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Short communication

Adrenomedullin modulates COX-2 and HGF expression in reserpine-injuried gastric mucosa in the rat

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Abstract

Here we show the increased hepatocyte growth factor (HGF) and cyclooxygenase-2 (COX-2) expression in gastric mucosa of rats which have developed a reserpine-induced ulcer. Such an increase of HGF and COX-2 expression was blunted in rats pretreated with adrenomedullin. Pretreatment with adrenomedullin and the adrenomedullin$_{22-52}$ fragment did not result in changes of HGF and COX-2 expression, compared to the reserpine and adrenomedullin treated group. Pretreatment with adrenomedullin and the calcitonin gene-related peptide$_{8-37}$ fragment (CGRP$_{8-37}$) increased HGF and COX-2 expression, compared to the reserpine and adrenomedullin treated group. Our results suggest that the inhibitory effect of adrenomedullin on the expression of HGF and COX-2 is mediated by CGRP receptors.

Keywords: Gastric damage; Hepatocyte growth factor; COX-2 (cyclooxygenase-2); Adrenomedullin; CGRP (calcitonin gene-related peptide) receptor

1. Introduction

Adrenomedullin, a 52-amino acid peptide isolated for the first time from human pheochromocytomas by Kitamura and colleagues (1993), is a multifactorial regulatory peptide synthesized from its larger precursor preproadrenomedullin (Kitamura et al., 1993). Adrenomedullin-like immunoreactivity has been detected in various tissues, including normal adrenal medulla, pancreas, kidney and the gastrointestinal tract (Ichiki et al., 1994; Sakata et al., 1998). Evidence suggests that adrenomedullin influences gastrointestinal activity. Central administration of adrenomedullin prevents ethanol-induced gastric damage (Kaneko et al., 1998) and also adrenomedullin is involved in repair mechanisms related to ethanol-induced gastric mucosal damage. In fact, an increase of both adrenomedullin and its receptors has been shown during gastric ulcer healing in the rat (Wang et al., 2000).

We have shown that adrenomedullin, administered subcutaneously (s.c.), prevents reserpine-induced gastric mucosal damage in the rat, either by decreasing gastric acid secretion (Clementi et al., 2002), or by increasing blood flow in the gastric mucosa (Salomone et al., 2003a). While the anti-secretory effect of adrenomedullin concerns the calcitonin gene-related peptide (CGRP) receptors, its effects on microcirculation are mediated by adrenomedullin receptors (Salomone et al., 2003b). Although a cross-reactivity between these peptides has been reported, each of them binds specific receptors (Poyner et al., 2002). The seven trans-membrane domain protein, calcitonin-receptor-like receptor, can in fact serve as a receptor for either CGRP or adrenomedullin.

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Recently, we have shown an increase in the c-Met hepatocyte growth factor (HGF) receptor expression during reserpine-induced gastric damage in the rat, suggesting that adrenomedullin and c-Met act in parallel as defence mechanisms during pharmacologically induced gastric mucosa injury (Martinez et al., 2004).

Among molecules involved in gastric ulcer healing (Tarnawski, 1993; Schmassmann et al., 1997), prostaglandins have been demonstrated to play an important role in the local inflammatory response (Reinhard et al., 1983) modulated by the rate-limiting enzyme cyclooxygenase (COX) (Vane, 1971). COX exists in two isoforms, COX-1, which is constitutively expressed, and COX-2, inducible during inflammation (Battistini et al., 1994). HGF is a peptide exerting mitogenic effects on hepatocytes and other epithelial cells which play a relevant role in the repair process related to gastric damage (Schmassmann et al., 1997). Interestingly, prostaglandins induce HGF expression in gastric fibroblasts in vitro (Takahashi et al., 1996).

