Metoclopramide stimulates catecholamine and granin-derived peptide secretion from pheochromocytoma cells through activation of serotonin type 4 (5-HT$_4$) receptors

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Abstract

Context: The gastroprokinetic agent metoclopramide is known to stimulate catecholamine secretion from pheochromocytomas.

Objective: To investigate the mechanism of action of metoclopramide and expression of serotonin type 4 (5-HT_4) receptors in pheochromocytoma tissues.

Design and Setting: In vitro study performed in an academic research laboratory.

Material: Tissue explants obtained from 18 pheochromocytomas including the tumor removed from a 46-yr-old female patient who experienced life-threatening hypertension crisis after metoclopramide administration and 17 additional pheochromocytomas (9 benign and 8 malignant).

Intervention: Cultured pheochromocytoma cells derived from the patient who previously received metoclopramide were incubated with metoclopramide and various 5-HT_4 receptor ligands. In addition, total mRNAs were extracted from all the 18 tumors.

Main outcome measure: Catecholamine and granin-derived peptide concentrations were measured in pheochromocytoma cell incubation medium by high performance liquid chromatography and radioimmunological assays. In addition, expression of 5-HT_4 receptor mRNAs in the 18 pheochromocytomas was investigated by use of reverse transcriptase-polymerase chain reaction.

Results: Metoclopramide and the 5-HT_4 receptor agonist cisapride were found to activate catecholamine and granin-derived peptide secretions by cultured tumor cells. Metoclopramide- and cisapride-evoked catecholamine and granin-derived peptide productions were inhibited by the 5-HT_4 receptor antagonist GR 113808. 5-HT_4 receptor mRNAs were detected in the patient's tumor and the series of 17 additional pheochromocytomas.

Conclusions: This study shows that pheochromocytomas express functional 5-HT_4 receptors that are responsible for the stimulatory action of metoclopramide on catecholamine and granin-derived peptide secretion. All 5-HT_4 receptor agonists must therefore be contra-indicated in patients with proven or suspected pheochromocytoma.
Introduction

The worldwide used gastroprokinetic and antiemetic agent metoclopramide is known to exert stimulatory effects on prolactin and aldosterone secretions in human subjects (Norbiato et al. 1977; Harrington et al. 1983). These two endocrine actions are usually attributed to the antidopaminergic properties of the compound (Fraser 1987; Molitch 1992). Metoclopramide has also been shown to induce acute hypertensive crisis and adrenergic myocarditis in patients with pheochromocytoma and metoclopramide administration has been used as a provocative test in the diagnosis of pheochromocytoma (Abe et al. 1984; Plouin et al. 1976; Agabiti-Rosei et al. 1977; Sever 1977; Hsu et al. 1993; Maxwell et al. 2001; Leow & Loh 2005). In some cases, metoclopramide-induced adrenergic myocarditis presented as a dilated cardiomyopathy with profound left ventricular akinesia (Maxwell et al. 2001; Leow & Loh 2005). The mechanism of metoclopramide-evoked hypertensive crisis in these patients likely involves an increase in norepinephrine release via presynaptic D_2 receptor blockade, an inhibition of the vasodilatory effect of dopamine leading to potentiation of the hypertensive action of norepinephrine and epinephrine, and a direct stimulatory effect of the drug on pheochromocytoma cells (Abe et al. 1984; Plouin et al. 1976; Adler-Graschinsky et al. 1984). Although the occurrence of dopamine D_2 receptors has been clearly established in pheochromocytoma tissues (Wu et al. 2001), the molecular events involved in the action of metoclopramide on pheochromocytoma cells are not fully elucidated.

