INSULIN RECEPTOR-RELATED RECEPTOR MESSENGER RIBONUCLEIC ACID LEVELS ARE UP-REGULATED IN ENTEROCHROMAFFIN-LIKE CELL PATHOPHYSIOLOGY

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Physiology University of Toronto

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ABSTRACT


The insulin receptor-related receptor (IRR), an orphan member of the insulin receptor family, is selectively localized in the stomach to a subset of neuroendocrine cells, the enterochromaffin-like (ECL) cells. ECL cells are the pivotal regulators of gastric acid secretion. The physiological role of IRR was investigated by analyzing its mRNA levels using semi-quantitative RT-PCR in two states of ECL cell pathophysiology in the rat. First, hypergastrinemia which stimulates ECL cell activity and replication was induced by gastric gavage of omeprazole which blocks acid release. Second, a hypogastrinemic state in which ECL cell activity was reduced was achieved by fasting. As expected, mRNA levels of the ECL cell markers histidine decarboxylase and gastrin receptor increased in hypergastrinemia and decreased with fasting. IRR mRNA levels also were shown to parallel the increase in ECL cell activity in hypergastrinemia. In fasting, however, like the insulin and IGF-I receptors, IRR mRNA levels increased despite the decrease in ECL cell activity. Since IRR mRNA levels were not exclusively conditional on ECL cell activity, they may represent a response to the decreased acid secretion or responses more specific to hypergastrinemia and fasting, for example to increased ECL cell activity and loss of ligand, respectively.
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<tr>
<td>AIDS</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>bp</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>[Ca],</td>
<td>intracellular calcium concentration</td>
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<td>CAG</td>
<td>chronic atrophic gastritis</td>
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<td>CCKβ</td>
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<td>cDNA</td>
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<tr>
<td>DNA</td>
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<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
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<td>ECL cell</td>
<td>enterochromaffin-like cell</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>FGF</td>
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<td>galanin receptor 1</td>
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<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GAPDH</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>H₂</td>
<td>type 2 histamine receptor</td>
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<td>H₃</td>
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<td>HDC</td>
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<td>hsp</td>
<td>heat shock protein</td>
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IDDM  insulin-dependent diabetes mellitus
Ig       immunoglobulin
IGFBP    insulin-like growth factor binding protein
IGF-I    insulin-like growth factor 1
Il       interleukin
IRR      insulin receptor-related receptor
IRS-1    insulin receptor substrate 1
kb       kilobase pairs
MAP      mitogen activated protein
MEN      multiple endocrine neoplasm
mRNA     messenger ribonucleic acid
NGF      nerve growth factor
NIDDM    noninsulin-dependent diabetes mellitus
NPY      neuropeptide Y
NT       neurotrophin
PACAP    pituitary adenylate cyclase activating peptide
PBS      phosphate-buffered saline
PCR      polymerase chain reaction
PI-3K    phosphotidylinositol 3'-kinase
PKA      protein kinase A
PKC      protein kinase C
PLC      phospholipase C
PPI      proton pump inhibitor
PTB      phosphotyrosine binding
PYY      peptide YY
RNA      ribonucleic acid
rRNA     ribosomal ribonucleic acid
RTK      receptor protein-tyrosine kinase
RT-PCR   reverse transcription - polymerase chain reaction
SEM      standard error of the mean
SH-2  Src homology 2
SNAP-25  synaptosome-associated protein of 25 kDa
SSTR₂  somatostatin-2 receptor
Taq  Thermus aquaticus
TGFα  transforming growth factor α
VIP  vasoactive peptide
VMAT₂  vesicular monamine transporter, type 2
Y₁  NPY/PPY receptor, type 1
ZES  Zollinger-Ellison syndrome
I. INTRODUCTION

A. Protein Tyrosine-Kinase Receptors

Protein tyrosine-kinase receptors (RTKs) activate signal transduction pathways that regulate integral cell functions such as growth, differentiation and migration. Every RTK subunit has an extracellular ligand-binding domain, single transmembrane domain and intracellular tyrosine kinase domain. The usual sequence of events in the signaling of an RTK begins with the binding of a soluble ligand, often a paracrine or autocrine factor, to the extracellular domain, causing homo- or hetero-dimerization of two or more receptor subunits. The resultant close proximity of the intracellular domains permits autophosphorylation of tyrosine residues in the tyrosine kinase domain. Phosphorylation may increase the catalytic activity of the domain itself and/or provide docking sites for intracellular proteins that have specific domains - Src homology 2 (SH-2) or phosphotyrosine binding (PTB) domains - that activate downstream signaling pathways. RTKs are deactivated by dephosphorylation which may be accompanied by receptor internalization and recycling or degradation (reviewed in Heldin, 1996).

The more than 50 known RTKs have been organized into subfamilies on the basis of structural and functional similarity (reviewed in Heldin, 1996; van der Geer et al., 1994). Members of each RTK family have similar extracellular domains which usually bind structurally similar ligands. There is some redundancy within subfamilies in that ligands may bind to more than one receptor and a receptor may bind more than one ligand (Heldin, 1996). As well as extracellular similarities, the intracellular tyrosine kinase domains of the members of each RTK subfamily share greater similarity within the subfamily than with members of other subfamilies. In fact, they can often activate the same downstream signaling pathways and also produce related physiological effects (van der Geer et al., 1994). I have been investigating a novel RTK, the insulin receptor-related receptor (IRR), which is an orphan member of one of the most well-known and well-studied RTK subfamilies, the insulin receptor family.
A.1. The Insulin Receptor Family

The insulin receptor family consists of three highly similar members: the insulin receptor, the insulin-like growth factor I (IGF-I) receptor and IRR. While it is known that the insulin receptor binds insulin to regulate metabolism and the IGF-I receptor binds IGF-I and IGF-II to promote mitogenic signaling, both the ligand and the function of IRR remain unknown (McInnes & Sykes, 1997). All members of this family are disulfide bonded tetramers, containing 2α and 2β chains. The α and β chains are proteolytically cleaved from a common precursor. Unlike most RTKs, the receptor is incorporated into the cell membrane as a dimer in the absence of ligand. The α chain contains most of the extracellular domain, including the cysteine rich region which is thought to be important for ligand binding (McInnes & Sykes, 1997). The β chain contains some of the extracellular domain, the membrane-spanning domain and the tyrosine kinase site which has three tyrosine residues in close proximity to each other. Phosphorylation of these residues, in response to ligand binding at the α subunit, increases the catalytic activity of the kinase domain. The kinase phosphorylates an effector protein, such as insulin receptor substrate (IRS) 1 or 2, which in turn provides docking sites for SH-2 containing signal transduction molecules that activate downstream pathways. Physiological effects of the insulin receptor family are produced by signal cascades that include growth factor receptor-bound protein 2 (Grb2)-SOS activation of mitogen-activated protein (MAP) kinase and the phosphotidylinositol 3'-kinase (PI-3K) pathway (Jones & Clemmons, 1995; White & Yenush, 1998).

A.1.a. The Insulin Receptor

The insulin receptor is probably the most studied RTK, but unlike other RTKs, including the IGF-I receptor, its primary role is in metabolism rather than propagation or cell growth (McInnes & Sykes, 1997). Insulin receptors are present on a wide variety of tissues, especially liver and muscle. They bind circulating insulin that is released from pancreatic β cells in the Islets of Langerhans. Insulin receptor tyrosine kinase activity initiates the signal transduction cascades that stimulate anabolic processes like glucose and amino acid uptake and glycogen and lipid synthesis. Also, DNA synthesis and cell
proliferation are stimulated to some extent. These effects may be seriously altered in diabetes mellitus, a disease of impaired insulin signaling. Diabetes mellitus can be caused by autoimmune destruction of the pancreatic β cells (Type I, insulin-dependent diabetes mellitus, IDDM; Tisch & McDevitt, 1996) or defective insulin release and action (Type II, noninsulin-dependent diabetes mellitus, NIDDM; Kahn, 1998).

A.1.b. The IGF-I Receptor

Like most RTKs, the primary function of the IGF-I receptor is growth. Pituitary growth hormone (GH) stimulates IGF-I production and release from the liver into the blood. IGF-I may circulate with one of six known binding proteins which either inhibit or enhance ligand binding to the receptor (Jones & Clemmons, 1995). IGF-I is also synthesized in many tissues other than the liver and can interact in an autocrine or paracrine way with its receptor to have a more local effect on growth. Like the insulin receptor, IGF-I has effects on a wide variety of tissues. Aberrant IGF-I receptors, like many RTKs, may be oncogenic and contribute to tumor formation. Thus, the IGF-I receptor is found to be overexpressed in certain tumors. It is postulated that elevated numbers of receptors increase the activity of SH-2 domain-containing PI-3K which overstimulates the Ras pathway, leading to unrestricted cell growth (reviewed in Blakesley et al., 1997).

B. The Insulin Receptor-Related Receptor

IRR was discovered in our laboratory (Shier & Watt, 1989) by low stringency hybridization screening with human insulin receptor DNA of a guinea pig genomic library. Its predicted amino acid sequence is as similar to that of the insulin and IGF-I receptors as they are to each other (Figure 1; Shier & Watt, 1989). IRR is located on chromosome 1 (Shier et al., 1990) where it is closely linked to the high-affinity nerve growth factor receptor, Trk A (Rainey et al., under revision). To this day, neither the physiological function nor ligand for IRR is known. However, the cell- and tissue-specific expression of the orphan receptor indicate that it may have a more specific function than those of the relatively widespread insulin and IGF-I receptors.
Figure 1. Structure of the insulin receptor-related receptor (Chan & Watt. 1995). The scale along the bottom represents protein length in amino acids. The extracellular region is to the left of the membrane and the intracellular region is to the right.
B.1. **Functional Structure of IRR**

Since neither the ligand nor physiological function of IRR is known, studies into the binding properties and signaling pathways have been challenging. Since RTKs within a family usually bind related ligands and have similar functions, most studies compare IRR to the other members of the insulin receptor family. The process of elimination has been used to look for potential ligands of the insulin family and downstream signaling pathways have been identified by comparison to the insulin and IGF-I receptors. IRR may diversify insulin receptor family function through its capability for hybrid formation and expression of different IRR isoforms.

B.1.a. **Ligand Binding**

Most of the information we have about ligand binding comes from the process of elimination of potential ligands. The similarity between the putative ligand-binding extracellular domains of IRR, IGF-IR and the insulin receptor suggest that they all bind ligands from the same family. In fact, when a specific part of the insulin receptor ligand-binding domain that was thought to be determinant for insulin binding was replaced with the analogous region of IRR, insulin binding was not decreased (Watt et al., 1994; Zhang & Roth, 1991). There must be other determinants involved in ligand binding because whereas the endogenous insulin and IGF-I receptors can bind each other’s ligand, albeit less stringently than their own, the α subunit of IRR shows no binding to the known members of the insulin receptor family including insulin, IGF-I, IGF-II or relaxin (Zhang & Roth, 1991). Furthermore, none of proinsulin, insulin, IGF-I and IGF-II, (Jui et al., 1994), nerve growth factor (NGF; Kovacina and Roth, 1995) or bombyxins II and IV, molluscan insulin-like peptide and growth hormone (Zhang & Roth, 1992) possesses the ability to stimulate endogenous IRR (first two sets of ligands) or a chimera containing the IRR α subunit and IR β subunit (latter set), as measured by tyrosine phosphorylation.
B.1.b. Receptor Activity