The present study was aimed to evaluate the possible gastroprotective effect of adrenomedullin in the rat using the reserpine-induced ulcer model. To do this, we explored changes in some events associated with gastric ulcer healing, as the expression of either COX-2 and HGF, and investigated whether the latter were correlated to either CGRP or adrenomedullin receptors. With this aim we have studied the effects of pretreatment with either $CGRP_{8-37}$ or $adrenomedullin_{22-52}$, two peptide fragments acting respectively as specific antagonists for the CGRP and the adrenomedullin-receptors.

2. Material and methods

2.1. Animals

Male Sprague–Dawley rats weighing 180–220 g were housed in individual cages under constant environmental conditions (22±1 °C; 65±5% relative humidity; 12-h light/dark cycle). The animals, with free access to tap water until 1 h before testing, were fasted for 36 h before experiments. Adrenomedullin, $adrenomedullin_{22-52}$ and $CGRP_{8-37}$ were dissolved in normal saline and the injection volume was 1 ml/kg. Control animals received the same amount of vehicle.

2.2. Reserpine-induced gastric lesions

The method of Lau and Ogle (1981) was used. Reserpine was administered intraperitoneally (i.p.) at the dose of 25 mg/kg in 0.5% acetic acid solution. Adrenomedullin was injected s.c. at a dose of 100 ng/kg immediately before reserpine administration. This dose of adrenomedulin was selected because in our previous experiments in the rat (Clementi et al., 2002) it assured a maximal protection against reserpine-induced gastric injury. $Adrenomedullin_{22-52}$ or $CGRP_{8-37}$ was injected 10 min before adrenomedullin, at the doses of 1 μg/kg i.p. The doses of $adrenomedullin_{22-52}$ or $CGRP_{8-37}$ were selected according to previous data (Salomone et al., 2003b). Four hours later, the animals ($n=8$ for each group) were euthanized by decapitation and the stomachs were removed, opened along the greater curvature and immediately and quickly examined under a threefold magnifier. The number and the severity of lesions in the mucosa were scored blind from 0 to 5 as illustrated in the Table 1.

Once the damage was verified, four gastric tissue samples for each experimental group, were snap-frozen in liquid nitrogen and stored at −80 °C for protein extraction. The remaining four stomachs were stored at 4 °C in buffered paraformaldehyde 4% (pH 7.4) for histological and immunohistochemical procedures. Thereafter, the same samples were either processed with standard hematoxylin/eosin staining or with the dichromic method, according to Tandler et al. (1997).

2.3. Immunohistochemistry

Samples were fixed in 10% neutral buffered formalin. After an overnight wash, each tissue was dehydrated in graded ethanol and embedded in paraffin, with the anatomic orientation preserved. Sections of 3–4 μm thickness were cut according to routine procedures, mounted on silicone-coated slides and air dried. Slides were subsequently de-waxed in xylene and hydrated through graded ethanol. Sections were then incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, then rinsed for 20 min with phosphate-buffered saline (PBS) (BIO-Optica M107, Milan, Italy). High temperature antigen unmasking was conducted by microwave.

For localization of COX-2, a mouse monoclonal anti-COX-2 antibody (BD Transductions Laboratories, New Jersey, USA) was used, with a working dilution of 1:20. It was applied directly onto the sections and the slides were incubated overnight (4 °C) in a humidified chamber. Immune complexes were subsequently treated with the secondary antibody (containing anti-rabbit and anti-mouse immunoglobulins) and then detected by Streptavidin peroxidase treatment, both applied for 10 min at room temperature (LSAB 2 System-HRP, Dako-Cytomation, California, USA). After rinsing sections with three changes of PBS, the immunoreactivity was visualized by development with 20 μl 3,3′diaminobenzidine in 1ml of buffer substrate (Imidazole-HCl buffer pH 7.5 containing hydrogen peroxide) for 10 min (DAB substrate chromogen system, Dako-Cytomation, California, USA). After rinsing in distilled water, some sections were counterstained with haematoxylin. Positive controls consisted of tissue samples with known positive antigens and included sections of prostate epithelium. Negative controls were sections incubated with PBS, omitting the primary antibody.

