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Increased gastrin gene expression provides a physiological advantage to mice under hypoxic conditions

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Laval M, Baldwin GS, Shulkes A, Marshall KM. Increased gastrin gene expression provides a physiological advantage to mice under hypoxic conditions. Am J Physiol Gastrointest Liver Physiol 308: G76–G84, 2015. First published November 13, 2014; doi:10.1152/ajpgi.00344.2014.—Hypoxia, or a low concentration of O2, is encountered in humans undertaking activities such as mountain climbing and scuba diving and is important pathophysiologically as a limiting factor in tumor growth. Although data on the interplay between hypoxia and gastrins are limited, gastrin expression is up-regulated by hypoxia in gastrointestinal cancer cell lines, and gastrins counterbalance hypoxia by stimulating angiogenesis in vitro and in vivo. The aim of this study was to determine if higher concentrations of the gastrin precursor progastrin are protective against hypoxia in vivo. hGAS mice, which overexpress progastrin in the liver, and mice of the corresponding wild-type FVB/N strain were exposed to normoxia or hypoxia. Iron status was assessed by measurement of serum iron parameters, critical regulatory genes by PCR, and tissue iron homeostasis appears to be physiologically important, as hematopoiesis was disturbed in gastrin-deficient mice subjected to dietary iron deficiency (19).

Hypoxia, or reduced O2, increases expression of the gastrin gene in rats (36), newborn calves (26), and humans (1, 7, 18, 31). Translation of the gastrin mRNA continues in several gastrointestinal cancer cell lines during hypoxia, while translation of other peptides is inhibited because of the presence of an internal ribosomal entry site (15). The gastrin gene is upregulated by hypoxia in the human gastric carcinoma cell line AGS by a mechanism independent of hypoxia-inducible factors (35). In fact, gastrins may play a protective role during hypoxia, as knockdown of the gastrin gene in the human colorectal cancer cell line LoVo increased apoptosis in vitro and necrosis in xenografts in vivo (34).

Thus there is abundant circumstantial evidence that gastrins may be involved in the compensatory changes in iron homeostasis in response to hypoxia. The aim of this study was to investigate the physiological significance of the upregulation of gastrins in response to hypoxia. The effect of hypoxia on the body weight and health of hGAS mice, which overexpress progastrin in the liver, was compared with the effect of hypoxia on mice of the parental FVB/N strain. The mechanisms of the effects were investigated by measurement of hematological parameters, critical regulatory genes by PCR, and tissue iron concentrations by Perls’ stain and atomic absorption spectrometry. The results are the first report of a link between progastrin overexpression, hypoxia, and iron homeostasis.
MATERIALS AND METHODS

**Mouse strains.** hGAS mice, which overexpress human progastrin in the liver, were developed on the FVB/N background and kindly provided by Professor T. Wang (Columbia University, New York, NY) (33). Wild-type FVB/N mice were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia). All mice were fed a commercial pellet diet supplemented with sunflower seeds and hydrogel (rodent recovery gel) and allowed free access to water. Mice [8–12 wk old, ≥10 mice/group (≥5 males and 5 females)] were acclimatized to hypoxic or normoxic containers 3 days prior to the start of the experiment. Hypoxia [10% O2, automatically adjusted by an electronic O2 controller (ProOx model 110, Biospherix, Redfield, NY)] was obtained by injection of compressed N2 into an almost-airtight container. A similar container that allowed the circulation of normal air was used to obtain normoxia (21% O2). Both chambers were used under normobaric conditions and were opened once a day to release any accumulated CO2 and to weigh mice and evaluate their health. Sickness scores were determined using a combination of clinical observations, such as mouse activity, breathing, eating, drinking, movement, body condition, body weight, dehydration, and vocalization. After 10 days, the animals were given an anesthetic overdose of isoflurane before exsanguination by cardiac puncture. Fresh blood (250 µl) was placed into tubes containing EDTA as an anticoagulant. Tissue was collected and flash-frozen in liquid nitrogen or fixed in 10% formalin. All animal work was approved by the Austin Health Animal Ethic Committee (AEC2013-04957).

**Full blood examination.** Hematological parameters were determined by the Austin Pathology Laboratory (Heidelberg, VIC, Australia) using an automated hematological analyzer (Advia 120, Bayer, Tarrytown, NY).