The three members of the insulin receptor family share the most similarity (greater than 75% conservation) in their tyrosine kinase domains, suggesting that they may potentiate some of the same signaling pathways. Since the ligand for IRR remains unknown, activation of the receptor must be achieved by means other than activation of its α subunit. Jui et al. (1994) stimulated the IRR β subunit directly with vanadate and hydrogen peroxide, which had been shown to cause tyrosine phosphorylation of the insulin receptor (Heffetz et al., 1992). Chimeric studies incorporating the ligand-binding domains of other receptors into IRR also demonstrate tyrosine phosphorylation of the orphan receptor and further show that it can potentiate some of the same downstream signaling pathways as the other members of the insulin receptor family. Stable transfectants of chimeras containing the intracellular domain of IRR and the extracellular domain of the insulin receptor into Chinese hamster ovary cells showed IRR capable of activating to a comparable degree many of the same endogenous and exogenous substrates as the insulin receptor family, including IRS-1, PI-3K, Shc and GTPase-activating protein (GAP)-associated p60 protein, Adt/PKA (a serine/threonine kinase) and (Danielsen et al., 1995; Zhang & Roth, 1992). This signaling of IRR is inhibited by protein kinase C α which is also an antagonist of the signaling activity of other members of the insulin receptor family (Danielsen et al., 1995). Presumably through its activation of downstream signaling molecules, IRR is capable of mediating two biological responses to insulin receptor activation, thymidine incorporation and 2-deoxyglucose uptake (Zhang & Roth, 1992). Glucose uptake as well as phosphorylation, increased glycogen synthesis and inhibition of lipolysis were also demonstrated to be at similar levels as stimulated by the insulin receptor in 3T3-L1 adipocytes transfected with colony stimulating factor-1/IRR chimeras (Dandekar et al., 1998). Finally, Kovacina and Roth (1995) were able to increase MAP kinase activity in IMR-5 neuroblastoma cells by stimulating IRR with an agonist monoclonal antibody to the orphan receptor. IRR signaling is inhibited by phospholipase Cγ (PLCγ) which also decreases insulin and IGF-1 receptor signaling (Danielson et al., 1995).
B.1.c. Heterodimerization

IRR is colocalized with other members of the insulin receptor family in some tissues and may form heterodimers with insulin or IGF-I receptors and serve to diversify the signaling capabilities of the insulin receptor family in these locations. It was first shown by Soos et al. (1989) that hybrid receptors containing one $\alpha$ and $\beta$ set of the insulin receptor and one $\alpha$ and $\beta$ set of the IGF-I receptor were endogenously present. These hybrids bind insulin and IGF-I but exhibit different behavior than either of the insulin and IGF-I receptors on their own. Kovacina and Roth (1995) confirmed that IRR hybrids with the insulin and IGF-I receptors were also naturally present in neuroblastomas. However, these hybrids were not activated by insulin, IGF-I or the more distantly related NGF. Although IRR is selectively co-expressed in specific neuronal cells with the high affinity receptor for the latter ligand (the Trk A receptor), IRR did not form hybrids with Trk A (Kovacina & Roth, 1995). In NIH-3T3 human fibroblast cells co-expressing IRR and the insulin receptor, only a small proportion of IRR was present as part of an insulin receptor hybrid and there was no detectable increase in the phosphotyrosine content of IRR upon insulin stimulation (Jui et al., 1996). This led to the proposal that one of IRR's functions is to prevent insulin or IGF-I receptor function by binding up their receptor subunits in inactive heterodimers.

B.1.d. Isoforms

The first Northern blot analyses of IRR showed two different sized transcripts at 6 and 2 kb (Shier & Watt, 1992). The larger transcript encodes a full-length membrane-bound receptor with tyrosine kinase activity. The shorter transcript is a truncated form encoding a potentially secreted protein that lacks transmembrane and kinase domains (Jui et al., 1993; Shier & Watt, 1992). This secreted IRR may compete for ligand and prevent it from stimulating the membrane-bound receptor. Alternatively, it could be a binding protein that facilitates presentation of the ligand to the full-length IRR. An insulin-responsive fragment corresponding to the $\alpha$ subunit of the insulin receptor was detected in human plasma and could represent a secreted form of that receptor as well (Pezzino et
al., 1992). There are also alternatively spliced variants of both forms of IRR; a 24 amino acid insertion between exons 13 and 14 of full length IRR (Jui et al., 1994) and a 59 amino acid insertion in the C-terminal region of truncated IRR (Itoh et al., 1993). The former splice variant is also found in the human IGF-I receptor, equally distributed with the regular transcript (Yee et al., 1989). While in rat kidney less than 10% of the IRR transcripts include the splice variant (Jui et al., 1994), the IGF-I receptor alternate splice site does not exist at all in the rat (Pedrini et al., 1994). Both rat (Goldstein & Dudley, 1990) and human (McClain, 1991) insulin receptor genes have an alternate splice site at exon 11 that is not seen in either IRR or the IGF-I receptor transcripts.

B.2. Unique Expression Pattern of IRR

Shier and Watt (1992) first confirmed that IRR was an expressed gene and at the same time showed that, in contrast to the relatively widespread expression of the insulin and IGF-I receptors, IRR expression is limited to selected tissues. This differential expression was later quantified (Mathi et al., 1995) using quantitative-competitive reverse-transcription polymerase chain reaction (RT-PCR), and revealed a large range (about 150-fold) in IRR mRNA levels among a variety of human tissues. IRR transcripts are most abundant in kidney, thymus, heart and stomach. Even more specifically, IRR is only expressed in certain cells of selected tissues. For instance, IRR expression was recently shown in the pancreas, specifically in the islets of Langerhans where it co-exists in β cells with the insulin receptor and Trk A (Ozaki, 1998).

B.2.a. Gastric Localization to the ECL Cell

In the stomach, IRR was localized to the acid-producing oxyntic mucosa by in-situ hybridization (Mathi et al., 1995). All of the cells that showed IRR hybridization also demonstrated immunoreactivity for a neuroendocrine cell marker called chromogranin A (CGA). This subset of neuroendocrine cells was distinguished morphologically as enterochromaffin-like (ECL) cells. Subsequently, Tsujimoto et al. (1995) confirmed their identity by demonstrating coexpression of IRR with histidine
decarboxylase (HDC), a specific marker for ECL cells. ECL cells are the most numerous neuroendocrine cell population in the stomach; they are the pivotal regulators of gastric acid secretion from parietal cells (reviewed in Modlin & Tang, 1996).

B.2.b. Renal Localization to B Intercalated Cells

IRR expression is most abundant in the kidney (Shier and Watt, 1992; Kurachi et al., 1992; Mathi et al., 1995). It was localized to distal tubules (Reinhardt et al., 1993), then showed to be more specifically expressed in a sub-population of epithelial cells beyond the macula densa (Mathi et al., 1995). This cell type was recently identified. Both IRR transcripts and protein are localized to non-A intercalated cells (Bates et al., 1997) or more specifically to the basolateral membrane of B intercalated cells which secrete bicarbonate into the urine (Ozaki et al., 1997). As in the stomach, IRR in the kidney may be involved in pH regulation. Furthermore, the B intercalated cells may have the ability to switch phenotypes in response to the acid/base status of the body and become A intercalated cells, which secrete acid into the lumen (Al-Awqati et al., 1998).

B.2.c. Neural Colocalization with Trk A

IRR is colocalized with the high affinity nerve growth factor receptor, Trk A, in specific neuronal populations at the same stages in development. Both transcripts and proteins are present in subpopulations of trigeminal, dorsal and sympathetic ganglia (Reinhardt et al., 1993 & 1994), forebrain cholinergic neurons (Tsujimoto et al., 1995) and brainstem and cerebellar nuclei (Tsuji et al., 1996). The highly restricted distribution of IRR in the brain is in direct contrast to the virtually ubiquitous insulin and IGF-I receptors in the brain (Reinhardt et al., 1993). It suggests that IRR has a very specific function in the nervous system. This function must be intimately linked with the development and growth functions of TrkA. The two are closely linked in the genome (Rainey et al., under revision) which may facilitate their co-regulation in the brain.
I chose to focus my work on the expression of IRR in the stomach. The cell type that IRR is localized to - the ECL cell - is the most abundant and clinically significant neuroendocrine cell in the stomach. The experimental manipulation of ECL cell activity has generated considerable insight into the cell’s role in gastric acid secretion; I hypothesized that similar experiments would provide insight into the function of IRR in these cells.

C. The Enterochromaffin-Like Cell

The ECL cell was recognized as a distinct cell type present in the fundic mucosa of the stomach in 1971 (Hakanson et al.; Capella et al.). Unfortunately, it was only named on the basis of its similar staining properties to the enterochromaffin cell which had previously been thought to be the only endocrine cell type to be found in the gastric mucosa. Despite the undefined character of their name, ECL cells are the most numerous neuroendocrine cells in the stomach and the most important regulators of parietal cell gastric acid secretion (Modlin & Tang, 1996).

C.1. Morphology of ECL Cells

ECL cells are small (8-10 μm) and irregularly shaped cells with many prominent cytoplasmic extensions (reviewed in Solcia et al., 1987). They are rich in secretory vesicles, microvesicles and electron-dense granules which indicate the importance of secretory activity to their function (Modlin & Tang, 1996). Since all endocrine cells account for less than 2% of the total volume of cells in the rat fundic mucosa (Solcia et al., 1987), identification of ECL cells can be difficult. Furthermore, while ECL cells can be identified by silver and chromium staining, these reactions also identify chromaffin cells (Solcia et al., 1987). Similarly, while ECL cells can be identified with antibodies to histamine or hybridization to HDC, mast cells may also be identified by these means (Modlin & Tang, 1996). It seems that the selective presence of IRR could be a more specific marker for ECL cells.
A critical tool for the specific investigation of ECL cells has been their isolation by counter-flow elutriation. This technique separates cells on the basis of their density by running them through a centrifugal field at different flow rates. Resultant fractions provide a powerful tool for ECL cell studies; they are up to 95% pure with greater than 95% viability and can undergo short-term culture (eg. Lindstrom et al., 1997).

C.2. Function of ECL Cells

ECL cells are the pivotal regulators of gastric acid secretion in the stomach. While regulated by many hormones, gastrin from the antral G cell is the major stimulant of ECL cell histamine release. Histamine binds to histamine-2 (H₂) receptors on parietal cells to stimulate H⁺/K⁺ ATPase proton pump activation and acid release into the stomach lumen (Sachs et al., 1997). Gastric acid serves three functions in the lumen: initial breakdown of stomach contents, activation of the gastric enzyme pepsin and destruction of ingested bacteria.

ECL cells contain and release a number of proteins that have specific functions. For instance, CGA and its derivative, pancreastatin, are involved in the sequestering of histamine into granules; peptide YY (PYY) has a negative feedback autocrine effect on the ECL cell; gastrocalcin is involved in bone homeostasis and calbindin is involved in calcium-mediated ECL cell stimulation (Modlin & Tang, 1996). However, more is known about the physiological relevance and regulation of histamine release. In the stomach, histamine represents the final common pathway in the stimulation of gastric acid secretion (reviewed in Hocker et al., 1996). Histamine is also involved in a number of other physiological functions including inflammation, allergic reactions, neurotransmission and growth and repair (Rangachari, 1992).

Histamine is synthesized from the amino acid L-histidine exclusively by the enzyme HDC (Rangachari, 1992). HDC is present in mast cells, skin, platelets and basophils, but in the adult mammal it is most abundant in ECL cells (Rangachari, 1992). The coincidence of mast cells in the stomach does present a potential source of histamine in ECL cell studies. However, mast cells are scarce in rodent models, found only in the submucosa and uppermost layer of the mucosa (Hakanson & Sundler, 1991).
Furthermore, their histamine pool is much less mobile than that of ECL cells (Andersson et al., 1992). Mast cells are more numerous in the mucosa of larger mammals (Hakanson & Sundler, 1991), but Soll et al. (1989) showed that they do not release histamine in response to gastrin, at least in the canine model.

In the ECL cell, depletion of histamine, which presumably abolishes histamine autofeedback inhibition, is a major stimulant of HDC synthesis (Andersson et al., 1996). In addition, expression of HDC may be regulated by factors that affect ECL cell activity. For instance, the HDC gene contains a gastrin response element which promotes expression through a protein kinase C (PKC) and AP-1 (fos/jun) dependent pathway (Hocker et al., 1997).

Pre-formed histamine in the cytoplasm is actively taken into small, electron dense granules by a vesicular monoamine transporter (VMAT₂) that is exclusively expressed in the fundic mucosa of the stomach (Dimaline & Struthers, 1996). VMAT₂ uses ATP and the electrochemical gradient of the vacuole to counter-transport histamine and protons. These vesicles also contain granular proteins, ECL cell prohormones and CGA which is cleaved in situ into smaller peptides like pancreastatin. The osmotic force generated by these proteins and the high concentration of histamine causes the vesicles to accumulate fluid and become enlarged (Modlin & Tang, 1996).

