Secretion of soluble leptin receptors by exocrine and endocrine cells of the gastric mucosa

Philippe G. Cammisotto,1 Diane Gingras,1 Christian Renaud,1 Emile Levy,2 and Moïse Bendayan1

1Département de Pathologie et Biologie Cellulaire, and 2Département de Nutrition, Université de Montréal, Montréal, Québec, Canada

Submitted 19 July 2005; accepted in final form 19 September 2005

Leptin is a hormone secreted by the gastric mucosa into the lumen of the stomach. It is present in its intact form in the intestine where it regulates nutrient absorption and intestinal mucosa integrity. We have identified the binding protein that protects leptin from the harsh conditions of the gastric juice. Immunoprecipitations and Western blot analyses demonstrated that leptin is present in the gastric mucosa and the gastric juice, bound to a protein corresponding to the extracellular domain of the leptin receptor. In the absence of this soluble receptor, leptin is rapidly degraded. Immunocytochemistry on rat gastric mucosa identified the cells and intracellular compartments involved in secretion of this complex. Leptin receptor extracellular domain and leptin are present along the rough endoplasmic reticulum-Golgi-granules secretory pathways and form a complex in the secretory granules of Chief and specific endocrine cells. The long-form membrane leptin receptor OB-Rb, the protease activator furin, and proprotein convertase 7 were found in Chief cell granules but not in those of endocrine cells. The shedding of the receptor occurs in the immature granules. It is concluded that in the immature secretory granules of Chief cells, furin activates proprotein convertase 7 that, in turn, cleaves the extracellular portion of membrane-bound leptin receptors. Leptin bound to its soluble receptor forms a complex that is resistant to the gastric juice. Endocrine cells, on the other hand, generate a soluble leptin receptor by mechanisms different from those of the exocrine cells.

gastric juice; exocrine secretion; endocrine secretion

LEPTIN IS AN ADIPOKINE SECRETED by mature white adipocytes as well as by several other tissues (2, 28, 36). It acts in several hypothalamic nuclei to regulate appetite, energy expenditure, and activity of the sympathetic nervous system (11, 18, 20). Leptin is also involved in a large variety of physiological functions (1, 18). Besides adipose tissue, the stomach has been shown to be another major source of leptin (2). Exocrine and endocrine cells of the gastric mucosa secrete leptin along a regulated rough endoplasmic reticulum (RER)-Golgi-granules secretory pathway (7). On fasting and refeeding, gastric endocrine cells secrete leptin, which may account for the quick increase of plasma leptin levels (2, 8, 17). Because leptin and pepsinogen are secreted in tandem (7), we can assume that leptin levels in gastric juice increase following food intake. In accordance with this dual exocrine-endocrine secretion, leptin receptors have been identified on the luminal and basolateral membranes of intestinal enterocytes and have been shown to regulate several intestinal activities, including absorption of nutrients (3, 7, 10).

Despite the strongly acidic nature of the gastric juice and the presence of proteolytic enzymes, leptin has been detected intact in duodenal juice (17). It was reported to be part of a large complex of high molecular mass that is thought to confer a protective role (16, 17, 31). The nature of this putative leptin binding protein is so far still unknown. On the other hand, it has been well established that plasma leptin circulates associated to a soluble isoform of its receptor (OB-Re), which has the double role of protecting leptin and regulating its interactions with membrane receptors (21, 23, 25, 26, 35). The receptor OB-Re is present in tissues secreting leptin, such as white adipose tissue and salivary glands (5, 6, 9, 25). Similarly, leptin receptor mRNA for long (OB-Rb) and short isoforms (including OB-Re) have been detected in the gastric mucosa by several groups (14, 27, 31, 33). However, only the long form OB-Rb has been studied so far (17, 27).

In the present study, we wanted to elucidate how leptin resists gastric juice conditions and characterize the binding protein that confers a protecting activity. We now report for the first time that leptin is present in the gastric juice, bound to a protein with immunochemical similarities to the soluble leptin receptor. Soluble forms of the leptin receptor were detected in the secretory granules of both endocrine and exocrine cells of the gastric mucosa. Exocrine cells produce the soluble receptor by processing membrane-bound forms of the receptors. Endocrine cells synthesize a soluble receptor by different mechanisms. These results suggest that endocrine and exocrine gastric cells secrete leptin-receptor complexes that protect leptin. Endocrine and exocrine cells display different leptin-receptor complexes processing.

