.4 and homogenized, in ratio 1:10 w/v, by using a Teflon pestle. After centrifugation at 3000 × *g* for 10 min at 4 °C, the clear homogenate supernatant was used for the biochemical assay.

Briefly, 0.65 ml of 10.3 mM *N*-methyl-2-phenyl-indole in acetonitrile was added to 0.2 ml of homogenate supernatant. After vortexing and adding 0.15 ml of HCl 37%, samples were mixed well, closed with a tight stopper and incubated at 45 °C for 60 min. The samples were then cooled on ice, centrifuged and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard malondialdehyde solution (from 2 to 20 nmol/ml) was also run for quantitation (Campo et al., 1998).

2.2.4. Statistical analysis

All results are presented as means ± standard deviation (S.D.). Data were compared by one-way analysis of variance (ANOVA) with Bonferroni correction and with Duncan post hoc analysis. Statistical significance was set at a value of $P<0.05$.

3. Results

3.1. Histology

The histological results are summarized in Fig. 2. Sham-operated groups (1 and 4) did not show any mucosal damage, as expected.

The results of treatment with compound IA on the extent of tissue damage (mean of grades as defined by Chiu et al., 1970) and the comparison among groups using analysis with one-way ANOVA are shown in Table 1.

The 60 min occlusion of superior mesenteric artery in groups (2) and (5) induced moderate tissue damage and

Table 2
Multiple comparisons between groups when ischemia, and/or ischemia–reperfusion were performed

	Mean difference	Std. error	Sig.	95% Confidence interval	
				Lower bound	Upper bound
(2) Control–ischemia vs. (5) IA–ischemia	0.483	0.227	N.S.[a]	−0.13	1.09
(3) Control–ischemia/reperfusion vs. (6) IA–ischemia/reperfusion	0.950	0.161	<0.0005[b]	0.52	1.38
(2) Control–ischemia vs. (3) control–ischemia/reperfusion	−0.570	0.254	N.S.[a]	−1.24	0.17
(5) IA–ischemia vs. (6) IA–ischemia/reperfusion	−0.100	0.175	N.S.[a]	−0.57	0.37

Analysis with Bonferroni post hoc correction.
[a] Not significant.
[b] The mean difference is significant at the 0.0005 level.