Table 1
Evaluation of reserpine-induced gastric damage

Score	Macroscopic gastric damage (small=<2 mm; marked=>2 mm)
0	No lesions
0.5	Diffuse hyperemia
1	1 to 2 small erosions
1.5	3 to 6 small erosions
2	7 to 10 small erosions
2.5	More than 10 small erosions
3	1 marked erosion plus 0 to 4 small erosions
3.5	1 marked erosion plus 5 or more small erosions
4	2 marked erosions plus 0 to 4 small erosions
4.5	2 marked erosions plus 5 or more small erosions
5	3 or more marked erosions

2.4. Protein extraction

Gastric tissues were homogenized with a Polytron homogenizer in a lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na3VO4, 30 mM Na pyrophosphate, 50 mM NaF, 1 acid mM phenyl–methyl–sulfonyl–fluoride, 5 Ag/ml aprotinin, 2 Ag/ml leupeptin, 1 Ag/ml pepstatin, 10% glycerol and 0.2% Triton X-100. The homogenates were then centrifuged at 14,000 rpm for 10 min at 4 °C. The protein concentration of the supernatant was determined by the Bradford (1976) method.

2.5. Western blot analysis

Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and then transferred onto Hybond ECL nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK) for 1 h and analyzed by immunoblotting with a primary monoclonal mouse anti-COX-2 antibody (BD Transductions Laboratories, New Jersey, USA) (1:1000), and with a secondary peroxidase-conjugated anti-mouse (Amersham Life Science, Buckinghamshire, UK) (1:10,000) or with a rabbit polyclonal anti-HGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) followed by secondary peroxidase-conjugated anti-rabbit (Amersharm Life Science, Buckinghamshire, UK) (1:10,000). Detection was performed with a chemoluminescence assay (ECL; Amersham Italia, Milan, Italy).

All the experiments were repeated at least three times and the intensity of the signal was analysed using a digital imaging analysis system (1D Image Analysis Software; Scientific Imaging Kodak Company). β-Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal control to validate the right amount of protein loaded in the gels. Densitometric analysis is expressed as integrated density by normalizing the sample value towards the corresponding β-tubulin expression level panel C (data not shown).

2.6. Drugs

Human adrenomedullin, adrenomedullin$_{22-52}$ and CGRP$_{8-37}$ were purchased from Peninsula Laboratories Europe (Merseyside, England). Reserpine was purchased from Sigma (Milan, Italy).

2.7. Statistical analysis

Data were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Kruskall–Wallis non-parametric test, followed by Mann–Whitney post hoc *U*-test for ulcer score. *P* values lower than 0.05 were considered statistically significant.

3. Results

Morphological examination of gastric mucosa of the rats ($n=8$ for each group) showed the gastroprotective activity of s.c. adrenomedullin pre-treatment in reserpine-induced damage (ulcer score: reserpine, 3.4 ± 0.2; reserpine + adrenomedullin, 1.6 ± 0.2*; *$P<0.01$ vs. reserpine). Pre-treatment with 1 μg/kg CGRP$_{8-37}$ abolished the anti-ulcer effect of adrenomedullin, whereas pre-treatment with adrenomedullin$_{22-52}$, at the dose of 1 μg/kg, resulted less effective in such protective effect (ulcer score: reserpine + adrenomedullin + CGRP$_{8-37}$, 3.2 ± 0.2**, **$P<0.01$ vs. reserpine + adrenomedullin; reserpine + adrenomedullin + adrenomedullin$_{22-52}$, 1.8 ± 03*, *$P<0.01$ vs. reserpine).

Treatment with the gastrolesive agent reserpine resulted in increased expression of both HGF and COX-2 in the gastric mucosa. Pretreatment with adrenomedullin (100 ng/kg), in rats treated with reserpine, resulted in decreased expression of both HGF and COX-2 in the gastric mucosa compared to the reserpine-treated group (Fig. 1A,B).

