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### Expression of Reactive Oxygen Species in Reflux Disease

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#### 1. Introduction

Reflux of acidic gastric contents, or bile and pancreatic enzymes into the esophagus may cause mucosal inflammation (esophagitis or red streak). This disorder is commonly called gastroesohageal reflux disease (GERD), and in signs of esophageal mucosal injuries the disorder is called 'erosive reflux disease' (ERD). If the reflux is frequent and long standing such episodes can elicit severe inflammation or damage of the esophageal squamous epithelium (1-2).

Oxygen is a requirement for life but oxygen metabolites can cause serious tissue injuries. It is normal for the immune system to respond to injury to the mucosa or pathogens by producing oxygen and nitrogen radicals. Reactive oxygen species (ROS) are an often-used term that includes true radicals that have unpaired electrons as well as chemicals that can gain or loose electrons. Oxidative stress is a general term used to describe the steady state of oxidative damage in a cell, tissue or organ, caused by ROS. If there is an unbalance between the production of ROS and the systems ability to detoxify the reactive species or easily repair the resulting damage, oxidative stress is caused and this is a reality in most living organisms. ROS are used in immune system to attack and eliminate pathogens but ROS are also involved in the development of many diseases such as atherosclerosis and cancer (3-5).

There are many different sources by which ROS are generated. Among a lot of enzymes and molecules that cause oxidative stress there are three major enzymes; myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and nitric oxide syntase (NOS) that produce the products superoxide ( $O_{2^-}$ ), hydrogen peroxide ( $H_2O_2$ ), nitric oxide (NO) and hypocloric acid (HOCl).

We have previously shown that extremely high levels of NO are formed when nitrite in swallowed saliva meets acidic refluxates in the distal esophagus (6). NO has radical-characteristics and has been suggested to initiate esophageal carcinogenesis (7). This view may be questioned because luminally formed NO appears to be quite harmless and is rapidly eliminated during normal tissue conditions. However, in inflamed tissue with production of ROS it is reasonable to assume that luminal NO will react with particularly  $O_{2^-}$  and form the extremely labile oxidising compound peroxynitrite (ONOO-). Such oxidative species have potential roles in all steps of carcinogenesis including DNA

mutation, activation of proto-oncogenes and inactivation or loss tumor suppressor genes (5, 7).

The aim of the present study is to elucidate the presence of radical producing enzymes, represented by NADPH oxidase, MPO and iNOS and also the expression of radical formation marker for ONOO- activity as well as nitro radical end products; nitrotyrosine. The second aim is to elucidate the histological changes and number of inflammatory cells as well as the expression of inflammatory markers IL1β and IL6 in human esophageal biopsies from healthy volunteers and patients with ERD.

#### 2. Material and methods

#### 2.1 Subjects and tissue

Biopsies were taken from healthy controls (n=7, mean age 36, 4 female) and patients with ERD (n=13, mean age 48, 3 female). The biopsies were collected during endoscopy and specimens were immediately frozen in liquid nitrogen (for later western blot analysis) or snap frozen in RNA STAT-60 (Nordic Bio Site AB, Stockholm, Sweden) (for later rt-PCR analysis) and subsequently stored in liquid nitrogen or fixed in buffered 4% formaldehyde (for later histo-morphology evaluation). The biopsies were taken in the 3 o'clock position 2 cm proximal to the gastro esophageal junction, and in the altered area for the esophagitis (red streak)(8). Protein expressions of MPO, NADPH oxidase, iNOS and nitrotyrosine were assessed by western blot technique and immunohistochemistry. Expression of the IL1 $\beta$  and IL6 were assessed by both western blot and rtPCR.

The Los Angeles classification was used by the endoscopist to decide the degree of esophageal inflammation (1). All patients who contributed for this study were classified as LA-A.

*Ethics.* All participants had given informed consent and the study had been approved by Ethical Committee of Göteborg University and was performed in accordance with the Declaration of Helsinki.

