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Insights in the mechanisms underlying the anti-ulcer activity of nicorandil

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This study was conducted to investigate possible mechanisms underlying the gastroprotective effect of nicorandil on experimentally-induced gastric lesions in rats. The rats were randomly assigned to vehicle (saline or tween 80), nicorandil (2 mg/kg), glibenclamide (2 mg/kg), nicorandil plus glibenclamide- and cimetidine (50 mg/kg)-pretreated groups, in addition to the non-stressed control group, to demonstrate whether the K_{ATP} channel opening activity contributed to nicorandil's gastroprotection. Gastric lesions were induced by water immersion-restraint stress (WIRS) and ulcer indices were determined. Gastric juice parameters (pH, free and total acid output, and pepsin and mucin concentrations) were determined for each group. Another group of rats was divided into control, saline-pretreated and nicorandil (2 mg/kg)-pretreated subgroups. The rats were subjected to 5 h of WIRS and the stomachs were used for determination of gastric mucosal levels of lipid peroxides, histamine, prostaglandin E_2 (PGE_2) and total nitrites. Nicorandil displayed significant protection against gastric lesions formation. Glibenclamide, when administered concomitantly with nicorandil, abolished its protective effects. Nicorandil significantly reduced gastric acid secretion and pepsin concentration, but upon co-administration with glibenclamide, these effects were blocked. Additionally, nicorandil significantly reduced gastric mucosal lipid peroxides and total nitrites back to near normal levels and significantly increased gastric mucosal PGE_2, but did not alter significantly histamine levels. The results confirm a gastroprotective effect for nicorandil, the mechanism of which comprises K_{ATP} channel opening, free radical scavenging, PGE_2 elevation, decrease of proteolytic activity and acid output and prevention of the detrimental increase of nitric oxide during WIRS, probably, by inhibiting iNOS activity.

1. Introduction

Although the introduction of histamine H_2 receptor blockers and proton pump inhibitors has allowed great progress in the treatment of peptic ulcer, search for new drugs continues, in order to achieve greater drug efficacy and safety (Patel et al. 2001). Interest in modulating ion channels has increased over the past few years following the success of calcium channel antagonists in gastroprotection, and now considerable attention has been focused on potassium channel openers, especially the ATP-sensitive potassium (K_{ATP}) channel openers. Different researchers have demonstrated an anti-ulcer activity for some K_{ATP} channel openers like cromakalim (Goswami et al. 1997), diazoxide (Toroudi et al. 1999) and nicorandil (Sakai et al. 1999; Patel et al. 2001). Nicorandil is unique among the K_{ATP} channel openers in that its chemical structure includes a "nitrate" group. As a result, this agent is considered a hybrid between an ATP-sensitive potassium channel opener and a nitric oxide (NO) donor (Taira 1987, 1989). Nicorandil has been clinically used for the treatment of ischemic heart disease and its usefulness has been widely accepted (Akai et al. 1995).

In the light of the previous reports on the gastroprotective effects of some of the K_{ATP} channel openers and due to the unique nature of nicorandil among the K_{ATP} channel openers, this study was initiated to investigate the mechanism underlying this gastroprotection in the water immersion-restraint stress (WIRS)-induced gastric ulcer model.

2. Investigations and results

The tween 80-pretreated WIRS group did not show any significant differences as compared to the saline-pretreated WIRS group in any of the parameters investigated in this study (unpublished data), therefore the results obtained with this group were omitted from the figures for convenience.

2.1. Investigation of the involvement of K_{ATP} channels in nicorandil's gastroprotective effect on WIRS-induced gastric lesions

2.1.1. Effect of the various pretreatments on the gastric mucosal lesions formation

Fig. 1 shows the effect of WIRS on gastric lesions development and its alteration by various pretreatments. The WIRS induced marked ulcerative lesions achieving an

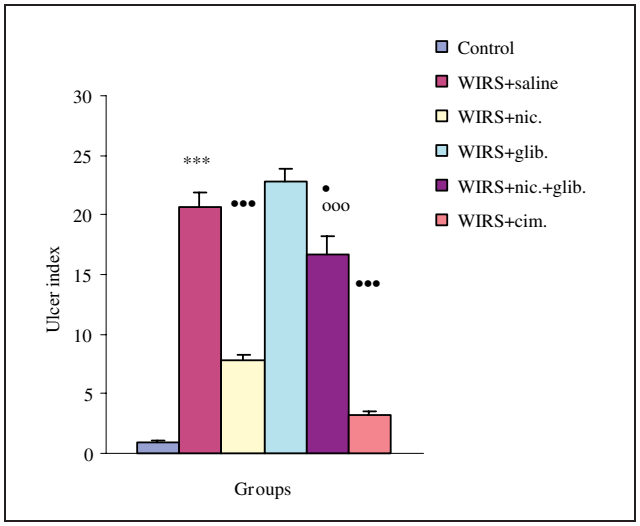

Fig. 1: Effect of WIRS on gastric lesions development and its alteration by the various pretreatments **significantly different from control group at $p < 0.001$, • significantly different from WIRS + saline group at $p < 0.05$, ••• significantly different from WIRS + saline group at $p < 0.001$ and ooosignificantly different from WIRS + nic. group at $p < 0.001$. WIRS = Water Immersion-restraint stress, glib. = glibenclamide, nic. = nicorandil and cim. = cimetidine. Values represent themean ± S.E. for 10 observations