The exocytosis of histamine seems to involve vesicular docking and fusion proteins that were originally identified in neurons. Synaptobrevin and synaptosome-associated protein of 25 kDa (SNAP-25) are present in ECL cells and are necessary for histamine release (Hohne-Zell et al., 1997). ECL cell vesicles also contain V-type H⁺-transporting adenosine triphosphatases (ATPases) on their membranes. These pumps generate a proton gradient facilitated by a parallel chloride ion intake. When vesicles fuse with the ECL cell membrane, this chloride current is thought to be responsible for the ECL cell depolarization seen upon histamine release. A potassium current is subsequently activated to repolarize the cell (Loo et al., 1996). Histamine is released at
basal levels (Loo et al., 1996), but its stimulated release, especially by gastrin, is more physiologically relevant. The pathway involved in stimulation of granule exocytosis remains to be defined, but is known to be calcium-dependent and to involve PKC and cAMP (Prinz et al., 1993).

C.3. Regulation of ECL Cells

ECL cell activity is influenced by a number of modulators, the primary one of which is gastrin (Figure 2). Since histamine secretion represents the final common pathway for gastric acid stimulation (Hocker et al., 1996), the ECL cell is the site through which most transmitters that influence gastric acid secretion relay their effect.

C.3.a. Gastrin Stimulation

Gastrin is the primary stimulant of ECL cells. A “resting state” whereby ECL cells are not subject to gastrin stimulation is considered to be prolonged fasting. For example, the hypogastrinemia caused by a 24 hour fast in rats is considered to diminish gastrin stimulation of ECL cells (Chen et al., 1996). Gastrin is a heptadecapeptide released from the antral G cell that activates ECL cells by binding to the gastrin/cholecystokinin β (CCKβ) receptor. The relationship between this hormone and its receptor is unique in that gastrin has a positive-feedback effect on the gastrin receptor (Takeuchi et al., 1980). The gastrin receptor is a G protein receptor that regulates internal calcium concentrations ([Ca]i) via a Gq subtype protein (Prinz et al., 1994a). The effect on [Ca]i, a typical G-7 receptor-mediated change, is a transient then sustained elevation corresponding to release of intracellular calcium stores and receptor-mediated extracellular calcium influx, respectively (Sachs et al., 1997). This is thought to cause activation of calcium-dependent signaling pathways including PKC (Prinz et al., 1993) and Ras-MAP kinase (Kinoshita et al., 1998) that up-regulate histamine exocytosis, HDC activity and expression and ECL cell growth (Prinz et al., 1994a).
Figure 2. Modulation of ECL cell activity (modified from Sachs et al., 1997; Sandor et al., 1996). The ECL cell is shown in the fundic mucosa of the stomach, the top of the diagram is the gastric lumen. $\beta_3$, $\beta_3$ adrenergic receptor; CCK-B, gastrin/cholecystokinin B receptor; CGRP, calcitonin gene-related peptide; CGRP1, CGRP type 1 receptor; D cell, somatostatin cell; ECL cell, enterochromaffin-like cell; GalR1, type 1 galanin receptor; $H_2$, histamine type-2 receptor; $H_3$, histamine type-3 receptor; $M_1$, muscarinic cholinergic receptor; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase activating peptide; PYY, peptide YY; SSTR2, somatostatin Type 2 receptor; VIP, vasoactive intestinal peptide; $Y_1$, NPY/PYY receptor type 1.
C.3.b. Other Stimulation of Activity

Positive modulation of ECL cells is also provided by pituitary adenylate cyclase activating peptide (PACAP) and vasoactive intestinal peptide (VIP), vagal acetylcholine and epinephrine. PACAP is a hypothalamic peptide that is also present in the neurons of the gastric mucosa (Sundler et al., 1992). It is a powerful cAMP stimulant that is more potent than gastrin and stimulates histamine secretion with the same efficacy (Lindstrom et al., 1997). Although VIP is a closely related peptide, it increases basal and stimulated histamine secretion to a lesser extent than PACAP (Lindstrom et al., 1997). This may be because VIP also stimulates D cells to release somatostatin which inhibits histamine secretion and in this way VIP attenuates its own stimulatory effect (Schubert, 1991). Acetylcholine stimulates ECL cell activity in the same way as gastrin. Whereas all ECL cells are responsive to gastrin, only 10 - 30% of ECL cells, perhaps those that are vagally innervated, respond to acetylcholine (Zeng et al., 1996). Epinephrine acts upon β1-adrenergic receptors and stimulates ECL cell activity by a mechanism that involves adenylyl cyclase signaling (Prinz et al., 1993).

C.3.c. Inhibition of Activity

ECL cell function is down-regulated by somatostatin. PYY, histamine, galanin and calcitonin gene-related peptide (CGRP). Somatostatin, the major inhibitor of gastric acid secretion, is released from antral and oxyntic D cells and gastric nerve terminals. It is released from D cells upon stimulation by gastrin and acetylcholine (Zeng et al., 1996) and has paracrine inhibitory effects on at least three mucosal cells, the antral G cell, the fundic ECL cell and the parietal cell itself (Prinz et al., 1994b). On the ECL cell, somatostatin binds to the somatostatin-2 receptor subtype (SSTR2) and blocks stimulated intracellular calcium release and extracellular calcium entry, as well as directly inhibiting histamine secretion via a G1-like coupling protein (Prinz et al., 1994b).

PYY and neuropeptide Y (NPY) are released by gastric endocrine cells and nerve afferents, respectively. Both peptides act on the same Y1 receptor on ECL cells to selectively block the plateau phase of the change in [Ca2+], thus inhibiting only the sustained phase of stimulated histamine release (Zeng et al., 1997). This regulation may
be autocrine since PYY is contained in and potentially released by the ECL cell (Solcia et al., 1991). Similarly, histamine itself appears to have an autocrine negative feedback effect on its own release. ECL cells contain a histamine type-3 (H₃) receptor that is activated only at high histamine concentrations (Prinz et al., 1993).

Galanin is a neuropeptide that colocalizes with NPY and VIP in the submucosal plexus near the mucosal epithelium (Ekblad et al., 1985). It activates the type 1 galanin receptor (GalR1), a Gₛ₃α protein-coupled receptor which inhibits the gastrin-induced increase in [Ca²⁺], as well as directly suppressing histamine release (Zeng et al., 1998). However, its effect is transient and has only a modest effect on sustained histamine release (Zeng et al., 1998). CGRP is also released from primary afferents that innervate the corpus and antrum. It directly inhibits basal and stimulated histamine release but may also act indirectly via stimulation of somatostatin release (Sandor et al., 1996).

C.3.d. Regulation of Growth

While gastrin is the primary regulator of both ECL cell function and growth, other growth factors also play physiological, as well as pathological, roles in ECL cell growth. Transforming growth factor α (TGFα) is an endogenously produced growth factor that has a protective and reparative effect on the stomach (Coffey et al., 1995). It is produced by parietal and ECL cells, so it may have endocrine and autocrine effects on the ECL cell. TGFα activates the epidermal growth factor (EGF) receptor, a RTK, on ECL cells, stimulating growth but inhibiting gastrin stimulated histamine release (Tang et al., 1996). Activation of the EGF receptor by TGFα and of the fibroblast growth factor (FGF) receptor, another RTK, by basic FGF increases ECL number by inhibiting cytokine-induced apoptosis or programmed cell death (Mahr et al., 1998). ECL cell tumors stimulate their own growth in an autocrine manner by producing excessive amounts of growth factors including TGFα and bFGF, as well as IGF-1 (Townsend et al., 1993). The IGF-I receptor, a member of the insulin receptor family, has only been reported in malignant ECL cells (Townsend et al., 1993).
Conversely, interleukin (II)-1, a cytokine that mediates many of the physiological responses to inflammation, has a negative effect on ECL cell growth. II-1 can completely inhibit gastrin-stimulated histamine release and de novo synthesis of histamine (Prinz et al., 1997) and cause ECL cell apoptosis (Mahr et al., 1998). The effects of II-1 may be relevant considering the frequency of gastric epithelial inflammation in the human population, as in the case of gastric and duodenal ulcers caused by Helicobacter pylori (H. pylori; Graham, 1989).

C.4. Clinical Relevance of ECL Cells

ECL cells in humans are the most numerous of all endocrine cells in the oxyntic mucosa (Fave & Annibale, 1996). Therefore, it is not surprising that gastric carcinoid tumors are usually composed of ECL cells (Waldum et al., 1998). Such tumors can develop spontaneously or may be the climax of a long-term gastric disease such as Zollinger-Ellison Syndrome (ZES) or chronic atrophic gastritis (CAG; Modlin & Tang, 1996). These gastric diseases involve increased ECL cell activity due to hypergastrinemia. Conversely, ECL cell function is decreased in the hypogastrinemia of fasting. The pathophysiological states of hypergastrinemia and fasting in the rat have been useful tools for studying ECL activity. I also chose to use these models in my investigation of IRR in rat ECL cells.

D. Hypergastrinemia

Hypergastrinemia is a pathophysiological state in which sustained high levels of gastrin cause continued stimulation of ECL cell activity. In response, ECL cells release large amounts of histamine which, under normal circumstances, stimulate the parietal cell to secrete more acid into the gastric lumen. In addition to secretory activation, hypergastrinemia also causes ECL cell hyperplasia and self-replication.
D.1. **Etiology of Hypergastrinemia**

Hypergastrinemia can be caused by pathological conditions or pharmaceutical intervention. To some extent, the specific mode of its induction controls the downstream effects of hypergastrinemia. For instance, hypergastrinemia may be accompanied by excessive acid secretion in ZES or after pharmacological infusion of gastrin. Alternatively, there may be reduced or absent acid secretion (hypochlorhydria or achlorhydria) as in CAG or with administration of proton-pump inhibitors (PPIs). Pharmaceutical treatment with gastrin or PPIs provide a mechanism for inducing controlled hypergastrinemia in experimental animal models.

D.1.a. **Pathological**

Pathological states such as those encountered in ZES and CAG involve hypergastrinemia. ZES is caused by a gastrinoma most commonly in an area known as the gastrinoma triangle, bordered by the duodenum, bile duct and pancreas (Howard & Passaro, 1990). The gastrinoma may be caused by a genetic disease, Multiple Endocrine Neoplasm type I, that gives rise to multiple tumors in different endocrine organs. The increased gastrin released by the gastrinoma causes hyperacidity as well as ECL and parietal cell proliferation. Hyperchlorhydria can cause persistent and therapy-resistant peptic ulcers, reflux esophagitis, diarrhea and vitamin B₁₂ malabsorption (Qureshi & Rashid, 1998). CAG, on the other hand, involves low or no gastric acid secretion and is often associated with autoimmune disease or infection with H. pylori (Modlin & Tang, 1996). It is usually confined to the oxyntic mucosa where there are suppressed parietal and chief cell populations (Bordi et al., 1995). Hypochlorhydria causes antral G cell hyperplasia, hypergastrinemia and increased ECL cell activity. H. pylori infection may also cause peptic ulcer disease (Graham, 1989). The products of the bacteria and IL-8, a recruited inflammatory mediator, decrease D cell somatostatin release which is the major inhibitor of gastrin release (Sumii et al., 1994), and directly stimulate G cell gastrin
secretion (Beales et al., 1995). While hypergastrinemia is a major factor in the presentation of these diseases, in some circumstances it is also present in other pathological states like diabetes mellitus, rheumatoid arthritis and renal insufficiency (Qureshi & Rashid, 1998).

D.1.b. Pharmaceutical

Hypergastrinemia can be more intentionally induced by the use of drugs that decrease gastric acid secretion. The resulting increase in stomach lumen pH has a positive effect on G cells, permitting sustained elevated levels of gastrin. Omeprazole is the most potent member of a family of substituted benzimidazoles that are used to block the H⁺/K⁺ ATPase proton pump in parietal cells. Alternatively, H₂ antagonists, such as ranitidine and cimetidine, block parietal cell histamine receptors and prevent the action of histamine, the primary stimulant of gastric acid release. As well, peroxisome proliferators (eg. ciprofibrate), used primarily to reduce blood lipids, cause hypergastrinemia and stimulate ECL cells (Eason, 1988). Their mode of action is unknown, but does not involve reduction of gastric acid secretion like the H⁺/K⁺ ATPase or H₂ receptor blockers previously mentioned (Hammer et al., 1998). Since it has an independent mode of action, ciprofibrate potentiates the effect of omeprazole, increasing hypergastrinemia 6-fold (Hammer et al., 1998).

PPIs such as omeprazole are commonly used to decrease acid secretion in patients with ulcers and reflux esophagitis. They are preferred over H₂ antagonists which fail to control the direct stimulatory effects of gastrin and acetylcholine on parietal cell gastric acid release (Massoomi et al., 1993). Furthermore, omeprazole is very specific for the parietal cell H⁺/K⁺ ATPase proton pump and does not affect any other similar ATPases in the body (Andersson, 1996). There is one reported case of a patient who developed an ECL tumor after three years of treatment with two different gastric acid suppressers, omeprazole and ranitidine (Haga et al., 1998).

