Modulation of protein tyrosine phosphorylation in gastric mucosa during re-epithelization processes

Olena V Bogdanova, Larysa I Kot, Kateryna V Lavrova, Volodymyr B Bogdanov, Erica K Sloan, Tetyana V Beregova, Ludmyla I Ostapchenko

AIM: To investigate the role of protein tyrosine phosphorylation in gastric wound formation and repair following ulceration.

METHODS: Gastric lesions were induced in rats using restraint cold stress. To investigate the effect of oxidative and nitrosative cell stress on tyrosine phosphorylation during wound repair, total activity of protein tyrosine kinase (PTK), protein tyrosine phosphatase (PTP), antioxidant enzymes, nitric oxide synthase (NOS), 2',5'-oligoadenylate synthetase, hydroxyl radical and zinc levels were assayed in parallel.

RESULTS: Ulcer provocation induced an immediate decrease in tyrosine kinase (40% in plasma membranes and 56% in cytosol, \( P < 0.05 \)) and phosphatase activity (threefold in plasma membranes and 3.3-fold in cytosol), followed by 2.3-2.4-fold decrease (\( P < 0.05 \)) in protein phosphotyrosine content in the gastric mucosa. Ulceration induced no immediate change in superoxide dismutase (SOD) activity, 30% increase (\( P < 0.05 \)) in catalase activity, 2.3-fold inhibition (\( P < 0.05 \)) of glutathione peroxidase, 3.3-fold increase (\( P < 0.05 \)) in hydroxyl radical content, and 2.3-fold decrease (\( P < 0.05 \)) in zinc level in gastric mucosa. NOS activity was three times higher in gastric mucosa cells after cold stress. Following ulceration, PTK activity increased in plasma membranes and reached a maximum on day 4 after stress (twofold increase, \( P < 0.05 \)), but remained inhibited (1.6-3-fold decrease on days 3, 4 and 5, \( P < 0.05 \)) in the cytosol. Tyrosine phosphatases remained inhibited both in membranes and cytosol (1.5-2.4-fold, \( P < 0.05 \)) in the cytosol. Tyrosine phosphatases remained inhibited both in membranes and cytosol (1.5-2.4-fold, \( P < 0.05 \)). NOS activity remained increased on days 1, 2 and 3 (3.8-, 2.6-, 2.2-fold, respectively, \( P < 0.05 \)). Activity of SOD increased 1.6 times (\( P < 0.05 \)) days 4 and 5 after stress. Catalase activity normalized after day 2. Glutathione peroxidase activity and zinc level decreased (3.3- and 2-fold, respectively, \( P < 0.05 \)) on the last day. Activity of 2',5'-oligoadenylate synthetase increased 2.8-fold (\( P < 0.05 \)) at the beginning, and 1.6-2.3-fold (\( P < 0.05 \)) during ulcer recuperation, and normalized on day 5, consistent with slowing of inflammation processes.

CONCLUSION: These studies show diverse changes in total tyrosine kinase activity in gastric mucosa during re-epithelization processes.
the recovery process. Oxidative and nitrosative stress during lesion formation might lead to the observed reduction in tyrosine phosphorylation during ulceration.

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Key words: Protein tyrosine kinase; Protein tyrosine phosphatase; Antioxidants; Gastric ulcer; Wound repair

Peer reviewer: Martin Götte, PhD, Head of Research, Department of Obstetrics and Gynecology, Münster University Hospital, Albert-Schweitzer-Str. 33, D-48149 Münster, Germany

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INTRODUCTION

Gastric mucosa regeneration following wounding is controlled by growth factors that coordinate cell proliferation and migration after binding specific receptors on the cell surface[6]. Growth factor receptors are mostly transmembrane protein tyrosine kinases (PTKs). In gastric mucosa, physiological activity of PTKs is 20-40-fold higher than in liver or pancreas[6]. Increased tyrosine phosphorylation of proteins is necessary for physiological and pathological regeneration[5].

Intracellular gastric mucosal PTKs have been understudied, with a notable exception being cytosolic Src kinase, which regulates secretion of mucus and phospholipids - the main protective molecules of the gastric surface[6]. During ulcer recovery, Src kinase is activated by the epidermal growth factor (EGF) cascade and regulates cell migration[6]. Phosphorylation by cytosolic PTKs regulates nuclear factor κB transcription factor activity.