ulcer index of 20.7 ± 1.16. Nicorandil and cimetidine significantly mitigated the development of gastric lesions by WIRS and decreased the ulcer index to 7.75 ± 0.49 and 3.15 ± 0.31, respectively. Glibenclamide alone did not significantly modify gastric lesions formation and when co-administered with nicorandil significantly attenuated the protective effect of nicorandil raising the ulcer index to 16.7 ± 1.44.

2.1.2. Effect of the various pretreatments on gastric juice pH, free and total acid outputs

Fig. 2 shows that WIRS significantly reduced the pH of gastric juice from 3.08 ± 0.08 for the control non-stressed rats to 2.12 ± 0.04. Pretreatment of stressed rats with cimetidine or nicorandil significantly elevated the pH of gastric juice to 2.78 ± 0.07 and 2.69 ± 0.06, respectively, as compared to the non-pretreated WIRS group. Glibenclamide did not significantly alter gastric juice pH of the WIRS group as compared to the non-pretreated WIRS group.

Meanwhile, concomitant administration of glibenclamide with nicorandil significantly reduced the elevated gastric juice pH achieved by the nicorandil pretreated WIRS group.

Fig. 3 illustrates the effect of WIRS on gastric juice free and total acid outputs and their alteration by various pretreatments. The WIRS significantly increased gastric juice free and total acid outputs as compared to the control non-stressed group (99.3 ± 2.66 and 109 ± 2.51 versus 61.2 ± 0.69 and 76.2 ± 2.05 μEq/5 h, respectively).

Pretreatment with cimetidine or nicorandil significantly reduced both free and total acid outputs (13.5 ± 0.47 and 18.5 ± 0.39, and 47.2 ± 2.01 and 54.4 ± 2.25 μEq/5 h for cimetidine and nicorandil, respectively) as compared to the non-pretreated WIRS group. Glibenclamide administration was injurious as it markedly increased both free and total acid outputs to 210 ± 10.5 and 241 ± 9.82 μEq/5 h. Coadministration of glibenclamide with nicorandil abolished the decrease in both free and total acid outputs induced by administration of nicorandil alone.

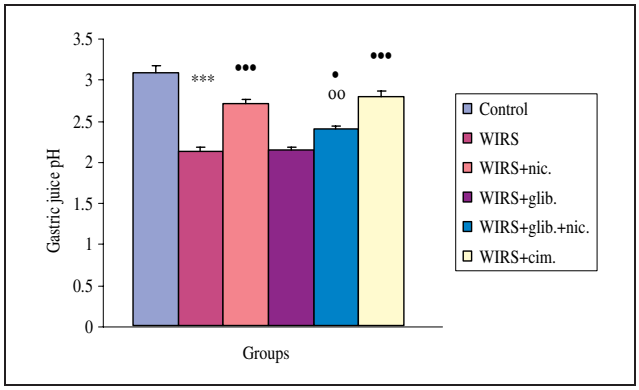

Fig. 2: Effect of WIRS on gastric juice pH and its alteration by various pretreatments **significantly different from control group at $p < 0.001$, • significantly different from WIRS + saline group at $p < 0.05$, ••• significantly different from WIRS + saline group at $p < 0.001$ and ooosignificantly different from WIRS + nic. group at $p < 0.01$. WIRS = Water immersion-restraint stress, glib. = glibenclamide, nic. = nicorandil, cim. = cimetidine. Values represent the mean ± S.E. for 10 observations

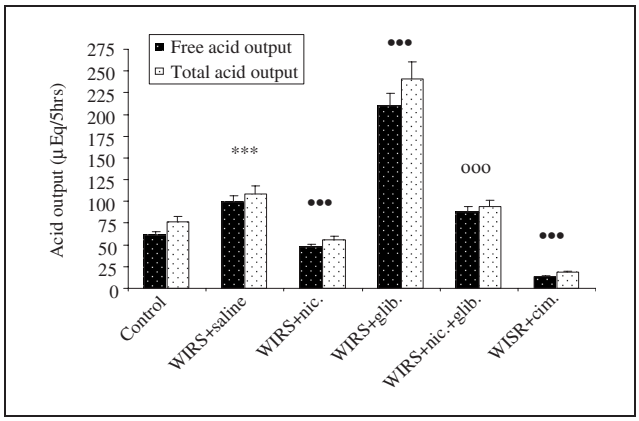

Fig. 3: Effect of WIRS and the various pretreatments on the free and total acid outputs. **significantly different from control group at $p < 0.001$, ••• significantly different from WIRS + saline group at $p < 0.001$ and ooosignificantly different from WIRS + nic. group at $p < 0.001$. WIRS = Water immersion-restraint stress, glib. = glibenclamide, nic. = nicorandil, cim. = cimetidine. Values represent the mean ± S.E. for 10 observations

