Dramatic diurnal variation in the concentration of the human trefoil peptide TFF2 in gastric juice

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Abstract

Background—TFF2, a member of the trefoil factor family of proteins, is a glycosylated protein of 106 amino acids. It is secreted by gastric antral and pyloric glands and by Brunner's glands of the duodenum. TFF2 is found in high concentrations around sites of ulceration. It stimulates cell motility and is probably the principal cytoprotective trefoil peptide in the stomach.

Aims—To determine if production of TFF2 follows a circadian rhythm and to measure changes in secretion of TFF2 in response to food intake and during sleep.

Subjects—Young healthy adults were recruited. They were asymptomatic and were not receiving medication. The 24 hour regimen was designed to allow normal stimulation of gastric secretion in response to food intake and sleep. Gastric juice was collected two hourly via a nasogastric tube.

Methods—Glycosylated and non-glycosylated TFF2 proteins were measured by quantitative western transfer analysis. The results were analysed statistically using SPSS software.

Results—There was a dramatic diurnal variation in the concentration of TFF2. The mean concentration was lowest in the early evening (0.29 µg/ml), increased gradually during the evening, and then sharply during the night to reach 7.9 µg/ml. The ratio of glycosylated to nonglycosylated TFF2 varied and was higher during the night than in the afternoon. pH, total protein, and pepsin concentrations in gastric juice did not vary significantly over 24 hours.

Conclusion—The data suggest that diurnal variations in TFF2 secretion occur independently of pepsin and gastric acid secretion. The concentration of glycosylated TFF2 in the gastric lumen falls in response to food intake. TFF2 secretion increases during inactivity and sleep. These results suggest that secretion of TFF2 in the stomach is highest during the night and that the cytoprotective effects of TFF2 on the gastric mucosa occur mainly during sleep.

Keywords: trefoil protein; human TFF2; gastric acid; diurnal; pepsin; circadian rhythm

TFF2, originally called human spasmylytic polypeptide, was the second member of the human family of trefoil peptides to be identified. There are three human trefoil proteins whose genes are clustered on chromosome 21, with TFF1 12.5 kb upstream of TFF2 which is ~30 kb upstream of TFF3. TFF2 is the second member of the diurnal; pepsin; circadian rhythm

The first indications of the biological role of trefoil peptides came from immunohistochemical and in situ hybridisation studies of their expression in various pathologies of the gastrointestinal tract. High concentrations of TFF2 mRNA are found in cells adjacent to sites of mucosal injury in the duodenum, small intestine, and colon. This ectopic expression suggested that trefoil peptides have a role in tissue repair. Following acute mucosal injury, repair of the mucosa of the gastrointestinal tract is initiated within a few minutes. One of the earliest processes is rapid migration of cells from the margins of the damaged region over the denuded area to re-establish epithelial continuity, a process called epithelial restitution. The possibility that trefoil peptides stimulate this migration was suggested by the observation that TFF2 was motogenic for cells in culture. Recombinant TFF2 stimulated human colonic carcinoma cells to migrate across and invade into collagen gel, and stimulated their migration across plastic in an in vitro assay of wound repair. TFF2 may be of particular importance among the trefoil peptides in the repair process as it is produced...
within 30 minutes after cryoprobe induction of gastric ulceration in the rat.17

Studies with recombinant human TFF214 have shown that TFF2 protects rats both from indomethacin and restraint induced, and from ethanol induced gastric mucosal damage.15 18 19 Further evidence for the protective role of TFF2 is provided by the demonstration from Ussing chamber experiments that TFF2 reduces proton permeation through gastric mucus in vitro and in vivo.20 It has also been suggested that the normal role of trefoil peptides may be to maintain the integrity of the gastrointestinal epithelial layer.14 15

We have shown recently that significant quantities of 12 kDa TFF2 are present in human gastric juice.21 Larger quantities of a protein of higher apparent molecular mass were also detected. This was shown to be N-glycosylated TFF2, glycosylated on Asn15 which forms part of the single N-glycosylation consensus site in the TFF2 protein (fig 1A). The majority of human TFF2 present in gastric mucosa is glycosylated.21 Glycosylation may be of functional importance and it has been shown that glycosylated recombinant TFF2 is more potent than the non-glycosylated protein in protection against indomethacin induced gastric damage in vivo.21 22 Secretion by gastric cells of acid, pepsin, intrinsic factor, and gastrointestinal regulatory peptides is tightly regulated. Acid and pepsin are secreted following food intake while secretion of other proteins follows a circadian pattern. In the present study, TFF2 protein has been quantified in gastric juice collected from young healthy subjects over a 24 hour time period. Meals were standardised to allow measurement of changes in TFF2 levels in response to food intake. The changes in TFF2 concentrations were related to pH, and to total protein and pepsin concentrations in gastric juice.

