Orexins Acting at Native OX1 Receptor in Colon Cancer and Neuroblastoma Cells or at Recombinant OX1 Receptor Suppress Cell Growth by Inducing Apoptosis*

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Patricia Rouet-Benżineb§, Christiane Rouyer-Fessard‡, Anne Jarry§, Virgile Avondo‡, Cécile Pouzet†, Masashi Yanagisawa**, Christian Laboisse‡, and Thierry Voisin‡‡§§

From [1]INSERM U410, Neuroendocrinologie et Biologie Cellulaire Digestives and [2]IIFR 02 Claude Bernard, Faculté de Médecine Xavier Bichat, 16 Rue Henri Huchard, BP 416, 75870 Paris Cedex 18, [3]INSERM U539, Faculté de Médecine, 44035 Nantes, France, and [4]Howard Hughes Medical Institute and Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9050

Screening of 26 gut peptides for their ability to inhibit growth of human colon cancer HT29-D4 cells grown in 10% fetal calf serum identified orexin-A and orexin-B as anti-growth factors. Upon addition of either orexin (1 μM), suppression of cell growth was total after 24 h and >70% after 48 or 72 h, with an EC50 of 5 nM peptide. Orexins did not alter proliferation but promoted apoptosis as demonstrated by morphological changes in cell shape, DNA fragmentation, chromatin condensation, cytochrome c release into cytosol, and activation of caspase-3 and caspase-7. The serpentine G protein-coupled orexin receptor OX1R but not OX2R was expressed in HT29-D4 cells and mediated orexin-induced Ca2+ transients in HT29-D4 cells. The expression of OX1R and the pro-apoptotic effects of orexins were also indicated in other colon cancer cell lines including Caco-2, SW480, and LoVo but, most interestingly, in normal colonic epithelial cells. The role of OX1R in mediating apoptosis was further demonstrated by transfecting Chinese hamster ovary cells with OX1R cDNA, which conferred the ability of orexins to promote apoptosis. A neuroblastoma cell line SK-N-MC, which expresses OX1R, also underwent growth suppression and apoptosis upon treatment with orexins. Promotion of apoptosis appears to be an intrinsic property of OX1R regardless of the cell type where it is expressed. In conclusion, orexins, acting at native or recombinant OX1R, are pro-apoptotic peptides. These findings add a new dimension to the biological activities of these neuropeptides, which may have important implications in health and disease, in particular colon cancer.

Classical growth factors for colon cancer cells have been extensively described including agonists of tyrosine kinase receptors such as epidermal growth factor and related proteins (1) or insulin-like growth factors (2). More recently, some G protein-coupled receptor (GPCR)‡ agonists such as peptide hormones (3–5), prostaglandins (6), or serine proteases (7, 8) have been shown also to promote colon cancer cell proliferation often through transactivation of the epidermal growth factor receptor (6, 8). These GPCRs are expressed in both normal colonic epithelium and colon tumors (9) or even ectopically expressed by cancer cells such as in the case of the neurotensin receptor NT1 (10) or the thrombin receptor protease-activated receptor 1 (7). Whatever their expression pattern, they probably all contribute to the growth of colon tumors because of the presence of abundant ligands in the neuroendocrine environment of colonic tumors and/or to the production of receptor ligands by the tumor itself (11, 12).

Our knowledge of receptor agonist suppressing colon cancer cell growth is much more limited apart from a few observations regarding transforming growth factor-β (13) or Fas ligand (14). We reasoned that among the very rich environment of peptide hormones and neuropeptides in the gut, we should be able to find natural agonists behaving as suppressors of colon cancer growth. In order to test this hypothesis, we developed a very simple assay by using human colon adenocarcinoma cells HT29-D4 grown in 10% FCS and screened for various peptides by their ability to inhibit cell growth. We made two dramatic hits with orexin-A and orexin-B, which appear to be robust growth inhibitors as shown here.

Orexin-A and orexin-B (15), also named hypocretin-1 and hypocretin-2 (16), were discovered in 1998 by orphan receptor technologies (15) or subtractive CDNA cloning (16). They are encoded by a single gene that drives the synthesis of preproorexin that is subsequently matured into the 33-amino acid orexin-A and the 28-amino acid orexin-B, sharing 46% amino acid identity in humans (reviewed in Ref. 17). Two orexin receptor subtypes OX1R and OX2R have been cloned (15). They are serpentine GPCRs that bind both orexins with poor selectivity and are coupled to Ca2+ mobilization (15). Orexins were initially characterized as neuropeptides restricted to hypothalamic neurons that project in the brain to nuclei involved in the

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§ Present address: INSERM U460, Bat 13, 46 Rue Henri Huchard, BP 416, 75870 Paris Cedex 18, France.

** To whom correspondence should be addressed: INSERM U410, Faculté de Médecine Xavier Bichat, 16 Rue Henri Huchard, BP 416, 75870 Paris Cedex 18, France. Fax: 33-0-42288765; E-mail: tvosin@bichat.inserm.fr.

† The abbreviations used are: GPCR, G protein-coupled receptor; OX1R, human orexin receptor type 1; OX2R, human orexin receptor type 2; TUNEL, TdT-mediated dUTP digoxigenin nick-end labeling; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; RT, reverse transcription; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LDH, lactate dehydrogenase; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Pipes, 1-piperazineethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; h, human.
control of feeding, sleep-wakefulness, neuroendocrine homeostasis, and autonomic regulation (17). Genetic or experimental alterations of the orexin system have been shown to be associated with narcolepsy (18, 19). More recent observations indicated that orexins and their receptors are not restricted to the hypothalamus but are also expressed in a few peripheral tissues (17), including the gastrointestinal tract (20).