2.1.3. Effect of the various pretreatments on gastric juice pepsin concentration

Fig. 4 shows that WIRS significantly increased the gastric juice pepsin concentration as compared to the control group from 123 ± 12.6 to 236 ± 21.7 μg/ml tyrosine. Pretreatment with cimetidine and nicorandil significantly reduced gastric juice pepsin concentration to 131 ± 4.42 and 139 ± 12.1 μg/ml tyrosine, respectively. Glibenclamide pretreatment did not significantly alter the gastric juice pepsin concentration as compared to the non-pretreated WIRS group. Concomitant administration of glibenclamide with nicorandil attenuated the decrease in pepsin concentration obtained with pretreatment of nicorandil alone.

2.1.4. Effect of the various pretreatments on gastric juice mucin concentration

Fig. 5 shows the effect of WIRS on the gastric juice mucin concentration and its alteration by various pretreatments. WIRS significantly reduced gastric juice mucin

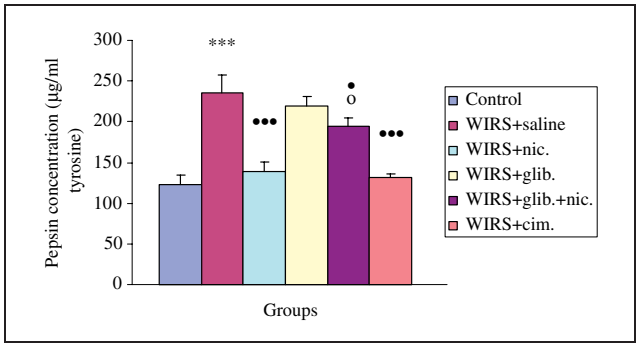

Fig. 4: Effect of WIRS and the various pretreatments on the gastric juice pepsin concentration. **significantly different from control group at $p < 0.001$, • significantly different from WIRS + saline group at $p < 0.05$, ••• significantly different from WIRS + saline group at $p < 0.001$ and °significantly different from WIRS + nic. group at $p < 0.05$. WIRS = Water immersion-restraint stress, glib. = glibenclamide, nic. = nicorandil, cim. = cimetidine. Values represent the mean ± S.E. for 10 observations

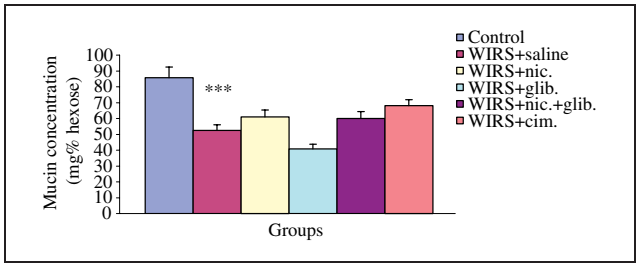

Fig. 5: Effect of WIRS on the gastric juice mucin concentration and its alteration by various pretreatments. ***significantly different from control group at $p < 0.001$ and • significantly different from WIRS + glib. Group at $p < 0.05$. WIRS = Water immersion-restraint stress, glib. = glibenclamide, nic. = nicorandil and cim. = cimetidine. Values represent the mean ± S.E. for 10 observations

concentration from 85.7 ± 6.87 mg% hexose for the non-stressed control group to 52.4 ± 3.67 mg% hexose. None of the pretreatments was able to, significantly, alter gastric juice mucin concentration as compared to the non-pretreated WIRS group.

2.2. Investigation of the involvement of other mechanisms in nicorandil's gastroprotective effect on WIRS-induced gastric lesions

2.2.1. Effect of nicorandil on the gastric mucosal lipid peroxides

Fig. 6 shows that WIRS significantly elevated the gastric mucosal MDA concentration to about three folds the value observed for the non-stressed group, reaching 65.3 ± 5.85 nmol/g wet tissue as compared to 22.7 ± 2.17 nmol/g wet tissue for the non-stressed control group. Nicorandil pretreatment significantly reduced the gastric mucosal MDA concentration to 46.0 ± 3.18 nmol/g wet tissue.

2.2.2. Effect of nicorandil on the gastric mucosal histamine concentration

Fig. 7 shows that WIRS significantly increased gastric mucosal content of histamine from 172 ± 13.2 µg/g tissue for the non-stressed control group to 262 ± 19.9 µg/g tissue. Nicorandil pretreatment did not bring about any significant change.