**Tissue staining.** Paraffin-embedded, formalin-fixed liver and spleen sections were stained with hematoxylin and eosin or Perls’ Prussian blue according to standard techniques. Stained tissue sections were imaged using a Coolscope (Nikon, Lidcombe, NSW, Australia) and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD).

**Iron measurements.** Serum was prepared from the remaining mouse blood (~700 µl) for measurement of serum iron, ferritin, and transferrin concentrations. Serum iron levels were quantitated by the Austin Pathology Laboratory using the Roche/Hitachi cobas c system (Castle Hill, NSW, Australia), and ferritin levels were detected using a Roche ferritin kit. Total transferrin concentrations were determined using an iron/total iron-binding capacity (TIBC) reagent set (Pointe Scientific, Canton, MI). Total serum iron and TIBC were calculated according to the manufacturer’s protocol. Transferrin saturation was calculated with the following equation: transferrin saturation = (total serum iron/TIBC) × 100. Hepatic iron levels were quantitated by Regional Laboratory Services (Benalla, VIC, Australia) using atomic absorption spectrometry on liver mouse samples after digestion in nitric/perchloric acid.

**Serum erythropoietin quantification.** Serum concentration of erythropoietin (EPO) was determined with an EPO ELISA kit (R & D systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Dmt-1 and Hamp mRNA quantification.** Total RNA was isolated from ~100 mg of frozen liver or duodenum of all mice using TRIzol reagent (Life Technologies, Melbourne, VIC, Australia) according to the manufacturer’s instructions. Total RNA (5 µg) was used for cDNA synthesis with the SuperScript II First Strand Synthesis system (Life Technologies). The resulting cDNA transcripts of hepatic or duodenal mRNA were used for real-time PCR amplification using a PRISM 7700 sequence detector (Applied Biosystems, Melbourne, VIC, Australia) and TaqMan chemistry. The following primers were used: 5'-AGCACCATCTATCCATCAACA-3' (forward) and 5'-GCTTCTTCCGGCGTGGAA-3' (reverse) for Dmt-1, and 5'-CTGCACTCTGATGCT-3' for Hamp. 5'-CAGCTTATTCGACACTTA-3' (forward) and 5'-GTCCGGCCATGAGTGA-3' (reverse) for Dmt-1 MGB probe. Gene expression was quantitated relative to 18S RNA expression.

**Statistics.** Values are means ± SE. Significance was determined by one-way ANOVA followed by Student’s t-test with a Holm-Sidak correction. Statistics were analyzed using SigmaStat and graphed using SigmaPlot (Jandel Scientific, San Rafael, CA).

**RESULTS**

To test the hypothesis that overexpression of the gastrin precursor progastrin protects mice against systemic hypoxia, responses of hGAS mice and mice of the corresponding wild-type strain (FVB/N) to normoxia (21% O2) and hypoxia (10% O2) were compared.

**Increased progastrin protects mice against hypoxia.** hGAS and FVB/N mice lost significantly more weight and had significantly higher sickness scores under hypoxia than normoxia (Fig. 1). However, FVB/N mice lost weight at a faster rate and had higher sickness scores than hGAS mice. Mice with gastrin gene overexpression appeared to cope better under hypoxic conditions than control mice under the same conditions.
hGAS mice have significantly lighter spleens and livers than FVB/N mice. Previous work from our group demonstrated that dietary iron deficiency in gastrin-deficient mice could cause gross splenomegaly (19). To determine whether O2 status in combination with altered gastrin expression could alter this tissue, the weight and histological sections of the spleen were examined. No difference was found between hGAS and wild-type FVB/N mice in spleen weight, expressed as a percentage of total body weight, when data from male and female animals

Fig. 2. Spleen weight and histology during hypoxia. A: spleen size was determined as a percentage of total body weight for FVB/N and hGAS mice exposed to normoxia (white) and hypoxia (gray) for 10 days. B: spleen size for female (white) and male (gray) mice in A. C: spleen morphology was not substantially different between groups. Top: representative hematoxylin-eosin-stained section for each group. Bottom: magnified regions from within each image, as indicated by matching numbers. Scale bar = 100 μm. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Liver weight and histology during hypoxia. A: liver size was determined as a percentage of total body weight for FVB/N and hGAS mice exposed to normoxia (white) or hypoxia (gray) for 10 days. B: liver size for female (white) or male (gray) mice in A. C: liver morphology was not substantially different between groups. Top: representative hematoxylin-eosin-stained section for each group. Bottom: magnified regions from within each image, as indicated by matching numbers. Scale bar = 100 μm. *P < 0.05; **P < 0.01.
were combined (Fig. 2A). However, when the sexes were analyzed separately, spleens from normoxia-exposed FVB/N and normoxia- or hypoxia-exposed hGAS male mice were significantly lighter than spleens of female mice under the corresponding conditions (Fig. 2B). Spleens from male hGAS mice were also lighter than spleens from male FVB/N mice under normoxia, but splenic architecture did not appear to be affected (Fig. 2C). hGAS mice had significantly lighter livers than FVB/N mice, regardless of O2 levels (Fig. 3A). When the sexes were analyzed separately, no difference in hepatic weight was observed for either strain under hypoxia, except livers were lighter in male than female hGAS mice exposed to hypoxia (Fig. 3B). No morphological difference was observed between the liver sections (Fig. 3C).

