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Short communication

Effect of adrenomedullin on c-Met receptor expression after reserpine-induced gastric damage in the rat

Giuseppa Martinez[a], Giuseppina Cantarella[b], Vincenza Maria Cutuli[b], Carla Loreto[a], Agata Prato[b], Laurence Lempereur[b], Maria Luisa Carnazza[a], Matilde Amico-Roxas[b], Renato Bernardini[b], Giuseppe Clementi[b,*]

[a] *Department of Anatomy, Diagnostics Pathology, Forensic Medicine, Public Health, School of Medicine, University of Catania, Italy*
[b] *Department of Experimental and Clinical Pharmacology, School of Medicine, University of Catania, Italy*

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Abstract

Here, we show an increase in c-Met receptor expression during reserpine-induced gastric damage in the rat, as assessed by immunohistochemistry. Pretreatment of animals with adrenomedullin prevented this increase in c-Met expression. c-Met immunoreactivity was localized in gastric glands. c-Met immunoreactivity was associated with increased phosphorylation of c-Met receptor and extracellular signal-regulated kinase ($ERK_{1/2}$). Our results suggest that both adrenomedullin and c-Met act as parallel defence mechanisms during pharmacologically induced gastric mucosa injury.

Keywords: Ulcer; Adrenomedullin; Gastroprotection; Hepatocyte growth factor; Receptor

1. Introduction

Adrenomedullin, with 52 amino acid residues, is a potent vasodilator peptide isolated from acid extracts of human pheochromocytoma (Kitamura et al., 1993). Immunoreactive adrenomedullin has been detected in a number of tissues, including adrenal medulla, heart, kidney, pancreas and bowel (Ichiki et al., 1994). Adrenomedullin is considered to be a member of the calcitonin gene-related peptide (CGRP) super-family, which also includes CGRP, amylin and calcitonin (Wimalawansa, 1997). Some studies have shown that adrenomedullin influences gastrointestinal activity. In fact, its intracerebroventricular administration inhibits gastric emptying (Martinez et al., 1997) and prevents ethanol-induced gastric injury (Kaneko et al., 1998). It has been suggested that adrenomedullin is involved in repair mechanisms after ethanol-induced gastric mucosal damage. Interestingly, an increase in adrenomedullin and its receptor expression has been shown during gastric ulcer healing in the rat (Wang et al., 2000).

We have shown that adrenomedullin injected subcutaneously prevents reserpine-induced gastric mucosal damage in the rat, probably by either reducing gastric acid secretion (Clementi et al., 2002) or increasing blood flow in the gastric mucosa (Salomone et al., 2003a). We have recently shown that the anti-secretory effect of adrenomedullin involves CGRP receptors, whereas its effect on the microcirculation involves adrenomedullin receptors (Salomone et al., 2003b).

It is well known that the epithelial cells of the stomach are continuously exposed to various toxic stimuli that may cause mucosal injury. During injury, the epithelial lining is rapidly replaced by cells that migrate from the proliferative zone of the gastric mucosa to the surface, a repair mechanism that has not yet been clarified (Terano et al., 2001). An important role in these repair processes is exerted by hepatocyte growth factor (HGF), a peptide which stimulates the proliferation of hepatocytes and other epithelial cells (Schmassmann et al., 1997). HGF elicits its biological effects by binding to and activating its receptor, c-Met (Comoglio, 1993), which is also expressed in gastro-intestinal tissues (Nishimura et al., 1998; Hori et al., 2000).

* Corresponding author. Dipartimento di Farmacologia Sperimentale e Clinica, Facoltà di Medicina, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy. Tel.: +095-7384084; fax: +095-7384226.
E-mail address: gclemen@unict.it (G. Clementi).

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In light of the gastroprotective activity of adrenomedullin and the mitogenic properties of HGF, the present study aimed to verify the activity of adrenomedullin on c-Met receptor expression in the gastric mucosa of rats undergoing reserpine-induced injury, in order to investigate a possible reciprocal role of adrenomedullin and c-Met in the repair mechanisms of the gastric mucosa in the rat.