MATERIALS AND METHODS

Biological material. Sprague-Dawley male rats (290–305 g) were handled following the guidelines of the Canadian Council on Animal Care. Rats were fasted for 18 h and anesthetized with urethane before surgery. Gastric juice was collected during 30 min on carbachol (Sigma-Aldrich, Oakville, ON Canada) (12 mg/kg body wt ip). Gastric mucosa from another group of animals was scraped off and homogenized in RIPA buffer (50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.25% deoxycholate; 1 mM sodium orthovanadate, and 1% Nonidet P-40). Anti-protease inhibitors cocktail (Complete Mini, Roche Diagnostics, Laval, QC, Canada) was added to samples.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Antibodies. Rabbit antibody against leptin (Ob A20) and goat antibody against the extracellular domain of leptin receptor (K20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibody against the leptin receptor OB-Rb (OBR-13) COOH-terminal portion was purchased from Alpha Diagnostic (San Antonio, TX). Corresponding blocking peptides were purchased from the same companies. Proprotein convertase 7 (PC7) rabbit antibody was kindly provided by Dr. N. Seidah (IRCM, Montreal, QC, Canada). Rabbit antibody against furin was from Alexis Biochemicals (San Diego, CA). FITC anti-rabbit antibody (AP187F) and rhodamine anti-goat antibody (TRITC) (AP184R) were purchased from Chemicon (Temecula, CA).

Light microscopy. Small fragments of the gastric wall were fixed in Bouin’s fixative and embedded in paraffin. Sections (5 μm) were mounted on Superfrost slides (Fisher Scientific, Montreal, QC, Canada) and processed for immunohistochemistry. After being deparaffinized, tissue sections were washed twice in 10 mM phosphate buffer (pH 7.4), and 150 mM NaCl (PBS). After incubation with pepsin (1 mg/ml in 10 mM HCl), sections were washed with PBS and covered with 1% ovalbumin for 30 min. Incubation with the primary antibody was carried out overnight at 4°C. Sections were then washed in PBS, incubated with the secondary antibody, washed with PBS, and mounted with 1% DABCO in 50% glycerol. The sections were examined with a LEICA DM-IRBE inverted confocal laser microscope (Leica, St.-Laurent, QC, Canada).

Fluorescence resonance energy transfer. Images of FITC and rhodamine were obtained with LEICA DM-IRBE inverted confocal laser microscope using ×100 oil-immersion objectives. Argon-krypton laser lines of 488 and 568 nm were used to excite FITC and rhodamine, respectively. The emission filters used were a double dichroic filter DD/488/568 for FITC and rhodamine channels. For each experiment, imaging conditions were optimized to prevent saturation, to minimize signal contamination, and to prevent photo bleaching. The average intensity of the fluorescence resonance energy transfer (FRET) signals on single cells was evaluated with the LEICA confocal software SP1. Apparent FRET efficiency (Ea) was computed using the following formula: Ea = (Fd post – Fd pre)/(Fd post), where Fd post is the fluorescence of the donor (FITC) after bleaching and Fd pre is the fluorescence of the donor before bleaching (15). Bleaching was carried out at 588 nm by 240 cycles of 1.7 s each. The percent

Fig. 1. Western blot analysis of leptin (Lep) and leptin receptor in gastric juice (GJ) and gastric mucosa (GM) extract. GJ, GM, extract and recombinant leptin were loaded on a 10% SDS polyacrylamide gel, transferred, and revealed with the specific anti-leptin antibody. A: leptin was found in all samples as monomers (16 kDa) and dimers (32 kDa). Immunoprecipitations (IP) using the antibody against leptin were carried out on GM extract and GJ and immunoblotted. Bands similar to those in A and one at 19 kDa for the leptin precursor were obtained with the leptin antibody while the anti-leptin receptor antibody revealed an 80-kDa band in the gastric juice (B). Conversely, immunoprecipitation with the anti-leptin receptor antibody (K20) and immunoblotting with the anti-leptin antibody yielded results similar to those of B/C. Removal of leptin receptors from the gastric juice by immunoprecipitation with an excess of the K20 anti-leptin receptor antibody led to the disappearance of the added recombinant leptin in acidic conditions D.