Changes in the level of gastric protein phosphorylation accompany numerous physiological[6] and pathological processes[7]. Protein tyrosine phosphatases (PTPs) dephosphorylate tyrosine residues and modulate proliferative signal transduction. Probably, transmembrane PTPs regulate the first steps of proliferative and migration signaling, whereas intracellular enzymes are involved in blocking signal transduction and differential regulation of nuclear factor activity. Gastric PTPs have different functions in maintaining epithelial integrity, and might be involved in malignant transformation[8].

The enzymes that control protein tyrosine phosphorylation are themselves regulated by other enzymatic systems. That regulation greatly increases under nitrosative and oxidative stress[10-12], which follows inflammation during ulcer formation. Oxidative and nitrosative cell stress leads to apoptotic or necrotic cell death, which results in epithelium desquamation, weakening of mucosal defense, and deepening of the ulcer lesion. Activation of antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase counteract oxidative stress[13]. Zinc compounds are an important component of antioxidant protection of the cell[14]. Serum zinc levels are decreased in patients with peptic ulcer[15], and introduction of zinc derivatives promotes ulcer healing[16], stimulates migration and proliferation of intestinal epithelial cells[17], and has gastroprotective actions[18].

Activation of inducible nitric oxide synthase (iNOS) occurs during inflammation. High concentrations of NO from iNOS exhibit toxic effects through nitrosative and oxidative stress[19]. Excess NO reacts with reactive oxygen species (ROS) to form highly toxic peroxynitrite. Peroxynitrite attacks intracellular structures, which affects nucleic acids, membranes, and proteins. Tyrosine nitrosylation by NO is a recently discovered mechanism of protein regulation. NO covalently modifies tyrosine residues in proteins and changes their conformation and function[20].

Inflammation during ulcerogenesis is controlled by specific cytokines, including interferons. Following binding to its receptor transmembrane PTK, interferon activates signaling pathways including the oligoadenylate cascade. A 42-kDa isoform of 2′,5′-oligoadenylate synthetase is present in the surface and glandular gastric cells[21].

Thus, proliferative signaling in gastric mucosa via protein tyrosine phosphorylation is influenced by oxidative and nitrosative cell stress caused by ulcer formation and recuperation. The study of total activities of enzymes that regulate phosphorylation of protein tyrosine residues during re-epithelization is very important for a more complete biochemical understanding of the regulation of tyrosine phosphorylation during gastric wound repair, and could contribute to improvement of ulcer treatment.

The aim of our work was to investigate the total PTK and PTP activity in gastric mucosa of rats with stress-induced gastric ulcer during the first 5 d after lesioning, and examine the activity of antioxidant enzymes, NOS and 2′,5′-oligoadenylate synthetase and levels of hydroxyl radicals and zinc.

MATERIALS AND METHODS

Reagents

Assay kits, monoclonal anti-phosphotyrosine antibodies, Fast Blue BB Salt, UDP-Glc pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St Louis, MO, USA). Other chemicals were analytical grade.

Animals

Animal experiments were carried out following the guidelines of the Taras Shevchenko National University Biological Faculty committee for ethics. Male Wistar rats (200-250 g) were maintained in experimental groups of six in a controlled environment (12 h light/dark cycle, 22°C room temperature). The control group (n = 6) was
kept in its home cage for the duration of the study. All the other animals were fasted for 12 h with water ad libitum, and underwent ulcer provocation by cold restraint stress. Gastric lesions in rats were provoked as described previously[21]. Immobilized animals were placed for 3.5 h at 4°C. Rats were sacrificed either immediately after cold restraint stress, or on consecutive days (1-5) after cold restraint stress. After sacrificing by decapitation, area and number of stomach lesions, erosions, and hemorrhages were calculated. Gastric lesions were no longer visible at 5 d after ulcer provocation.