Here we show that orexins acting at the OX₁R suppress the growth of human colon cancer cells HT29-D4 by promoting apoptosis through cytochrome c release from mitochondria and caspase activation. We further expand upon these data by showing that activation of native OX₁R in other colon cancer cell lines and neuroblastoma cells or activation of recombinant OX₁R expressed in CHO cells also leads to strong apoptosis and subsequent growth suppression. The OX₁ receptor-mediated apoptosis therefore appears to be an intrinsic property of the receptor regardless of the cell type where the receptor is expressed.

**EXPERIMENTAL PROCEDURES**

**Materials—**Orexin-A, orexin-B, and other peptides were from Neo
ystem (Strasbourg, France) with the exception of cholecystokinin-8 and gastrin-1 which were from Sigma. Rabbit polyclonal anti-OX₁R antibodies (297980A) were from Alpha Diagnostic International (San Antonio, TX). Mouse monoclonal anti-cytochrome c antibodies 6H2-B4 (immunoprecipitation experiments) and TH8–2C12 (Western blot) were from Pharmingen. Mouse monoclonal anti-β-actin antibodies (AC-74) were from Sigma. Rabbit polyclonal anti-caspase-3 antibodies, anti-cleaved caspase-3 antibodies (Asp-175), anti-caspase-7 antibodies, and anti-cleaved caspase-7 antibodies (Asp-198) were from Cell Signaling Technology/Ozyme (Saint-Quentin en Yvelines, France). Human cytochrome c ELISA kit was from Bender MedS
systems (San Bruno, CA). The In Situ Cell Death Detection kit and the M30 antibody were from Roche Diagnostics. The Enzyline LDH kit was from Biome ´rieux (Marcy l'Etoile, France). The ATPlite kit was from PerkinElmer Life Sciences. The Guava Nexin™ assay was from Guava Technologies (Hayward, CA).

**Cell Culture—**Chinese hamster ovary (CHO-K) cells were grown as described (21). Recombinant CHO/hOX₁R and CHO/hOX₂R cell lines stably expressing human orexin receptor type 1 (OX₁R) or human orexin receptor type 2 (OX₂R) were grown in F-12 medium containing 1-glutamine, supplemented with 10% FCS, 100 units/ml penicillin, and 0.7 mg/ml geneticin. The human colon cancer cell line HT29-D4 (22) was obtained from Dr. J. Marvaldi (CNRS, Marseilles, France). The human colon cancer cell lines Caco-2, SW480, HCT116, and SK-N-MC cells (seeded at 2 × 10⁵ cells/well) were grown in 24-well plates for 24 h in standard culture conditions with 10% FCS (23). Recombinant CHO/hOX₁R and CHO/hOX₂R cell lines were maintained in serum-free medium. The OX₁R or OX₂R-transduced CHO cells were stably expressing human orexin receptor type 1 or type 2, respectively.

**Isolation of Colon Epithelial Cells from Normal Human Colon—**Fresh normal human colons with no digestive disease were collected with the assistance of France-Transplant following French bioethic law. The colons were removed from small intestine and then immediately carried from the operating room to our laboratory in an isothermic box on ice. It usually took 30–60 min from the colon collection to the beginning of the epithelial cell isolation procedure. The colons were gently washed with water, and normal colon epithelial cell isolation was performed as reported previously (23).

**Explant Culture of Human Colonic Mucosa—**Fragments of human normal sigmoid colon taken at about 10 cm downstream to the tumor were obtained from a patient undergoing surgery for moderately differen
tiated colon carcinoma. The tissue fragments were processed according to the Guidelines of the French Ethics Committee for Research on Human Tissues. A sample, taken adjacent to the explants, was submit
ted to histological analysis and subsequently reported as normal by the pathologists. Immediately after removal, the tissues were placed in 4 °C oxygenated Krebs solution and processed as described previously (24). The colons were cut into 5-mm-thick segments and then incubated for 30 min in Krebs solution. They were then washed with Krebs solution, incubated in 10% Triton X-100, and put in 2.5 ml of scintillation fluid for counting incorporated radioactivity. For each point, cells excluding trypan blue were counted in a hemocytometer. The experiments were performed in triplicate wells and at least repeated twice. Results are expressed in disintegrations/min/10⁶ viable cells.

**Characterization of Apoptosis in Cultured Cell Lines—**Three methods were used for the characterization of apoptosis in cultured cell lines.
For the TUNEL method, cells (7 × 10^5) were seeded on Lab-Tek chamber coverglasses and grown for 24 h in standard culture medium. The culture medium was then replaced with fresh culture medium containing or not containing orexins. After 24 h, cells were lysed by adding Chaps cell extract buffer containing 50 mM Pipes/NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cells were resuspended in the buffer and frozen and thawed three times, and the lysate was centrifuged for 30 min at 14,000 rpm. After addition of SDS-PAGE sample buffer to the supernatant (30 μg of protein), the samples were separated by electrophoresis on 16% SDS-polyacrylamide gel and then transferred to nitrocellulose membrane. Blots were probed with rabbit polyclonal anti-cleaved caspase-3 antibodies (Asp-175, dilution 1:1000) or anti-cleaved caspase-7 antibodies (Asp-198, dilution 1:1000) that specifically recognize cleaved enzyme isoforms. Subsequently, blots were probed with rabbit polyclonal anti-caspase-3 antibodies (1:1000), which principally detect the full-length pro-caspase-3 (32 kDa) and the fragment of cleaved caspase-3 following cleavage at Asp-175 (17 kDa), or with rabbit polyclonal anti-caspase-7 antibodies (1:1000), which detect the full-length pro-caspase-7 (35 kDa). Blots were standardized by using the mouse monoclonal anti-β-actin antibodies (AC-74). Immune complexes were revealed with secondary peroxidase-conjugated antibodies using a chemiluminescent kit.