Fig. 2. Immunohistochemical staining for leptin (A) and leptin receptor (B) in the GM. Cells from the lower half of the mucosa display positive reactions for leptin (A) as well as for its receptor (B). The staining is located around the gastric pits. Omission of the primary antibody leads to a complete disappearance of the staining (C).
change of FRET was computed using the initial value as 100% fluorescence.

Electron microscopy. Tissues samples were fixed by immersion with 1% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), for 2 h at 4°C and embedded in Lowicryl at −30°C (K4M CANEMCO, St.-Laurent, QC, Canada) (4). For embedding in Epon 812, the tissue fragments were postfixed with 1% osmium tetroxide for 1 h at 4°C before being embedded. Thin sections were mounted on Parlodion and carbon-coated nickel grids. Grids were incubated on a drop of a saturated sodium metaperiodate solution and then on glycine (150 mM in PBS), followed by 1% ovalbumin in PBS. Incubation with the antibody was carried out overnight at 4°C. Tissue sections were then washed and incubated on protein A-gold complex (5 nm) or anti-goat IgG-gold complex (10 nm). Grids were then washed thoroughly, stained with uranyl acetate, and examined with a Philips 410LS electron microscope (FEI Systems Canada, St.-Laurent, QC, Canada). Control experiments for light and electron microscopy were performed by omission of primary antibodies or by using blocking peptides.

Immunoprecipitation and Western blot analysis. Preclearing of samples was performed with protein A-Sepharose beads (Sigma-Aldrich) (10 mg/ml). Suprnatants were incubated overnight with specific antibodies. Protein A-Sepharose or protein G-agarose was added and left for 4 h at 4°C with gentle rocking. Beads were then centrifuged and washed six times in RIPA buffer and one time in Tris-buffered saline. Total protein content was assessed using a standard bicinchoninic acid protein assay kit from Pierce (Rockford, IL). Samples (20–30 μg protein) were heated at 90°C for 5 min in Laemmli buffer and then resolved by 10% SDS-PAGE. Proteins were transferred to nitrocellulose sheets. The blots were blocked with 1% nonfat dry milk in 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 (TBST buffer) and probed with the anti-leptin (A20) or anti-leptin receptor K20 antibodies overnight. After incubation with their respective secondary antibodies, the immune complexes were

Fig. 3. Double immuno-staining of leptin and leptin receptor in the GM. Sections of the GM were incubated with anti-leptin (A) and anti-leptin receptor (B) antibodies, followed by FITC-conjugated (green) and rhodamine-conjugated (red) secondary antibodies, respectively. Chief cells are positively stained for both leptin and its receptors (orange staining; C). Colocalization was also found in the small endocrine cells (arrow). These cells yield a brighter intensity (green) due to their high content in leptin. In double labeling, these cells appear yellow (C).

Fig. 4. Immunogold detection of leptin receptor in gastric Chief cells. Rough endoplasmic reticulum (RER; A) and Golgi apparatus (G) and secretory granules (g) (B) display a positive labeling by gold particles. Control of specificity using the blocking peptide showed very few gold particles (C). Nuclei (N) were rather devoid of labeling.
revealed by the enhanced chemiluminescence detection system (Lumi-Light Plus, Roche Diagnostics, Laval, QC, Canada).

RESULTS

Presence of leptin in the gastric juice. Leptin was previously reported to be present in exocrine secretory granules of the gastric mucosa Chief cells (7) and in gastric juice (17). First, to confirm these observations, Western blot analyses were carried out on pure gastric juice, gastric wall extracts (see MATERIAL AND METHODS), and recombinant leptin on the same gel. Leptin was found in gastric juice and mucosa, under various forms close to 16 and 32 kDa, which correspond, respectively, to monomers and dimers of leptin (Fig. 1A). Recombinant leptin (Sigma-Aldrich) displayed two bands similar to those of the gastric juice. Second, to isolate the binding protein linked to leptin, immunoprecipitations using an antibody against leptin

Fig. 5. Labeling of leptin receptor in gastric endocrine cells. Some endocrine cells (A) are labeled for leptin receptor. Secretory granules in particular are heavily labeled by gold particles (B). Particles are absent from nucleus. Control with blocking peptide showed no gold particles in the granules (C).