Methods

COLLECTION OF GASTRIC JUICE SAMPLES

Twelve volunteers aged 20–24 years (mean age 21) were recruited into the study. Four were female and eight were male. All volunteers were asymptomatic, were not receiving medication, and had no significant past medical history. They were all non-smokers. All volunteers gave written informed consent. Ethics permission was obtained.22 No alcohol consumption was permitted. The volunteers went to bed at 11:30 pm and were all asleep at 1:00 am.

Samples of gastric juice were aspirated via the nasogastric tube using a 20 ml sterile syringe at two hourly intervals. Samples were filtered to remove particulate material. This did not affect the TFF2 concentration. The pH of each sample was measured and 1 ml of each sample was then diluted 10-fold with 55 mM sodium chloride, 55 mM sodium acetate, and acetic acid, pH 4.1, to inactivate but not denature pepsin. This stabilises both TFF2 and pepsin and does not interfere with the subsequent assay of either protein. Each diluted sample of gastric juice was divided into four aliquots for storage at −20°C.

MEASUREMENT OF TFF2

Aliquots of diluted gastric juice were passed into sterile water by gel filtration over Sephadex G25 (Pharmacia) and concentrated by vacuum evaporation. Samples were electrophoresed on polyacrylamide gels that contained 0.1% sodium dodecyl sulphate as described previously.23 The stacking gels contained 10% (w/v) acrylamide, the separating gels contained 20% (w/v) acrylamide, and both contained 10% glycerol. Samples to be fractionated were brought to 62.5 mM Tris HCl, pH 6.8, 12.5 mM EDTA, 100 mM β-mercaptoethanol, and 0.005% bromophenol blue and boiled for five minutes prior to loading. Molecular weight markers and recombinant TFF2 were included in all gels.

The separated proteins were transferred from the gels to 0.2 µm pore size PVDF membranes (Schleicher and Schuell) using a semi dry transfer apparatus (Schleicher and Schuell) for 15 minutes at 100 mA, and left uncovered at room temperature overnight. They were fixed in 0.2% glutaraldehyde for 45 minutes, blocked with 3% (w/v) bovine serum albumin in phosphate buffered saline for one hour at 37°C, and incubated with a 1:1000 dilution of mouse polyclonal anti-TFF2 antisera in 3% (w/v) bovine serum albumin and phosphate buffered saline for two hours at 37°C. The mouse antisera was raised against the corrected 16 amino acid carboxy terminal peptide EVP-WCFFPKSVEDCHY of human TFF2 (see fig 1A) conjugated to keyhole limpet haemocyanin.24 The membranes were incubated for a further two hours at 37°C with alkaline phosphatase conjugated rabbit antimouse IgG in phosphate buffered saline containing 0.1%
Results

MEASUREMENT OF GLYCOSYLATED AND NON-GLYCOSYLATED TFF2 IN GASTRIC JUICE

To detect human TFF2 in the gastric juice samples, proteins from equal volumes of each sample were separated by denaturing polyacrylamide gel electrophoresis, transferred to PVDF membranes, and reacted with antiserum as described in the methods section. An example of the results obtained for one of the 12 individuals is shown in fig 1B. Two major TFF2 immunoreactive protein bands were detected. The heterogeneous protein that migrates more slowly with an apparent molecular weight of ∼20 kDa has been shown previously to be mature secreted TFF2 that is glycosylated via an N-linkage on the first asparagine in loop 1 (fig 1A). The less prominent protein that migrates faster, has an apparent molecular weight of 12 kDa, and co-migrates with recombinant TFF2 is unmodified mature TFF2. Glycosylated TFF2 was detected in a higher proportion of the samples than non-glycosylated TFF2.