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 were fasted for 36 h before experiments, but had free access to tap water until 1 h before testing. Adrenomedullin was 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 at the dose of 25 mg/kg in 0.5% acetic acid solution. Adrenomedullin was injected subcutaneously at the dose of 100 ng/kg immediately before reserpine administration. Four hours later, the animals were euthanized by decapitation and the stomachs were removed, opened along the greater curvature and examined under a threefold magnifier. The number and the severity of lesions in the mucosa were scored blind from 0 to 5.

Gastric tissue samples, including ulcer and immediately adjacent tissue (ulcerated tissue), and gastric tissue from sham-operated rats (vehicle) were snap-frozen in liquid nitrogen and stored at − 80 °C for protein extraction.

The stomachs were stored at 4 °C in buffered paraformaldehyde 4% (pH 7.4) for histological and immunohistochemical studies. Thereafter, samples were either processed with standard hematoxylin and eosin stain or with the dichromic method, according to Tandler et al. (1997).

2.3. Immunohistochemical staining

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).

Non-specific protein binding was reduced by incubation for 30 min with normal horse serum (5% in PBS) (Vectastain ABC kit, Vector Laboratories, Burlingame CA, USA). For localization of c-Met, a monoclonal mouse anti-c-Met antibody (Novocastra Laboratories) was used, with a working dilution of 1:20. The antibodies, stored in PBS containing bovine serum albumin and 0.05% sodium azide, were prediluted products. They were applied directly to 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 incubated for 30 min at room temperature (Vectastain ABC kit, Vector Laboratories). After rinsing of sections with three changes of PBS, the immunoreactivity was visualized by development with 0.1% 3,3′ diaminobenzidine and 0.02% hydrogen peroxidase for 30 min (DAB substrate kit, Vector Laboratories). After rinsing of sections in distilled water, some sections were counter-stained with hematoxylin.

Positive controls consisted of tissue samples with known positive antigens and included sections of prostate epithelium. Negative controls were sections incubated with normal horse serum only.

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 Na_3VO_4, 30 mM Na pyrophosphate, 50 mM NaF, 1 acid mM phenyl-methyl-sulfonyl-fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/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. Immunoprecipitation and immunoblot analysis

Equal amounts of protein were incubated overnight at 4 °C with rabbit polyclonal anti-c-Met. Complexes were precipitated with protein A-Sepharose (Sigma-Aldrich) for 2 h at 4 °C. After three washes with lysis buffer, the conjugated material was eluted from the beads by boiling in sodium dodecyl sulfate (SDS) loading buffer (20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 100 mM Tris–HCl pH 6.8, 0.2% bromophenol blue), 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 specific antibody rabbit polyclonal anti-c-Met (1:1000), the same used for immunoprecipitation, and with the secondary peroxidase-conjugated anti-rabbit. For c-Met phosphoryla-

tion, the membrane was incubated with a monoclonal anti-phosphotyrosine (PY) antibody (Amersham Life Science) diluted 1:1000. Detection was performed with a chemoluminescence assay (ECL; Amersham Italia, Milan, Italy).

2.6. Western blot analysis

For determination of ERK phosphorylation, 50 μg of protein from tissue lysate was separated by SDS–PAGE (12%), transferred to a nitrocellulose membrane and probed