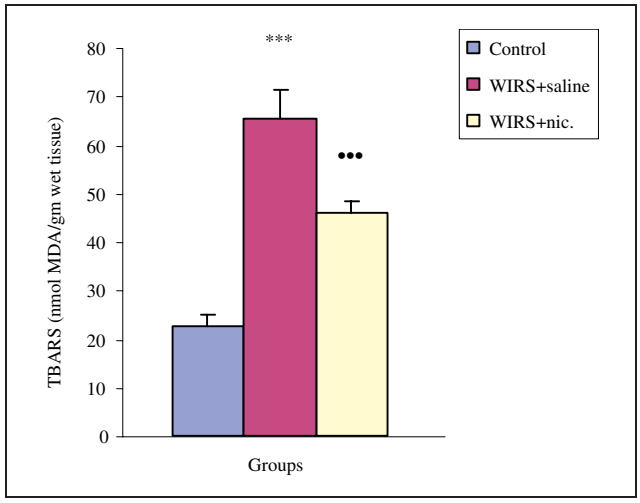

Fig. 6: Effect of nicorandil pretreatment on the gastric mucosal MDA concentration in WIRS ***significantly different from control group at $p < 0.001$, •• significantly different from WIRS + saline group at $p < 0.01$ and ••• significantly different from WIRS + saline group at $p < 0.001$. WIRS = Water immersion-restraint stress and nic. = nicorandil. Values represent the mean ± S.E. for 6 observations

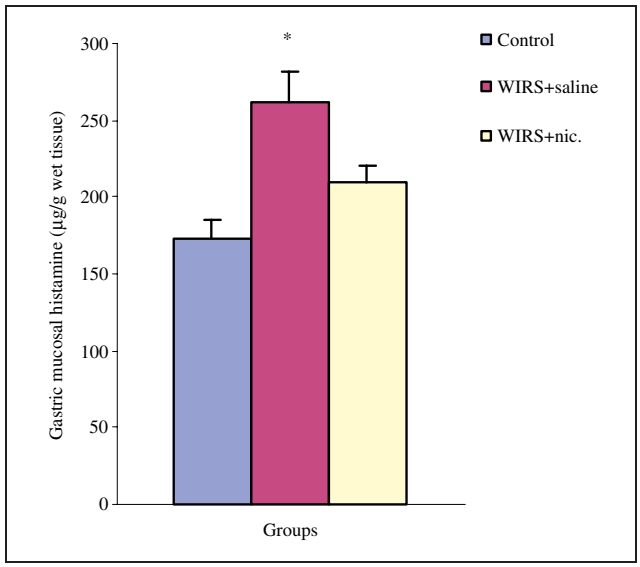

Fig. 7: Effect of nicorandil pretreatment on the gastric mucosal histamine level in WIRS *significantly different from control group at $p < 0.05$. WIRS = Water immersion-restraint stress and nic. = nicorandil. Values represent the mean ± S.E. for 6 observations

2.2.3. Effect of nicorandil on the gastric mucosal PGE_2 concentration

Fig. 8 shows that WIRS did not significantly alter gastric mucosal PGE_2 content as compared to the non-stressed control group. Administration of nicorandil significantly increased PGE_2 level to, approximately, double the control value.

2.2.4. Effect of nicorandil on the gastric mucosal nitrite concentration

Fig. 9 shows that WIRS significantly increased gastric mucosal nitrites from 168 ± 11.7 nmol/g wet tissue for the non-stressed control group to 376 ± 10.9 nmol/g wet tissue. Pretreatment with nicorandil significantly reduced gastric mucosal nitrites level to 216 ± 18.3 nmol/g wet tissue, returning it back to near normal (control) value.

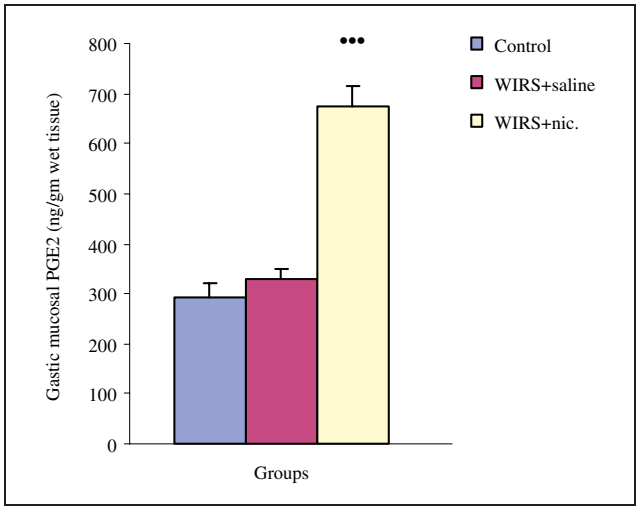

Fig. 8: Effect of nicorandil on the gastric mucosal PGE_2 level in WIRS. ••• significantly different from WIRS + saline group at $p < 0.001$. WIRS = Water immersion-restraint stress and nic. = nicorandil. Values represent the mean ± S.E. for 6 observations