Figure 1B shows that there was a dramatic variation in the concentration of TFF2 present in the gastric juice of an individual over 24 hours. TFF2 concentrations were lowest during the afternoon and early evening and rose during the night to reach their highest concentrations in the early morning. The amount of TFF2 detected in the first samples, and for some individuals in the second samples, of gastric juice may have been reduced due to dilution of the gastric contents by the method used to position the nasogastric tube. This involved volunteers drinking 20 ml of water, which was then aspirated from the stomach.

The amounts of glycosylated and non-glycosylated TFF2 in the gastric juice samples of the 12 individuals were quantified by comparison with known amounts of glycosylated and non-glycosylated recombinant TFF2. Mean (SEM) concentrations of glycosylated and non-glycosylated TFF2 in the gastric juice samples for the 12 volunteers are shown in fig 2. The concentration of glycosylated TFF2 recovered during the morning but then fell to reach its lowest mean concentration at 7:00 pm. Subsequently it rose, at first gradually and then sharply, to reach a maximum mean concentration of approximately 6 µg/ml at 5:00 am. The mean concentration of non-glycosylated TFF2 was much lower (below 0.1 µg/ml) during the morning, barely detectable during the afternoon and evening, but rose dramatically during the early hours of the morning. The concentration of glycosylated TFF2 became significantly higher (p=0.004) than the concentration of glycosylated TFF2 present in the gastric juice at 7:00 pm by 11:00 pm. The concentration of non-glycosylated TFF2 also became significantly higher (p=0.046) at 11:00 pm.

Parametric and non-parametric statistical tests were used for analysis of the data shown in fig 7, as detailed in the text.
Diurnal variation in gastric TFF2

Levels of glycosylated, non-glycosylated, and total TFF2 rose dramatically between 9:00 pm and 5:00 am (fig 2). Glycosylated TFF2 rose from 0.29 (0.09) to 5.91 (0.73) µg/ml (p<0.0001), non-glycosylated TFF2 rose from undetectable levels to 1.4 (0.3) µg/ml (p=0.002), and total TFF2 rose from 0.29 (0.09) to 7.32 (1.02) µg/ml (p<0.0001). The initial more gradual increases in TFF2 concentrations occurred between 7:00 pm and 11:00 pm while volunteers were resting and the more dramatic increases occurred between 1:00 am and 5:00 am while they were asleep.

Concentrations of glycosylated and non-glycosylated TFF2 rose during the night in all 12 individuals but there was considerable variation both in TFF2 concentration and in the extent of the increase. TFF2 concentrations were highest in one individual at 3:00 am, in five individuals at 5:00 am, and in six individuals at 7:00 am. The extent of variation is illustrated in fig 3 which shows TFF2 concentrations in gastric juice at 7:00 pm and after they woke at 7:00 am. Concentrations of glycosylated TFF2, non-glycosylated TFF2, and total TFF2 concentrations were measured as described in the methods. The values obtained for all volunteers are shown.

The data shown in fig 1B and fig 2 suggest that there may be a decrease in TFF2 concentration following food intake, which occurred at 1:15 pm and 5:15 pm. Concentrations of glycosylated TFF2 and total TFF2 were significantly lower following lunch (table 1). Very little non-glycosylated TFF2 was detected at these times and the decreases after each meal were not significant. The decreases in glycosylated and total TFF2 following supper were statistically significant (table 1). Emptying of the liquid phase after food consumption occurs within 45 minutes and it is unlikely that the reduction in TFF2 concentration following food intake is a dilution effect. This suggests that ingestion of food may suppress TFF2 secretion.

Mean TFF2 concentrations mask considerable variability both in TFF2 concentrations and the effect of food intake. The changes induced in each of the 12 volunteers by food ingestion are illustrated in fig 4. After lunch, total TFF2 concentration decreased in nine subjects between twofold and 10.5-fold, with TFF2 becoming undetectable in one individual, and increased in three subjects between 1.3-fold and 1.6-fold. After the second meal, TFF2 concentrations fell in eight subjects between 1.3-fold and 7-fold, with TFF2 becoming undetectable in three individuals, remained unchanged in three individuals, and rose in only one subject 1.2-fold.