Fig. 6. Double labeling of the leptin-leptin receptor complex in exocrine cells. Confocal microscopy using FRET analysis (A) was carried out on double immunostaining for leptin (green, FITC) and leptin receptor (red, rhodamine) on the cytoplasm of single cells. Rhodamine associated to receptor molecules was bleached using 568-nm wavelength, leading to an increase in FITC-associated leptin fluorescence with an apparent FRET efficiency of 13.4%. Double-immunogold labeling of leptin (5-nm small particles) and leptin receptors (10-nm large particles) shows a colocalization of both types of gold particles and thus association of both proteins in the secretory granules of Chief cells (B).
were carried out on the same samples. Two bands at 16 (leptin) and 19 kDa (leptin precursor) for the gastric wall and a single band at 16 kDa for the gastric juice were found by Western blot analysis (Fig. 1B). Because it is well demonstrated that leptin is protected in the plasma by a soluble isoform of its membrane receptors (24, 26, 35), the same immunoblots of the gastric juice were revealed with an antibody specific for the extracellular domain of the leptin receptor (K20). A band at 80 kDa, which is close to the molecular mass of the soluble form of the leptin receptor in the rat, was detected (Fig. 1B). Plasma analyzed under the same conditions also yielded a band at 80 kDa (results not shown). Conversely, immunoprecipitations were conducted with the leptin receptor antibody, immunoblotted, and revealed with the anti-leptin antibody. Bands for leptin appeared at 16 kDa for the gastric juice and at 16 and 19 kDa for the gastric wall extract (Fig. 1C). Finally, when the leptin receptor was removed from the gastric juice by immunoprecipitation, the supernatants reacidified (pH 2), and in samples left at 37°C for 30 min, recombinant leptin in the gastric juice was not found, whereas the same recombinant leptin in PBS remained intact (Fig. 1D). These results demonstrate that a complex formed by leptin and a binding protein is present in the gastric juice and mucosa. The molecular mass of this binding protein is close to that of the plasma soluble isoform of the leptin receptor and reacts with the anti-receptor antibody, which indicates that it may correspond to the leptin soluble receptor isoform or a fragment of the extracellular portion of the leptin receptor. Once complexed to this protein, leptin appears to acquire resistance to the acidic and proteolytic conditions existing in the gastric juice.

Detection of leptin and leptin receptors in exocrine and endocrine cells of the gastric mucosa. To determine the origin of the leptin-leptin receptor complex present in the gastric juice, paraffin sections of gastric mucosa were incubated with an antibody directed against the NH2-terminal extracellular portion of leptin receptors. Localization of leptin was realized in parallel as previously described (7). At low magnification, the lower half of the gastric mucosa appeared positive for leptin (Fig. 2A) as well as for leptin receptors (Fig. 2B). The staining was located around the gastric pits. Controls without

---

Fig. 7. Immunogold labeling of leptin receptor in immature and mature secretory granules of Chief cells. The immature exocrine granule (ig) displays a labeling by gold particles, with most of them associated to the granule limiting membrane (arrows; A). In mature granules (mg), the gold particles are rather centrally located within the secretory granules (B).
specific primary antibodies or by adsorption with corresponding peptides display no staining (Fig. 2C). Similar results were obtained using the peroxidase method (results not shown). To further define localization of leptin receptors, double immunolabelings were carried out. Leptin (Fig. 3A) and its receptor (Fig. 3B) were found colocalized in Chief and endocrine cells (Fig. 3C). The yellow color of endocrine cells comes from the high concentration of leptin in these cells (7). To confirm these observations, ultrathin sections of the gastric mucosa were processed by immunogold using antibodies to the receptor and immunogold complexes. Secretory granules of Chief as well as those of the endocrine cells were heavily labeled by gold particles (Figs. 4B and 5B). Parietal and mucus cells were negative for both proteins (results not shown). Mitochondria and nuclei were devoid of any specific labeling. The use of an antibody-blocking peptide complex led to absence of specific staining in both type cells (Figs. 4C and 5C). These results confirmed the presence of soluble leptin receptors inside the secretory granules of exocrine and endocrine cells.