**Morphological Analysis of Apoptotic Cell Death in Human Colonic Mucosa Explante**—Morphological analysis of apoptotic cell death was assessed on paraffin sections by using two assays. The DNA-specific dye Hoechst 33342 (Calbiochem), which visualizes all the steps of the apoptotic process, was applied on deparaffinized sections (1 μg/ml) and incubated for 30 min at 37 °C. DNA was then precipitated, washed, resuspended in Tris-EDTA buffer, and incubated for 1 h with 0.1 mg/ml RNase A as described (25). DNA fragmentation was analyzed on 1.5% agarose gels in the presence of 0.5 mg/ml ethidium bromide.

**Cytochrome c Release**—HT29-D4 cells were seeded in 75-cm² culture dishes (10^6/dish) and grown in standard culture medium for 24 h. The medium was then replaced by a fresh culture medium containing or not containing orexins, and cells were further grown for 24 h. After cell lysis, cytochrome c was measured in cytosol (14,000 × g supernatant after 15 min centrifugation) by using an ELISA kit (see above) according to the manufacturer’s instructions. Under these experimental conditions, the cytosolic fraction did not contain mitochondria as verified by electronic microscopy. Alternatively, cytochrome c was characterized by immunoprecipitation followed by Western blot. Briefly, cytochrome c in cytosol was immunoprecipitated using the 6H2-B4 monoclonal anti-cytochrome c antibodies (2 μg/ml) and protein G-Sepharose beads. After addition of SDS-PAGE sample buffer to the washed beads, the samples were separated by electrophoresis on 10% SDS-polyacylamide gel and then transferred to nitrocellulose membrane. Total cytochrome c present in the cellular extract before cytosolic separation was characterized as described above. The blot was analyzed with the computerized analysis system. Results are expressed as the percentage of apoptotic annexin V-phycoerythrin-positive cells and are the means of four analyses.

**Immunocytochemical Studies of Cleaved Forms of Caspase-3 and Caspase-7**—HT29-D4 cells (7 × 10^5) were seeded on Lab-Tek chamber coverglasses and grown for 24 h in standard culture medium. The culture medium was then replaced with fresh culture medium containing or not containing orexins. After 24 h, the coverslips were probed with rabbit polyclonal anti-cleaved caspase-3 antibodies (Asp-175, dilution 1:100) or anti-cleaved caspase-7 antibodies (Asp-198, dilution 1:100) that specifically recognize cleaved enzyme isoforms. Antibodies do not recognize full-length caspase-3 or full-length caspase-7 or other cleaved caspases. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin IgG was used as the secondary antibody. Vectashield mounting medium containing propidium iodide was added, and coverslips were observed by confocal microscopy.

**Western Immunoblotting Studies of Caspase-3 and Caspase-7**—HT29-D4 cells were seeded in 75-cm² culture dishes (10^6/dish) and grown in standard culture medium for 24 h. The culture medium was then replaced with fresh culture medium containing or not containing orexins. After 24 h, cells were lysed by adding Chaps cell extract buffer containing 50 mM Pipes/NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cells were resuspended in the buffer and frozen and thawed three times, and the lysate was centrifuged for 30 min at 14,000 rpm. After addition of SDS-PAGE sample buffer to the supernatant (30 μg of protein), the samples were separated by electrophoresis on 16% SDS-polyacrylamide gel and then transferred to nitrocellulose membrane. Blots were probed with rabbit polyclonal anti-casparase-3 antibodies (Asp-175, dilution 1:1000) or anti-cleaved caspase-7 antibodies (Asp-198, dilution 1:1000) that specifically recognize cleaved enzyme isoforms. Subsequently, blots were probed with rabbit polyclonal anti-caspase-3 antibodies (1:1000), which principally detect the full-length pro-caspase-3 (32 kDa) and the fragment of cleaved caspase-3 following cleavage at Asp-175 (17 kDa), or with rabbit polyclonal anti-caspase-7 antibodies (1:1000), which detect the full-length pro-caspase-7 (35 kDa). Blots were standardized by using the mouse monoclonal anti-β-actin antibodies (AC-74). Immune complexes were revealed with secondary peroxidase-conjugated antibodies using a chemiluminescent kit.

**RT-PCR**—For cultured cell lines (HT29-D4, Caco-2, SW480, LoVo, and HCT116) or for epithelial cells isolated from normal human colons, total RNA (RNA prep) was extracted from cells by using Trizol® reagent according to the manufacturer’s instructions. Five μg of RNA were reverse-transcribed by using oligo(dT) primers. Twenty percent of the cDNA mixture was amplifed by using human OX_R sense primer (5'-GCCTGTGCCTCAGCATGTA-3') and OX_R antisense primer (5'-ACACTGCTGCTCATTCTG-ug-3'), or OX_R antisense primer (5'-CGCTCCTAGTGGTCT-3'), or OX_R antisense primer (5'-CGCTCCTAGTGGTCT-3'), or OX_R antisense primer (5'-CGCTCCTAGTGGTCT-3'). The OX_R amplification (500 bp) obtained from HT29-D4 RNA prep was subcloned by the pGEM®-T Easy vector system (Promega Corp.) and sequenced.

**Immunocytochemical Detection of OX_R**—Cells (2 × 10^5) were cultured on coverslips, washed with cold PBS, and immediately fixed in ice-cold 4% paraformaldehyde in PBS for 1 h followed by three washings in PBS. Samples were incubated for 1 h at 4 °C with 10% newborn calf serum in TBST buffer (10 mM Tris, pH 8, 150 mM NaCl and 0.1% Tween 20) and then incubated overnight at 4 °C with rabbit polyclonal anti-oxycortex receptor antibody OX_R (dilution 1:1000). Staining was revealed using FITC-conjugated goat anti-rabbit IgG by confocal microscopy.