On the other hand, the administration of either the adrenomedullin receptor antagonist adrenomedullin$_{22-52}$ (1 μg/kg), or the CGRP receptor antagonist CGRP$_{8-37}$ (1 μg/kg) 10 min prior to

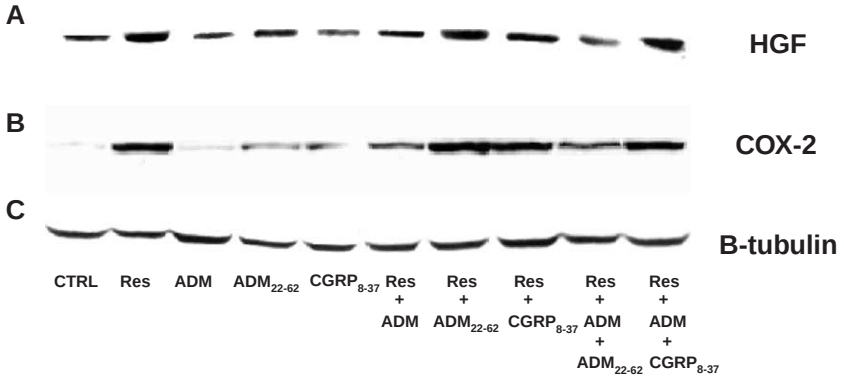

Fig. 1. Panel A. Western blot analysis of HGF in the gastric mucosa of: untreated rats (lane 1); reserpine (Res; 25 mg/kg)-treated rats (lane 2); adrenomedullin (ADM) (100 ng/kg)-treated rat (lane 3); ADM$_{22-52}$ (1 μg/kg)-treated rat (lane 4); calcitonin gene-related peptide fragment (CGRP$_{8-37}$) (1 μg/kg)-treated rat (lane 5); or rats treated with the following combinations: Res plus ADM (lane 6); Res plus ADM$_{22-52}$ (lane 7); Res plus CGRP$_{8-37}$ (lane 8); Res plus both ADM and ADM$_{22-52}$ (lane 9); or Res plus both ADM and CGRP$_{8-37}$ (lane 10). Panel B. Western blot analysis of COX-2 in the gastric mucosa of: untreated rats (lane 1); reserpine (Res; 25 mg/kg)-treated rats (lane 2); ADM (100 ng/kg)-treated rat (lane 3); ADM$_{22-52}$ (1 μg/kg)-treated rat (lane 4); CGRP$_{8-37}$ (1 μg/kg)-treated rat (lane 5); or rats treated with the following combinations: Res plus ADM (lane 6); Res plus ADM$_{22-52}$ (lane 7); Res plus CGRP$_{8-37}$ (lane 8); Res plus both ADM and ADM$_{22-52}$ (lane 9); or Res plus both ADM and CGRP$_{8-37}$ (lane 10). Panel C. β-Tubulin.

reserpine, did not affect either reserpine-induced HGF or COX-2 expression (Fig. 1A,B).

Nevertheless, when adrenomedullin was added to the combined treatment schedule consisting of reserpine and adrenomedullin$_{22-52}$, the effect of reserpine upon both HGF and COX-2 expression appeared attenuated. However, adrenomedullin was not effective in attenuating either HGF or COX-2 expression in the gastric mucosa of rats receiving a combined treatment with both reserpine and CGRP$_{8-37}$ (Fig. 1A,B).

To localize COX-2 immunoreactivity in the gastric mucosa of the small curvature, we also performed immuno-histochemical analysis. Immunohistochemistry data concur with biochemical data. In fact COX-2-like immunoreactivity was not present in the gastric mucosa of untreated animals (Fig. 2A). In the rats treated with reserpine, COX-2-like immunoreactivity was localized in the mucosal surface in the mucoid cells (green arrow), as well as in the neck gland cells (Fig. 2B). In animals treated with both reserpine and adrenomedullin (Fig. 2C), COX-2-like immunoreactivity was detected exclusively at the mucosal microvessels level (red arrow). Such immunoreactivity was extended to the submucosal connective tissue in animals receiving reserpine in combination with adrenomedullin and the adrenomedullin$_{22-52}$ fragment (green star, Fig. 2D). In rats receiving reserpine in combination with adrenomedullin and CGRP$_{8-37}$ fragment, higher magnification showed specific immunoreactivity with special regards to the bottom of mucinous glands (green arrow, Fig. 2E). Similar data were obtained using an anti-HGF antibody (data not shown).