Beside its antagonistic action at D_2 receptors, metoclopramide is able to act as a partial agonist at the serotonin4 (5-HT_4) receptor type (Dumuis et al. 1989). The 5-HT_4 receptor is a 7-transmembrane domain receptor positively coupled to adenylyl cyclase (Bockaert et al. 2004; Brattelid et al. 2004). Its primary transcript is alternatively spliced to produce nine receptor isoforms that differ in the length and structure of their C-terminal tail (a-g, i, n) and one variant which exhibits a 14-aminoacid insertion within the second extracellular loop (h) (Bockaert et al. 2004; Brattelid et al. 2004). It is now recognized that the 5-HT_4 receptor mediates metoclopramide-evoked aldosterone secretion as well as the abnormal response of plasma cortisol to metoclopramide observed in some patients with ACTH-
independent macronodular adrenal hyperplasia (Lefebvre et al. 1993; Rizzi et al. 1997; Cartier et al. 2003; Christopoulos et al. 2005).

In the present report, a pheochromocytoma removed from a patient who experienced hypertension crisis after metoclopramide administration, was used as a model for the study of the mechanism of action of metoclopramide on pheochromocytoma cells. In vitro experiments were conducted in order to examine the effect of metoclopramide on the secretion of catecholamine and granin-derived peptides, including the secretogranin II-derived peptide EM66 and the chromogranin A-derived peptide WE14, by cultured pheochromocytoma cells. In addition, RT-PCR analysis was used to investigate the expression of 5-HT4 receptor mRNA in the patient's tumor and a retrospective series of 17 pheochromocytomas.

Methods

Patients and Materials

A 46-yr-old woman was referred to our department of endocrinology for a recently discovered left adrenal tumor measuring 7 cm of diameter. She had a history of labile hypertension and paroxysmal spells of headache, palpitations and cold sweating, and was therefore highly suspected of pheochromocytoma. Immediately after admission, she complained of nausea and unfortunately received 10 mg metoclopramide orally. Thirty minutes later, her blood pressure and pulse rose to 180/100 mmHg and 150/min, respectively, and signs of cardiac insufficiency appeared. She was then transferred to the cardiology intensive care unit. Ventriculography showed profound hypokinesia and a large thrombus of the left ventricle. Treatment with low-molecular-weight heparin, aspirin and prazosin led to recovery of normal ventricular function, regression of the ventricular thrombus and normalization of blood pressure allowing surgical removal of the adrenal mass. Pathological examination of the tumor confirmed the diagnosis of pheochromocytoma. All clinical signs of catecholamine excess completely resolved after surgery.

Fragments of the tumor were obtained at surgery and either immediately transported to the laboratory for culture experiments or frozen on dry ice and stored at –80°C until RT-PCR experiments.
RT-PCR studies were also performed in a retrospective series of 17 pheochromocytomas (9 benign and 8 malignant) provided by a French endocrinological network for collection of adrenal tumors (Réseau COMETE) and stored at -80°C until utilization. The tumors (mean diameter: 64 ± 25 mm, range: 30-120 mm) had been obtained from 8 women and 9 men (mean age: 47.6 ± 16.5 yr, range: 13-68 yr). Five tumors were of extra-adrenal localization. The diagnosis of benign tumor was assessed on the absence of any histological criteria of malignancy, mutation in the succinate dehydrogenase B (SDHB) gene, AND tumor recurrence or metastatic diffusion during a follow-up of at least two years. Malignancy was established by the presence of at least one metastasis. Normal adrenomedullary tissue explants (control tissues) were obtained from two brain-dead organ donors. The adrenal medulla was carefully dissected from the cortex with a scalpel by scraping off the brown interdigitated islets of chromaffin cells, as previously described (Cavadas et al. 2001). This method allows to obtain a degree of purity of 95% for chromaffin cells when the tissues are used for cell culture experiments. The protocols of collection of the tissues and the experimental procedures were approved by the regional ethics committee and informed consent was obtained from all patients with pheochromocytoma.