**[Ca²⁺]** Measurements by Confocal Fluorescence Imaging—HT29-D4 cells (2.5 × 10⁵ cells/cm²) were seeded onto Lab-Tek. When cells achieved near-confluency (24 h before the experiment), the medium was replaced with free red phenol DMEM without serum. Cells were incubated for 30 min at 37 °C (5% CO₂ and darkness) in serum-free medium containing 6.7 μM Fluo-4 acetoxyethyl ester (Fluo-4AM) and rinsed twice with

2 G. Peranzi and M. Ostuni, unpublished results.
Orexins Inhibit Cell Growth in Human Colon Cancer Cells

**RESULTS**

**Orexins Inhibit Cell Growth in Human Colon Cancer Cells HT29-D4 in Culture**—Human colon cancer HT29-D4 cells grown in standard medium in the presence of 10% FCS were treated for 24 h with a variety of peptide hormones or neuropeptides present in the gut. Among the 26 peptides tested, orexin-A and orexin-B were the only peptides inhibiting cell growth, other peptides being without any effect or even stimulating cell growth such as in the case of ghrelin (Table I). In the presence of 10% FCS, which triggers a strong mitogenic effect on HT29-D4 cells, orexin-A and orexin-B elicited a dramatic decrease in cell number (Fig. 1, A and B). This inhibition of serum-induced increase in cell number, referred to as suppression of cell growth, was almost total after 24 h (Fig. 1, A and B) or 72 h (Fig. 1B) of treatment, i.e. >70% suppression. Orexins were active in the range of concentrations between 1 nM and 1 μM, with half-maximal responses being obtained for 5 nM for both orexins (Fig. 1C). Similar dose-response curves were observed after challenging cells with orexins for 24 h (Fig. 1C) or 48 h (not shown). After 1–2 days of challenge with orexins some morphological changes in cell shape were observed, in particular the cells looked rounder and shrunken (Fig. 1A, insets f and g). Although no cell detachment was observed, these morphological changes were reminiscent of apoptosis.

**Orexins Promote Cell Apoptosis but Do Not Alter Cell Proliferation in Human Colon Cancer Cells HT29-D4 in Culture**—Next we determined whether suppression of HT29-D4 cell growth by orexins was related to inhibition of cell proliferation and/or induction of cell apoptosis. Flow cytometric data of cell cycle analysis of control cells cultured in standard medium with 10% FCS indicated that 69.5 ± 4.5% of HT29-D4 cells are in G1/G0 phase; 15.6 ± 2.9% are in S phase, and 13.2 ± 2.2% are in G2/M phase (n = 6). When HT29-D4 cells were treated with 1 μM orexin-A or orexin-B for 24 h, 69.0 ± 5.3 and 70.2 ± 5.9% of cells were found in G1/G0 phase, 14.8 ± 2.2 and 13.4 ± 2.1% in S phase, and 12.3 ± 3.6 and 14.0 ± 2.5% in G2/M phase, respectively (n = 6). These data indicated that orexins have no significant effect on the HT29-D4 cell cycle. This was further confirmed when experiments were carried out with HT29-D4 cells that were synchronized by serum deprivation for 48 h. Indeed treatment of synchronized cells with 10% serum in the absence or presence of 1 μM orexin-B (Fig. 2A) or orexin-A (data not shown) provided identical flow cytometric data. Direct assessment of DNA synthesis by [methyl-3H]thymidine incorporation into DNA of HT29-D4 cells further supported the idea that orexins did not alter cell proliferation. Indeed, treatment of synchronized HT29-D4 cells with orexin-B (1 μM) did not modify serum-induced [methyl-3H]thymidine incorporation into DNA (Fig. 2B).

In sharp contrast with the proliferation data, a body of evidence supported that HT29-D4 cells undergo apoptosis upon orexin-A or orexin-B treatment. Fluorescence microscopic analysis of DNA-staining patterns with DAPI (Fig. 3A–F). TUNEL-positive cells were observed upon orexin-A or orexin-B treatment, whereas no labeling was detected in control cells. Apoptosis was further indicated by typical DNA ladder corresponding to cleavage of genomic DNA upon cell treatment with orexins (Fig. 3B).

**Orexin-induced Cell Apoptosis in Human Colon Cancer HT29-D4 Cells Is Associated with Cytochrome c Release and Caspase Activation**—Orexin-induced apoptosis was shown to be associated with cytochrome c release into cytosol. By using a confocal laser-scanning microscope (LSM 510 META, Zeiss) with a ×40 objective, Fluor-AM was excited by the 488-nm argon laser line, and emission was collected through a 505–530-nm bandpass filter.

**Miscellaneous Procedures**—Routine procedures such as radioimmunoassay of intracellular cAMP content (30) and measurement of protein content (31) were performed as described. Statistical Analysis—All data were expressed as mean ± S.E. values and analyzed by analysis of variance and Student’s t test for statistical significance. A p value of <0.05 was considered as statistically significant.

**Pro-apoptotic Role of Human OX1R Orexin Receptor**

Human colon cancer HT29-D4 cells grown in standard medium containing 10% FCS were treated for 24 h with peptide hormones or neuropeptides present in the gut. Cells were seeded at low density in 48-well plates and grown at 70–80% confluence (about 90,000 cells per well). The culture medium was then replaced with fresh medium containing 10% FCS without (none) or with 1 μM peptide. After 24 h, cells were harvested by trypsinization and counted. Cells produced over 24-h period of treatment (number of cells/well) are expressed as means ± S.E. (six determinations).