**Plasma membranes and cytosol separation**

The gastric mucosa was separated from the stomach by mechanical scraping. Plasma membranes and cytosol of gastric mucosa cells were used for estimation of PTK and PTP activities and level of protein phosphorysity. Isolated gastric mucosa was homogenized in ice-cold 0.14 mmol/L NaCl. Homogenate was centrifuged at 20000 g for 30 min. The supernatant was applied to a 30% sucrose gradient (density 1.127) and the pellet was discarded. After centrifugation at 105 000 g for 1 h, the plasma membrane fraction was collected from the gradient. The cytosolic fraction was collected from the top of the tube. All the procedures were carried out at 4°C[22].

**Biochemical procedures**

All assays were done in triplicate. PTK and PTP activities were measured using Sigma-Aldrich Assay Kits PTK101 and PTP101, following the manufacturer’s instructions. PTK activity was calculated using standard purified EGF receptor and expressed in pmol inorganic phosphate formed per minute per milligram total protein. PTP activity was calculated using a standard calibration curve, using 0-4000 pmol standard curve, and expressed in pmol inorganic phosphate formed per minute per milligram total protein. Total protein concentration was determined using the Bradford assay[23].

The level of phosphorylated tyrosine in proteins was assayed by immunoprecipitation. The samples that contained the plasma membrane and cytosolic fractions were applied to nitrocellulose filters. After three washings, the filters were incubated with monoclonal peroxidase-conjugated anti-phosphotyrosine antibodies. Antibody hybridization was visualized using peroxidase conjugate 3,3’-diaminobenzidine tetrahydrochloride and band intensities calculated using Image J1.36b (NIH, Bethesda, MD, USA). Phosphotyrosine content in plasma membrane and cytosol proteins was calculated according to the band intensity, and presented as deviations from control values.

Hydroxyl radical levels were determined by spectrophotometric measurement of methanesulfonic acid, the product of hydroxyl radical reaction with dimethylsulfoxide (DMSO)[24]. One milliliter of 1 mol/L DMSO was injected into animals as the radical trapping agent at 30 min before sacrifice[25]. The concentration of methanesulfonic acid formed in the reaction was determined by reaction with Fast Blue BB salt, by absorbance at λ420 nm against a negative control. The quantity of hydroxyl radical was calculated against a methanesulfonic acid standard curve[26] and expressed in nmol of hydroxyl radical per milligram total protein.

SOD activity was estimated by improved spectrophotometric assay based on epinephrine auto-oxidation[27]. The oxidized epinephrine product has an absorption maximum of λ347 nm. The activity was expressed in relative units per minute per milligram total protein, where one relative unit means 50% delay in oxidized epinephrine formation. Catalase activity was determined spectrophotometrically by decomposition of hydrogen peroxide, which was visualized by complex formation with molybdate salts with an absorption maximum at λ420 nm[27]. The activity was expressed in μmol of H2O2 cleaved per minute per milligram total protein. Glutathione peroxidase activity was determined spectrophotometrically by accumulation of oxidized glutathione which has an absorption maximum at λ260 nm, and expressed in nmol of glutathione oxidized per minute per milligram total protein[28]. Activity of NOS was measured by L-arginine-dependent oxidation rate of reduced NADPH[29]. The quantity of reduced NADPH was analyzed in spectrophotometric assay at λ340 nm. The activity of NOS was expressed in nmol NADP oxidized per minute per milligram total protein.

A spectrophotometric assay was used to assay 2’,5’-oligo(gadenylate synthetase activity[30]. The inorganic pyrophosphate production during oligoadenylate synthesis has been coupled to enzymatic reaction results in a mole to mole formation of NADPH compared to the inorganic pyrophosphate through the use of the three enzymes: UDP-Glc pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The activity of 2’,5’-oligo(gadenylate synthetase was expressed in nmol inorganic pyrophosphate formed per minute per milligram total protein.

Zinc concentration was measured using an atomic absorption spectrometer (C115-M1) with acetylene-air gas mixture. Zinc concentrations were determined using a zinc standard curve[31] and expressed in micrograms per milligram total protein.

**Statistical analysis**

The data were analyzed using one-way ANOVA and Student’s t test in Statistica 5.0 software and expressed as a percentage change from the control level; *P* < 0.05 compared to the control group; ′*P* < 0.05 compared to the previous timepoint.