**Primary culture of pheochromocytoma cells**

Pheochromocytoma fragments were immersed in culture medium (Dulbecco's modified Eagle Medium, DMEM, Sigma-Aldrich, St-Quentin-Fallavier, France), transported to the laboratory and processed as previously described (Guillemot et al. 2006). Briefly, the tissues were minced with scissors and tumor cells were enzymatically dispersed in DMEM containing 0.1% collagenase (Serlabo, Bonneuil-sur-Marne, France) and 30 U/ml DNase I (Sigma-Aldrich). Isolated cells were cultured in DMEM supplemented with 5% calf serum (Biowhittaker Europe, Verviers, Belgium), 10% horse serum (Invitrogen, Cergy Pontoise, France), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone (Life Technologies, Cergy-Pontoise, France). Tumoral chromaffin cells were purified by overnight differential plating to remove adherent non chromaffin cells and then plated at a density of 10^6 cells/ml per well. After one day, pheochromocytes were treated with metoclopramide (Sigma-Aldrich), dopamine (Sigma-Aldrich), cisapride (10^7 M; Laboratoire Janssen, Boulogne-
Billancourt, France), domperidone (Sigma-Aldrich) and/or the 5-HT\textsubscript{4} receptor antagonist GR113808 (10\textsuperscript{-7} M; GlaxoSmithKline, Greenford, England, UK). Cells were incubated with each secretagogue (3 wells for each concentration tested) for 24 h at 37°C. The culture medium was then collected and immediately frozen at −80°C until HPLC or at −20°C until RIA.

6 Catecholamine and granin-derived peptide assays

Characterization and quantitation of norepinephrine and epinephrine in cell culture medium were carried out by high performance liquid chromatography combined with electrochemical detection after extraction on alumina, using a method adapted from that previously used for measurement of plasma catecholamine concentrations (Joannides et al. 1998).

The concentrations of EM66 and WE14 were measured by RIA. Purified recombinant EM66 and synthetic [Tyr0] WE14 were iodinated by the chloramine-T method and separated from free iodine on Sep-Pak C18 cartridges using a gradient of acetonitrile (0-100%) in 0.1% TFA, as previously described (Guillemot et al. 2006; Montero-Hadjadje et al. 2002). The RIA was performed in veronal buffer (pH 7.4) supplemented with 0.4% bovine serum albumin (BSA, Roche Diagnostics, Mannheim, Germany) and 0.1% Triton X-100. The EM66 and WE14 antisera, used at a final dilution of 1:60,000, and 1:100,800, respectively, were incubated with 7,000 cpm of tracer/tube in the presence of graded concentrations of standard (purified EM66 or synthetic WE14), or culture medium. After a 2-day incubation at 4°C, the antibody-bound fraction was immunoprecipitated by addition of 100 µl of 20% bovine β-globulins and 1 ml of 5% polyethylene glycol 8000. After a 20 min incubation at room temperature, the mixture was centrifuged (5,000 X g, 30 min, 4°C) and the pellet containing the bound fraction was counted on a gamma-counter (LKB, Wallack, Rockville, MD). The standard curve was set up with concentrations of EM66 or WE14 ranging from 5-10,000 pg/tube.

24 RNA extraction and real time RT-PCR

Total RNA from the 18 PCs including the patient’s tumor, and 2 normal adrenal medulla was extracted by the acid guanidium-thiocyanate-phenol-chloroform procedure by using Tri Reagent
The concentration of total RNA was determined by measuring the optical density at 260 nm. Real-time RT-PCR analysis was carried out as described by Fink et al. (1998) in order to quantify 5-HT$_4$ receptor mRNA in both pheochromocytomas and normal adrenal medulla. The primers and fluorogenic TaqMan probe used for these experiments hybridized to all 5-HT$_4$ receptor splice variants as previously published (Cartier et al. 2005). Briefly, 1 µg of total RNA from each tissue was converted to single stranded cDNA using SuperScript II from Life Technologies (Eragny, France) with oligo (dT)12-18 primer (0.5 µg/ml), and the cDNA was diluted and aliquoted into microtitre plates. For each 25-µl TaqMan reaction, 5 µl cDNA was mixed with 1 µl water, 12.5 µl TaqMan Universal PCR Master Mix 2X (Applied Biosystem, Courtaboeuf, France), 2 µl sense primer (2 µM), 2 µl antisense primer (2 µM), and 2.5 µl TaqMan probe (2 µM). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Parallel assays using the same cDNA pools were carried out using primers and probe to the housekeeping gene porphobilinogen deaminase. Quantitative RT-PCR was performed using an ABI Prism 7700 sequence detector system (Applied Biosystem) and analyzed using relative expression to PBGD, as previously described (Cartier et al. 2005). Briefly, the level of expression in each sample was normalized by dividing copies/ng total RNA of 5-HT$_4$ receptor gene by copies/ng total RNA of porphobilinogen deaminase gene, and expressed as a percentage. This mode of calculation allows to correct for both RNA quality and quantity.