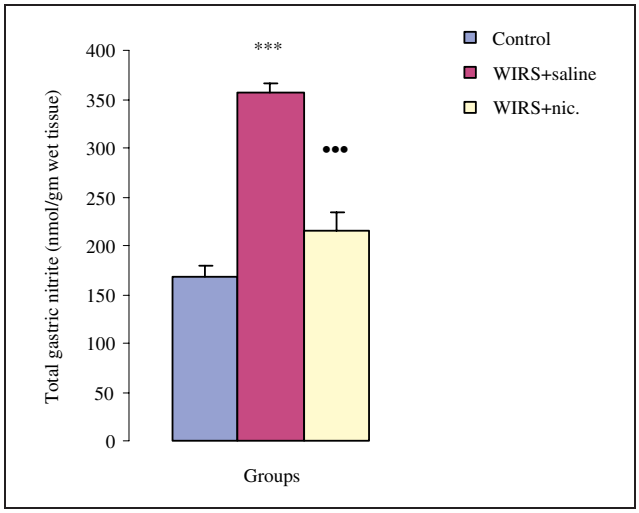

Fig. 9: Effect of nicorandil on the total gastric mucosal nitrite level in WIRS *** significantly different from control group at $p < 0.001$ and ••• significantly different from WIRS + saline at $p < 0.001$. WIRS = Water immersion-restraint stress and nic. = nicorandil. Values represent the mean ± S.E. for 6 observations

3. Discussion

This investigation examined the possible involvement of K_{ATP} channels, nitric oxide, histamine, free radical scavenging and PGE_2 in the mechanism underlying the anti-ulcer activity of a unique K_{ATP} channel opener and nitric oxide donor, nicorandil, in a popular experimental ulcer model.

The present results revealed that nicorandil, administered intraperitoneally in a dose of 2 mg/kg, significantly reduced the formation of gastric lesions in WIRS; cimetidine, also, significantly reduced the ulcer index of the gastric lesions. This protection afforded by nicorandil was abolished when coadministered with the K_{ATP} channel blocker, glibenclamide. These results suggest that nicorandil possesses a gastroprotective effect that depends, totally or partly, on its K_{ATP} channel opening activity, and that K_{ATP} channels play an important role in gastric homeostasis.

How K_{ATP} channel opening is linked to gastroprotection is still not fully understood, but K_{ATP} channel modulation has been reported to influence smooth muscle motility and gastric mucosal blood flow (GMBF), two factors that were suggested to contribute significantly to stress-induced gastric lesions formation (Garrick et al. 1986; Kitajima et al. 1991; Ito et al. 1993; Nielsen-Kudsk 1996; Sakai et al. 1999). Therefore, K_{ATP} channel openers, by preventing gastric hypermotility that is associated with WIRS and by increasing GMBF (and thus increasing oxygen and nutrient delivery to tissues allowing more resistance to injury), may protect the gastric mucosa against ulcer formation.

Gastric acid is a cardinal factor in peptic ulcer formation, hence verify the saying: "no acid no ulcer". That is the reason why regimens for peptic ulcer prevention and treatment always include acid suppressing drugs. This study clearly demonstrated that nicorandil decreased significantly gastric acid secretion during WIRS, an effect that was blocked by glibenclamide that, by itself, caused an in increase in gastric acid secretion. Similar results have been reported for cromakalim (Goswami et al. 1997). A plausible explanation for this lies in the fact that some potent gastric acid inhibitors or bicarbonate secretion enhancers act, partly, through K_{ATP} channels; those include prostaglandins (Peskar et al. 2002), adrenomedullin and calcitonin gene-related peptide (CGRP) (Rossowski et al. 1997; Sakai et al. 1999). Thus, it is reasonable to consider that nicorandil inhibited gastric acid secretion by its own K_{ATP} opening activity or by cooperating with the aforementioned mediators through opening of K_{ATP} channels, and glibenclamide prevented this effect by blocking K_{ATP} channels, thus inhibiting their action. Moreover, the results of this study indicate that gastric acid secretion may be regulated, directly or indirectly, by K_{ATP} channels. Therefore, another mechanism by which K_{ATP} channel openers may protect against gastric lesion formation is by acid suppression.

Additionally, PGE_2, that has been found to significantly increase in the gastric mucosa by nicorandil pretreatment in this study, is potent stimulator of bicarbonate secretion (Kauffman et al. 1980), therefore this might contribute to nicorandil's acid suppressing effect.