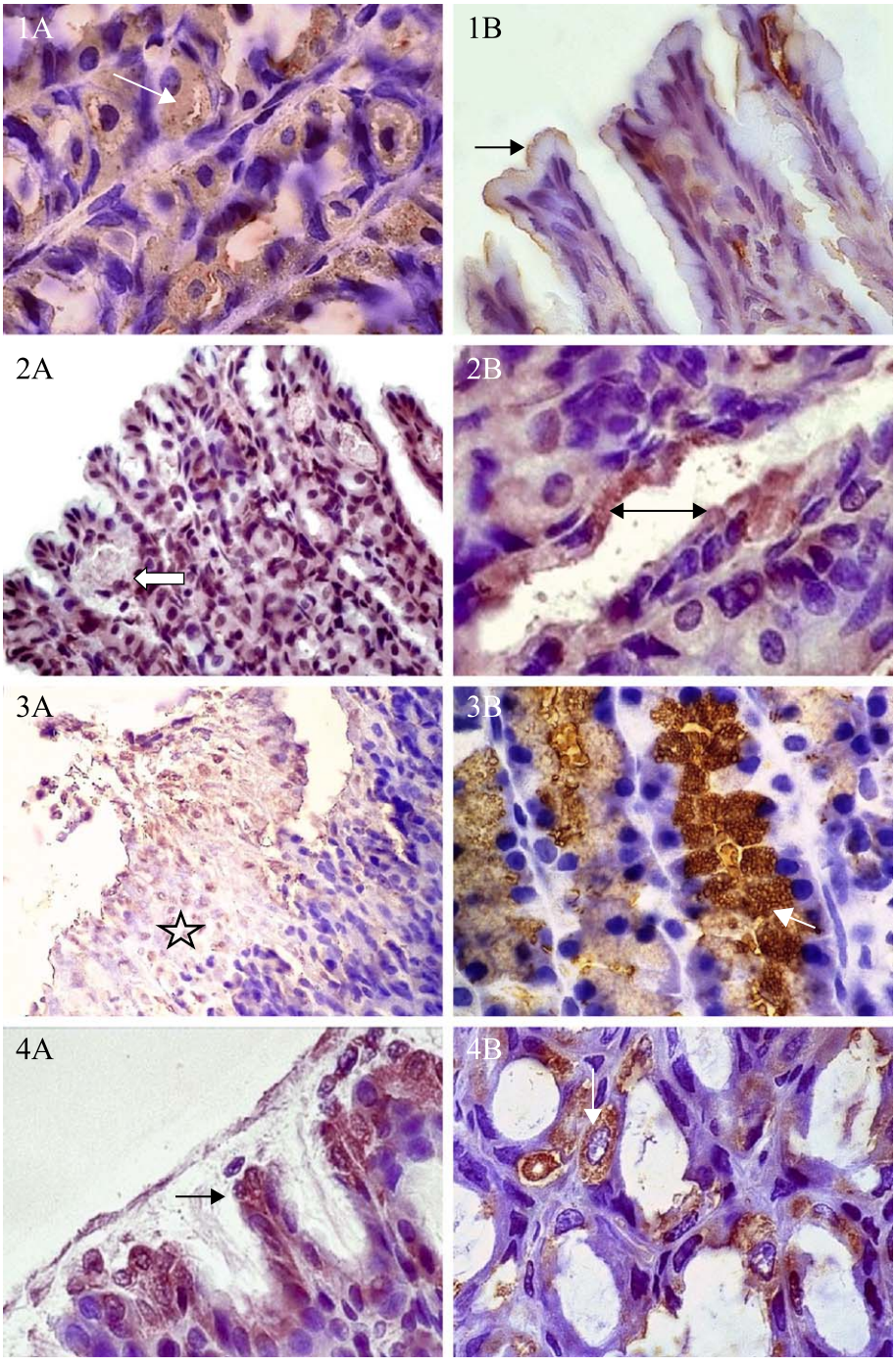

Fig. 1. Immunohistochemical expression of c-Met receptor in gastric mucosa of normal rats and after pharmacological treatment (2A, 3A, 4A) ×40; (1A, 1B, 2B, 3B, 4B) ×100. Control: (1A) White arrow indicates parietal cells showing moderate c-Met positive immunostaining. (1B) Black arrow indicates superficial epithelium. Adrenomedullin treatment: (2A) White arrow indicates vasodilatation of microvessels. (2B) Black arrows indicate c-Met expression in neck gland cells. Reserpine treatment: (3A) Black star indicates micro-hemorragic area. (3B) White arrow indicates intracellular canaliculi of parietal cells showing hypertrophy. Reserpine + Adrenomedullin: (4A) Black arrow indicates a significant mucoid cell integrity. (4B) White arrows indicates the normal function of parietal cells (transversal section).

with specific rabbit polyclonal anti-phospho-mitogen-activated protein kinase (anti-pMAPK) (New England BioLabs, Beverly, MA) (1:1000) and with the secondary peroxidase-conjugated anti-rabbit Ab (1:10 000), which was finally detected by enhanced chemiluminescence (ECL; Amersham Italia). β-Tubulin was used as a quantitative internal control.

2.7. Drugs

Human adrenomedullin was purchased from Peninsula Laboratories Europe (Merseyside, England). Reserpine was purchased from Sigma (Milan, Italy).