**Characterization of 5-HT$_4$ receptor isoforms by RT-PCR**

Total RNA was extracted and reverse transcribed as described above. Amplification of the cDNAs encoding the different 5-HT$_4$ receptor C-terminal splice variants was performed by PCR using primer S1, which hybridizes to all 5-HT$_4$ receptor messengers, and splice variant-specific reverse primers as previously published (Cartier et al. 2005). The 5-HT$_{4(h)}$ variant was amplified using the forward primer Fwh, which is specific for cDNAs containing the 5-HT$_{4(h)}$ exon, and the reverse primer Revh which hybridizes to all 5-HT$_4$ receptor messengers. All PCR-based procedures were performed in a final volume of 50 µl containing 10% of reverse transcription mixture, 3 U DNA Taq Polymerase (Life Technologies), DNA Polymerase buffer (Life Technologies), 1.5 mM MgCl$_2$, 0.4 mM dNTP and 20
pmol of each primer. The PCR reactions were performed for 40 cycles (94°C, 40 s; 50°C, 60 s; 72°C, 90 s). The PCR products were analyzed in 1.5% agarose gels, blotted on a nylon membrane and hybridized with the [32P]ATP-labeled oligonucleotide S2. In addition, PCR products were subcloned into pGEM-T (Promega, Charbonnières, France) and sequenced, using the Thermosequenase kit (Amersham, Orsay, France) on a Li-Cor 4200L DNA sequencer (ScienceTec, Les Ulis, France) using fluorescent T7 and T3 primers (MWG-Biotech, Courtaboeuf, France).

Statistical analysis

All results of cell incubation studies were expressed as mean ± SEM and statistical analysis was performed using Dunnett’s test after one-way ANOVA. Potencies and efficacies of the test substances are expressed as concentrations that produced half-maximum responses (EC50) and maximum stimulations (Emax), respectively. Expression levels of 5-HT4 mRNAs in the benign and malignant pheochromocytomas were compared using the Mann-Whitney U test. Probability values less than 0.05 were considered significant. Data were analyzed with the Prism program (GraphPad Software, San Diego, CA).

Results

Effect of metoclopramide on catecholamine and granin-derived peptides from cultured pheochromocytoma cells

Administration of graded concentrations of metoclopramide (10^-9 M to 10^-6 M) to pheochromocytoma cells in primary culture induced a dose-dependent increase in norepinephrine and epinephrine secretion (Fig. 1A and 1B). The potencies (EC50) and efficacies (Emax) of metoclopramide to stimulate catecholamine production were 1.44 x 10^-8 M and +44±12%, respectively, for norepinephrine, and 1.23 x 10^-8 M and +43±12%, respectively, for epinephrine. Interestingly, a concentration of 10^-7 M of metoclopramide, which corresponds to the level of metoclopramide commonly observed in plasma after oral intake of 10 mg of the drug (Lamparczyk et al. 2001), was sufficient to trigger a significant increase (i.e. +20±4% for norepinephrine and +23±4% for
epinephrine) in catecholamine secretion. Metoclopramide also induced a concentration-dependent
increase in EM66 and WE14 productions (Fig. 1C and 1D) with EC50 reaching respectively 4.03 x 10^{-9} M and 3.84 x 10^{-8} M, and Emax of +46±4% and 78±12%, respectively. The norepinephrine, epinephrine, EM66 and WE14 responses to metoclopramide were all inhibited by GR 113808 (10^{-7} M), a 5-HT_{4} receptor antagonist devoid of any dopaminergic properties (Grossman et al. 1993) (Fig. 1).