Peptic activity is an indispensable factor in the pathogenesis of ulcers is gastric acid; without pepsin gastric acid has little digestive power (Samloff 1989). In the present work, nicorandil was found to decrease significantly pepsin concentration. This effect was antagonized when glibenclamide was concomitantly administered. Glibenclamide alone did not increase pepsin concentration as was expected, but it increased significantly pepsin output (unpublished data). This may be due to the marked increase in gastric juice volume induced by glibenclamide (unpublished data) i.e. although pepsin secretion (output) has actually increased, the concentration did not increase because there was a corresponding increase in the gastric juice volume. These results may suggest that K_{ATP} channels have some direct or indirect regulatory function on pepsin secretion and that suppression of pepsin secretion might be an additional mechanism by which K_{ATP} channel openers protect against gastric lesions formation, especially in WIRS. In addition, CGRP (which acts partly through K_{ATP} channels) was found to inhibit pepsin secretion (Kraenzlin et al. 1985), therefore, similarly, modulators of K_{ATP} channels may affect pepsin secretion. Moreover, nicorandil is known to be an antagonist of the mobilization of calcium bound to intracellular storage sites (Miyamoto et al. 1992), leading to reduction of intracellular free calcium and consequently, inhibition of calcium-dependent pepsin secretion. Whether this property is

linked to nicorandil's K_{ATP} channel opening activity (and thus might be the link between K_{ATP} channel modulation and pepsin secretion) or not, is not known.

Neither nicorandil nor cimetidine caused any significant alteration in the gastric juice mucin concentration in comparison to the saline-pretreated WIRS group. This reveals that the gastroprotective action of nicorandil does not involve strengthening the mucus barrier, although an increase in PGE_2, which is reported to increase mucus secretion (McQueen et al. 1983), was observed with nicorandil administration in this study; rather, it acts to attenuate the aggressive factors like acid and pepsin.

In the second part of this work, attempts were made to discover mechanisms other than K_{ATP} channel opening that might contribute to nicorandil's gastroprotective effect. Nicorandil was reported to have free radical scavenging activity *in vitro* (Pieper and Gross 1992; Pogrebniak et al. 1992; Naito et al. 1994; Mano et al. 2000), but no reports have been published on the possible contribution of this property to its gastroprotective effect. In the present work, it has been found that nicorandil pretreatment reduced significantly the gastric mucosal MDA level, as compared to the saline-pretreated WIRS group. This suggests that the free radical scavenging property of nicorandil may be part of nicorandil's armamentarium against WIRS-induced gastric lesions; especially that free radical production has been proposed by more than one author to be a key detrimental factor in stress-induced ulcers (Nishida et al. 1997; Kwiecien et al. 2002).

Nicorandil's ability to decrease lipid peroxidation may be because it possesses in its chemical structure a nicotinamide moeity, which is a known hydroxyl radical scavenger (Pieper et al. 1992). Additionally, nicorandil possesses a nitrate moeity, which releases nitric oxide (NO) upon metabolism. Nitric oxide has been shown to act as a free radical scavenger (Rubanyi et al. 1991), and NO-donors were found to minimize free radical-induced lipid peroxidation and leukocyte-endothelial cell interactions and to hinder oxidative bursts produced by activated neutrophils and microglia (Forslund and Sundqvist 1995; Johnston et al. 1996; Kiprianova et al. 1997; Kim and Kim 1998). Indeed, nicorandil was found to have neutrophil modulating activity (Pieper et al. 1992) and to reduce lipid peroxidation *in vitro* (Naito et al. 1994) and *in vivo* in this study. In addition, nicorandil was reported to inhibit TNF-α release from macrophages (Pogrebniak et al. 1992; Heywood and Thomas 2002; Wei et al. 2003). TNF-α is a potent stimulator of neutrophil infiltration, which has been implicated in the pathogenesis of WIRS-induced gastric lesions (Hamaguchi et al. 2001). Consequently, if TNF-α release is inhibited by nicorandil, neutrophil infiltration and the oxidative burst of reactive oxygen species are inhibited.

In the present work, all pretreatments failed to change significantly the gastric mucosal histamine level in comparison to the non-pretreated WIRS group. The initial notion was that, since nicorandil is an intracellular calcium mobilization antagonist (Miyamoto et al. 1992), it might inhibit calcium-dependent histamine release through inhibiting the increase in intracellular free calcium, but no significant reduction in histamine was observed. This might be due to absence of the intracellular receptor for nicorandil in enterochromaffin-like (ECL) cells, which has not yet been known, but could be related to intracellular K_{ATP} channels that nicorandil opens.

Nicorandil significantly increased the PGE_2 level in the gastric mucosa as compared to the saline-pretreated WIRS group. Prostaglandins influence virtually every component of mucosal defense: stimulating mucus and bicarbonate secretion, maintaining mucosal blood flow, enhancing the resistance of epithelial cells to injury induced by cytotoxins and inhibiting leukocyte recruitment (Wallace and Granger 1996). The relationship between PGE_2 increase and the opening of K_{ATP} channels by nicorandil is not yet understood. Moreover, this increase in PGE_2 may be merely an indirect consequence of nicorandil gastroprotective effect and not a direct effect of the drug. Interestingly, Peskar et al. (2002) reported that prostaglandin-mediated gastroprotection is, partly, dependent on K_{ATP} channels.