Animal models of hypergastrinemia are usually developed by treatment with high concentrations of PPIs or direct administration of gastrin itself. High doses of omeprazole are delivered daily; in rats, 400 μmol/kg blocks gastric acid secretion for 24
hours (Larsson et al., 1986). Omeprazole is usually delivered to animals by gastric gavage to ensure its complete ingestion. It is dissolved in a buffered pH basic solution because it is both less stable and soluble at lower pH values (Massoomi et al., 1993). Alternatively, hypergastrinemia may be induced by direct administration of very high levels of gastrin itself. Human Leucine$^{12}$-gastrin-17 is infused intravenously or continuously infused subcutaneously by an osmotic minipump. Both omeprazole treatment and gastrin infusion elevate plasma gastrin levels to the same extent. However, omeprazole increases the proportion of ECL cell granules and large vacuoles to a greater extent than gastrin infusion (Bottcher et al., 1989). The molecular size of the endogenous gastrin peptide stimulated with omeprazole treatment is larger than the synthetic peptide that is infused which may have a different and perhaps less relevant effect on ECL cells (Bottcher et al., 1989).

D.2. Effects of Hypergastrinemia on ECL Cells

ECL cell stimulation may be considered as a time course of events occurring with acute (minutes or hours), intermediate (days), maximal (2 weeks) and long-term (months) hypergastrinemia. Briefly, the ECL cell undergoes secretory activation, hyperplasia and increased self-replication in an effort to maximize its histamine release and increase parietal cell gastric acid secretion.

D.2.a. Acute

The acute response to hypergastrinemia within minutes or hours includes release of histamine and pancreastatin and accelerated protein synthesis of HDC and CGA. Within the first hour of gastrin infusion to fasted rats, the concentrations of histamine and pancreastatin in the ECL cell decline in parallel with the number, density and size of the secretory vesicles (Chen et al., 1994). While these effects on the secretory vesicles are also seen in gastrin-infused fed rats, the decline in histamine concentration is not (Chen et al., 1996); pancreastatin concentration is still decreased probably because it has a relatively less important function and is not replenished as quickly as histamine (Kimura et al., 1997). The activity of HDC slowly increases during the first hour of gastrin
infusion and is enhanced more rapidly in the next few hours until it reaches maximum activity at six hours (Chen et al., 1994). The enhancement of HDC activity facilitates repletion of ECL histamine stores to pre-stimulation levels at four hours in fasted gastrin-infused rats. Furthermore, an increase in HDC gene expression at six hours helps maintain the elevated secretory activity of the hypergastrinemic ECL cell in this model (Chen et al., 1994).

D.2.b. Intermediate

Within the first week of hypergastrinemia, there is a sustained high level of ECL cell activity as demonstrated by a transient exhaustion of secretory vesicles and increased HDC activity. The cells hypertrophy and accumulate more vacuoles and microvesicles (Chen et al., 1996). The reduction in secretory vesicles, seen previously to commence within the first hour of hypergastrinemia (Chen et al., 1994) is maintained for six days of gastrin infusion to fed rats. The enhanced HDC activity is also maintained but reaches a plateau at two days (Chen et al., 1996), later than the plateau seen at four hours in fasted rats (Chen et al., 1994). At the same time there is a sustained increase in vacuoles, thought to store excess histamine by fusion of secretory vesicles, and microvesicles that may retrieve vesicle membrane after exocytosis or transport newly synthesized proteins (Bottcher et al., 1989; Chen et al., 1996). The volume density of granules declines, but there is probably not an absolute change in granule number, instead the ECL cell increases in size (Chen et al., 1996). At one week a new "steady state" of enhanced ECL cell activity is attained. This is characterized by hypertrophy, elevated HDC activity and vacuole and microvesicle accumulation. The ECL cell labeling index, which indicates DNA synthesis or preparation for mitosis, also increases by the end of this period (Hakanson et al., 1993; Tielemans et al., 1989).

D.2.c. Maximal

The effects of hypergastrinemia on ECL cell activity are maximal at two weeks. The increased labeling index reaches a maximum and ECL cell density, which lags behind, begins to increase (Hakanson et al., 1993). At two weeks of hypergastrinemia
induced with omeprazole, there is an increase in HDC, CGA and gastrin receptor mRNA levels (Andersson et al., 1996; Kimura et al., 1997). Furthermore, serum pancreastatin and oxyntal mucosal histamine concentrations and HDC activity remain elevated (Kimura et al., 1997) and the ultrastructural modifications of increased vacuoles and microvesicles, and decreased granules are maintained (Chen et al., 1996).

D.2.d. Long-Term

After the maximal response to hypergastrinemia, although the numbers of ECL cells steadily increase to a maximum at twenty weeks (Hakanson et al., 1993), the activity of each individual ECL cell is impaired. While absolute levels of HDC activity, histamine content and HDC and CGA mRNA expression remain elevated, when adjusted to the increased number of ECL cells they decline towards pre-stimulation levels after one month of omeprazole-induced hypergastrinemia (Kimura et al., 1997). However, expression of the gastrin receptor only approaches normal levels when adjusted for ECL cell volume rather than number (Kimura et al., 1997).

ECL cell carcinoids begin to form after one and a half years of sustained hypergastrinemia induced by omeprazole in rats (Hakanson et al., 1990). Another model of ECL cell tumorigenesis is the African rodent mastomys. These rodents have a genetic susceptibility to gastric carcinoids; up to 80% develop gastric lesions by the age of two years (Modlin et al., 1994) and ECL cell tumors present after just four months of sustained hypergastrinemia (Modlin et al., 1989).

D.2.e. Reversibility

Upon removal of omeprazole, hypergastrinemia ceases within a few days (Tielemans et al., 1992). As well, most of the ultrastructural features of elevated ECL cell activity promptly return to normal; the numbers and volume densities of vacuoles, secretory vesicles and microvesicles return to pre-stimulation values within a week after stopping omeprazole treatment (Zhao et al., 1998). There are instances of sustained over-compensation in response to the removal of omeprazole. Granule number and density surpass normal levels, perhaps because less histamine is available to be taken in
and transform these granules into secretory vesicles (Zhao et al., 1998). On the other hand, cell size, HDC activity and replication rate decline to values below normal (Zhao et al., 1998). While the ECL cell labeling index decreases to abnormally low values, ECL cell numbers do not return to pre-stimulation levels until all of the surplus cells undergo apoptosis, after about 100 days (Tielemans et al., 1992). The suppression of mitotic activity could be achieved by an autocrine signal from the still elevated numbers of ECL cells, suppressing self-replication, cell growth and HDC activity to compensate for their presence (Tielemans et al., 1992).

E. Fasting

Fasting is also a pathophysiological state that alters the activity of ECL cells, this time down-regulating them via hypogastrinemia. But the known effects of fasting are not limited to gastrin levels; starvation also has effects on growth and metabolism that involve members of the insulin receptor family.

E.1. Clinical Significance of Fasting

In nature, the threat of insufficient caloric intake is a common challenge. Unfortunately, the danger of starvation is also present in human populations as an aspect of the following: malnutrition caused by unequal food distribution, wasting illnesses like acquired immune deficiency syndrome (AIDS) and cancer, endocrine disorders including diabetes mellitus and Addison’s disease, and eating disorders such as anorexia nervosa and bulimia (Schwartz & Seeley, 1997). Food deprivation triggers neuroendocrine mechanisms that suppress nonessential functions and at the same time stimulate survival mechanisms. Under normal conditions, circulating levels of the hormone leptin relate adipose storage levels to the hypothalamus by inhibiting NPY (Fruhbeck et al., 1998). However, during fasting leptin levels under-represent fat stores (Boden et al., 1996) and NPY levels rise due to the resultant loss of leptin inhibition. Heightened NPY release from the arcuate nucleus stimulates pituitary release of corticotrophins which trigger
adrenal release of glucocorticoids (Schwartz & Seeley, 1998). While this pathway inhibits nonessential processes like reproduction, immunity and growth, it also promotes the utilization of alternative energy stores and prepares the body to maximize caloric intake upon refeeding by increasing the drive to eat (Schwartz et al., 1995).

E.2. Effects of Fasting on the Stomach

In the absence of nutrients, levels of the hormone gastrin decline and digestive function is turned off. Intestinal DNA, RNA and protein content decrease to a greater extent than body weight in fasting (Lichtenberger et al., 1976). While most tissues in the body, including skeletal muscle, heart and liver, adopt a protein-sparing strategy (Cherel et al., 1991), the gastrointestinal tract undergoes protein wasting in response to fasting (Samuels et al., 1996). In the stomach there is decreased protein mass due to a reduction of protein synthesis (Samuels et al., 1996). It is thought that since the function of the stomach, and the rest of the gastrointestinal tract, is not essential to survival when there is no food, the materials and energy for protein synthesis are diverted to more critical organs such as skeletal muscle, heart and liver. As cellular activity diminishes in the stomach, there is a reduction in the mRNA levels of housekeeping genes such as β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Yamada et al., 1997). On the other hand, mRNA levels of heat shock protein (hsp) 70, an intracellular protein involved in the cellular response to stress, increase in an effort to maintain some gastric integrity for when refeeding occurs (Ehrenfried et al., 1996). Hsp70 is the most prominent of a group of chaperones that protect proteins by binding to them, transporting them between organelles and aiding in their refolding after denaturation (Craig et al., 1994).

E.2.a. Digestive Function and ECL Cells

ECL cell function is depressed in fasting by low levels of its primary stimulant, gastrin, and high levels of its primary inhibitor, somatostatin. Upon fasting and the absence of nutrients in the stomach lumen, serum gastrin levels decline (Takeuchi et al., 1979) as do levels of its receptor (Takeuchi et al., 1979), antral gastrin (Schwarting et al., 1986), antral gastrin mRNA (Wu et al., 1991) and G cell density (Schwarting et al.,
1986). Granules in the G cells aggregate and display obvious lysosome and secretory granule fragments (Zaviacic et al., 1976). On the other hand, antral (but not fundic) somatostatin mRNA (Sandvik et al., 1995) and protein (Schwarting et al., 1986) levels are increased. As well, the somatostatin receptor does not undergo ligand induced down-regulation and is instead elevated in parallel with somatostatin (Sandvik et al., 1995). Although D cell density declines in the antrum (Schwarting et al., 1986), there is increased secretory activity of the remaining cells (Zaviacic et al., 1976). Increased somatostatin and decreased gastrin suppress ECL cell activity as seen by the decrease in HDC mRNA levels (Dimaline et al., 1997; Kondo et al., 1995). Presumably, the reduction in histamine secretion as well as direct effects of reduced gastrin and increased somatostatin decrease parietal cell gastric acid secretion.

E.3. Effects of Fasting on the Insulin Receptor Family

Fasting involves the suppression of energy storage and growth, the major functions of the insulin and IGF-I receptors, respectively. The suppression is achieved by decreased levels of insulin and IGF-I and increased counter-regulatory hormones, glucagon, epinephrine and cortisol (reviewed in Macdonald & Webber, 1995; Schwartz & Seeley, 1997). The limited amount of glucose that is stored as glycogen in liver and muscle is quickly exhausted in the absence of caloric intake. To spare lean mass and prolong survival, catabolism of muscle protein is prevented by the oxidation of free fatty acids and ketone bodies, the major sources of energy in fasting (Cahill et al., 1966). Insulin and IGF-I levels are suppressed so that their anabolic effects, including amino acid and glucose uptake and glycogen and lipid synthesis, are not maintained (Macdonald & Webber, 1995). Furthermore the mitogenic effects of IGF-I and insulin, including DNA synthesis and cell proliferation, are depressed in order to conserve material and energy for more essential functions. But paradoxically, the decreased hormone levels relieve ligand-mediated receptor down-regulation and the corresponding receptor levels increase.
E.3.a. The Insulin Receptor

In the hypoinsulinemia of fasting, low plasma insulin levels relieve ligand-induced receptor down-regulation so that there is increased insulin receptor expression in tissues such as liver (Tozzo & Desbuquois, 1992), muscle and fat (Knott et al., 1992) and intestine (Ziegler et al., 1995), but not brain (Marks et al., 1989). The increased number of insulin receptors causes increased insulin binding (Saad et al., 1992; Tozzo & Desbuquois, 1992; Ziegler et al., 1995) but there is no increase in insulin receptor affinity (Balage et al., 1990; Marks et al., 1989). Since there is low circulating insulin, basal levels of autophosphorylated insulin receptor are very low, for example in the liver (Ito et al., 1997a). However, when insulin is administered to fasted rats, insulin receptor autophosphorylation is greater than normal. In the liver this is due to the increased number of insulin receptors and when adjusted, the efficiency of phosphorylation is at control values (Ito et al., 1997b) or reduced (Saad et al., 1992). Regardless, an absolute increase in liver insulin receptor phosphorylation, and phosphorylation of substrate (Ito et al., 1997b), seems contrary to the state of insulin resistance known to occur in the liver and fat of fasted animals. In the liver, there is reduced glucose production (Penicaud et al., 1985), lipid synthesis (Caro & Amatudro, 1982), amino acid uptake (Cech et al., 1980) and glycogen synthesis (Trowbridge et al., 1984). If the liver is insulin resistant during fasting then increased insulin receptor activity must be attenuated at some point along the signaling pathway. While this is not seen at the level of insulin receptor substrate in rats, IRS-1 phosphorylation increases (Ito et al., 1997b), it is seen in a different animal model. IRS-1 phosphorylation decreases in the livers of fasted chickens (Dupont et al., 1998). In the rat, another insulin receptor substrate increases to a much greater extent than IRS-1 with fasting; this 195 kDa protein might attenuate the insulin signaling pathway in the liver (Ito et al., 1997b).