#### 2.2 Western blot analyses

The frozen specimens were sonicated in a PE buffer (10 mM potassium phosphate buffer, pH 6,8 and 1mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1propane sulphonate (CHAPS: Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete (Roche Diagnostics AB, Stockholm, Sweden). The homogenate was then centrifuged (10,000 g for 10 min at 4°C) and the supernatant was analysed for protein content by the Bradford method and stored at -80°C (9). Samples were diluted in SDS buffer and heated at 70°C for 10 min before they were loaded on a NuPage 10% Bis-Tris gel, and electrophoresis run using a MOPS buffer (Invitrogen AB, Lidingo, Sweden). One lane of each gel was loaded with prestained molecular weight standards (SeeBlue, NOVEX, San Diego, CA, USA). A positive control was loaded on each gel (Table 1). After the electrophoresis the proteins were transferred to a polyvinyldifluoride transfer membrane, Hybond, 0.45µm, RPN303F, (Amersham, Buckinghamshire, UK) using an iBlot (Invitrogen AB). Membranes were than incubated with polyclonal specific antibodies directed at the MPO, NADPH oxidase (p47phox-subunit), iNOS, nitrotyrosine, IL-1β and IL-6 respectively (Table 1). An alkaline phosphatase conjugated goat anti-mouse or goat antirabbit IgG antibody (Santa Cruz) and CDP-Star (Tropix, Bedford, MA, USA) were used as a

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substrate to identify immunoreactive proteins by means of chemiluminescense. Images were captured by a Chemidox XRS cooled CCD camera, and analyzed with Quantity One software (BioRad laboratories, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Imgenex, San Diego, CA, USA) was used as control for equal loading, and for each tested sample the ratio of primarily antibody/GAPDH was used.

| Target protein                                 | Primary antibody   | Positive control                               |  |
|--|--|--|--|
| MPO  | Antimyeloperoxidase<br>07-496<br>Upstate/Millipore                 | Hl-60<br>sc-2209<br>Santa Cruz                 |  |
| Nitrotyrosine                                  | Antinitrotyrosine<br>06-284<br>Upstate/Millipore                   | Nitrotyrosine<br>12-354<br>Upstate             |  |
| iNOS   | Transinos<br>N 32030<br>TransductionLab/BioSite                    | RAW 264.7<br>sc-2212<br>Santa Cruz             |  |
| <b>NADPH-oxidase</b><br>(p47 <sup>phox</sup> ) | H-195<br>sc-14015<br>Santa Cruz                                    | Hl-60<br>sc-2209<br>Santa Cruz                 |  |
| GAPDH  | Glyceraldehyde-3-<br>phosphate<br>dehydrogenase<br>Imgenex/BioSite | Loading control<br>for western blot            |  |
| IL-1β  | Interleukin-1 Beta<br>Sc-52012<br>Santa Cruz                       | 1-1 Beta Serum was used as<br>positive control |  |
| IL-6   | Interleukin-6<br>Sc-28343<br>Santa Cruz                            | Serum was used as positive control             |  |

MPO; myeloperoxidase, iNOS; inducible nitric oxide synthase, NADPH-oxidase; nicotinamide adenine dinucleotide phosphate oxidase, GAPDH; Glyceraldehyde-3-phosphate dehydrogenase, IL; interleukin

Table 1. Antibodies and controls used in Western Blot analyses and immunohistochemistry

#### 2.3 Immunohistochemistry

The mucosal specimens were fixed in buffered 4% formaldehyde and embedded in paraffin. Sections for immunohistochemistry (3µm) were deparaffinized and then boiled for 15 min in 10mM citrate buffer (pH 6.0) for antigen retrieval. The Immunocruz TM Staining System (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the immunohistochemistry protocol. After inhibition of endogenous peroxidase activity, the slides were pre-incubated with serum block and then incubated with primary antibodies against MPO, NADPH oxidase iNOS and nitrotyrosine (see table 1) over night in dilutions of 1:200, 1:50, 1:1000 and

1:500 respectively. Control sections were incubated with normal rabbit or mouse IgG  $0.4\mu g/\mu L$  instead of the primary antibody. After being washed, the slides were incubated with biotinylated secondary antibody and the complex was detected using horseradish peroxidase (HRP)-streptavidin. The colour was developed using 3,3'-diaminobenzidine.