**Table I**

| Peptide (0.1 μM) | 24-h cell production |
|------------------|----------------------|
| None             | 193,559 ± 10,634     |
| Bombesin         | 178,773 ± 9875      |
| Cholecystokinin-8| 189,386 ± 12,469    |
| Galanin          | 217,723 ± 19,847    |
| Gastrin          | 174,448 ± 5888     |
| Gastric inhibitory polypeptide | 176,599 ± 9801 |
| Ghrelin          | 287,817 ± 27,718a   |
| Glucagon-like peptide-1 | 183,774 ± 6741 |
| Glucagon-like peptide-2 | 186,712 ± 9694 |
| Glucagon         | 190,456 ± 17,284    |
| Growth hormone-releasing factor | 187,317 ± 5066 |
| Neurotensin      | 187,657 ± 8510     |
| Neurotensin Y    | 187,975 ± 15,033   |
| Orexin-A         | 76,668 ± 3171a     |
| Orexin-B         | 46,717 ± 10,741a   |
| Putative adenylate cyclase-activating polypeptide | 190,692 ± 12,101 |
| Pancreastatin    | 181,506 ± 16,306    |
| Peptide histidine-methionine | 181,508 ± 5729 |
| Pancreatic polypeptide | 192,061 ± 15,920 |
| Prolactin-releasing peptide | 190,998 ± 1448 |
| Parathyroid hormone | 186,295 ± 6265 |
| Peptide YY       | 184,594 ± 7799     |
| Secretin         | 192,655 ± 10,372   |
| Somatostatin-14  | 208,654 ± 13,310   |
| Vasoactive intestinal contractor | 191,685 ± 25,573 |
| Vasoactive intestinal peptide | 189,207 ± 8802 |

*a p < 0.001 versus none.

**Figure 1**

**Figure 2**
chrome c after a 24-h challenge with orexins (Fig. 4B). Further experiments showed that orexin-induced apoptosis is associated with caspase activation. In situ caspase activation was followed by cleavage of a fluorogenic substrate (see “Experimental Procedures”). The fluorescent product was strongly labeled orexin-B (1 µM)-treated cells (Fig. 5A, b). Similar results were obtained with orexin-A (data not shown). A faint fluorescent labeling was also observed in control cells indicating a low background of caspase activation in the culture conditions used (Fig. 5A, a). To confirm caspase activation in orexin-induced apoptosis in HT29-D4 cells, we immunodetected cleavage of effector caspases downstream of the cytochrome c release, i.e. caspase-3 and caspase-7. By using cleaved caspase-3 (Asp-175) and cleaved caspase-7 (Asp-198) antibodies, endogenous levels of cleaved caspase-3 (Fig. 5A, d) and cleaved caspase-7 (Fig. 5A, f) were detected in HT29-D4 apoptotic cells upon 24 h of treatment with 1 µM orexin-B. Cleaved caspase-3 and caspase-7 detected upon HT29-D4 cell treatment with orexin-B were co-localized with fragmented nuclei (Fig. 5A, d and f). Similar results were obtained with orexin-A (data not shown). No cleaved caspase-3 (Fig. 5A, c) or caspase-7 (Fig. 5A, e) could be detected in control untreated cells. Further characterization of the effects of orexins on caspase cleavage was obtained by Western blot. As shown in Fig. 5B, treatment of HT29-D4 cells for 24 h with 1 µM orexin-B resulted in the appearance of 19- and 17-kDa forms of cleaved caspase-3 and the 20-kDa form of cleaved caspase-7 (Fig. 5B, right), whereas no cleaved caspases could be detected in control cells. Similar data were obtained with orexin-A (not shown). The pro-forms of caspase-3 (Fig. 5, left) and caspase-7 (Fig. 5B, right) were present in both control and orexin-treated cells.

**HT29-D4 Cells Express the OX1 Receptor Subtype**—Because reliable orexin tracers are still unavailable (17), the nature of orexin receptor subtype(s) expressed in HT29-D4 cells was determined by RT-PCR and immunocytochemistry. Total mRNA from HT29-D4 cells or control CHO cell lines expressing recombinant hOX1R or hOX2R were reverse-transcribed and amplified with specific couples of primers for the two subtypes of orexin receptors. Amplification products of the expected size were obtained in HT29-D4 cells with OX1R primers but not OX2R primers (Fig. 6A). Control recombinant CHO cells clearly express OX1R or OX2R transcripts (Fig. 6A), supporting the idea that the absence of OX1R mRNA in HT29-D4 cells was not related to limits of RT-PCR technology. The OX1R-amplified product obtained from HT29-D4 cells was sequenced and found to be identical to the hOX1R cDNA sequence (GenBankTM AF0412343). To provide evidence for OX1R protein expression in HT29-D4 cells, indirect immunofluorescence was performed by using rabbit polyclonal antibodies against hOX1R (Fig. 6B). Strong immunostaining with membrane localization was observed. This staining was completely abolished by co-incubating antibodies with the immunogen peptide.

Because orexins were shown previously to consistently induce Ca2+ transients in orexin receptor-expressing cells (15), we further investigated this second messenger as evidence for the presence of functional OX1R in HT29-D4 cells. The intracellular Ca2+ concentration was first monitored under a confocal microscope using Fluo-4AM dye. Fig. 6C shows confocal pictures before and after treatment with 1 µM orexin-B. The neuropeptide clearly induced Ca2+ transients as shown by confocal images. The effect of orexin-B on cytosolic calcium was further tested in Fura-2/AM dye-loaded HT-29-D4 cells by fluoroanalysis in a classical fluorimeter. Again orexin-B induced calcium transients with a time course of response similar to that observed with neurotensin (Fig. 6D), a well known inducer of calcium transients in human colon cancer cells (32). In sharp contrast, orexin-B failed to induce calcium transients in the human colon cancer HCT116 cells (Fig. 6D) that are not equipped with OX1R receptor (see below). As a control, we showed that neurotensin nicely induced calcium
transients in HCT116 cells. All these results clearly showed that functional OX₁ receptors are expressed in HT29-D4 cells.