The mechanism of insulin receptor attenuation is species-specific, as seen by the difference between the rat and the chicken, but it is also tissue-specific within a species. In fact, insulin receptor signaling capability is not attenuated at all in muscle. Muscle shows augmented insulin sensitivity with fasting (Goodman et al., 1979; Brady et al., 1981). The increase in insulin receptor activity in muscle is further augmented by an
increase in insulin receptor phosphorylation efficiency. This heightened responsiveness is thought to direct transient nutrient intake to the muscle and allow the liver to continue gluconeogenesis (Contreras et al., 1990).

E.3.b. The IGF-I Receptor

As for the insulin receptor, low levels of ligand allow IGF-I receptor mRNA levels to rise. Fasting reduces serum GH and its binding so that serum IGF-I levels also decline (Thissen et al., 1994). The decrease in IGF-I is not completely due to decreased GH stimulation since normal GH function is coincident with suppressed IGF-I during protein restriction (Thissen et al., 1990; Vance et al., 1992). There are low levels of IGF-I mRNA in the liver, the site of synthesis for the endocrine hormone, and other tissues like lung, kidney, muscle, stomach, brain and testes where IGF-I is thought to have paracrine and autocrine effects on local growth (Lowe et al., 1989). Decreased IGF-I levels relieve ligand-induced receptor down-regulation so that IGF-I receptor mRNA levels increase in lung, kidney, muscle, testes, brain and stomach (Lowe et al., 1989). This increase is tissue specific because it is not seen in the intestine (Winsett et al., 1995; Ziegler et al., 1995), pituitary or hypothalamus (Olchovsky et al., 1993). In the jejunum, the abundance of IGF-I binding protein-3, a protein that limits the bioavailability of IGF-I, is reduced (Winesett et al., 1995). It remains reduced during initial refeeding and may represent an attempt to restore intestinal mass when nutrients are once again present. Similarly, increased IGF-I receptor signaling potential provided by increased receptor expression, content and binding may prepare the tissue for refeeding when IGF-I levels will rise and thus help to restore growth.

F. Summary & Objectives

IRR is an orphan member of the insulin receptor family of RTKs. It is expressed selectively in the ECL cell, the primary regulator of parietal cell gastric acid secretion. ECL cell activity can be elevated or suppressed in the pathophysiological models of hypergastrinemia and fasting, respectively. Additionally, the low levels of insulin and IGF-I in fasting increases the mRNA levels of both of the other members of the insulin
receptor family by relieving ligand-induced receptor down-regulation. It was therefore of interest to see how IRR mRNA levels changed in these two states. While it could be predicted that IRR mRNA levels increase with hypergastrinemia, like the mRNA levels of HDC and the gastrin receptor, other markers for ECL cells, it was more difficult to predict the response of IRR mRNA levels to fasting. In fasting, IRR seemed to be potentially controlled by two opposing forces; the decrease in ECL cell activity and ECL cell markers versus the increased mRNA levels of other insulin receptor family members. The evaluation of these effects of these opposing forces on IRR mRNA levels would suggest physiological functions in which the orphan receptor may be involved, such as gastric acid secretion, growth or metabolism.
II. MATERIALS & METHODS

A. Experimental Design

A.1. Hypergastrinemia

Twenty-five female Sprague-Dawley rats (Charles River Canada Ltd., St. Constant, PQ, Canada) weighing 250-290 g at the start of the experiment were used. The rats were paired as one control and one experimental in plastic cages with free access to standard rodent chow and water. They were housed under controlled light (12 h light and 12 h dark) and temperature (22 °C) conditions. The experiments were approved by The University of Toronto Animal Care Policies and Guidelines.

Twelve rats received daily omeprazole by gastric gavage (400 μmol/kg; Astra Hässle, Mölndal, Sweden) between 9:30 and 10:30 each morning. Controls received vehicle (buffered methocel 0.5% Solution, pH 9.0). Two groups - experimental and control - of 3-5 rats each were sacrificed 4 h, 4 days and 2 weeks after initiation of omeprazole or vehicle treatment. The latter two sets were sacrificed at 9:00 h; they received their last treatment 24 h prior to death.

A.2. Fasting

Thirty-two female Sprague Dawley rats (Charles River) weighing 230-330 g at the start of the experiment were used. The rats were housed as pairs in plastic cages with free access to water and controlled light (12 h light and 12 h dark) and temperature (22 °C) conditions. The experiments were approved by The University of Toronto Animal Care Policies and Guidelines.

Eight control rats had free access to standard rodent chow while the other 24 were fasted beginning at 9:00 h. Controls and 8 fasted rats were sacrificed 48 h after the induction of fasting while the remaining 16 were refed with free access to standard rodent chow for 4 h or 2 days.
B. Tissue Collection

Rats were sacrificed by cardiac puncture under halothane anesthesia. Stomachs were removed by excision distal to the cardiac and pyloric sphincters. They were cut open along the major line of curvature and rinsed with ice cold phosphate-buffered saline (PBS; Sambrook et al., 1989). For the hypergastrinemia study, stomachs were symmetrically divided in half. Tissue was quick-frozen in liquid nitrogen and stored at -75 °C.

C. RNA Extraction

Total RNA was extracted in guanidine thiocyanate, purified on a cesium chloride gradient and re-extracted with buffer-saturated phenol (Gibco BRL Life Technologies, Inc., Gaithersburg, MD, USA):chloroform:isoamyl alcohol (25:24:1; Chirgwin et al., 1979). The RNA was inspected for quantity by absorbance at 260 nm and quality by agarose gel electrophoresis. It was precipitated under ethanol and stored at -20 °C.

D. cDNA Synthesis

Five μg of total RNA were primed with 250 ng random hexamers (Gibco BRL) to synthesize single-stranded complimentary DNA (cDNA) for 1 h at 50 °C using 200 U Superscript II reverse-transcriptase (Gibco BRL) in the recommended buffer with 10% glycerol and 10 nmol sodium-salt dNTPs (Boehringer-Mannheim GmbH, Mannheim, Germany). The reaction was stopped by heating to 70 °C for 15 min, diluted 1:25 with sterile water and stored at -20 °C.

E. PCR Analysis

The diluted cDNA reaction (10 μL) was prepared for PCR with 1 μg of each primer and 20 nmol sodium-salt deoxynucleic triphosphates (dNTPs; Boehringer-Mannheim) in the recommended buffer (QIAGEN GmbH, Hilden, Germany). PCR
amplification was initiated at 96 °C for 5 min and 72 °C for 1.5 min in a temperature cycler (Amplitron II or Ericomp) before enzyme addition (1.5 U Thermus aquaticus. Taq. DNA polymerase, QIAGEN), followed by a program developed specifically for each primer set (Table 1).

Aliquots (15 μL) were taken after DNA extension (72 °C) at five different points along the cycle course of amplification for each sample. A volume of 5 μL from each aliquot, shown to be within the linear range of the quantitation process, was subjected to agarose gel electrophoresis. The gel was stained with SYBR green dye (Molecular Probes, Eugene, OR, USA), scanned onto the STORM Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA) and analyzed using ImageQuant software for the Apple Macintosh computer (Molecular Dynamics). Integrity of product was verified by comparison to the DNA marker (low DNA mass ladder; Gibco BRL) The linear range of amplification was titrated for each sample and a cycle number common to this range for all the samples was chosen for quantitation of mRNA expression.

G. Statistics

All data are expressed as mean ± SEM. Statistical significance was determined by ANOVA followed by a Tukey post-hoc test using a SAS program for IBM computers (Statistical Analysis Systems, Cary, NC, USA).
Table 1. PCR primers and thermal amplification profiles.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primers</th>
<th>Location</th>
<th>Thermal Amplification Profile</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRR(^a)</td>
<td>5'-TTCTGTGTGGAGAAGAACCAG-3'**</td>
<td>814-833</td>
<td>93°C 1 min 62°C 2 min</td>
<td>28-36</td>
</tr>
<tr>
<td></td>
<td>5'-TATAGAAT-</td>
<td>8 bp EcoR I</td>
<td>then 1304-1327</td>
<td>72°C 2 min</td>
</tr>
<tr>
<td></td>
<td>TCCGGGTGAAGCCTGGAGGGCCTGA-3'***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(\beta)-actin(^b)</td>
<td>8 bp EcoR I</td>
<td>then 1304-1327</td>
<td>72°C 2 min</td>
</tr>
<tr>
<td></td>
<td>5'-TGC-</td>
<td>3 bp spacer</td>
<td>93°C 1 min</td>
<td>19-23</td>
</tr>
<tr>
<td></td>
<td>TCGACAACGGCTCCGGCATGT-3'**</td>
<td>then 1272-1292</td>
<td>56°C 2 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-CCAGCCAGGTCGAGCAGAGGAT-3'***</td>
<td>2317-2339</td>
<td>72°C 2 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18S rRNA(^c)</td>
<td>355-372</td>
<td>93°C 30 sec</td>
<td>13-21</td>
</tr>
<tr>
<td></td>
<td>5'-CTGCCCTATCAACTTCG-3'**</td>
<td>854-871</td>
<td>62°C 45 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-CATTATCTCCTAGCCTGCGG-3'***</td>
<td>then 1272-1292</td>
<td>72°C 1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HDC(^d)</td>
<td>10 bp Hind III</td>
<td>94°C 1 min</td>
<td>28-33</td>
</tr>
<tr>
<td></td>
<td>5'-AGAGATGGTGGATTACATCT-3'**</td>
<td>then 120-139</td>
<td>55°C 2 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-TACTTGTCCTGACCCAGAA-3'***</td>
<td>1030-1049</td>
<td>72°C 2 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gastrin</td>
<td>10 bp Hind III</td>
<td>14 bp Hind III</td>
<td>as above</td>
</tr>
<tr>
<td></td>
<td>5'-ATATAAGCTT-</td>
<td>then 952-974</td>
<td>13 bp Hind III</td>
<td>for IRR</td>
</tr>
<tr>
<td></td>
<td>Receptor(^e)</td>
<td>9 bp Eco R I</td>
<td>then 1491-1514</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCAGACACCAGGCGCTGCCACCA-3'**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-AGTGAATTC-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTCTCCAATCTCCTCAACCCTCAG-3'***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Shier & Watt, 1992  
\(^b\) Nudel et al., 1983; Salvatore et al., 1995  
\(^c\) Chan et al., 1984; Dupont et al., 1998  
\(^d\) Kondo et al., 1995; Joseph et al., 1990  
\(^e\) Prinz et al., 1997; Wank et al., 1992  
* sense primer ** anti-sense primer
III. RESULTS

A. Hypergastrinemia

To study the effects of elevated ECL cell activity on IRR mRNA levels, hypergastrinemia was induced in rats by gastric gavage of high levels of the PPI omeprazole (400 μmol/kg). The acute, intermediate and maximal effects of this treatment were examined at four hours, four days and two weeks. As well, the levels of HDC and gastrin receptor mRNA were measured at these times to assess the elevated ECL cell activity.