#### 2.4 Reverse transcriptase polymerase chain reaction

The biopsy was snap frozen in liquid nitrogen. Frozen tissue was homogenised and total RNA was extracted according to the methods suggested by the manufacturer, following phenol-chloroform extraction and ethanol precipitation. Reverse transcription from 2.5 μg of total RNA was carried out using the SUPERSCRIPT<sup>TM</sup> First-Strand Synthesis System (Invitrogen, Lidingö, Sweden) with Oligo (dT) Primers (Life Technologies, Täby, Sweden). Resulting cDNA was stored at – 20°C until use.

Lightcycler Q-PCR (Roche Diagnostics AB, Stockholm, Sweden) was performed using the FastStart DNA Master SYBR Green I (Roche Diagnostics AB, Stockholm, Sweden). PCR was performed containing 2  $\mu$ l of each RT sample using the hot-start technique. MgCl2 concentration was optimised to 4 mM to obtain the highest signal intensity and lowest background. For each tested sample the copy number of the PCR products was calculated by dividing these values by the genometric mean copy number of the reference gene (GAPDH). The quantification was performed by the software supplied by Roche Diagnostics (Mannheim, Germany). The primer sequences, PCR products sizes and references are listed in Table 2.

|       | rtPCR  |           |           |
|-------|--|-----------|-----------|
|       | Primer sequences   | Size (bp) | Reference |
| IL-1β | F:5´- aaacagatgaagtgctccttccag-3´<br>R:5´- tggagaacaccacttgttgctcca-3´ | 388       | 10        |
| IL-6  | F:5´- ggtcaggggtggttattgcatc -3´<br>R:5´- tgtgtgaaagcagcaaagaggc-3´    | 276       | 10        |
| GAPDH | F:5´- cccatcaccatcttccaggag-3´<br>R:5´- gttgtcatggatgaccttggcc-3       | 284       | 11        |

IL; interleukin, GAPDH; Glyceraldehyde-3-phosphate dehydrogenase

Table 2. rt-PCR-related information

#### 2.5 Histology

The fixed biopsy were dehydrated and embedded in paraffin. A histo-pathologist evaluation of mucosal inflammation and histological changes were performed in coded three-micron sections stained with eosin-hematoxylin. Histological evaluation of inflammation (number of mucosal lymphocytes, plasma cells and eosinophilic granulocytes) and morphometric investigations concerning: basal cell layer thickness (BCL), papillary length (PL), total epithelium thickness, and dilatation of intracellular spaces (DIS), were performed on the mucosal specimens.

#### 3. Statistics

Significant differences for multiple independent groups of observation were identified using Kruskal-Wallis and contrasted by Mann-Whitney U-test. Significant differences for dependent

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group of observation was identified using Wilcoxon's signed rank test. Nonparamitric correlation analysis was performed by Spearman's rank correlation test. A p-value  $\leq 0.05$  was considered to be of statistical significance.



Fig. 1. Typical western blot for MPO (Panel A), NADPH oxidas (Panel B), iNOS (Panel C), nitrotyrosine (Panel D), IL-1 $\beta$  (Panel E) and IL-6 (Panel F) with a band at 60 kDa, 66 kDa, 130 kDa, 47 kDa, 31 kDa and 21.5 kDa respectively, in the positive control cell lysate HL-60, cell lysate 12-354, mouse macrophage cell line RAW 264.7 and serum, and in human esophageal mucosal biopsy retrieved during endoscopy