Expression of OX₁R and Anti-growth and Pro-apoptotic Effects of Orexins Are Observed in Other Colon Cancer Cell Lines but Not in Normal Colonic Epithelium—As shown in Fig. 7, the OX₁R-mediated effects of orexins are observed in three of four other human colon cancer cell lines tested. RT-PCR experiments showed that amplification products of the expected size were obtained in Caco-2, SW480, and LoVo cells with OX₁R primers (Fig. 7A). In contrast, no OX₁R mRNA could be detected in the HCT116 cell line. In good agreement with the RT-PCR data, orexin-B strongly inhibited FCS-stimulated cell growth in Caco-2, SW480, and LoVo cells but not in HCT116 cells (Fig. 7B). Finally, the effect of orexin-B on apoptosis was tested in the four colon cancer cell lines. Apoptosis was clearly indicated by a typical DNA ladder corresponding to cleavage of genomic DNA upon cell treatment with orexin-B in Caco-2, SW480, and LoVo but not HCT116 cells (Fig. 7C). Altogether these data indicated that expression of OX₁R and OX₁R-mediated anti-growth and pro-apoptotic effects of orexins are frequent in colon cancer because they are observed in four of five human colon cancer cell lines originating from different patient tumors, i.e. HT29-D4, Caco-2, SW480, and LoVo.

In this context, we explored the status of OX₁R in normal human colonic mucosa. RT-PCR experiments using total RNA extracted from pure epithelial cell preparations isolated from three normal human colons failed to indicate OX₁R mRNA (not shown) under conditions in which specific amplification products were clearly detected in human colon cancer cell lines (see Figs. 6A and 7A). Because the long term culture of isolated human colonic epithelial cells still remains an elusive task and the existence of normal intestinal epithelial cell lines is still a debated question, we used explant cultures of dissected human normal colonic mucosa to assess the effects of orexin on apoptosis in normal colon. These mucosal explants (polarized epithelial barrier and underlying lamina propria) can be maintained in good viability conditions, as assessed by a multiparametric approach (see “Experimental Procedures”). Mucosal explants maintained their morphological integrity over a 24-h culture period as shown by standard histology (Fig. 8). Spontaneous apoptosis occurred in a minority of epithelial cells (less than 1%). Indeed, a few apoptotic epithelial cells were visualized by the M30 antibody immunostaining specific for caspase-3-cleaved cytokeratin 18 and by the Hoechst dye, which reveals DNA condensation and fragmentation. They were preferentially located at the tip of the surface epithelium,
undergoing exfoliation, and occasionally in the lower region of the crypt (Fig. 8). A few lamina propria macrophages underlying the epithelial barrier occasionally contained M30 antibody-positive cells. Most interestingly, a 24-h treatment with 1 μM orexin-B neither altered the morphological integrity of the colonic crypts nor increased the number of apoptotic cells (Fig. 8).

The OX1R-mediated Anti-growth and Pro-apoptotic Effects of Orexins Are Intrinsic Properties of the OX1R and Not Dependent on Cell Context—All the actions of orexins described herein were observed in human colon cancer cell lines. We next asked the question of whether the OX1R-mediated anti-growth and pro-apoptotic effects of orexins are restricted to colon cancer cells or are intrinsic properties of the OX1R. Because OX1Rs were initially described in the brain (15), we first tested the human neuroblastoma cell line SK-N-MC which expresses OX1R (35). As shown in Fig. 9, orexin-A and orexin-B, in the range of concentrations between 1 nM and 1 μM, strongly reduced SK-N-MC cell growth (Fig. 9A, top). The maximal effect observed at 1 μM peptide represented 75% inhibition of growth as compared with control cells. Half-maximal inhibitions were obtained for 5 nM for both orexins (Fig. 9A, top). The neuropeptides also induced SK-N-MC cell apoptosis as shown by propidium iodide staining, which revealed condensed nuclei upon cell death detection kit (36), typical DNA strand breaks (in green) in cells that were stained with propidium iodide (red nuclei). Cells are examined and visualized by confocal microscopy. B, typical DNA ladder obtained after agarose gel electrophoresis staining with ethidium bromide and photographed under UV light (*, 123-bp DNA marker; lane 1, extracted DNA from control cells; lane 2, extracted DNA from orexin-A 1 μM-treated cells; lane 3, extracted DNA from orexin-B (1 μM-treated cells).

In order to better quantitate the apoptotic rate of SK-N-MC and CHO/hOX1R cells upon orexin challenge, we analyzed annexin V binding (Guava annexin assay), which reveals phosphatidylserine externalization in apoptotic cells. After a 24-h treatment of cells with 1 μM orexin-B, the percentage of apoptotic cells strongly increased up to 11 and 27% in SK-N-MC and CHO/hOX1R cells, respectively (Table II). In sharp contrast, orexin-B had no significant effect on the percentage of apoptotic cells in the parent CHO-K cell line (Table II). Altogether these results supported the idea that OX1R mediates inhibition of cell growth and induction of apoptosis independently of the cell environment.