**RATIO OF GLYCOSYLATED AND NON-GLYCOSYLATED TFF2**

We have reported previously that the ratio of glycosylated TFF2 to non-glycosylated TFF2 in gastric juice varies between 5:1 and 10:1. The possibility that this ratio may change during the day was considered. As shown in fig 5, the means of the relative proportions of glycosylated and non-glycosylated TFF2 changed...
Table 1  Effect of food intake on mean TFF2 concentrations in gastric juice

<table>
<thead>
<tr>
<th>Meal</th>
<th>Time</th>
<th>Glyc. TFF2 (µg/ml)</th>
<th>TFF2 (µg/ml)</th>
<th>Total TFF2 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lunch</td>
<td>1:00 pm</td>
<td>0.72 (0.14)</td>
<td>0.08 (0.27)</td>
<td>0.8 (0.16)</td>
</tr>
<tr>
<td></td>
<td>3:00 pm</td>
<td>0.32 (0.10)</td>
<td>0.05 (0.04)</td>
<td>0.37 (0.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.04</td>
<td>p=0.38</td>
<td>p=0.05</td>
</tr>
<tr>
<td>Supper</td>
<td>5:00 pm</td>
<td>0.45 (0.08)</td>
<td>0.02 (0.01)</td>
<td>0.47 (0.08)</td>
</tr>
<tr>
<td></td>
<td>7:00 pm</td>
<td>0.29 (0.09)</td>
<td>0 (0)</td>
<td>0.29 (0.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.025</td>
<td>p=0.167</td>
<td>p=0.017</td>
</tr>
</tbody>
</table>

Gastric juice was collected 15 minutes before and one hour and 45 minutes after both lunch and supper. Concentrations of glycosylated TFF2 (Glyc. TFF2), non-glycosylated TFF2 (TFF2), and total TFF2 were measured. Mean (SEM) values are shown. Significant differences were tested using the paired sample t test.

During late morning and afternoon, the percentage of non-glycosylated TFF2 fell to reach 0% at 7:00 pm. The proportion of non-glycosylated TFF2 present at 1:00 pm was still significantly higher than the proportion at 7:00 pm (p=0.002) and the proportion became significantly higher again at 1:00 am (p=0.003). Thereafter the percentage of non-glycosylated TFF2 rose to reach approximately 15% again by 3:00 am (p<0.0001). The ratio of glycosylated TFF2 to non-glycosylated TFF2 at 7:00 am, when non-glycosylated TFF2 concentrations were highest, varied between 2.7:1 and 7:1.

Figure 4  Effect of eating on gastric luminal TFF2 concentrations. Gastric juice was collected from 12 healthy volunteers at two hourly intervals for 24 hours and concentrations of TFF2 were measured as described in the methods. Values obtained for all volunteers are shown.

Figure 5  Proportion of TFF2 that is not glycosylated. Gastric juice was collected from 12 healthy volunteers at two hourly intervals for 24 hours and concentrations of TFF2 were measured as described in the methods. Mean (SEM) percentages of the total TFF2 that was not glycosylated are shown.

It is noteworthy that the proportion of non-glycosylated protein is significantly higher during the night and early morning. At the start of the 24 hour study, the percentage of non-glycosylated TFF2 was approximately 15%.
in TFF2 concentration facilitated by a diurnal decrease in pepsin activity.

Finally, diurnal changes in TFF2 concentration were compared with the pH of the gastric juice. Mean (SEM) pH values for the 12 gastric juice samples at each time are shown in fig 6D. The pH of the first samples was relatively high (pH 4.4 (0.67)), probably due to dilution of the gastric contents caused by the method used to position the nasogastric tube. By 11:00 am pH had fallen to 2.4 (0.5) and thereafter it did not vary significantly. There was no discernible effect of food intake or of rest on gastric acid secretion. Diurnal changes in TFF2 secretion do not, therefore, appear to be related to gastric acid secretion.

RELATIONSHIP BETWEEN TFF2 CONCENTRATION AND PEPsin
Changes in mean pepsin activity did not accompany the diurnal variation in mean TFF2 concentration but there was a striking inverse correlation between TFF2 concentration and pepsin activity in individual gastric juice samples. Figure 7A shows the relationship between TFF2 and pepsin concentrations. Gastric juice was collected from 12 young healthy volunteers at 7:00 am. TFF2 concentrations and pepsin activities in gastric juice were measured as described in the methods. Values for TFF2 concentration in gastric juice from each individual are shown plotted against pepsin activity. The fold increases in TFF2 concentration over this time were calculated and are plotted against the average pepsin activity for each individual.
that TFF2 secretion is lower in individuals with higher levels of pepsin secretion.