#### 4. Results

#### 4.1 Biochemical signs of inflammation

A typical western blot for MPO, NADPH oxidase, iNOS, nitrotyrosine, IL-1 $\beta$  and IL-6 are shown in figure 1A-F. A significant increase of MPO protein expression (ERD, p=0.001, red streak, p=0.005) as well as a significant increase of nitrotyrosine expression (ERD, p=0.05, red streak, p=0.05) was detected in ERD-patients compared healthy controls using western blotting (figure 2A and D). No significant differences in expression of NADPH oxidase, iNOS (figure 2B and C), IL1 $\beta$  or IL6 were detected (data not shown). However at gene expression level using rt-PCR technique, IL6 was significantly increased in ERD-patients (p=0.01), whereas there was no difference in IL1 $\beta$  between the groups at gene level (data not shown).

Immunohistochemistry performed on endoscopically retrieved mucosal biopsies revealed a distinct staining for MPO in epithelial lymphocytes and also weak staining in the squamous epithelial cells (figure 3 A). Immunostaining for NADPH oxidase was detected in basal layer of the epithelium and papillae (figure 3B), whereas iNOS was localized mainly to the upper and mid-zone layer in the epithelium (figure 3C). Nitrotyrosin immunoreactivity was detected in the epithelium in both the basal and in the upper layer (figure 3D).

#### 4.2 Histological signs of inflammation

No signs of active inflammation, defined as presence of lymphocytes, amount of eosinophiles granulocyts or plasmacells, were detected in the esophageal mucosa (table 3).



A. Control; B. EKD; C. Red Streak

Fig. 2. Western blot analysis of the MPO (Panel A), NADPH oxidas (Panel B), iNOS (Panel C) and nitrotyrosine (Panel D) in human esophageal biopsies taken from macroscopically normal squamous mucosa in healthy subjects (n=7), patients with erosive reflux disease (ERD)(n=13) and from the red streak areas in ERD-patients (n=13). Tissue sample were taken in the 3 o'clock position 2 cm proximal to the gastro esophageal junction. Significant differences are indicated with asterisks (\*=p≤0.01; Mann-Whitney U-test). The median value in each group is indicated

|                           | Control  | ERD     | Red streak     |
|---------------------------|----------|---------|----------------|
| Mucosal lymphocytes       | 6.29±2.0 | 5.8±0.9 | 8.9±2.1        |
| Eosinophilic granulocytes | 0        | 4.6±3.1 | $0.67{\pm}0.4$ |
| Plasma cells              | 0        | 0       | 0              |

ERD; Errosive reflux disease

Table 3. The number of inflammatory cells in human esophageal mucosa



Fig. 3. Immunostaining of the human esophageal epithelium at X40 magnification with brown indicating positive immunoreactivity. (Panel A) Staining for MPO was localized in lymphocytes (arrow) as well as in upper layer epithelial cells. (Panel B) Immunostaining for NADPH oxidas is most obvious in basal epithelial cells and around the papillae (see arrows). (Panel C) Staining for iNOS was localized in stratum superficiale and spinosum (arrow). (Panel D) Immunoreactivity for nitrotyrosine is most obvious in upper layer epithelial cells and around the papillae

#### 4.3 Histology

Histological signs of erosive mucosal disease were confirmed. In the red streak, the squamous epithelium showed a significantly thicker basal cell layer (p=0.016), longer papillae (p=0.001), thicker total epithelium thickness (p=0.016) and wider intercellular space (p=0.003) compared to biopsies taken in healthy control subjects (Figure 4A-D). Furthermore, also when compared to unaffected squamous mucosa in ERD-patients the epithelium showed a significantly thicker basal cell layer (p=0.022), longer papillae (p=0.022), and thicker total epithelium thickness (p=0.001), compared to biopsies from healthy controls (Figure 4A-D).