In the present work, WIRS lead to a significant rise in the gastric mucosal nitrites level. Such increase during WIRS was also reported by Nishida et al. (1998) and Ohta and Nishida (2001). The previous authors also observed a drastic increase in iNOS activity associated with WIRS, and attributed the increase in mucosal nitrite level to this marked increase in iNOS activity. They also suggested that this increase in nitrites (via iNOS stimulation) contributes to gastric lesions development during WIRS.

In the present work, upon pretreatment with nicorandil, the nitrites level returned to near normal (control non-stressed) value. This was quite unexpected because nicorandil, being a NO-donor, was expected to increase the nitrite concentration, not to decrease it. What might have happened is that nicorandil could have prevented the deleterious increase in nitrites level, probably, through inhibition of the inducible nitric oxide synthase (iNOS) induction, since induction of iNOS was reported to be the source of the detrimental elevation of nitrites (Nishida et al. 1998) but certainly further studies are needed to investigate this. The TNF-α, a proinflammatory cytokine that is stimulated during WIRS (Hamaguchi et al. 2001; Kwiecien et al. 2002), is a potent stimulator of iNOS activity (Calatayud et al. 2001). Nicorandil was found to inhibit TNF-α secretion from macrophages by a mechanism that is dependent on K_{ATP} channel opening (Pogrebniak et al. 1992; Heywood et al. 2002; Wei et al. 2003), and therefore, it might inhibit iNOS through this pathway. Additionally, NO released from nicorandil might have been consumed in free radical scavenging, hindering lipid peroxidation, as observed with nicorandil pretreatment in this study. Therefore, it is suggested that nicorandil beside being a NO donor, might be an iNOS inhibitor and these two mechanisms might be involved in the gastroprotective effect of nicorandil against WIRS-induced gastric lesions by decreasing lipid peroxidation and preventing detrimental increase in iNOS activity.

The results obtained in this study confirm a gastroprotective effect for nicorandil in the studied model and the mechanism underlying this gastroprotective effect comprises K_{ATP} channel opening, free radical scavenging, decrease of both proteolytic activity and acid output, increase in PGE_2, in addition to, prevention of the detrimental increase of nitric oxide during WIRS, probably, by inhibiting iNOS activity.

4. Experimental

4.1. Drugs

Nicorandil was obtained from Torrent Pharmaceuticals (India). Cimetidine hydrochloride was obtained from Amoun Pharmaceuticals (Egypt), and glibenclamide was obtained from ICN (USA). Glibenclamide was suspended in 5% dextrose solution containing 1% tween 80. Nicorandil and cimetidine hydrochloride were dissolved in saline. Concentrations of the drugs were prepared to administer solutions or suspensions at a volume of 1 ml/kg intraperitoneally. All the drug solutions and suspensions were

always freshly prepared. Drug doses, as well as, dosage schedules were selected based on the previous studies done by Toroudi et al. (1999) and Patel et al. (2001).

4.2. Chemicals

Prostaglandin E2 kit and total nitric oxide assay kit were obtained from R & D systems, (USA). Thiobarbituric acid, bovine serum albumin, tyrosine and histamine dihydrochloride were obtained from Fluka (Switzerland); 1,1,3,3-tetramethoxypropane, orcinol and ophthaldialdehyde were obtained from Sigma-Aldrich (USA). Trichloroacetic acid, perchloric acid and diethyl ether were obtained from SDFine chemicals (India).

4.3. Animals

Male Sprague Dawley rats weighing 170–230 g were purchased from Othman Animal House (Abu Rawash, Giza, Egypt). Rats were fed a standard diet of commercial rat chow and water and left to acclimatize to the environment for at least one week prior to inclusion in the experiments. They were kept on a 12 h light/dark cycle and under conditions of controlled temperature (24 ± 1 °C).The rats were fasted for 24 h prior to the experiment in mesh-bottomed cages to minimize coprophagia. Except for the last hour before ulcer induction, water was supplied *ad libitum*. All experiments were performed during the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric functions.

4.4. Experimental procedure

4.4.1. Investigation of the involvement of K_{ATP} channels in nicorandil's gastroprotective effect on WIRS-induced gastric lesions

4.4.1.1. Pyloric ligation

For the sake of gastric juice collection, rats were pylorically-ligated under light ether anesthesia. The anterior abdominal wall was incised and the pyloric portion of the stomach was gently mobilized and ligated with a silk ligature around the pyloric sphincter taking great care not to interfere with the blood supply of the stomach and the abdominal wall incision was closed. Rats were allowed to recover from anesthesia for about 5 minutes.