Association of leptin to the extracellular domain of its receptor. Coming back to light microscopy, we proceeded with double-labeling confocal microscopy using FRET, with TRITC as the acceptor and FITC as the donor. FRET was carried out on the cytoplasm of single Chief cells positive for leptin and their receptor (Fig. 6A). Bleaching of TRITC (leptin receptor) led to an increase in the FITC signal (leptin) with an apparent Ea of 13.4%, which does confirm an association between leptin and its receptor (Fig. 6A). Double-immunogold labeling using antibodies against leptin and its receptor (K20) revealed an association between these two proteins inside the granules (Fig. 6B). Morphometric evaluations in Chief cell secretory granules showed that ~40% (38.73 ± 12.54%) of the gold particles assigned to the leptin receptor are closely associated to the gold particles assigned to leptin (Fig. 6B). These results indicate that soluble leptin receptors are bound to leptin inside the secretory granules of Chief as well as endocrine cells.

Processing of membrane leptin receptor in exocrine cells. The immunogold labeling for the leptin receptor in secretory granules of Chief cells was found to be associated with the limiting membrane of the granules. However, mature and immature granules displayed different distributions of the gold particles. Most of the gold particles were located on or close to the membrane of the immature granules (73.64 ± 21.05% of total gold particles per granules), whereas their distribution was more central in mature granules (45.49 ± 11.03% of total gold particles were close to the membrane; Fig. 7, A and B). This observation suggests that membrane-associated leptin receptor might undergo proteolysis leading to the release of their extracellular domain and becoming a soluble form of the receptor. To investigate whether membrane-bound leptin receptors are cleaved, ultrathin sections of the gastric mucosa were tested for the presence of proteases. After a few were tested, interesting results were obtained with furin and PC7. Furin is a widely expressed protease that has the ability to activate a series of proteases and proprotein convertases, including PC7 (30, 34). PC7 possesses numerous proteolytic sites on the leptin receptor molecule, as determined by amino acid sequence analysis (30, 32, 33). These two proteases were indeed found in the secretory granules of the Chief cells (Fig. 8, A and B) but absent in secretory granules of endocrine cells (results not shown). Double immunogold labeling for leptin receptor and PC7 showed that these two proteins are colocalized in the granules of Chief cells (Fig. 8C). Several large particles labeling the receptor were found associated with the small particles labeling the PC7, which strengthens the hypothesis that leptin receptors are a substrate for PC7 (Fig. 8C). These results suggest that in Chief cells, furin activates PC7, which in turn cleaves membrane-bound receptors to release soluble receptors that bind to leptin. On the other hand, we found that in rats, leptin endocrine secretory granules do not contain furin or PC7, which suggests that endocrine cells synthesize a soluble receptor by different mechanisms, perhaps similar to that of other tissues secreting leptin (12, 25, 27).

Presence of membrane-bound leptin receptor. We subsequently wanted to confirm that soluble leptin receptors in Chief...
cells are originating from a membrane-bound receptor. Due to the scarcity of specific antibodies against each of the various leptin receptor isoforms (32), we were limited to the long isoform receptor OB-Rb. With the use of an antibody specific to the intracellular COOH-terminal portion of OB-Rb (OB-R13), labeling was found in the lower half of the gastric mucosa, displaying a pattern similar to that generated by the previous antibody K20 (results not shown). Immunogold revealed the presence of this long-form receptor mainly in the RER, Golgi, and immature secretory granules but not in the mature ones of the Chief cells (Fig. 9). This long form of the receptor was also absent from endocrine cells. Immunoblotting using OBR-13 antibody revealed a band close to the molecular mass (120 kDa) of OB-Rb in gastric mucosa but not in gastric juice (results not shown). These results confirm that soluble leptin receptors in Chief cells originate, at least in part, by shedding of membrane-bound receptor OB-Rb. Endocrine cells produce soluble receptors by a different mechanism.

**DISCUSSION**

We have previously reported that leptin is present in secretory granules of exocrine and endocrine cells of the gastric mucosa of normal rats. We have now extended these results by identifying the protein conferring to leptin its resistance in the gastric juice. We report for the first time that exocrine cells of the gastric mucosa secrete leptin bound to a protein, which is structurally similar to the extracellular domain of the leptin receptor. These conclusions are based on the following observations.