A. Control; B. ERD; C. Red streak

Fig. 4. Histological appearance in biopsies from macroscopically normal squamous mucosa of control subjects and patients with erosive reflux disease (ERD), and from the red streak areas in ERD-patients. Tissue sample were taken in the 3 o´clock position 2 cm proximal to the gastro esophageal junction. (Panel A) thickness of basal cellular layer, (Panel B) length of intraepithelial papillae, (Panel C) total epithelial thickness, and (Panel D) dilatation of intracellular space. Significant differences are indicated with asterisks (\*=p $\leq$ 0.01; Mann-Whitney U-test, #=p<0.01; Wilcoxon's signed rank test). Data is showed as means±SEM

## 4.4 ROS expression correlation analysis with histopathological alterations and immunocells

Correlation analysis revealed a positive correlation of increased expressions of nitrotyrosine with the histopathological alteration PL (r=0.65, p=0.05) and total epithelium thickness (r=0.73, p=0.025) in biopsies taken in the red streak area from ERD-patients (Figure 5A, B). No other correlation was found nor for the histopathological alterations or the number of inflammatory cells (data not shown).

#### 5. Discussion

This study is attempted to elucidate the total generation of ROS that is produced by enzymes and molecules in the esophageal mucosa. In the present exploration of the human

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Fig. 5. Positive correlation of nitrotyrosine expression with histopathological changes papillary length (A) and total epithelium thickness (B) in biopsies taken in the red streak area from patients with erosive reflux disease

esophageal mucosa a number of reactive changes were observed. Firstly, the histological signs of erosive mucosal diseases were confirmed, also in macroscopically normal squamous epithelium from ERD-patients were changed. Secondly, there were clear signs of increased radical forming capacity in the epithelium despite absence of histological inflammation. Taken together these findings indicate that a significant change of the esophageal mucosa had occurred in association to ERD.

Nitration of tyrosine results in nitrotyrosine. The nitrate is mainly donated by peroxynitrite, however, nitrotyrosine formation is not solely generated by ONOO-. NADPH oxidase is a transmembrane electron transport chain, and the active NADPH oxidase catalyzes the production of O<sub>2</sub>- that serves as starting material for the production of different ROS (12). Oxidation of arginine by NOS creates the gas NO. NO reacts rapidly with O<sub>2</sub>- to produce the extremely reactive radical ONOO- which also can protonate and dissociate to give nitrogen (NO<sub>2</sub>) (5). MPO reacts with H2O2 formed by the respiratory burst to generate HOCl that further can form nitryl chloride (NO<sub>2</sub>Cl) (13). NO<sub>2</sub> and NO<sub>2</sub>Cl can then nitrogenate tyrosine to form nitrotyrosine. It follows that all enzymes used in the study; MPO, NADPH oxidase, and iNOS activity may be involved in the formation of nitrotyrosine.

Nitrotyrosine is a very stable altered amino acid (an addition of a nitro group to the benzene ring of tyrosine) that can be found both as a single amino acid and belong to a complete protein in the cell. It has been found in elevated levels in a lot of inflammatory conditions like atherosclerosis, rheumatoid arthritis, influenza, pancratitis, cholecystitis, Diabetes Mellitus, ulcerative colitis and Crohn's disease (5, 14-15) and is therefore a good indicator of the radical production. In the present study nitrotyrosine showed elevated levels in both the red streak and in the squamous epithelium taken from the ERD-patients compared to controls. A number of studies have shown that MPO is elevated in reflux esophagitis, Barrett's esophagus and adenocarcinoma (16-17). Eero *et al.* suggest that MPO is a key component of the pathway leading to oxidative stress and damage in the esophageal mucosa. They shown a step-by-step increase in MPO activity related to the severity of reflux disease (17). The expression of MPO was significant increased in the present study in both the red streak and

in the squamous epithelium from ERD-patients, however no correlation was found between MPO and the morphological changes.