**RESULTS**

Cold restraint stress induced immediate focal hemorrhaging (average lesion size: 12.78 ± 1.66 mm) inside the gastric lumen (Table 1). Local bleeding stopped over 1-2 d and lesions resolved within 5 d (Table 1).
To investigate the effect of cold stress induced ulceration and wound repair on phosphorylation of gastric proteins, we used biochemical assays to examine changes in activity of proteins that regulate tyrosine phosphorylation. Cold stress resulted in immediate 40% inhibition of PTK activity in gastric mucosal plasma membranes (Figure 1A). Plasma membrane PTK activity increased over subsequent days, and peaked at twofold higher than in control rats on day 4 after cold stress. By 5 d after lesioning, plasma membrane PTK activity returned to control levels. Intracellular PTK activity (cytosolic fraction) decreased by 54% after ulcer provocation by cold stress (Figure 1A), and then returned to baseline for the first 2 d after ulcer formation. However, threefold inhibition of cytosolic PTKs was observed on days 3 and 5 of investigation, which corresponded to resolution of wound healing. Of note, both cytosolic and membrane-bound PTK activity increased in parallel at 4 d after ulcer formation.

Cold-induced ulcer formation immediately inhibited PTP activity, and PTP activity did not return to the control levels over the following 5 d of wound recovery (Figure 1B). The greatest decrease in PTP activity was observed immediately after lesioning (plasma membrane: 2.5-fold decrease, cytosol: threefold) with significant reductions also observed on day 1 (plasma membrane: 1.9-fold, cytosol: 2.5-fold) and day 5 after lesioning (plasma membrane: 2.3-fold).

Protein tyrosine phosphorylation changed significantly during stress-induced stomach ulcer formation. Lesioning induced an approximately 60% decrease in phosphotyrosine content in both plasma membrane and cytosolic proteins of the gastric mucosa (Figure 1C). During recovery, phosphorylation of tyrosine residues in plasma membrane proteins remains reduced except on days 2 and 4. Changes in plasma membrane tyrosine phosphorylation immediately after ulcer provocation, and on days 4 and 5 were consistent with plasma membrane PTK activity changes in those days. Phosphotyrosine level in cytosolic proteins increased (30%) on day 1, returned to baseline on day 2 of recovery, decreased on day 3, normalized on day 4, and decreased on day 5 with resolution of the lesions. The changes in phosphotyrosine content in cytosol proteins were consistent with intracellular PTK activity (Figure 1A).

To investigate the stress-induced changes in oxidative and nitrosative ability, we examined activity of antioxidant enzymes and levels of hydroxyl radicals and zinc at lesioning and during recovery (Figure 2A). SOD

**Table 1** Average area (mm²) of gastric mucosa lesions after ulcer provocation (mean ± SE, mm²)

| Time after ulcer provocation | Immediately | 1 d | 2 d | 3 d | 4 d | 5 d |
|-----------------------------|------------|-----|-----|-----|-----|-----|
| 12.78 ± 1.66                | 8.30 ± 1.10 | 6.50 ± 0.72³ | 5.03 ± 0.50³ | 3.15 ± 0.28³ | 1.09 ± 0.09³ |

³P < 0.05 vs the group of animals sacrificed immediately after ulcer provocation.
activity increased 1.6 times on days 4 and 5 after ulcer provocation. Activity of catalase increased in gastric mucosa immediately and 2 d after stress (30% and 32%, respectively), but decreased at 1 d after ulceration and on the day of recovery (28% and 16%, respectively).

Decreased glutathione peroxidase activity and zinc content was observed in gastric mucosa immediately after lesioning (both 2.3-fold) and 5 d later (3.3- and twofold, respectively). Levels of hydroxyl radicals increased with a 3.3-fold maximum in the mucosa in the first 3 d after cold stress.

Threefold activation of NOS was observed in gastric mucosa on the day of ulcer formation (Figure 2B). In the following days, NOS activity remained increased, especially at 1 d after stress (3.8-fold increase over control levels), but returned to control levels by day 4 after lesioning. Activity of interferon-induced 2',5'-oligoadenylate synthetase (Figure 2B) increased up to 280% after ulcer formation, and remained increased over the following 4 d, and returned to baseline by day 5, which corresponded to resolution of the lesions.