A.1. HDC & Gastrin Receptor

To verify that ECL cell activity was elevated, HDC mRNA levels were assessed. HDC is a marker for ECL cells that is commonly used to reflect their activity (Kimura et al., 1997). A 929 bp fragment of HDC cDNA was amplified that included exons 3 to 9 of the rat HDC gene. The inclusion of an intron-exon boundary within the amplified region made it possible to discriminate between the amplification of cDNA and that of any contaminating genomic DNA which would yield a larger fragment. Genomic DNA, accidentally co-extracted with RNA, would consume reactants such as primers, dNTPs and Taq polymerase during the PCR reaction, limiting amplification of the desired cDNA fragment. Genomic DNA contamination was not detected at the size predicted from the human gene (~10 kb; Yatsunarni et al., 1994). At 2 weeks of omeprazole treatment, HDC levels were almost 75% greater than control (171 ±2 %; p<0.05; Figure 3; Table 2).

The gastrin receptor is another indicator of hypergastrinemia that is expressed in both ECL and parietal cells. Its levels were assessed using primers that amplified a 540 bp fragment. Since this part of the gene did not span an intron-exon boundary, PCR was performed on the RNA template that had been used for cDNA synthesis. There was no amplification of the gastrin receptor, confirming that genomic DNA did not contribute to the results. Gastrin receptor expression increased by 25% after two weeks of hypergastrinemia (125 ±9 % of control; p<0.05; Figure 3; Table 2).
Figure 3. Gastric HDC & gastrin receptor mRNA levels in hypergastrinemia. Stomach HDC (A) and gastrin receptor (B) mRNA levels were determined by semi-quantitative RT-PCR at cycles 28 and 30, respectively in CON (solid bars) and OMP (open bars) rats treated for 4 days (n=4 for each group) and 2 weeks (n=4 for CON and n=3 for OMP). Values are expressed in relation to β-actin and as % of time-specific CON. 100%.

* P<0.05 vs. time-specific CON.
Table 2. Changes in gastric mRNA levels with hypergastinemia.

<table>
<thead>
<tr>
<th></th>
<th>4 h</th>
<th>4 d</th>
<th>2 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>OMP</td>
<td>CON</td>
</tr>
<tr>
<td><strong>IRR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRR</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>± 30</td>
<td>± 20</td>
<td>± 6</td>
<td>± 6</td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>97</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>± 10</td>
<td>± 9</td>
<td>± 10</td>
<td>± 6</td>
</tr>
<tr>
<td><strong>18S rRNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>105</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>± 5</td>
<td>± 5</td>
<td>± 7</td>
<td>± 10</td>
</tr>
<tr>
<td><strong>HDC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>112</td>
</tr>
<tr>
<td>± 20</td>
<td>± 30</td>
<td>± 8</td>
<td>± 9</td>
</tr>
<tr>
<td><strong>Gastrin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>120</td>
<td>100</td>
<td>101</td>
</tr>
<tr>
<td><strong>Receptor</strong></td>
<td>±8</td>
<td>± 20</td>
<td>± 7</td>
</tr>
</tbody>
</table>

**IRR, HDC** and **gastrin receptor adjusted to β-actin amplification.**

All values expressed as % of time-specific **CON**, 100%.

*P < 0.05  **P < 0.001 vs. time-specific **CON**

**CON**-control; **OMP**-omeprazole
The RT-PCR amplification was compared to a control, β-actin (Figure 4; Table 2). For each sample, the amplification of β-actin was used to normalize amplification of the other investigated transcripts, HDC and the gastrin receptor as well as IRR. Primers amplified a 456 bp region that spanned exons 2 to 4 of the rat β-actin gene. No genomic DNA contamination was present to confound amplification of β-actin cDNA: the larger sized genomic amplicon of 1102 bp was not visualized upon agarose gel electrophoresis of the PCR reactions. β-actin expression remained constant between control and omeprazole-treated rats at each time point so the housekeeping gene was used to control for the semi-quantitative RT-PCR procedure.

The 18S ribosomal RNA subunit was analyzed as a supplementary control point (Figure 4; Table 2). Primers amplified a 517 bp region near the beginning of the transcript. Since the 18S rRNA gene does not have intron/exon organization, the absence of contaminating genomic DNA was demonstrated by negative results when PCR was performed on the RNA template used for cDNA synthesis. Although 18S rRNA levels did not change significantly between control and omeprazole-treated rats at any time, β-actin was used as the control since it is more common in the literature (Chen et al., 1994) and has an intron-exon organization that allows easier detection of contamination.

A.2. IRR

To determine whether alterations of IRR mRNA levels were associated with increased ECL cell activity, IRR mRNA levels were quantitated using semi-quantitative RT-PCR (Figure 5; Table 2). Primers amplified a 489 bp region at the beginning of the rat IRR gene, encompassing exons 2 and 3, so that both the truncated and full length forms of IRR were included. No genomic DNA was detected in the PCR products at ~2.1 kb (Figure 5.A). The amplification profile was graphed (Figure 5.B) and a cycle number that was within the linear range of amplification for all of the samples was chosen to represent relative mRNA levels. There were no changes in IRR mRNA levels in the
Figure 4. Gastric β-actin mRNA & 18S rRNA levels in hypergastrinemia. Stomach β-actin mRNA (A) and 18S rRNA (B) levels were determined by semi-quantitative RT-PCR at cycles 22 and 17, respectively in CON (solid bars) and OMP (open bars) rats treated for 4 days (n=4 for each group) and 2 weeks (n=4 for CON and n=3 for OMP). Values are expressed as % of time-specific CON, 100%.
Figure 5. Gastric IRR mRNA levels in hypergastrinemia. A. Representative semi-quantitative RT-PCR titrations of control (CON) and omeprazole (OMP) -treated rats at 2 weeks. Agarose gel electrophoresis of PCR titrations (cycles 27, 29, 31, 33, 35) stained with SYBR green dye. Marker corresponds to top, 2000, 1200, 800 and 400 bp, bottom DNA size markers. B. PCR amplification curves for the same representative samples (■ = CON; ○ =OMP). IRR mRNA levels are represented by arbitrary volume units as determined by scanning of the stained agarose gel onto the STORM Fluorimager and quantitation with ImageQuant software; they are plotted against cycle number. C. Stomach IRR mRNA levels were determined by semi-quantitative RT-PCR at cycle 31 in CON (solid bars) and OMP (open bars) rats treated for 4 days (n=4 for each group) and 2 weeks (n=4 for CON and n=3 for OMP). Values are expressed in relation to β-actin and as % of time-specific CON, 100%. ** P<0.01 vs. time-specific CON.
stomach during the acute or intermediate stages of hypergastrinemia (4 h, 4 d). At the maximal stage after two weeks of hypergastrinemia, however, IRR mRNA levels in the stomach rose significantly to a value that was 50% greater than control levels (148 ±4 %; p<0.01).

A.3. Growth

Rat weights were recorded to see if the experimental manipulations had any effects on growth. The weights were taken one day prior to the start of the treatment and at the time of sacrifice. Surprisingly, none of the rats followed the expected growth pattern for female Sprague-Dawley rats (Harlan Sprague-Dawley Inc., 1997); both control and experimental groups maintained a state of negative energy balance for the duration of the experiment (Figure 6). Just four hours after the administration of vehicle (buffered HPMC 0.5% Solution, pH 9.0, with or without omeprazole) by gastric gavage, the rats lost on average 3.3 ±0.6 % of their body weight. There was a significant difference between the groups at four days; omeprazole-treated rats lost more than twice the weight of vehicle-treated rats (5.6 ±0.8 % compared to 2.2 ±0.8 %; p<0.05). Within two weeks however, the control rats had experienced similar weight loss as experimental rats: 6 ±1% and 5 ±2 %.

B. Fasting

To study the effects of suppressed ECL cell activity on IRR mRNA levels, rats were deprived of food for two days. In addition, the recovery from starvation was assessed at four hours and two days after refeeding. As for hypergastrinemia, the levels of HDC and gastrin receptor mRNA were measured to assess the suppression of ECL cell activity.

B.1. Growth

Fasting caused a notable state of negative energy balance (Figure 7). Weights were recorded two days before the start of the experiment and at the time of sacrifice. Control rats followed the expected growth curve for female Sprague-Dawley rats (Harlan
Figure 6. Body weight in hypergastrinemia. Weight loss, expressed as a percentage of original body weight, for control (CON; solid bars) and omeprazole-treated (OMP; open bars) female Sprague-Dawley rats at 4 hours (n=5 for both groups), 4 days (n=4 for each group) and 2 weeks (n=4 for CON and n=3 for OMP). * P<0.05 vs. time-specific CON.
**Figure 7.** Body weight in fasting. Weight loss, expressed as a percentage of original body weight, for female Sprague-Dawley rats (control; n=7) that were fasted for 48 h (n=8) and refed for 4 h (n=8) or 2 d (n=8). *****P<0.001 vs. Controls.
Sprague-Dawley Inc., 1997), gaining 5.1 ±0.9 % over the 2 days of the experimental period. However, food deprivation for this time reduced body mass by 9.0 ±0.1 % in fasted rats (p<0.001 compared to control). At 4 hours of refeeding, rats did not regain a significant amount of weight and were still 6.2 ±0.6 % (NS compared to fasted; p<0.001 compared to control) below their original body weight. While the original body weight was achieved with 2 days of refeeding (1 ±1 % weight loss), the fasted then refed (2 days) rats were still below the weight of controls (p<0.001 compared to control). In summary, rats lost almost 10% of their body mass after a two day fast and succeeded in regaining their initial weight with two days of refeeding.

B.2. HDC & Gastrin Receptor

The measurement of HDC mRNA levels was used to confirm the state of decreased ECL cell activity. HDC expression in rat stomach was halved after a 48 hour fast (53 ±5 %; p<0.001; Figure 8; Table 3) and did not significantly increase with 4 hours of refeeding (58 ±6 %; p<0.001 compared to control and NS compared to fasted). After two days of refeeding it had returned to normal (89 ±9 %; p<0.01 compared to fasted; NS compared to control).

Gastrin receptor mRNA levels were analyzed to see if they paralleled depressed ECL cell activity and low levels of their ligand, gastrin. As for HDC, the mRNA levels of the gastrin receptor declined in the fasting paradigm, this time to 58 ±5 % of control (p<0.01; Figure 8; Table 3). Normal receptor levels were restored with 2 days of refeeding (91 ±7 %; p<0.05 compared to fasted).

Initially, β-actin was chosen to normalize amplification of transcripts for the semi-quantitative RT-PCR procedure in fasting. However, it was shown to decrease almost by half (55 ±7 % of control; p<0.001; Figure 9; Table 3) with fasting. Its expression remained suppressed even after four hours (53 ±2 % of control; p<0.001) and two days (74 ±8 % of control; p<0.05) of refeeding.
**Figure 8.** Gastric HDC & gastrin receptor mRNA levels in fasting. Stomach HDC (A) and gastrin receptor (B) mRNA levels were determined by semi-quantitative RT-PCR. The samples were analyzed as two equal sets and measurements were taken at cycles 29 and 30 for HDC or cycles 30 and 31 for the gastrin receptor in rats (control; n=8) that were fasted for 48 h (n=8) and refed for 4 h (n=8) or 2 d (n=8). Values are expressed in relation to 18S rRNA and as % of Controls, 100%. *P<0.05 **P<0.01 ***P<0.001 vs. Controls.
Table 3. Changes in gastric mRNA levels with fasting.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>FASTED</th>
<th>REFED 4h</th>
<th>REFED 2d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IRR</strong></td>
<td>100</td>
<td>170***</td>
<td>111</td>
<td>104</td>
</tr>
<tr>
<td>± 5</td>
<td>± 10</td>
<td>± 7</td>
<td>± 9</td>
<td></td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td>100</td>
<td>52***</td>
<td>54***</td>
<td>74*</td>
</tr>
<tr>
<td>± 4</td>
<td>± 7</td>
<td>± 2</td>
<td>± 8</td>
<td></td>
</tr>
<tr>
<td><strong>18S rRNA</strong></td>
<td>100</td>
<td>97</td>
<td>103</td>
<td>97</td>
</tr>
<tr>
<td>± 5</td>
<td>± 7</td>
<td>± 6</td>
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<td><strong>HDC</strong></td>
<td>100</td>
<td>53***</td>
<td>58***</td>
<td>89</td>
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<tr>
<td>± 6</td>
<td>± 5</td>
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<td>± 9</td>
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<tr>
<td><strong>Gastrin Receptor</strong></td>
<td>100</td>
<td>58**</td>
<td>70*</td>
<td>91</td>
</tr>
<tr>
<td>± 10</td>
<td>± 5</td>
<td>± 7</td>
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IRR, HDC and gastrin receptor adjusted to 18S rRNA amplification. All values expressed as % Control, 100%. *P<0.05 **P<0.01 ***P<0.001
Figure 9. Gastric β-actin mRNA and 18S rRNA levels in fasting. Stomach β-actin mRNA (A) and 18S rRNA (B) levels were determined by semi-quantitative RT-PCR at cycles 21 and 15, respectively in rats (control; n=4, β-actin; n=8, 18S) that were fasted for 48 h (n=4, β-actin; n=8, 18S) and refed for 4 h (n=4, β-actin; n=8, 18S) or 2 d (n=4, β-actin; n=8, 18S). Values are expressed as % of Controls, 100%. *P<0.05  ***P<0.001 vs. Controls.
A

\[ \text{\(\beta\)-actin mRNA levels (\% control)} \]

<table>
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B

\[ \text{18S rRNA levels (\% control)} \]

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Subsequently, the 18S ribosomal RNA subunit was used to normalize the amplification of IRR since there were no changes in its mRNA levels with fasting or refeeding (Figure 9; Table 3). Each sample’s specific 18S rRNA amplification value was used to normalize its amplification with the other primer sets: HDC, the gastrin receptor, and IRR.