The expression of NADPH oxidase was not increased in the ERD-patients compared to the healthy individuals. This could be compared with studies made on gastritis caused by Helicobacter Pylori where NADPH oxidase was significant higher in infected patients compared to healthy volunteers (18). Our finding may perhaps suggest the possibility that the reflux of acid is not strong enough as triggering, compared to pathogens. A recent study made by Feagins LA et al. have found that different components of gastric juice, acidic media or acidic bile acid media, induce ROS production through different mechanisms (19). Moreover, no signs of active inflammation were detected in the esophageal mucosa, defined as presence of lymphocytes, amount of eosinophiles granulocyts or plasmacells. The present study is also made on patients with mild esophagitis, classified to LA-A by the endoscopist (1). Several studies have looked at pro-inflammatory cytokines expression along the inflammation- metaplasia- dysplasia- adenocarcinoma sequence in the esophagus and have also found a stepwise-elevated expression correlate to grade of severity of the disease (20-21). Such association was not made in the present study for IL-1 $\beta$  and IL-6 at protein level, whereas gene transcript for IL-6 was increased in ERD-patients indicating somewhat small alteration may exist beyond the detections level for protein.

We have previously shown that two sources of NO formation exist in esophagus, both dependent on the presence of acid in the esophageal lumen; enzymatic degradation of L-arginine by NO synthase and non-enzymatic NO-production their nitrite from the salvia is reduced when it meets the extremely low pH in refluxate, a mechanism related to dietary intake of nitrate (6). The sources of body nitrate are intake through drinking water and vegetables, and endogenous synthesis (22). Vegetables vary greatly in their nitrate content, and water nitrate content also varies with geographical location (22). It follows that luminal NO formations differ between individual dependent on nitrate intake during acidic reflux.

Enzymatic NO formation is constantly expressed but may be activated upon presence of acid in esophageal lumen (6). Several studies have described the expression of iNOS in esophageal squamous epithelium which have been associated with pathological condition such as cell transformation but also suggested a function related to epithelial integrity (6, 23-24). Thus, our results confirm the expression of iNOS in the surface epithelium. However, neither the biopsies taken from ERD-patients nor the red streak areas in ERD-patients were significant different in iNOS expression compared to controls.

The topographical organisation of the iNOS in the epithelium in combination with luminal non-enzymatic NO, may create particular conditions for NO gradients through the mucosa. Immunoreactivily to MPO and NADPH oxidase was also found in the surface epithelium. Therefore we could assume that during gastric acidic reflux huge level of NO is formed simultaneous with production of epithelial mucosal ROS leading to increased formation of ONOO-, which may contribute to cellular injury and DNA damage (7, 25).

MPO, NADPH oxidase and iNOS are usually found in phagocytes including neutrophils, eosinophils, monocytes and macrophages with the primary function of phagocytosis and destruction of microorganisms (26). However, except MPO that was localized in both lymphocytes and epithelial cells, immunostaining for NADPH oxidase and iNOS was *only* found in the esophageal epithelial cells. Thus, the presently increased radical forming capacity in the epithelium is independent of inflammatory cells.

In the morphological investigation of reflux signs we found that all parametric used, dilatation of intracellular spaces, papillary length, basal cell layer thickness, and total epithelium thickness were significantly increased in the distal esophagus in ERD-patients

compared healthy subjects. Moreover, in this study the parametric dilatation of intracellular space was significantly increased at the "red streaks" of the distal esophagus of ERD-patients compared to biopsies from adjacent normal-locking epithelium. The above findings confirm previous observations for biopsies taken in the red streak and in 3 o'clock position 2 cm proximal to the gastro esophageal junction (27). Strong support for the involvement of nitrotyrosine in the pathogenesis of mucosal abnormalities in the red streak was obtained using correlation analysis.

In conclusion, there were clear signs of increased radical forming capacity in the epithelium despite absence of histological inflammation, in association to ERD. The histomorphological changes in ERD associating with nitrotyrosine expression, thus mirroring the foregoing radical formation, may suggest a role in the pathogenesis of esophagus. During acidified refluxes, epithelial ROS production in combination with luminal NO formation, may constitute aggravated factors in the carcinogenic process.

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