**DISCUSSION**

To understand better the role of tyrosine phosphorylation in formation and repair of peptic ulcers, we investigated the effect of cold stress induced ulceration on the activity of PTK, PTP and antioxidant enzymes in the gastric mucosa.

The dynamics of PTK modulation are consistent with a key role for growth factors in mucosal regeneration. In our study, ulcer provocation led to an immediate decrease in plasma membrane PTK activity, which then steadily increased during wound healing and mucosal recovery (Figure 1A). Consistent with this, other studies have shown maximal expression of the important mucosal growth regulator EGF at 3-4 d after gastric lesioning and decreased PTK activity in the gastric mucosa cells at 1 d after ulcer provocation. Decreased cytosolic PTK activity in the later stages of wound healing (days 3-5) might reflect recruitment and translocation of cytosolic PTK to the plasma membrane, which results in enzyme inactivation in the cytosol. Normalization of PTK activity in plasma membranes and its decline in cytosol by day 5 could reflect decreased signaling from early growth factors with resolution of the gastric lesions.

Membrane PTP activity showed parallel dynamics to membrane PTK activity (Figure 1B). Activity of both enzyme types declined considerably after stress, then gradually increase to day 4 (but not to fully restored PTP activity), and on day 5, relative inhibition was found. The observed inhibition of PTPs might be caused by oxidation of amino acid residues in the catalytic center of the enzymes with the ROS. The ROS are produced in large amounts due to the inflammatory reaction during the stress-induced ulcer processing. In our study, lesioning coincided with the hydroxyl radical increase, which provides a possible mechanism for PTP inhibition (Figure 2A). In addition, nitrosylation of active centers of PTP by peroxynitrite might inhibit PTP activity. Peroxynitrite is produced by coupling of nitrogen oxide produced by NOS, with a hydroxyl radical. The decrease in the PTP activity was less pronounced in the case of membrane-linked enzymes that might be predefined by the necessity of plasma membrane PTP participation in proliferative signaling, dephosphorylation and activation of the c-src tyrosine kinase, especially during the early stages after lesioning. Inactivation of PTPs in the cytosol and plasma membranes was more pronounced on the fifth day of recovery, which together with the data from PTK activity, was consistent with reduced signaling processes.

Variation in gastric phosphotyrosine content following lesioning probably reflected the changes in PTks and PTPs described above. Cold stress induced ulceration led to an immediate decrease in phosphotyrosine content in gastric mucosal proteins (Figure 1B). Consistent with this, reduced tyrosine phosphorylation of extracellular signal-regulated kinase (ERK)1/2 has been shown within 30 min after gastric lesioning. Similarly, reduced tyrosine phosphorylation of other signaling enzymes, especially at 1 d after stress (3.8-fold increase over the previous timepoint).

**Figure 2** Activities of superoxide dismutase, catalase, glutathione peroxidase, zinc, hydroxyl radical content (A), nitric oxide synthase and 2',5'-oligoadenylate synthetase (B) after cold-stress-induced lesioning (% change from the control level). Activities of antioxidant enzymes, hydroxyl radical and zinc content were quantified in gastric mucosa cells from control rats and immediately after lesioning (day 0) or on consecutive days after lesioning (days 1-5).

| Time after ulcer provocation (d) | % from the control means |
|---------------------------------|--------------------------|
| 0                               | 100                      |
| 1                               | 300                      |
| 2                               | 200                      |
| 3                               | 150                      |
| 4                               | 100                      |
| 5                               | 50                       |

**A**

- SOD: Superoxide dismutase
- CAT: Catalase
- GP: Glutathione peroxidase
- Zn: Zinc
- OH: Hydroxyl radical
- NOS: Nitric oxide synthase
- OAS: 2',5'-oligoadenylate synthetase

**B**

- NOS
- OAS

| Time after ulcer provocation (d) | % from the control means |
|---------------------------------|--------------------------|
| 0                               | 100                      |
| 1                               | 350                      |
| 2                               | 300                      |
| 3                               | 200                      |
| 4                               | 150                      |
| 5                               | 100                      |