4. Discussion

It is well known that the epithelial cells of the stomach are exposed to various toxic stimuli that may cause mucosal damage. Thus, epithelial cell proliferation becomes a crucial component of gastric ulcer healing, because it provides the elements necessary to fill the

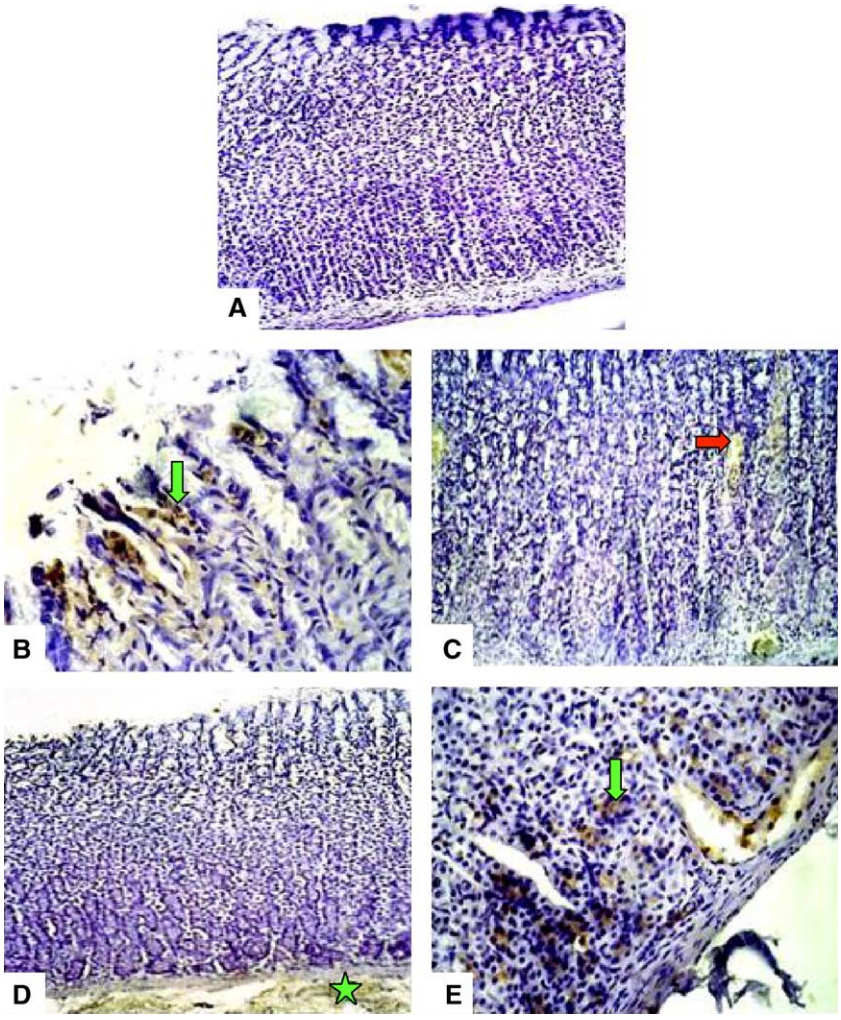

Fig. 2. A. Gastric mucosa from an untreated rat (20×). B. Gastric mucosa from a rat treated with reserpine. COX-2-like immunoreactivity is present onto the mucosa surface in both mucoid cells and in neck gland cells (40×). C. Gastric mucosa from a rat treated with both reserpine and adrenomedullin. Gastric mucosa does not show any COX-2-like immunoreactivity, whereas the latter is detected in microvessels (arrow; 20×). D. Gastric mucosa from a rat treated with the combination reserpine–adrenomedullin–adrenomedullin$_{22-52}$. COX-2-like immunoreactivity is almost exclusive to the subglandular soft connective tissue (star; 20×). E. Gastric mucosa from a rat treated the combination reserpine–adrenomedullin–CGRP$_{8-37}$. COX-2-like immunoreactivity is localized in the cells of gland bottom of the mucosa (40×).