3. Results

Morphological examination of gastric mucosa confirmed the previously reported (Clementi et al., 2002) gastroprotective activity of subcutaneous adrenomedullin pretreatment (Ulcer score; reserpine 3.18 ± 0.22; reserpine + adrenomedullin 1.51 ± 0.26; $P < 0.01$, $n = 6$ for group).

Immunohistochemical analysis of gastric mucosa of control animals showed c-Met immunoreactivity in the superficial epithelial cells and in the gastric gland (parietal cells) (Fig. 1: 1a–1b). In rats treated with adrenomedullin, there was vasodilatation of microvessels and the expression of c-Met was comparable to that in the control animals (Fig. 1: 2a–2b).

In stomachs from rats treated with reserpine, there were some lesions, mainly in the neck area of the gland, consisting of erosions and micro-hemorrhages (Fig. 1: 3a). Expression of c-Met in the parietal cells of the gastric gland was significantly increased (Fig. 1: 3b) and involved hypertrophy of the membrane of the intracellular canaliculus (Fig. 1: 3b).

The gastric mucosa of animals treated with reserpine and adrenomedullin appeared macroscopically intact, and, microscopically, the integrity of the epithelium was preserved (Fig. 1: 4a). c-Met immunoreactivity in this group was detectable in the gastric superficial epithelium, in the tubular area of glands, as well as in parietal cells (Fig. 1: 4b).

In order to explore the functional status of c-Met receptors in the gastric mucosa of rats treated with reserpine, we studied the expression of both unphosphorylated and phosphorylated c-Met protein by Western blot. The data obtained suggest that the c-Met receptor is phosphorylated under basal conditions (untreated animals), whereas phosphorylation increases in the gastric mucosa in animals treated with reserpine, probably reflecting an increased c-Met protein level (Fig. 2A). Injection of adrenomedullin in reserpine-treated animals resulted in a decrease in the phosphorylated fraction of c-Met in the gastric mucosa (Fig. 2B), reflecting a decrease in c-Met protein level.

Downstream signalling triggered by tyrosine kinase receptors encompasses the activation of mitogen-activated protein kinase with subsequent phosphorylation of extracellular signal-regulated kinases (ERKs). Phosphorylation of the c-Met receptor was followed by $ERK_{1/2}$ phosphorylation. Untreated rats had phospho-$ERK_{1/2}$ levels similar to those of rats treated with adrenomedullin alone (Fig. 2C). The latter was able to reduce the increased phospho-$ERK_{1/2}$ levels induced by treatment with reserpine. Levels of unsphosphorylated $ERK_{1/2}$ were comparable in all groups.

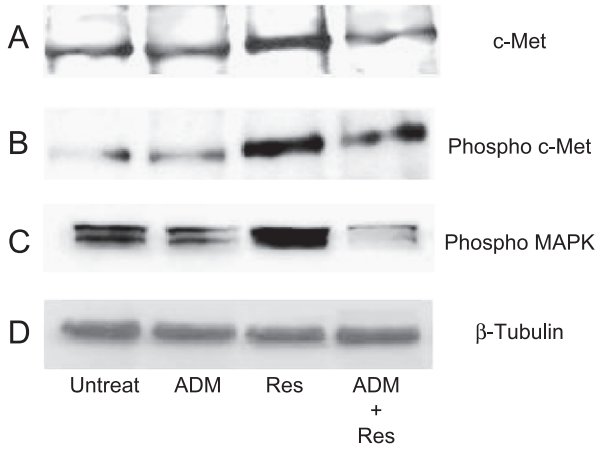

Fig. 2. (A) Western blot analysis of the c-Met receptor in untreated rats (lane 1), gastric mucosa of either adrenomedullin (ADM) (100 ng/kg)-treated rat (lane 2) or reserpine (Res) (25 mg/kg)-treated rats (lane 3) or rats treated with combination of adrenomedullin and reserpine (lane 4). (B) Western blot analysis of phosphorylated c-Met receptor in untreated rats (lane 1), gastric mucosa of either adrenomedullin (100 ng/kg)-treated rat (lane 2) or reserpine (25 mg/kg)-treated rats (lane 3) or rats treated with combination of adrenomedullin and reserpine (lane 4). (C) Western blot analysis of phosphorylated $ERK_{1/2}$ in untreated rats (lane 1), gastric mucosa of either adrenomedullin (100 ng/kg)-treated rat (lane 2) or reserpine (25 mg/kg)-treated rats (lane 3) or rats treated with combination of adrenomedullin and reserpine (lane 4). (D) β-Tubulin.