B.3. IRR

To determine the effect of reduced ECL cell activity on IRR mRNA levels, semi-quantitative RT-PCR was used to measure the changes in IRR mRNA levels. After a 48 hour fast, IRR mRNA levels were increased by 70 ±10% (p<0.001; Figure 10; Table 3). This enhancement was reduced with refeeding in just 4 hours (111 ±7%; p<0.01 compared to fasted and NS compared to control). Thus in fasting, IRR expression did not parallel the activity of ECL cells.
Figure 10. Gastric IRR mRNA levels in fasting. A. Representative semi-quantitative RT-PCR titrations of control, fasted, refed 4h and refed 2d. Agarose gel electrophoresis of PCR titrations (cycles 28, 30, 32, 34, 36) stained with SYBR green dye. Marker corresponds to top, 2000, 1200, 800 and 400 bp, bottom DNA size markers. B. PCR amplification curves for the same representative samples (□ = control; ○ = fasted; ¤ = refed 4h; ▼ = refed 2d). IRR mRNA levels are represented by arbitrary volume units as determined by scanning of the stained agarose gel onto the STORM Fluorimager and quantitation with ImageQuant software; they are plotted against cycle number. C. Stomach IRR mRNA levels were determined by semi-quantitative RT-PCR at cycle 32 in rats (control; n=7) that were fasted for 48 h (n=8) and refed for 4 h (n=8) or 2 d (n=8). Values are expressed in relation to 18S rRNA and as % of Controls, 100%. ***P<0.001 vs. Controls.
<table>
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<td>36</td>
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**A**

- genomic DNA
- IRR cDNA

**B**

Graph showing IRR mRNA levels (arbitrary volume units) vs. cycle number.

**C**

Bar chart showing IRR mRNA levels (% control) for different conditions: Control, Fasted, Refed 4h, Refed 2d.
IV. DISCUSSION

IRR, a member of the insulin receptor family, is selectively expressed in the most abundant neuroendocrine cell in the stomach, the ECL cell. This very specific localization suggests that this orphan receptor plays a role in ECL cell function, namely in the control of parietal cell gastric acid secretion. This suggestion has been substantiated by the demonstration that IRR mRNA is up-regulated at a time when there is increased ECL cell activity in hypergastrinemia. On the other hand, IRR is also up-regulated in the hypogastrinemic state of fasting when ECL cell activity is decreased. Fasting is also a hypoinsulinemic state in which the mRNA levels of other members of the insulin receptor family increase due to lack of ligand-induced down-regulation. In contrast, positive feedback was demonstrated on a molecular level by decreased gastrin receptor mRNA levels with the hypogastrinemia of fasting.

A. IRR

The increase in IRR mRNA levels in both hypergastrinemia and fasting suggests that the orphan receptor may be involved in one or more of the following: gastric acid secretion, metabolism and growth. IRR mRNA levels increased after two weeks of omeprazole administration, at a time when ECL cell activity and HDC and the gastrin receptor mRNA levels are also increased (Kimura et al., 1997; present study). No change in mRNA levels was apparent before this time. This is consistent with the thought that the initial enhancement of ECL activity mainly involves secretion of products and activation of proteins (Kimura et al., 1997). HDC activity has been previously shown to be greatly elevated before any detectable increase in HDC mRNA expression (Chen et al., 1994). Therefore, there may be significant modulation of the IRR protein and its activity at an earlier time in the progression of hypergastrinemia.

On the other hand, the increase in IRR mRNA levels with fasting is in contrast to the depressed state of ECL cell activity and decreased levels of the other ECL cell markers, HDC and the gastrin receptor. Since the other members of the insulin receptor family increase due to suppression of their ligands in fasting, IRR may bind a related
ligand that is also suppressed in fasting. The similar elevations in mRNA levels of other insulin receptor family members during fasting and the observation that most members within an RTK family share related functions (Heldin, 1996) suggest that IRR may be involved in aspects of fasting other than hypogastrinemia, for example metabolism or growth. During fasting, low circulating levels of insulin and IGF-I relieve the usual ligand-mediated receptor down-regulation of the insulin and IGF-I receptors. Insulin receptor mRNA levels increase 10-fold in the liver after just 24 hours of fasting (Tozzo & Desbuquois, 1992); less dramatic elevations occur in muscle and fat (Knott et al., 1992) and intestine (Ziegler et al., 1995). IGF-I receptor mRNA levels increase in the stomach almost 2.5-fold with a 48 hour fast; similar elevations occur in lung, testes, kidney and heart (Lowe et al., 1989). Levels of the ligand for IRR may also be low in fasting and permit enhanced levels of receptor transcripts in the same way. An elevated number of receptors may also have functional significance. For example, increased levels of insulin receptor augment insulin responsiveness in muscle upon fasting (Contreras et al., 1990). This is tissue-specific as increased insulin action is attenuated in the liver and fat, and is thought to direct any sporadically ingested nutrients to the muscle while maintaining liver gluconeogenesis. In a similar manner, increased IGF-I receptor expression may potentiate growth by facilitating a greater response to IGF-I when refeeding occurs. Conceivably, the increase in IRR mRNA could also have functional relevance while at the same time reflecting a change in ligand levels that results from the metabolic perturbation. Alternatively, if the ligand for IRR is produced in the stomach or intestine, its levels may decrease as a result of the dramatic wasting of these tissues during fasting (Samuels et al., 1996).

Since the trends for both omeprazole-induced hypergastrinemia and fasting involve an increase in IRR mRNA levels, IRR may be regulated in the same way by a common feature of the two paradigms. For example, both states involve an increase in stomach lumen pH. The absence of protein or lipid in the gut during fasting causes hypogastrinemia which decreases ECL cell stimulation of parietal cell gastric acid release (Ward & Coates, 1987; Wu et al., 1991) and omeprazole directly blocks parietal cell gastric acid release to cause hypergastrinemia. IRR and its ligand could be regulated by
lumenal acidity. If the up-regulation of IRR mRNA levels is, like the other members of the insulin receptor in fasting, due to lack of ligand-induced down-regulation, then an increase in pH may suppress the ligand for IRR. IRR is localized to another cell type involved in and regulated by acid/base balance in the collecting tubule of the kidney. The B intercalated cell secretes base into the urine via a chloride/bicarbonate (Cl/HCO₃⁻) exchanger on its apical membrane (Edwards et al., 1992) and acid into the circulation through an H⁺-ATPase on its basolateral membrane (Bastani et al., 1991). Under acidic conditions, B cells reverse their polarity to become A cells, secreting acid into the lumen via H⁺-ATPases now on the apical membrane (Al-Awqati et al., 1998). The mechanism for this phenotype switch involves the protein hensin. Hensin is thought to bind an RTK (Vijayakumar et al., unpublished observations in: Al-Awqati et al., 1998) that is on the basolateral surface of both types of intercalated cells (Takito et al., 1996). IRR is an RTK that has been localized to the basolateral membrane of B intercalated cells (Ozaki et al., 1997) and could be involved in the pH-responsive plasticity of at least the B intercalated cell. Since the collecting tubule dramatically increases acid secretion in response to metabolic acidosis (Cogan & Quan, 1992), it is likely that there is a switch from B to A intercalated cell phenotypes during fasting. IRR could respond to decreased pH and facilitate B cell plasticity, thus promoting acid secretion into the urine. Since IRR mRNA levels are increased when gastric acid secretion is suppressed in the stomach, the exact role of IRR in pH regulation may be tissue-specific. Furthermore, the increase in IRR mRNA levels in hypergastrinemia occurs well after the increase in stomach pH, suggesting that other factors may facilitate a more rapid enhancement of IRR mRNA in fasting.

Recently IRR levels in rat kidney have been shown to increase 2.5-fold with 48 hours of fasting (Chrysis et al., 1998). My lower IRR mRNA elevation in rat stomach (50% greater than control) could be a result of suppressed ECL cell activity that counteracts the stimulus for elevation of IRR mRNA levels. Alternatively, Chrysis et al. (1998) expressed their data relative to β-actin, the mRNA levels of which are decreased
in the fasted stomach. It has not been shown whether β-actin mRNA is regulated by prandial state in the kidney, so the reported 2.5-fold increase in IRR mRNA could be overstated. Yamada et al. (1997) showed that the mRNA levels of β-actin decrease by ~50% with fasting in the rat stomach. This suppression is tissue-specific, since β-actin mRNA levels remain constant in the fasted rat liver and duodenum (Hodin et al., 1995).

β-actin was initially chosen to normalize the RT-PCR procedures. While its mRNA levels did not change with hypergastrinemia, the dramatic halving of its expression in fasted rats was a major concern. While the rest of the body, especially essential organs like the liver, heart and skeletal muscle, adopts a protein sparing strategy during starvation (Cherel et al., 1991), the digestive system, including the stomach, undergoes protein wasting (Samuels et al., 1996). Thus housekeeping genes such as β-actin and GAPDH are less necessary and down-regulated to allow more materials and energy for functions critical to survival (Yamada et al., 1997). The demand for β-actin may also be decreased more directly by the hypogastrinemia of fasting. In the parietal cell, β-actin is localized to the apical plasma membrane where it is thought to be involved in the mechanism of gastric acid secretion (Yao et al., 1995). Since omeprazole-induced hypergastrinemia is also associated with low acid secretion, β-actin mRNA levels would not be expected to increase. Instead, they did not change with omeprazole administration and so the housekeeping gene was used as the internal standard for the hypergastrinemia study.

The up-regulation of IRR mRNA levels in both fasting and hypergastrinemia is similar to that of SSTR2, the somatostatin receptor. Levels of SSTR2 mRNA increase in both fasting and hypergastrinemia, at times when ligand levels are increased and decreased, respectively (Sandvik et al., 1995). This is thought to reflect the importance of the somatostatin receptor in controlling gastric acid secretion; while it depresses gastric function in fasting, it also attenuates the increased gastric function caused by hypergastrinemia (Sandvik et al., 1995). Thus it is possible that like SSTR2, IRR has an important function in the control of gastric acid secretion that warrants its augmentation in opposite states of ECL cell activity.
B. The Gastrin Receptor

While the gastrin receptor is located on parietal and somatostatin (D) cells, the proliferative response to gastrin is mostly produced by its presence on ECL cells (Nagata et al., 1996; Wank, 1998). The feedback regulation of the gastrin receptor by gastrin is unusual. Usually, ligand down-regulates expression of its receptor, adhering to the physiological principle of negative feedback. However there are rare cases, such as with prolactin (Bohnet et al., 1977), angiotensin II (Wagner & Kurtz, 1998) and vitamin D (Solvsten et al., 1997), in which a ligand increases the levels of its receptor. The positive feedback effect of gastrin on the gastrin receptor is also such a case. Scatchard analysis (Takeuchi et al., 1980) showed a 48% reduction in gastrin receptor levels after a 4 day fast. I have now demonstrated this on a molecular level; gastrin receptor mRNA levels paralleled the suppression of ECL cell activity and presumably gastrin levels during fasting. The 58% reduction in mRNA levels after 2 days of fasting is comparable to the reduction in protein shown almost 20 years ago. The positive regulation of the gastrin receptor by its ligand is opposite to the ligand-induced down-regulation seen for most receptors, including the previously discussed insulin receptor family.