**Discussion**

In this work, we discover and characterize a new function of the neuropeptides orexins as drastic pro-apoptotic peptides. We show that orexins acting at either native (human colon adenocarcinoma cells or neuroblastoma cells) or recombinant (CHO cells) seven transmembrane domain receptor OX1R have strong anti-growth properties by inducing cytochrome c- and caspase-dependent apoptosis. These data provide the first evidence that the OX1R and its natural agonists orexin-A and orexin-B are important players in the control of apoptosis. They may have future interesting applications in the treatment of apoptosis-resistant cancers such as colon cancer. They also promote the design of new studies to understand the physiological role of orexins in relation to apoptosis in OX1R-expressing tissues.
FIG. 5. Effect of orexins on in situ caspase activation and caspase-3 or caspase-7 cleavage in HT29-D4 cells. A, activated caspases are detected in situ by fluorescence labeling (in green) using caspase fluorescent isothiocyanate-VAD-fluoromethyl ketone, a cell-permeable caspase inhibitor that binds to activated caspases. HT29-D4 cells were grown on coverslips and then left untreated (a) or treated with 1 μM orexin-B (b) for 24 h at 37 °C, and then 10 μM of caspase fluorescent isothiocyanate-VAD-fluoromethyl ketone was added and incubated in darkness for 20 min. Cells were rinsed with PBS and then fixed in 10% buffered formalin for 30 min at room temperature under darkness and were observed by confocal microscopy. Confocal micrographs of immunostaining are shown for cleaved caspase-3 (Asp-175) antibody (green) in HT29-D4 cells treated for 24 h without (c) or with (d) 1 μM orexin-B. Confocal micrographs of immunostaining are shown for cleaved caspase-7 (Asp-198) antibody (green) in HT29-D4 cells treated for 24 h without (e) or with (f) 1 μM orexin-B. c–f, nuclei were stained in red with propidium iodide. B, immunoblot analysis of cell lysates from HT29-D4 cells treated or not by 1 μM orexin-B using caspase-3 antibodies and cleaved caspase-3 antibodies (left) or caspase-7 antibodies and cleaved caspase-7 antibodies (right). A β-actin antibody was used as a control.

Considering that many growth factors (1–8) but very few anti-growth factors for human colon cancer cells have been described during the last 2 decades (13, 14, 34), we tested a large array of gut peptide hormones and neuropeptides for their ability to inhibit cell growth in the human colon cancer HT29-D4 cell line cultured in the strong growth-promoting environment of 10% FCS. All peptides tested except orexins were without any effect in inhibiting cell growth. Among the peptides tested, some were described previously as growth-promoting such as neurotensin (3), glucagon-like peptide 1 (35), or gastrin/cholecystokinin (5). It is not surprising that they are inactive in our assay conditions because growth is already strongly stimulated by FCS. In this context, the ability of ghrelin, a peptide hormone produced by the stomach (36), to promote cell growth even in the presence of 10% FCS is amazing but is outside the scope of the present study. The screening assay identified only two anti-growth peptides, the closely related orexin-A and orexin-B encoded by the same gene (15, 17) and acting at common receptors (15). Initial experiments established that orexin-A and orexin-B are equipotent at suppressing HT29-D4 cell growth (see Fig. 1) with an ED50 of 5 nM. A body of evidence supports that orexins suppress HT29-D4 cell growth (e.g. inhibit serum-induced increase of cell number) by promoting apoptosis. (i) Orexins do not alter cell proliferation as indicated by flow cytometric data of cell cycle and [methyl-3H]thymidine incorporation into DNA. (ii) Upon cell treatment with orexins, morphological changes in cell shape,
reminiscent of apoptosis, are observed, in particular cells look rounder and shrunken. (iii) Direct evidence for orexin-induced apoptosis is provided by several techniques including DNA-staining patterns with DAPI, TUNEL assay, and DNA ladder. (iv) Orexins induce cytochrome c release into cytosol and activation of effector caspase-3 and caspase-7. The orexin-induced apoptosis in HT29-D4 cells is not associated with the observable cell detachment in contrast to Fas ligand-induced apoptosis (37), which is responsible for massive HT29-D4 cell detachment. Because cells do undergo apoptosis without detachment, we have to assume that apoptotic cells in culture are eliminated. Although not documented in this paper, a possible way is phagocytosis by adjacent nonapoptotic epithelial cells, a process demonstrated previously (38, 39).

FIG. 7. Expression of OX1R mRNA and anti-growth and pro-apoptotic effects of orexin-B in different human colon cancer cell lines. A, total RNA extracted from Caco-2, SW480, LoVo, and HCT116 cells were reverse-transcribed and PCR-amplified with hOX1R (top) or β-actin (bottom) primers. A single PCR-amplified product of the exact predicted size (500 bp) for hOX1R was visualized in Caco-2, SW480, and LoVo cells. B shows the effect of orexin-B on serum-induced cell number in Caco-2, SW480, LoVo, and HCT116 cell lines. Human colon cancer cells grown in standard medium containing FCS were treated for 24 h with orexin-B. Cells were seeded at low density in 48-well plates and grown at 70–80% confluence (about 90,000 cells per well). The culture medium was then replaced with fresh medium containing FCS without (−) or with (+) 1 μM orexin-B. After 24 h, cells were harvested by trypsinization and counted. Cells produced over a 24-h period of treatment (number of cells/well) are expressed as means ± S.E. (four determinations). **, p < 0.001 versus without orexin (-). C shows typical DNA ladders for Caco-2, SW480, LoVo, and HCT116 cell lines treated or not treated for 48 h with 1 μM orexin-B. Agarose gel stained with ethidium bromide was photographed under UV light. *, 123-bp DNA marker; −, extracted DNA from control cells; +, extracted DNA from orexin-B (1 μM)-treated cells.

FIG. 8. Absence of effect of orexin on apoptosis in explant cultures of human normal colonic mucosa. Explants of human normal colonic mucosa were microdissected and cultured for 24 h in the absence (control) or presence of 1 μM orexin-B. Morphological integrity of the explants was assessed by standard hematoxylin-eosin (HE) staining on paraffin sections. The crypt morphology was well preserved both in the control and orexin-treated explants. Epithelial apoptosis was evaluated by M30 immunostaining of caspase-cleaved cytokeratin 18 and by the Hoechst dye. M30-positive cells (*) (brown cytoplasmic staining) undergoing exfoliation were present near the surface epithelium or occasionally in the lower region of the crypts. Hoechst staining shows a few nuclei undergoing DNA condensation and fragmentation within the surface epithelium (*). Original magnification ×400 for hematoxylin-eosin and M30, and ×630 for Hoechst staining.