By Western blot analysis, leptin was found in its intact form (16 kDa) in the gastric juice and as both its precursor (19 kDa) and its mature form (16 kDa) in the gastric mucosa. It must be underlined that the precursor form is absent in the gastric juice. Immunoprecipitation with anti-leptin antibody coprecipitates leptin with a protein of ~80 kDa, which is recognized by an antibody directed against the extracellular NH2-terminal portion of the leptin receptor. Western blot analyses of plasma taken from the same animals also revealed a band of ~80 kDa with the same antibody, which corresponds to the soluble leptin receptor OB-Re. Conversely, immunoprecipitation of gastric juice with the anti-receptor antibody coprecipitated leptin. This suggests that the protein bound to leptin in the gastric juice is or has a strong structural homology with the plasma leptin soluble receptor OB-Re. This soluble receptor has been shown to circulate in plasma bound to leptin; it protects leptin and increases its half-life (21, 24). Most interestingly, when this soluble receptor was removed from the gastric juice, added recombinant leptin was unable to resist the acidic conditions of the gastric juice, demonstrating the fundamental role of the receptor in leptin survival.

Next, using immunocytochemistry, we revealed that the soluble receptor has a cellular distribution similar to the one reported previously for leptin, in the bottom half of the gastric mucosa (7). It is colocalized with leptin within the Chief and endocrine cells of the gastric mucosa. Electron microscopy confirmed these observations, demonstrating the presence of leptin and the soluble receptor in Chief and endocrine cells RER, Golgi apparatus, and inside secretory granules. Interestingly, the gastric mucosa of the ob/ob mice does not contain leptin, but we did observe that the leptin receptor is present in the secretory granules of exocrine and endocrine cells (results not shown), indicating that leptin and its receptor are synthesized independently.

A closer examination of the Chief cells showed that the leptin receptor is mainly associated with the limiting membrane in immature granules, whereas in the mature granules, the labeling is predominantly inside the granules. This suggests that in Chief cells, the soluble leptin receptor comes from a membrane-bound receptor that undergoes shedding. Indeed, generation of soluble receptors by proteolytic cleavage of membrane receptors is a common feature of numerous hormones (interleukin-1 and TNF) (13, 19, 23). When using an antibody specific for the cytoplasmic portion of the leptin receptor long isoform OB-Rb, the labeling was restricted to the RER, the Golgi apparatus, and the immature granules, fading in the mature ones. Subsequently, we searched for the presence of proteases that could be responsible for the processing of the membrane receptor. Furin is a widely expressed protease that has the ability to activate a series of other proteases and proprotein convertases, including PC7 (30, 34). PC7 possesses numerous proteolytic sites on the leptin receptor molecule as determined by amino acid sequence analysis (30, 32). Furin and PC7 were indeed found within the secretory granules of the Chief cells, but not in endocrine cells, and double immunolabeling showed an association between PC7 and leptin receptors. Taken together, these results indicate that 1) in Chief cells, PC7 cleaves membrane receptors to release a soluble one; and 2) rat endocrine cells may synthesize and secrete the soluble receptor as it is the case for other tissues (12, 27). The possibility remains that the soluble receptor in Chief cells comes from other leptin receptor shedding (OB-Ra, -Rc, -Rd, or -Rf) or may also be produced directly (OB-Re).

In summary, our results demonstrate that leptin is secreted by the gastric Chief and endocrine cells as a complex with the soluble form of its receptor. In Chief cells, such a complex is processed through the RER-Golgi-granules secretory pathway and allows leptin to resist the conditions of the gastric juice. The endocrine cells of the gastric mucosa also synthesized soluble receptors that is secreted in the blood, as it is the case for other tissues secreting leptin. Because leptin is an important factor for the integrity of the intestinal mucosa, the leptin-soluble leptin receptor complex may carry important significance in intestinal physiology.

**ACKNOWLEDGMENTS**

The authors give special thanks to M. Lauzon and E. Gervais for technical assistance.

**GRANTS**

This work was supported by a grant from the Canadian Institutes of Health Research.

**REFERENCES**