mucosal defect, thus restoring the mucosa within the scar (Tarnawski, 1993).

Cellular and molecular mechanisms of gastric ulcer healing have been extensively studied and well characterized (Tarnawski, 1993; Schmassmann et al., 1997). Even though HGF is known to play a primary role in this repair process, it has been suggested that also prostaglandins take part in these mechanisms (Reinhard et al., 1983).

In addition, it has been shown that adrenomedullin is also involved in repair mechanisms after pharmacologically induced gastric mucosal damage (Wang et al., 2000). This is in line with the described gastrointestinal effects of this peptide (Clementi et al., 2002; Martinez et al., 1997).

In the present study, we have shown that treatment with the gastrolesive agent reserpine determines an increase in HGF and COX-2 protein expression in the gastric mucosa and that pretreatment with adrenomedullin significantly reduces the gastric damage, an effect associated with decreased expression of HGF and COX-2 in the gastric mucosa. These findings were confirmed by macroscopic and histological examination of rat gastric mucosa, showing that the number of ulcers and the actual localization of COX-2-like immunoreactivity in the gastric mucosa of rats treated with the various combinations of pharmacological agents confirms the role of adrenomedullin as a physiological modulator of mucosal cells secretive function in response to reserpine-dependent stress.

It has been demonstrated that the expression of HGF in esophageal ulcers is proportionally related to the expression of both COX-2 and c-Met (Baatar et al., 2002). In recent work we have demonstrated that c-Met expression is increased in the reserpine-induced gastric mucosa damage in the rat (Martinez et al., 2004). On a similar line here we showed that the response to pharmacological treatment of both HGF and COX-2 expression is directly related.

Interestingly, such correlation between HGF and COX-2 is in accordance with evidence suggesting that prostaglandins induce HGF expression in gastric fibroblasts in vitro (Takahashi et al., 1996).

Both CGRP and adrenomedullin are known to interact with the same calcitonin-receptor-like receptor, which can function either as a CGRP receptor or an adrenomedullin receptor, depending upon the co-expression of particular receptor-activity-modifying proteins (McLatchie et al., 1998).

To characterize which receptor type mediates the effects of adrenomedullin, we first administered the adrenomedullin receptor antagonist adrenomedullin$_{22-52}$ in parallel with the CGRP receptor antagonist CGRP$_{8-37}$. The effects of adrenomedullin upon HGF and COX-2 expression were abolished after treatment of reserpine-damaged animals with the CGRP receptor antagonist CGRP$_{8-37}$. The fact that adrenomedullin, in our experimental conditions, was not able to maintain its protective effect upon reserpine-damaged gastric mucosa in rats pretreated with CGRP$_{8-37}$, suggests an involvement of the CGRP receptor in the protective effect of adrenomedullin on gastric mucosa. On the other hand, the fact that the peptide adrenomedullin$_{22-52}$ did not abrogate the beneficial effect of adrenomedullin, confirms the lack of involvement of the adrenomedullin receptor in such process. These results are in accordance with our previous study that showed that adrenomedullin, through CGRP receptor stimulation, inhibits gastric acid secretion and thereby protects against mucosal injury induced by reserpine (Clementi et al., 2002).

In conclusion, adrenomedullin exerts its protective effects on the reserpine-induced rat gastric mucosa damage with the mediation of the CGRP receptor. These data support the hypothesis that adrenomedullin is a physiological regulator of gastric mucosal function in the rat.

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