4. Discussion

Various growth factors, including epidermal growth factor and HGF, are implicated in the stimulation of epithelial cell mitogenesis during gastric healing (Tamawski et al., 1992; Schmassmann et al., 1997). Among others, HGF elicits its biological effects by binding to and activating its receptor, c-Met (Comoglio, 1993).

In this study, we show that adrenomedullin reduces the gastric damage induced by treatment with reserpine in the rat, an effect that appears parallel to the activation of the c-Met receptor. In fact, we reported here that c-Met-like immunoreactivity was increased after reserpine-induced gastric damage, while pretreatment with adrenomedullin, which possesses gastroprotective activity (Clementi et al., 2002), prevented this increase in c-Met immunoreactivity. Our results also demonstrate moderate c-Met expression in normal gastric mucosa. These data are in agreement with those of Kermorgant et al. (1997). It is plausible to hypothesize that both protective

effects are concurrent, but not overlapping, with the prevention of gastric damage.

Our data show that reserpine-induced gastric damage results in an increase in c-Met receptor expression at a gastric mucosal level, and particularly in parietal cells (acid secretion), supporting the evidence that c-Met has a role in gastric mucosa protection. However, it is well known that reserpine-induced gastric damage is due, among others, to an increase in cholinergic tone (Sandor and Cuparencu, 1977) and to a subsequent increase in acid secretion. Our data are in agreement with other evidence provided by Hori et al. (2000), suggesting that c-Met expression increases in the gastric mucosa after injury or during healing. Moreover, HGF is known to accelerate the repair of cultured gastric mucosal cells (Watanabe et al., 1994).

It has been suggested that the gastroprotective effect of adrenomedullin is mainly due to its inhibitory activity on the acid secretion, but also to its ability to improve the gastric microcirculation (Salomone et al., 2003a). Thus, we speculate that the improvement of the microcirculation induced by adrenomedullin in the mucosa bordering erosion attenuates other defence mechanisms which are normally set into motion when the gastric mucosa is damaged. This hypothesis is supported by the evidence that the increased expression of c-Met receptor did not occur in reserpine- and adrenomedullin-treated rats. The involvement of adrenomedullin in the repair mechanisms of gastric damage is suggested also by the increased expression of its receptors in the mucosa during gastric ulcer healing in the rat (Wang et al., 2000).

Since immunohistochemistry is not an appropriate method for quantitative evaluation of changes in protein expression, we additionally applied Western blot analysis. We found that the increase in c-Met-like immunoreactivity was accompanied by an increase in c-Met protein in the gastric mucosa, as assessed by Western blot analysis performed on the mucosa of rats treated with reserpine. c-Met is phosphorylated under basal conditions and in rats treated with reserpine, although to a greater extent in the latter case. Thus, an increase in c-Met phosphorylation reflects an increase in the amount of c-Met protein.

It has been observed that while adrenomedullin administered to untreated rats does not interfere with the phosphorylation of $ERK_{1/2}$, it is effective in decreasing the increase in the phosphorylated form of the kinase in rats treated with reserpine.

Interestingly, adrenomedullin has been reported to reduce $ERK_{1/2}$ activity in mesangial cells, a response associated with decreased cell proliferation (Parameswaran et al., 1999). Thus, it can be hypothesized that adrenomedullin may function as a molecule controlling the mitogenic activity of c-Met, by interfering with both c-Met receptors and $ERK_{1/2}$ phosphorylation, which are known to be activated in response to gastric tissue injury (Schmassmann et al., 1997; Baatar et al., 2002).

In conclusion, this study shows that pharmacologically induced injury of the gastric mucosa induces the expression of defence molecules, such as c-Met. This mechanism may be set in motion to trigger cell proliferation and growth to repair the damage. Taken together, our data show that gastric mucosa injury is followed, in the rat, by a repair mechanism involving parallel interactions between c-Met and adrenomedullin. These two molecules may be regarded as potential targets for novel pharmacological treatments for gastric ulcer.

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