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phosphorylation has been shown in other tissues following ischemia\[41\], which also accompanies stress-induced ulcer formation. Although it has been suggested that proliferative processes are accompanied by increased tyrosine phosphorylation\[12\], in our study, the absence of such an increase could be explained by the assay procedure, in which total fractions of plasma membrane and cytosol proteins were used. In that case, a relative increase in phosphotyrosine content following activation of growth factor receptors could plausibly have been compensated by decreased tyrosine phosphorylation in other cellular proteins, which was not detected due to analysis of total protein fraction. The increase in tyrosine phosphorylation of cytosolic proteins at 1 d after stress might have been due to intensive signaling through a phosphotyrosine-regulated regeneration pathway and/or inhibition of PTP (Figure 1). It is likely that activation of both cytosolic and membrane PTKs, observed at 4 d (compared with days 3 and 5), caused the relative increase of phosphotyrosine content in the proteins of gastric mucosa cells at this timepoint.

Data from our study of SOD activity in the gastric mucosal cells (Figure 2A) are consistent with previous studies that have shown activation of the enzyme in the stomach of rats after cold-induced stress\[40\]. Vascular endothelial growth factor (VEGF), “the second wave” growth factor, potentiates SOD expression\[40\], which could explain the increase in SOD activity on days 4 and 5 after ulcer provocation. It has been shown that overexpression of SOD results in decreased phosphorylation of c-Jun N-terminal kinase, p38\[45\] and ERK1/2\[45\] and also represses activating events in EGF-dependent cascades\[46\], which is consistent with the decrease in PTK activity observed by us on the fifth day after lesioning.

The role of catalase activity in the gastric mucosa during the ulcerative process is ambiguous. Uleration led to a slight increase in catalase activity in our study, and similar activation has been shown by chemical-induced ulcerative models\[47\]. Although other studies have shown decreased catalase activity in rat stomach tissues in response to cold stress\[44,49\], and patients with peptic ulcers have shown reduced gastric catalase activity\[49\], the reasons for these differences are not clear. Stress-induced changes in catalase might be linked to changes in ROS and PTK activation. Catalase activity is promoted by EGF\[49\], which is induced by stress conditions and might have been responsible for the increased catalase activity observed in the current study. Hydrogen peroxide is the substrate for catalase, and is important in receptor-like PTK activation\[10\]. Catalase-driven conversion of hydrogen peroxide prevents receptor-like PTK activation. Those enzyme dynamics could explain ulceration-induced inverse changes in activity of membrane PTKs and catalase immediately after lesioning and after 1 d (Figures 1A and 2A). Activation of catalase on day 2 after lesioning coincided with restoration of cytosol PTK activity on days 1 and 2 after stress, and might be linked to phosphorylation of catalase by non-receptor PTKs c-Abl and Arg, which is activated under ischemia-induced oxidative stress\[51\]. Similarly, p38 kinase - a downstream effector in PTK cascades - regulates the level of catalase, thereby promoting stability of its mRNA\[52\]. Normalization of catalase activity on day 3 after ulcer provocation did not prevent plasma membrane PTK activation during the later stages (days 4 and 5) of recovery.

Glutathione peroxidase is a highly sensitive antioxidant and detoxicant component of the cellular glutathione system that protects against oxidative stress\[53\]. Cold stress resulted in immediate inhibition of mucosal glutathione peroxidase activity (Figure 2A), consistent with other studies\[13,14\]. Glutathione peroxidase inhibition could result from site-specific inactivation by ROS that are produced during lesioning\[53\]. Also peroxinitrite causes the oxidation of the ionized selenol of the selenocysteine residue in the enzyme’s active site\[56\]. Selenium concentration in gastric biopsies from ulcer patients is lower than in biopsies from patients with other gastric disorders\[57\]. Non-receptor PTKs can activate glutathione peroxidase activity under oxidative stress\[48\]. In the current study, decreased activity of glutathione peroxidase immediately and 5 d after ulcer development coincided with more pronounced cytosol PTK inactivation in the gastric mucosal cells (Figures 1A and 2A).