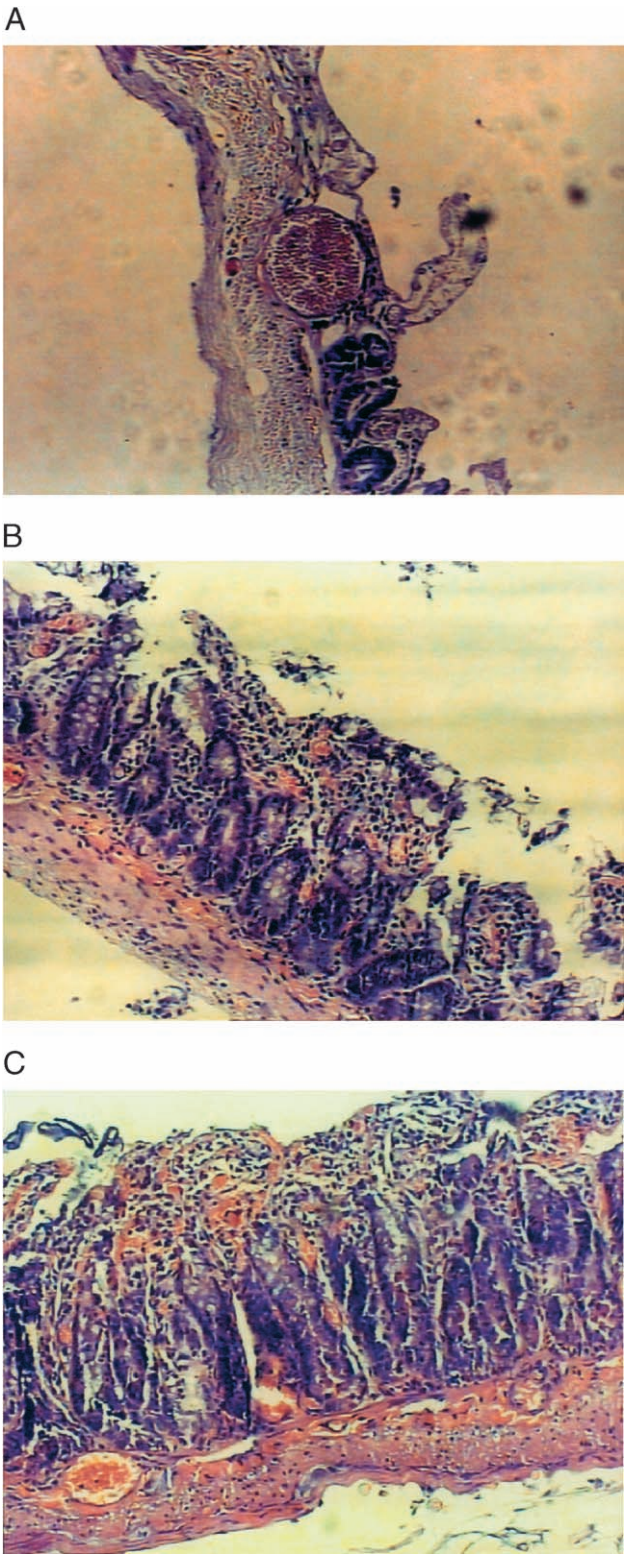

Fig. 3. (A) Control: ischemia–reperfusion. The overall cumulative score of abnormalities in the control group was higher (statistically very significant) than in IA group. (B–C) IA ameliorates the histopathological changes after 60 min of ischemia followed by 60 min of reperfusion of the intestinal tissue.

Table 3
Number of polymorphonuclear leukocytes (groups 3 and 6)

Groups	Number of polymorphonuclear leukocytes per high power field
3 (Control)	4–5
6 (IA)	1–2[a]

[a] Statistically significantly lower in IA group compared with control (Bonferroni and Duncan correction tests).

resulted in mucosa injury of mean grade, whether normal saline or compound IA was administered, respectively. There was no statistically significant difference between these groups (Table 2).

In groups (3) and (6) where 60 min of ischemia was followed by 60 min of reperfusion, the mucosal damage increased considerably. The difference in tissue injury between the IA and control group was statistically very significant ($P < 0.001$), (Table 2).

In histological sections of the groups in study (ischemia–reperfusion), we observe a complete destruction of mucosa in control group (Fig. 3A). When compound IA was administered, the mucosa remained less deranged with maintenance of normal architecture in a significant degree (Fig. 3B and C).

The number of polymorphonuclear leukocytes per high power field followed the same principals. Statistically, it was significantly lower in IA group compared with the control group (Table 3).

3.2. Protective effect of compound IA against oxidative damage in small intestine tissue after ischemia–reperfusion

The production of malondialdehyde was measured in all groups at the end of each experiment as an index of the occurrence of lipid peroxidation and the development of oxidative stress (Sato et al., 1999). The effect of the tested compound on malondialdehyde formation in small intestine tissue during ischemia–reperfusion is illustrated in Fig. 4.

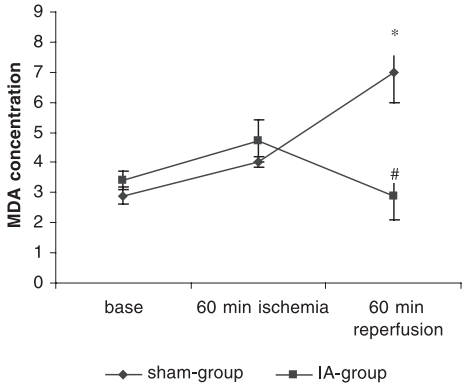

Fig. 4. Effects of compound IA on the malondialdehyde (MDA) formation during ischemia reperfusion at baseline, after 60 min of ischemia and after 60 min of ischemia followed by 60 min of reperfusion. *$P < 0.05$ versus baseline, #$P < 0.05$ compared with the control group after 60 min of ischemia followed by 60 min of reperfusion.

Increase in malondialdehyde concentration was found to be statistically significant at the end of reperfusion in the control group compared to the baseline. Upon application of compound IA, a statistically significant reduction of malondialdehyde levels was observed at the end of reperfusion ($P < 0.05$, Duncan post hoc analysis).

4. Discussion

Intestinal ischemia followed by reperfusion causes tissue damage considerably in excess of that induced by ischemia alone (Schoenberg and Beger, 1988). It has been shown that partial occlusion of the artery supplying blood to a segment of cat small intestine (hypoxia), followed by reperfusion, causes gross, histologically observable damage to the tissue and increases intestinal vascular permeability (Halliwell and Gutteridge, 1998). The potent ability of reactive oxygen species to induce injury during reperfusion in rat has been established (Osborne et al., 1994; Leung et al., 1992). Thus, research has focused on preventing ischemia–reperfusion injury by agents that inhibit or scavenge reactive oxygen metabolites (Nilsson et al., 1994). Previous studies have shown that intravenous administration of superoxide dismutase or oral administration of allopurinol (an inhibitor of xanthine oxidase) to animals before removal of the arterial occlusion decreases damage (Blikslager et al., 1997; Udassin et al., 1998).