On the other hand, there was an increase in gastrin receptor mRNA levels at 2 weeks of hypergastrinemia. Up-regulation of gastrin receptor mRNA levels at this time point in omeprazole treatment was also shown by Kimura et al. (1997), although to a larger extent, 213% compared to my 125% of control. This group, however, used male rats whereas I used female rats. Adult female rats have fewer gastrin receptors than males because of a negative effect of estrogen (Johnson et al., 1982). It is possible that the regulation of the gastrin receptor might also be limited by female hormones and so the enhancement of gastrin receptor mRNA levels with hypergastrinemia is curbed.

C. HDC

A change in HDC expression was used to confirm the effects of hyper- and hypogastrinemia on ECL cell activity. HDC mRNA levels increased more than any other transcript observed in the study of hypergastrinemia. The 71% increase at 2 weeks seen in female Sprague-Dawley rats is similar to an 87% increase seen by Kimura et al. (1997)
at the same time (14 days) in male rats of the same strain and a 66% increase seen by Andersson et al. (1996) four days earlier, at 10 days of omeprazole treatment in female Sprague-Dawley rats. The former study did not see any change in HDC mRNA levels after 1, 3 or 7 days of omeprazole treatment, also consistent with my findings. Other studies have, however, shown greater changes in HDC expression with hypergastrinemia. For instance, one study with female Sprague-Dawley rats observed increased HDC mRNA levels to be almost 2-fold at 3 days and almost 5-fold at 7 days compared to time-matched vehicle-treated controls (Swarovsky et al. 1994). Other studies can be excluded due to the use of different experimental controls, such as fasted male Sprague-Dawley rats (4-fold increase at 8 hours; Chen et al., 1994). Dimaline & Sandvik (1991) showed that fasted male Wistar rats have depressed HDC levels that, when used as controls, exaggerate the elevation of HDC mRNA levels as caused by hypergastrinemia (282% of fasted control vs. 85% of fed control at 48 hours).

The importance of choosing a suitable control procedure was also reflected by the measurement of growth. All of the rats in the hypergastrinemia study, including vehicle-treated controls, lost weight throughout the experiment. Vehicle administration by gastric gavage probably had an anorectic effect on the rats; either reflux of the unpalatable vehicle (bicarbonate-buffered Methocel) or the invasive procedure of probe insertion down the esophagus disturbed the feeding behavior of the rats. Since administration of the vehicle presumably has metabolic consequences, the inclusion of time-specific vehicle-administered controls is very important. Surprisingly, the study from Kimura et al. (1997) in which the elevation in HDC mRNA levels was very similar, used untreated controls sacrificed at the beginning of the experiment and age-matched controls that were not given vehicle. Obviously, the inconsistent use of controls makes it difficult to draw comparisons between studies. Nonetheless, the increase in HDC expression at 2 weeks agrees with the most current literature and demonstrates achievement of elevated ECL cell activity in the present study.

HDC mRNA levels were also employed to verify the achievement of a fasted state. The blunting of HDC mRNA levels by ~50% with fasting indicated suppressed ECL cell activity. However, the mRNA levels of this ECL cell marker were not as low as
has been previously published. Dimaline et al. (1997) showed a decrease in HDC mRNA to 32% of control and Kondo et al. (1995) saw a decrease to 39% of control after a 48 hour fast. Both of these groups used male Wistar rats whereas I used female Sprague-Dawley rats. The regulation of HDC mRNA levels in fasting may differ between sexes, as was suggested for the gastrin receptor in hypergastrinemia. Also, in this case there may be strain-specific differences, for instance Sprague-Dawley rats have more ECL cells than Wistar or Fischer rats (Tuch et al., 1992). Furthermore, while the treatment of Kondo et al. (1995) is not known, Dimaline et al. (1997) housed their rats in wire-bottomed cages. My rats were housed in plastic cages, having access to bedding and their feces. It is possible that stomach distension resulted from ingestion of these materials and stimulated digestive processes that curbed the reduction of HDC mRNA. However, gastrin release, the primary stimulant of ECL cell activity, is specifically stimulated by protein or lipid in the gut (Wu et al., 1991) of which there would have been minimal amounts if any in this case.

Body weight decreased by ~10% after 2 days of fasting. This is the same as reported by Chrysis et al. (1998), although they did not specify the type of housing that was used. However, Knott et al. (1989) housed fasted rats in wire-floored cages and reported ~17% weight loss with 40 hours of fasting. So it seems that although a fasting state was achieved, it may not have been as profound as those obtained by other groups who observed a greater decrease in HDC mRNA levels. The reduction in weight was due to decreased mass intake (ie. stomach contents), as well as retarded growth and utilization of carbohydrate, fat and protein stores to provide energy for life-sustaining activities. Four hours of refeeding did not significantly increase the body weight of the animals. This was probably because of inconsistent feeding amongst the rats for the short time period. For example, because rats were housed in pairs a dominant rat may have prevented its more timid partner from feeding as much as she desired. As well, more time was needed to consume and process enough nutrients for storage. After two days of refeeding, the rats regained their original weight but they were still underweight, compared to fed controls killed 2 days earlier.
V. CONCLUSIONS & FURTHER STUDIES

The mRNA levels of IRR, an orphan member of the insulin receptor family, are up-regulated in both the pathophysiological states of hypergastrinemia and fasting. In the former condition of increased ECL cell activity, the mRNA levels of IRR increase in concert with those of HDC and the gastrin receptor, suggesting that like these markers for ECL cell activity, IRR may play an important role in gastric acid secretion. On the other hand, in fasting IRR mRNA levels are opposed to the decreased ECL cell activity and instead increase, like those of other insulin receptor family members. In this case, low circulating levels of the ligand for IRR may relieve ligand-induced receptor down-regulation, as they do for the insulin and IGF-I receptors. Here, IRR and its ligand could play a role in some other aspect of fasting, such as metabolism or growth. The results suggest that IRR may be regulated by a number of different factors and furthermore, that the orphan receptor may have more than one physiological function in the stomach.

While this study evaluated the total amount of IRR mRNA, assessment of the different isoforms in fasting and hypergastrinemia may also provide insight into the function of IRR in ECL cells. The up-regulation of IRR mRNA levels in fasting and hypergastrinemia could produce different effects if the isoforms of IRR are discordantly regulated. For example, the increase in hypergastrinemia may be due to increased secretion of truncated IRR which may draw away circulating ligand, while the increase in fasting may be due to increased full-length receptors with tyrosine kinase activity, or vice versa. Such differential regulation has been shown for fasting with leptin receptor expression in the thalamus. While there is a decrease in the total amount of receptor transcripts, expression of the long form of the receptor increases and, presumably, it is expression of the short, inactive isoforms that declines (Bennett et al., 1998). Differential regulation of IRR isoforms could explain the paradoxical result that IRR mRNA levels are elevated in both states of increased and decreased ECL cell activity.
The mRNA levels of IRR that have been measured do not necessarily reflect the levels of functional protein in the individual ECL cell. Although Kimura et al. (1997) showed that the increase in HDC and gastrin receptor mRNA levels indicates increased ECL cell activity, ECL cell numbers should be ascertained to show whether IRR mRNA levels also indicate increased ECL cell activity. Then it should be determined whether levels of the IRR protein increase with the levels of mRNA. HDC and gastrin receptor levels decrease in fasting (Ohning et al., 1998; Takeuchi et al., 1980, respectively) with decreased mRNA levels, and HDC activity and gastrin receptor levels increase in hypergastrinemia (Kimura et al., 1997; Takeuchi et al., 1980, respectively) with increased mRNA levels. However, these parallel changes are not always coincident. In hypergastrinemia, the initial enhancement of ECL activity and activation of proteins occurs before elevations of mRNA levels (Kimura et al., 1997). IRR protein levels and activity could be enhanced at an earlier time than the mRNA levels, especially during hypergastrinemia. A specific antibody to IRR can be used to detect the orphan receptor using Western blotting or immunohistochemical techniques. Furthermore, IRR activity can be determined with anti-phosphotyrosine antibodies. These studies are not only necessary to show that increased functional IRR protein accompanies increased mRNA levels in fasting and hypergastrinemia, they may also demonstrate that IRR is more active in the earlier stages of hypergastrinemia, when its mRNA levels have not yet begun to rise.

The isolation of ECL cells using counter-flow elutriation has been achieved in the laboratory and is a powerful tool for investigating the physiology and pathophysiology of ECL cells more specifically (e.g. Lindstrom et al., 1997). A purified preparation of cells can be used to determine the changes in IRR mRNA, protein and receptor phosphorylation levels in a population of pure ECL cells from fasted or hypergastrinemic rat stomachs. The elimination of contaminating stomach material, such as mast cells which also contain HDC and histamine, will enable more specific inferences from the data. Also, by comparing the data from purified ECL cells to that from whole stomach studies, changes can be attributed to altered ECL cell number, altered ECL cell activity, or both. Additionally, purified ECL cells can undergo short-term culture, allowing
investigation into their specific regulation both in normal physiology as well as pathophysiology. There is no doubt that purification of ECL cells by counter-flow elutriation will allow more specific conclusions to be made about the presence and activity of IRR both in physiological as well as pathophysiological states.

The increase in IRR mRNA levels with fasting may be attributed to either metabolic state or gastric state, or both. Hypogastrinemia can be studied more independently by the administration of a gastrin receptor antagonist (Ding et al., 1997). Dissection of the gastric consequences of fasting from the metabolic consequences will indicate more specifically in which aspect of fasting the orphan receptor is involved. To see if IRR is affected by the increase in gastric lumen pH that accompanies fasting-induced hypogastrinemia as well as omeprazole-induced hypergastrinemia, a state of decreased lumenal pH can be examined. The administration of high levels of synthetic gastrin causes hypergastrinemia accompanied by excess acid secretion. The effects of pH on IRR mRNA levels will be seen through a comparison of the two hypergastrinemic states, gastrin-infusion which stimulates acid secretion and omeprazole-treatment which inhibits it.

Finally, the effects of hypergastrinemia on three other RTKs, the insulin and IGF-I receptors and TrkA, remain to be determined. To date it is not known whether any of the other members of the insulin receptor family are present in ECL cells. But since another member of their RTK family, IRR, is affected by hypergastrinemia, it is possible that they too are affected by this state. Furthermore, although the effects of fasting on the insulin receptor are widely known, it remains to be seen how its expression in the stomach is affected by fasting. Trk A is not a member of the insulin receptor family, but it is very closely linked to IRR; this high affinity NGF receptor is closely linked in the genome with the orphan receptor (Rainey et al., under revision), conceivably facilitating their co-expression in the brain (Reinhardt et al., 1994; Tsuji et al., 1996). In the stomach Trk A is not co-expressed with IRR and is instead localized to parietal cells (Shibayama & Koizumi, 1996), the downstream effectors of ECL cells. Trk A mRNA levels in stomach therefore need to be assessed for the pathophysiological states of fasting and hypergastrinemia. If the regulation of IRR and Trk A is still coordinate even when they
are in different locations, they may represent a signaling pathway that is conserved throughout the body. The determination of the changes in mRNA levels of Trk A as well as the other members of the insulin receptor family will provide insight into their involvement with IRR in the stomach.

In the stomach, there are now two ways in which the investigation of IRR may further medical knowledge. First, its specific presence on ECL cells and up-regulation of its mRNA levels with increased ECL cell activity imply that the orphan receptor may play a role in gastric acid secretion. Furthermore, that IRR is elevated in two states of increased lumenal pH, fasting and omeprazole-induced hypergastrinemia, suggests that it may be regulated by the pH of the stomach lumen. Pathological elevations of gastric acid are a consequence of several gastric diseases including ZES and gastric ulceration caused by H. pylori. Next, the up-regulation of IRR mRNA levels in concert with those of the insulin and IGF-I receptors suggests that there may be conservation of regulation as well as structure within the insulin receptor family. Since members of an RTK family often share conservation of function, this suggests that the orphan receptor could function in growth or metabolism. Thus IRR could also be involved in the pathogenesis of other disease states related to those of the insulin receptor, like diabetes mellitus, or the IGF-I receptor, like tumorigenesis.
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