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imunodetected at the cell surface of HT29-D4 cells by using a specific anti-OX1R antibody (20). In contrast, specific anti-
OX1R antibodies do not immunostain HT29-D4 cells under conditions in which control OX1R-expressing CHO cells are
nicely stained. The presence of a functional orexin receptor in HT29-D4 cells is known to promote intracellular Ca2+
release (33). In this context, our work shows that the OX1R-expressing SK-N-MC neuroblastoma cells (33) do undergo apoptosis upon treatment with orexins.

The pro-apoptotic activity of orexins demonstrated here in human colon cancer cells HT29-D4, human neuroblastoma
cells SK-N-MC and CHO cells expressing recombinant OX1R receptor, results in massive suppression of cell growth regardless of the OX1R-expressing cell. The mechanisms of coupling between activation of cell surface seven transmembrane domain OX1R and release of cytochrome c into cytosol remain to be elucidated. Activation of OX1R is known to result in mobilization of intracellular calcium through a Gq-dependent mechanism (15, 40, 45). This Ca2+ response also observed here in HT29-D4 cells is certainly not sufficient to explain the pro-apoptotic effect of orexins, although increases of cytosolic calcium are well known to occur during apoptotic cell death (46). Indeed, a variety of GPCRs in human colon cancer cells including HT-29 cells is known to promote intracellular Ca2+ mobilization (3, 7, 8, 10). These receptors such as NT1 receptors for neurotensin (3, 10), protease-activated receptors 1 (PAR1) for thrombin (7), protease-activated receptors 2 (PAR2) for trypsin (8), or muscarinic M3 receptors for acetylcholine (47) not only do not trigger apoptosis but rather stimulate cell proliferation. In this context, it is worth pointing out that up to now very few serpentine GPCRs have been shown to inhibit cell growth by promoting apoptosis e.g. endothelin receptor ETB (48), chemo-

**Table II**

| Cell line | % apoptotic cell (annexin V-positive) | Control | Orexin-B (1 μM) |
|-----------|------------------------------------|---------|----------------|
| SK-N-MC   | 0.2 ± 0.1                          | 11.4 ± 1.2* |
| CHO/hOX1R | 2.1 ± 0.8                          | 27.7 ± 0.9* |
| Parent CHO-K | 0.8 ± 0.3                      | 1.7 ± 0.7  |

*p < 0.001 versus control.

Fig. 9. Effect of orexins on cell growth and apoptosis in human neuroblastoma SK-N-MC cells and recombinant CHO/hOX1R cells. A, dose effect of orexin-A (●) or orexin-B (○) on the growth of SK-N-MC cells (top), CHO/hOX1R cells (middle), or parent CHO-K cells (bottom). For SK-N-MC cells grown in standard medium with 10% FCS, the dose response of orexins is shown after 24 h of treatment with peptides. For CHO/hOX1R cells or CHO-K cells grown in 10% FCS, cell counts are shown after cell treatment without (A) or with orexins for 1–3 days. B, SK-N-MC cells (top), CHO/hOX1R cells (middle), or parent CHO-K cells (bottom) were grown in standard culture medium containing 10% FCS and then treated for 24 h without (control) or with 1 μM orexin-A (●) or orexin-B (○). Condensed nuclei (arrows) were then visualized by propidium iodide staining.
kine CXCR4 receptor (49), β-adrenergic receptor (50), angiotensin AT2 receptor (51), somatostatin SST2 receptor (52), or parathyroid hormone receptor (53).

The OX₁R-mediated pro-apoptotic role of orexins described herein raises the question of its significance in health and disease. With respect to colon cancer, this study already shows that expression of OX₁R and OX₂R-mediated apoptosis are frequent in colon cancer cells because they are observed in five human colon cancer cell lines originating from different patients (9), i.e. HT29-D4, Caco-2, SW480, and LoVo. Most interestingly, our data also show that normal human colonic epithelial cells are not equipped with OX₁R, resulting in the absence of the pro-apoptotic effect of orexins in normal human colon epithelium. These observations suggest that OX₁Rs are aberrantly expressed in human colon cancer cells. Whether this aberrant expression represents an ectopic expression or the re-expression of receptors expressed in colonic epithelium during embryogenesis remains to be determined. Ectopic expression of G protein-coupled receptors in colon cancers has been reported previously for neurotensin receptor (10) and PAR1 for thrombin (7). However, these receptors were clearly shown to promote cell proliferation and colon cancer cell growth (7, 10). In that respect, this report presents one of the first examples of an ectopic expression of a receptor promoting apoptosis in colon cancer cells. Given the known resistance of colon cancer to apoptosis and chemotherapy (39, 54), OX₁R thereby represents an attractive new target for the development of instrumental orexin agonists in this cancer. On the other hand, because the pro-apoptotic role of OX₁R appears to be an intrinsic property of the receptor regardless of the nature of the OX₁R-expressing cell, we may speculate about the role of orexins in normal tissues expressing OX₁R. In that respect, a site of expression of orexin receptors in normal tissues is small intestinal epithelium (17, 20). Because this epithelium is a rapidly renewing tissue in which cell homeostasis is regulated by a balance among proliferation, growth arrest, differentiation, and apoptosis (39), the possibility that orexins, which are neuropeptides expressed in the small intestinal wall (20), may control apoptosis and cell homeostasis in this tissue is an attractive hypothesis. A major site of expression of OX₁R is the brain and more specifically the hypothalamus (15, 16). In this context, our studies raise the question of the possible role of orexins in neuronal apoptosis which is a major event during brain development and maturation (55) and also in neurodegenerative diseases (55).

In conclusion, this work characterizes for the first time the pro-apoptotic and subsequent anti-cell growth properties of orexins acting at the OX₁ receptor. Although the molecular mechanisms of the OX₁R-mediated pro-apoptotic effect of orexins remain to be elucidated, these findings add a new dimension to the biological activities of these neuropeptides that may have important implications in health and disease.

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