In the present study, we showed that administration of compound IA decreased considerably the mucosal damage in rats that were subjected to 60 min of ischemia followed by 60 min of reperfusion. It has been shown that this compound inhibited the in vitro lipid peroxidation in rat liver microsomes almost completely at a concentration of 25 μM. DL-(α)-tocopherol acetate, used as reference compound, looses this inhibitory activity at a concentration of 0.5 mM. In addition, compound IA protected from the oxidative damage in the small intestine of rats under ischemia–reperfusion by inhibiting lipid peroxidation, as confirmed by the reduction of malondialdehyde levels. Since lipid peroxidation is more likely to be an important factor in the development of tissue injury during reperfusion rather than during the ischemic period (Park et al., 1994), this very strong inhibition can be one of the mechanisms of action of IA in the protection during the acute phase of the mesenteric ischemia–reperfusion.

It has been shown that catalase, an enzyme that catalyzes the reduction of hydrogen peroxide to oxygen and water, attenuates reperfusion-induced tissue permeability and mucosal damage. Similar results were obtained with dimethyl sulfoxide (DMSO), a hydroxyl radical scavenger. The protection provided by superoxide dismutase, catalase and DMSO suggests that the oxygen species inducing tissue damage is the highly cytotoxic hydroxyl radical (Schoenberg and Beger, 1988). Compound IA is a very potent hydroxyl radical scavenger. It was found that it inhibited significantly the $HO^{\cdot}$ mediated oxidation of DMSO, demonstrating a very potent hydroxyl radical scavenging activity under the experimental conditions applied (Andreadou et al., 1997).

Generation of reactive oxygen species can also occur by activation of neutrophils entering (or already present within) reoxygenated intestine and other tissues (Halliwell and Gutteridge, 1998). Neutrophils can also synthesize and release arachidonic acid products, mainly leukotriene B4 and thromboxane A_2 which are potent chemo-attractants, induce adhesion of the neutrophil to the endothelium and activation of neutrophils to produce more oxygen radicals and proteolytic enzymes (Hernandez et al., 1987; Otamiri, 1989). On the other hand, it has been shown in vitro that, due to the generation of lipid peroxides, oxygen-free radicals indirectly stimulate arachidonic acid metabolism and lead to increased concentrations of prostaglandins, thromboxane and leukotrienes, which further contribute to permeability changes and micro- and macrocirculatory derangement. However, the enhanced prostaglandin metabolism seems to be independent of the generation of lipid peroxides by oxygen radicals. Superoxide dismutase and catalase treatment before reperfusion prevent the increase in conjugated dienes in intestinal tissue, but do not influence production of prostaglandins (Schoenberg and Beger, 1988). In our study, the number of polymorphonuclear leukocytes per high power field was lower in the group treated with compound IA when compared to the control group.

Compound IA has been proved to be a potent anti-inflammatory agent, reducing the carrageenan-induced rat paw edema (Andreadou et al., 1997). Since it has also been found to inhibit lipid peroxidation and scavenge $HO^{\cdot}$, it is considered to combine beneficial properties as an anti-inflammatory agent.

In our study, we tried to study the effect of compound IA, a novel compound with antioxidant properties, on transient intestinal ischemia and reperfusion in an imitated clinical setting. The majority of previous work on intestinal ischemia presented agents administered to subjects in varying time points before ischemia. In a recent review article, it was suggested that the previously used settings were not satisfactorily proportional to real clinical conditions (Collard and Gelman, 2001). Therefore, there was a place for studies where any substances with possible antioxidant effects should be administered after ischemia had been performed, just before reperfusion (Takahasi et al., 1998). This is a setting which could be characterized as almost clinical since in every day practice, the opportunity to begin treatment before the onset of intestinal ischemia is rare.

In conclusion, several mechanisms seem to be responsible for the development of post-ischemic lesions of the intestine. Firstly, oxygen radicals, generated initially by the hypoxanthine–xanthine oxidase system, are the ‘molecular triggers’. Secondly, inflammatory mediators mostly generated by activation of phospholipase A_2 constitute the ‘enzymatic trigger’. Both these pathways lead to the accumulation and activation of neutrophils in intestinal tissue. These cells seem

to be largely responsible for the induction of severe mucosal lesions. Compound IA combines both antioxidant and anti-inflammatory properties in one molecule. Thus, it seems that it can 'target' both the 'molecular' and 'enzymatic triggers'. This beneficial effect of the compound IA on intestinal viability after acute mesenteric ischemia and reperfusion needs further investigation in order to elucidate the exact mechanism of action of this novel anti-inflammatory antioxidant agent with basic character. In addition, the administration of the antioxidant molecule IA took place after ischemia and right before reperfusion, a setting that is closer to real clinical conditions and may be used as a potential therapeutic agent for post-ischemic injury.

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