Cold stress induced immediate hemorrhaging in the gastric mucosa. Lysed erythrocytes free hemoglobin and iron ions, which are catalysts for transformation of superoxide anion and hydrogen peroxide to hydroxyl radicals\[13,54\]. Consistent with this interaction, gastric hemorrhaging coincided with maximal increase in hydroxyl radical levels (Figure 2A). Gradual lowering of hydroxyl radical level might be explained by disappearance of mucous hemorrhaging over the next few days. Hydroxyl radical levels increased on day 5 (compared to days 3 and 4), which coincided with glutathione peroxidase inactivation and increased SOD activity, in line with the dynamics described above.

Cellular zinc levels are coupled to the activity of metallothionein proteins\[41\], which are protective during ulcerative stress\[43\]. Metallothioneins release zinc under oxidative stress, which allows oxidation of core protein and protection of the cell. Under conditions of zinc deficit, cell oxidants are released and hydrogen peroxide increases while proliferation decreases\[53\]. In the current study, cold stress induced a two-phase decline in zinc (Figure 2A). This could reflect depletion of the zinc-containing antioxidant system, which is likely to have undergone considerable oxidative damage immediately after cold stress. Zinc sequestration from other tissues could have led to the restoration of zinc content during days 1-4 after stress. The second decrease in zinc levels on day 5 might be due to exhaustion of zinc deposits in the organism. Application of zinc compounds during wound healing regulates growth factor expression and membrane stabilization\[40\]. Zinc-containing transcription factors are responsible for EGF-stimulated expression of gastrin, which promotes
proliferation of stomach cells[63]. This suggests that the reduction of membrane PTK activity immediately after stress and on day 5 might have been linked to reductions in zinc levels at these timepoints (Figures 1A and 2A). Zinc levels also correlated with glutathione peroxidase activity (Figure 2A) after lesioning. Consistent with this, zinc treatment has been shown to increase glutathione peroxidase and SOD activity in the stomach during ulceration[66]. However, in the current study, increased SOD activity was associated with reduced zinc content on day 5. This may have been due to VEGF-dependent activation of manganese-containing (rather than Zn-containing) isoforms of SOD during these stages of wound healing[64]. In the current study, reduced glutathione peroxidase activity and zinc content immediately after stress and on day 5 of wound healing coincided with reduced PTK and PTP activity and reduced phosphorysorine content in gastric mucosa (Figures 1 and 2A).

The system of nitrogen oxide synthesis is differentially regulated during ulcer development and recovery[67]. iNOS is predominantly located in inflamed ulcerated tissues, whereas constitutive forms are found in surface cells and capillaries[68,69]. Highly inducible NOS activity has been observed after formation of ulcerous lesions[65] and on days 1-3 of ulcer healing[68,69], whereas constitutive forms are inactivated. That could explain the less-pronounced increase in the activity of NOS immediately after stress than on the first day of recovery (Figure 2B). During the late stages of recovery from stress-induced gastric lesions in rats, a relative decrease in NOS activity in gastric mucosa was observed[70]. Consistent with this, total NOS activity was elevated immediately after stress and increased further on day 1 after stress, but declined with wound healing (Figure 2B).

Endothelial and neuronal NOSs are important for successful tissue healing[70]. Treatment with NO donors activates PTK-dependent protein kinases ERK and p38[71] and stimulates gastric mucin synthesis. Inhibition of p38 kinase results in a considerable delay in mucosal recuperation, and impairs NOS activity[72]. Consistent with this, the decrease in receptor-linked PTK activity on day 5 of recovery might have been a consequence of the relative decline in NOS activity on day 4 (Figures 1A and 2B). Ulceration led to opposite dynamics in PTP and NOS activity (Figures 1A and 2B). This might be due in part to oxidation of PTP catalytic residues by NO and its derivatives[73]. Alternatively, active PTPs are required for dephosphorylation and activation of constitutive NOSs that are necessary for tissue reconstitution in the later stages of healing[64]. It is plausible that the relative decline in NOS activity on days 4 and 5 of recovery might have been due to prolonged inactivation of PTPs, especially in the cytosolic fraction (Figures 1A and 2B). Nitrosylation of protein tyrosine residues by NO[71] prevents subsequent phosphorylation and impairs protein function. Consistent with this, NOS activity and phosphorysorine levels were closely coupled in the current study. NOS activity was reduced to the level of controls only on day 4. Phosphotyrosine content in membrane proteins also was normalized on that day. At other timepoints, the level of phosphorylated tyrosine in membrane proteins was lower (and NOS activity was higher) than in controls (Figures 1B and 2B). Phosphotyrosine content in cytosolic proteins decreased immediately after stress-induced lesioning and on days 3 and 5, then NOS activity is elevated; and returned to the level of controls on day 4 after stress, which coincided with NOS normalization.