Hypoxia-exposed hGAS mice have smaller red blood cells than hypoxia-exposed FVB/N mice. Hematopoiesis was also disturbed when gastrin-deficient mice were fed a low-iron diet (19). Full blood examinations were therefore done on all animals to see if increased circulating concentrations of gastrin altered blood parameters (Table 1). While differences in the number of red blood cells or hematocrit were not observed, mean corpuscular volume was significantly decreased in both strains under hypoxia compared with normoxia and in hGAS mice compared with FVB/N mice under hypoxia. However, no difference in mean corpuscular volume was observed between strains under normoxia. White blood cell counts were significantly increased in hGAS mice during hypoxia. Serum iron and total ferritin concentrations were reduced in hGAS mice under hypoxia. To determine if the selective advantage observed in hGAS mice was caused by changes in iron status, concentrations of hemoglobin, serum iron, serum ferritin, and transferrin saturation were measured. As expected, hemoglobin was significantly increased in FVB/N and hGAS mice exposed to hypoxia, but no differences were observed between strains (Fig. 4A). Interestingly, serum iron levels were

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Table 1. Hematological profile of hGAS on FVB/N background and wild-type FVB/N mice under normoxia or hypoxia

<table>
<thead>
<tr>
<th>Peripheral Blood</th>
<th>FVB/N</th>
<th>hGAS</th>
<th>FVB/N</th>
<th>hGAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC, 10^6/l</td>
<td>8.09 ± 0.20 (11)</td>
<td>8.67 ± 0.20 (12)</td>
<td>8.48 ± 0.35 (16)</td>
<td>8.91 ± 0.83 (10)</td>
</tr>
<tr>
<td>Hct, l/l</td>
<td>0.42 ± 0.01 (11)</td>
<td>0.42 ± 0.01 (12)</td>
<td>0.44 ± 0.02 (16)</td>
<td>0.46 ± 0.02 (10)</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>15.50 ± 0.16 (11)</td>
<td>14.85 ± 0.20 (12)</td>
<td>15.85 ± 0.31 (16)</td>
<td>14.74 ± 0.19** (10)</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>50.64 ± 0.36 (11)</td>
<td>48.58 ± 0.54** (12)</td>
<td>48.88 ± 0.26# (16)</td>
<td>51.70 ± 0.35## (10)</td>
</tr>
<tr>
<td>WBC, 10^9/l</td>
<td>2.00 ± 0.28 (11)</td>
<td>3.03 ± 0.24** (12)</td>
<td>1.86 ± 0.21 (16)</td>
<td>1.99 ± 0.31## (10)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>12.59 ± 3.50 (8)</td>
<td>11.38 ± 1.90 (12)</td>
<td>10.65 ± 1.88 (15)</td>
<td>6.88 ± 2.30 (6)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>81.80 ± 2.66 (10)</td>
<td>83.28 ± 1.84 (12)</td>
<td>70.91 ± 2.50## (16)</td>
<td>76.58 ± 2.77 (9)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>3.30 ± 1.13 (10)</td>
<td>3.80 ± 1.19 (12)</td>
<td>14.76 ± 2.67## (16)</td>
<td>9.96 ± 2.60 (9)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.09 ± 0.09 (9)</td>
<td>0.09 ± 0.07 (12)</td>
<td>0.49 ± 0.33 (16)</td>
<td>0.00 ± 0.00 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE of number of mice shown in parentheses. Serum samples were collected from hGAS and FVB/N wild-type mice under normoxia (21% O2) or hypoxia (10% O2). Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RBC, red blood cells; WBC, white blood cells. Significantly different from FVB/N: *P < 0.05, **P < 0.01; significantly different from normoxia: #P < 0.05, ##P < 0.01.
lower in hGAS than FVB/N mice and decreased further when the animals were exposed to hypoxia (Fig. 4B). The serum concentration of ferritin, a major iron storage protein, was also decreased in hGAS mice compared with FVB/N mice (Fig. 4C). Saturation of transferrin, the main iron transport protein, was decreased in hGAS mice compared with FVB/N mice (Fig. 4D).