4.4.1.2. Water immersion-restraint stress (WIRS)-induced gastric ulcers

Immediately after pyloric ligation, rats were subjected to restraining by fixing the four limbs to a board pre-designed for utilization in this study, and placed in a water bath maintained to the level of the xiphoid process at a temperature of 23 ± 1 °C for 5 h. Animals were divided into the following 7 groups (each group contained 10 rats):

1. Control non-stressed subgroup; in which animals were left freely wandering in their cages for 5 h after being subjected to pyloric ligation.
2. Saline-pretreated WIRS subgroup; in which rats received saline (1 ml/kg, i.p.) 30 min before WIRS.
3. WIRS + Tween 80 (vehicle of glibenclamide) subgroup; in which Tween 80 (1% v/v solution in saline, 1 ml/kg, i.p.) was administered 1 h prior to stress.
4. WIRS + nicorandil subgroup; in which nicorandil (2 mg/kg, i.p.) was administered 30 min prior to stress.
5. WIRS + glibenclamide subgroup; in which glibenclamide (2 mg/kg, i.p.) suspended in Tween 80 (1% solution, 1 ml/kg) was administered 1 h prior to stress.
6. WIRS + nicorandil + glibenclamide subgroup; in which glibendamide (2 mg/kg, i.p.) was injected. After 30 min, nicorandil (2 mg/kg, i.p.) was injected then stress was performed 30 min later.
7. WIRS + cimetidine subgroup; in which cimetidine (50 mg/kg, i.p.) was administered 30 min prior to stress.

After completion of the 5 h of stress (and pyloric ligation) rats were killed by an overdose of ether; their stomachs were removed and opened along the greater curvature and gastric content of each stomach was collected. The stomachs were washed with ice-cold saline and scored for macroscopic gross mucosal lesions.

4.4.1.3. Assessment of gastric mucosal lesions

Gastric mucosal lesions were expressed in terms of the ulcer index (U.I.) according to the method of Peskar et al. (2002) which depends on the calculation of a lesion index by using of a 0–3 scoring system based on the severity of each lesion. The severity factor was defined according to the length of the lesions. Severity factor 0 = no lesions; 1 = lesions <1 mm; 2 = lesions 2–4 mm and 3 = lesions > 4 mm. The lesions score for each rat was calculated as the number of lesions in the rat multiplied by their respective severity factor. The U.I. for each group was taken as the mean lesion score of all the rats in that group.

4.4.1.4. Collection and analysis of gastric juice

Stomachs of the rats were removed and opened along the greater curvature and the gastric juice of each stomach was collected. Gastric juice collected was centrifuged for 15 minutes at 3000 rpm to remove any solid debris and the volume of the supernatant was determined. Then the supernatant was used for the analysis of pH, free and total acid outputs, pepsin concentration (Sanyal et al. 1971) and mucin concentration (Winzler 1955).

4.4.2. Investigation of the involvement of other mechanisms in nicorandil's gastroprotective effect on WIRS-induced gastric lesions

Another group of rats was divided into 3 groups, each containing 12 rats; group 1: control non-stressed group, group 2: saline-pretreated WIRS group and group 3: nicorandil (2 mg/kg, i.p.)-pretreated group (like groups 1, 2 and 4 above but without pyloric ligation). After thirty minutes of intraperitoneal administration of saline or nicorandil (2 mg/kg) all three groups were subjected to WIRS for 5 hours (without pyloric ligation). At the end of the 5-hour stress rats were killed by an overdose of ether, their abdomens were opened and the stomachs were removed, opened along the greater curvature, washed with ice-cold saline. The stomachs for each group were divided into 2 subgroups each containing 6 stomachs: one group was reserved for determination of the malondialdehyde (MDA) concentration (Uchiyama and Mihara 1978) as a measure of lipid peroxidation, and histamine concentration (Shore et al. 1959) in the gastric mucosa and the other group for determination of total nitrites (total nitrites kit, catalog no. DE 1600, R & D systems) and PGE_2 concentrations (PGE_2 immunoassay kit, catalog no. DE0100, R & D systems) in the gastric mucosa. The stomachs of the latter group were immersed in indomethacin (10 μg/ml) and all stomachs were immediately stored at −80 °C until the time of the assays. Whenever assay kits were used, the procedures for sample preparation, extraction and assay provided by the kits manufacturers were strictly followed. Prior to the determination of total nitrites in the gastric mucosa, ultrafiltration of the samples was done using 10 000 Nominal Molecular Weight Limit (NMWL) centrifugal ultrafiltration units (ultrafree®-MC with PL-10 membrane, Millipore) as recommended by the manufacturer.

4.5. Statistical analysis of the data

Results were expressed as means ± standard error of the mean (SEM) and were analyzed for statistically significant difference using one-way analysis of variance (ANOVA) followed by the Tukey-Kramar multiple analysis post test; p values less than 0.05 were considered significant. Graph Pad Prism was used for statistical calculations (version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

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