Activation of 2',5'-oligoadenylate synthetase was associated with increased membrane PTK activity, especially during peak inflammation on days 3-4 after cold stress. A relative decrease in 2',5'-oligoadenylate synthetase activity during resolution of wound healing on the last day also coincided with the relative decrease in the PTK activity, possibly due to decreased phosphorylation of the enzyme by protein kinase C, one of the PTK cascade effectors[75].

In conclusion, despite modern treatment approaches peptic ulceration remains a major health problem, especially in developing countries. Enzymes that regulate tyrosine phosphorylation are crucial for tissue regeneration processes. The current study shows that interplay of multiple enzymes is crucial for coordinating repair processes during gastric mucosal wound repair. Despite repair of the physical lesion by day 5, the enzymes involved in protein phosphorylation and antioxidant metabolism had not normalized. Prolonged biochemical changes that lead to exhaustion of regenerative capacity of the gastric mucosa could be a key characteristic in the recurring chronic nature of peptic ulcer disease. Future therapies that provide support during this period could be essential for preventing acute ulcer disease from becoming chronic.

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COMMENTS

Background

Peptic ulcers are a common gastroenterological disease in developing countries in spite of development of modern therapies aimed at eradication. Gastric ulcer is a chronic disease with acute and recuperation phases. Enzymes regulating tyrosine phosphorylation are crucial for ulcer regeneration. However, little is known about the inter-communication between enzymes which regulate regenerative processes. To investigate the effect of oxidative and nitrosative cell stress on tyrosine phosphorylation during wound repair, total activity of protein tyrosine kinases (PTKs) and phosphatases as well as anti-oxidant enzymes was assayed in parallel.

Research frontiers

Gastric mucosal regeneration following peptic lesioning is controlled by growth factors through their specific receptors, which are mostly transmembrane PTKs. In the current study, the authors showed interplay of multiple enzymes, which
are crucial for modulation of protein tyrosine phosphorylation during the re-
epithelization process. **Innovations and breakthroughs**

These studies show diverse changes in total tyrosine kinase activity and
inhibition of protein tyrosine phosphatases in gastric mucosa during the
recovery process. Oxidative and nitrosative stress during lesioning may lead
to the observed reduction in tyrosine phosphorylation during ulceration.
Despite repair of the physical lesion, the enzymes involved in protein phosphorylation
and antioxidant metabolism did not return to baseline levels. Prolonged
biochemical changes that lead to exhaustion of regenerative capacity of the
gastric mucosa could be a key characteristic in the recurring chronic nature of
peptic ulcer disease.

**Applications**

The results of this study may provide a framework for development of
supportive therapies that target wound healing during the recuperation period,
and which may be essential for preventing acute ulcer disease from becoming
chronic.

**Terminology**

PTKs and phosphatases are enzymes that control phosphorylation of specific
tyrosine residues in target proteins in the cell. Protein tyrosine phosphorylation
is a common post-translational modification that can create novel recognition
motifs for protein interactions and cellular localization, affect protein stability,
and regulate enzyme activity.

**Peer review**

This is a potentially interesting study that investigated the activities of several
enzymes and modulators of tyrosine phosphorylation during gastric mucosa
re-epithelialization in a rat model, using classical biochemical techniques.
The strength of this study is the contribution to a more complete biochemical
understanding of the regulation of tyrosine phosphorylation during gastric
wound repair. The methods used are suitable to address this question. The
data demonstrate significant changes in several factors that modulate tyrosine
phosphorylation, and these data are largely well explained in the discussion.

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