**EPO is not increased by short-term hypoxia.** EPO is up-regulated in response to an increased need for erythropoiesis (3). As gastrin-deficient (Gas<sup>−/−</sup>) mice fed a low-iron diet developed splenomegaly, likely as a result of significantly increased circulating concentrations of EPO (19), EPO concentrations were measured in hGAS and FVB/N mice under normoxia and hypoxia. No significant differences in EPO concentrations were observed between strains or treatment groups (Fig. 5).

![Fig. 5](image1.png)

**Fig. 5.** Erythropoietin (EPO) concentrations are unchanged in hGAS mice exposed to hypoxia for 10 days. EPO concentrations were determined for FVB/N (solid bars) and hGAS (hatched bars) mice exposed to normoxia (white) or hypoxia (gray). No significant difference was observed.

![Fig. 6](image2.png)

**Fig. 6.** Iron staining in the spleen. A: representative images of spleen sections stained with Perls’ Prussian blue (top). Blue areas contain iron; pink areas are counterstained with hematoxylin. Bottom: magnified regions from within each image, as indicated by matching numbers. Scale bar = 200 μm. B: quantification of Perls’ Prussian blue staining using Image-Pro Plus. Area of blue staining for FVB/N (solid bars) or hGAS (hatched bars) mice exposed to normoxia (white) or hypoxia (gray) is expressed as a percentage of total area. *P < 0.05; **P < 0.01; ***P < 0.001.
Iron concentrations are reduced in the liver, but not the spleen, of hGAS mice. Iron concentrations in the spleen and liver were examined using Perls’ Prussian blue stain (Fig. 6A), and staining was quantitated using Image-Pro Plus. Iron concentrations were significantly greater in the spleen of hGAS than FVB/N mice under normoxia (Fig. 6B). The increase in normoxic hGAS mice was reversed under hypoxia. Surprisingly, Perls’ Prussian blue staining revealed a strikingly reduced amount of liver iron in hGAS mice compared with FVB/N mice (Fig. 7A and B). Quantification of iron in liver samples using atomic absorption spectrometry verified significantly less liver iron in hGAS than FVB/N mice, and the difference was independent of O2 status (Fig. 7C).

Hepcidin, but not DMT-1 concentrations, are increased in hGAS mice. Quantitation of the expression of Hamp [the gene coding for hepcidin, the master regulator of iron homeostasis (3)] by real-time PCR revealed the expected reduction in hepcidin mRNA expression in FVB/N mice exposed to hypoxia (Fig. 8A). Concentrations of hepcidin mRNA were higher in hGAS than FVB/N mice under normoxia (Fig. 8A), and, in contrast to FVB/N mice, no reduction in hepcidin mRNA expression was observed under hypoxia in hGAS mice. As duodenal iron uptake involves the divalent metal ion transporter DMT-1 (3), expression of the Dmt-1 gene was also measured. No difference was found for Dmt-1 mRNA expression in either strain under hypoxia or normoxia (Fig. 8B).

**DISCUSSION**

Hypoxia increases expression of the gastrin gene in rats (36) and newborn calves (26). In humans, exposure to high altitude and, hence, a lower O2 concentration also increased the amounts of circulating gastrins (1, 7, 18, 31). To investigate the...
hypothesis that increased gastrin gene expression would confer a selective benefit on O$_2$-stressed animals, hGAS mice, which overexpress the gastrin precursor progastrin in the liver, and mice of the corresponding wild-type FVB/N strain were exposed to normoxia or hypoxia, and physiological responses were investigated.