We also tested if the fold increase in TFF2 concentration observed during rest was related to levels of pepsin. The fold increases in TFF2 concentration between 7:00 pm and 7:00 am for each individual are shown against the average pepsin concentration during this time in fig 7B. A strong inverse correlation was observed with the greatest increase in TFF2 concentration in individuals with lower pepsin activities; Pearson’s two tailed p<0.05 and Spearman’s two tailed p<0.003 with a correlation coefficient of −0.781.

Discussion

This is the first study of diurnal variation in concentrations of a trefoil protein. The study was designed to permit quantification of the effects of food intake and sleep on TFF2 concentrations in gastric juice during a 24 hour period. Our results show that gastric luminal TFF2 concentrations vary dramatically over 24 hours, with highest concentrations detected during the night.

Taken together, the data suggest that the diurnal variation in gastric luminal TFF2 concentration is caused by increased secretion of TFF2. It is unlikely that the changes are due to dilution because the concentrations of other proteins, pepsin activity, and hydrogen ion concentrations do not show diurnal variation. An alternative explanation would be that the variations in TFF2 concentrations reflect differential proteolysis of TFF2. However, mean TFF2 concentrations did not show any relationship with mean pepsin concentrations over the 24 hour time course, and pepsin activity did not show diurnal variation. Proteases other than pepsin are unlikely to be responsible for the diurnal variation in TFF2 concentration because trefoil peptides, including TFF2, are characterised by resistance to proteolysis and TFF2 has been shown to be stable during incubation in gastric juice. Furthermore, in this study, TFF2 was measured by western transfer analysis, and as immunoreactive protein bands smaller than 12 kDa were not detected there was no indication that TFF2 proteolysis occurs (fig 1B). It seems probable that the diurnal variations in gastric luminal TFF2 concentration are due to increased synthesis and secretion.

Available evidence suggests that glycosylated TFF2 is the major form of human TFF2 and that it is more potent than the non-glycosylated protein. The proportion of non-glycosylated TFF2 was highest during the night and early morning, which is consistent with an increase in TFF2 synthesis at night. The altered ratio is unlikely to be due to preferential proteolytic degradation of glycosylated TFF2 as glycosylation normally protects proteins from proteolysis. The different proportions of the two proteins probably reflect alterations in their relative rates of synthesis and secretion. The TFF2 N-glycosylation consensus sequence Asn15Arg16Thr17 is predicted to be fully glycosylated and therefore the increased proportion of non-glycosylated TFF2 probably arises because the glycosylation machinery in the endoplasmic reticulum is overloaded during the synthesis and secretion of the large amounts of TFF2 that occur during the night.

The mechanisms that control TFF2 synthesis and secretion are unknown. It may be under the control of neurogastric peptides whose concentrations alter in response to food intake, sleep, or circadian rhythms. It is known that plasma concentrations of several gastrointestinal regulatory peptides are subject to diurnal variations; vasoactive intestinal polypeptide and cholecystokinin are highest during the evening and night. It has been shown that the neuropeptides somatostatin and vasoactive intestinal polypeptide, and carbonic anhydrase, stimulate TFF3 mRNA synthesis by the HT-29 human colon epithelial cell line. If TFF2 synthesis is also regulated by these regulatory molecules, this could be the mechanism responsible for the diurnal variations in TFF2 concentrations.

TFF2 is thought to be the principal cytoprotective trefoil peptide in the stomach and our data suggest that it performs this role during inactivity or sleep. It is interesting that in a study of ulcer incidence in 1480 patients, a strong diurnal pattern was found with a significant trough in ulcer presentation between 4:00 am and 8:00 am when TFF2 levels are highest. This suggests that TFF2 secretion increases specifically to facilitate repair of the gastric mucosa at night. In addition, our data indicate that TFF2 concentrations fall following food intake. It is possible that mucosal restitution may be suppressed during active digestion, and that TFF2 secretion may be reduced until the digestive functions of the stomach are accomplished and active restitution can resume.

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Diurnal variation in gastric TFF2