A higher concentration of progastrin decreased the amount of weight loss in animals under hypoxia and reduced daily sickness scores but was not able to restore health to normoxic control levels (Fig. 1). However, elevated progastrin concentrations still provided a significant selective health advantage in mice exposed to hypoxia. These data provide convincing evidence that gastrins may play a protective role in low-O$_2$ conditions. Previously, our studies with Gas$^{-/-}$ mice showed that gastrins protected against the adverse effects of a low-iron diet. Thus the health of Gas$^{-/-}$ mice deteriorated when the animals were fed a low-iron diet for 6 wk, and the mice developed severe anemia (19). The protective effects of gastrins were not the result of activation of the CCK2R, as Cck2r$^{-/-}$ mice had lower sickness scores (i.e., were healthier) than wild-type controls fed a low-iron diet (19). We therefore predict that the selective advantage in hGAS mice would also be independent of the CCK2R.

Kovac et al. (19) also reported that Gas$^{-/-}$ mice fed a low-iron diet developed splenomegaly, likely as a result of significantly increased circulating concentrations of EPO. Therefore, the effects of hypoxia on the spleen and liver of mice with normal or increased circulating gastrin concentrations were examined. Although the spleens from male animals were generally lighter than those from female animals and the spleens from male hGAS mice under normoxia were significantly lighter than the spleens of male FVB/N mice under the
same conditions, no significant differences in spleen weight were noted between strains when the sexes were combined (Fig. 2). Consistent with this observation, circulating concentrations of EPO were also unchanged (Fig. 5). In contrast, the livers of hGAS mice were significantly lighter than the livers of FVB/N mice, and this difference was independent of O2 status (Fig. 3). No differences were observed in spleen or liver architecture after exposure of either strain to hypoxia.

Subtle changes were noted in serum iron parameters. hGAS mice had significantly lower serum iron under normoxia and hypoxia (Fig. 4B), lower ferritin concentrations under hypoxia (Fig. 4C), and lower transferrin saturation under normoxia (Fig. 4D) than wild-type FVB/N controls. Despite this evidence of slightly impaired iron status, the hGAS mice maintained a normal circulating hemoglobin concentration, which could still be increased in response to hypoxia (Fig. 4A).

A striking and previously unreported finding was significantly lower concentrations of iron in the liver of hGAS mice than wild-type FVB/N mice (Fig. 7). The initial observation of a low level of Perls’ staining (Fig. 7, A and B) was confirmed by atomic absorption spectrometry (Fig. 7C). Previous work from our laboratory showed that gastrins can bind two ferric ions (4, 6, 27). Our model proposes that gastrins catalyze the uptake of apotransferrin for transport throughout the body. Iron-bound transferrin binds to transferrin receptor (TFR) 1 (TFR1), and the complexes are internalized to provide target cells with their iron requirements. We have developed a model (20) of how progastrin-derived peptides may participate in the control of iron homeostasis (Fig. 9). Circulating gastrins, such as Gamide, Ggly, and progastrin, bind ferric ions (4, 6, 27). Gamide and Ggly also bind apotransferrin, but not diferric transferrin (5, 21, 25). Our model proposes that gastrins catalyze the uptake of ferric ions by apotransferrin.

We previously reported that hypoxia-inducible gastrin gene expression protects against chronic hypoxia in the human colorectal cancer cell line LoVo in vitro and in vivo (34). The viability of LoVo cells, in which gastrin expression had been reduced by shRNA knockdown, was diminished after exposure to hypoxia (1% O2) in vitro because of loss of resistance against hypoxia-induced apoptosis, and the effect was partly reversed by treatment with nonamidated, but not amidated, gastrin. Furthermore, necrosis in LoVo xenografts in mice exposed to continuous hypoxia for 21 days was significantly increased by knocking down gastrin gene expression. Thus nonamidated gastrins also appear to play a crucial role in pathophysiological responses to hypoxia. The corollary of these observations is that nonamidated gastrin antagonists, such as bismuth ions (28), may be useful as a novel treatment for carcinoma of the colon.

This study investigated the effects of altered O2 concentrations on mice with normal or increased levels of nonamidated gastrins. A novel physiological connection between progastrin status, O2 deprivation, and iron homeostasis was discovered. The surprising finding that hGAS mice cope with hypoxic conditions better than FVB/N control mice appears to be a consequence of more efficient mobilization of hepatic iron stores, as manifested by a significant reduction in hepatic iron concentrations.

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
M.L. and K.M.M. performed the experiments; M.L. and K.M.M. analyzed the data; M.L. and K.M.M. prepared the figures; G.S.B., A.S., and K.M.M. developed the concept and designed the research; G.S.B. and A.S. edited and revised the manuscript; G.S.B. and A.S. approved the final version of the manuscript; K.M.M. interpreted the results of the experiments; K.M.M. drafted the manuscript.

REFERENCES