Effect of cisapride and domperidone on catecholamine and granin-derived peptides from cultured pheochromocytoma cells

Incubation of cultured pheochromocytoma cells with the 5-HT_{4} receptor agonist cisapride at the concentration observed in plasma (C_{max}) after oral administration of the drug, i.e. 10^{-7} M (Gross et al. 1999), induced a significant stimulation of norepinephrine (+23±4%), epinephrine (+24±1%), EM66 (+43±3%) and WE14 (+63±15%) production (Fig. 2). Cisapride-induced catecholamine and granin-derived peptide secretions were inhibited by GR 113808 (10^{-7} M). In contrast to cisapride, domperidone (10^{-6} M), a dopaminergic D_{2} receptor antagonist devoid of any agonistic activity at the 5-HT_{4} receptor, had no significant effect on catecholamine and granin-derived peptide release (Fig. 2).

Effect of metoclopramide on the catecholamine and granin-derived peptide responses to dopamine from cultured pheochromocytoma cells

Incubation of pheochromocytoma cells with graded concentrations of dopamine (10^{-9} M to 10^{-6} M) induced a dose-dependent inhibition of catecholamine production reaching -49±2% for norepinephrine and -51±3% for epinephrine (Fig. 3A and 3B). Interestingly, the inhibitory effect of dopamine on norepinephrine and epinephrine secretions was not significantly influenced by metoclopramide (10^{-6} M; Fig. 3A and 3B). In contrast to its negative action on catecholamine release, dopamine dose-dependently stimulated EM66 and WE14 production with maximum efficacies of +28±10% and +30±6% (Fig. 3C and 3D).
Quantitative expression of 5-HT₄ receptors in normal adrenal medulla and pheochromocytomas

We have investigated the quantitative expression of 5-HT₄ receptor mRNA by the tissues by real-time PCR using oligonucleotides hybridizing to all receptor variants. 5-HT₄ receptor mRNAs were detected in all the tissues (Fig. 4). When expressed as arbitrary units normalized to porphobilinogen deaminase, 5-HT₄ receptor expression levels were 3.61% and 1.23% in the two normal medulla explants, 0.13-207% with a median value of 2.01% in benign pheochromocytomas, and 0.28-25.4% with a median value of 2.22% in malignant pheochromocytomas. There was no significant difference in the expression rates of benign versus malignant pheochromocytomas. The patient's pheochromocytoma was one of the tissues that expressed the highest levels of 5-HT₄ receptor mRNA, i.e. 29.7%.

Characterization of 5-HT₄ receptor isoforms by RT-PCR in normal adrenal medulla and pheochromocytomas

RT-PCR amplification was applied to characterize 5-HT₄ receptor isoforms in both normal adrenal medulla and pheochromocytomas (Table 1). None of the known isoforms was detected in adrenomedullary explants. In contrast, pheochromocytoma tissues were found to frequently express isoforms (a), (b), (c), (i) and (n). Isoform (g) was present in two tumors, i.e. one benign (n°3) and one malignant (n°10). Two pheochromocytomas (n°6 and n°17) exclusively expressed isoform (b). There was no significant difference between the profiles of expression of 5-HT₄ receptor isoforms in benign and malignant pheochromocytomas, respectively.

Discussion

In the present study, we have used pheochromocytoma tissue removed from a patient who experienced hypertension crisis and acute adrenergic myocarditis after metoclopramide administration, as an in vitro model for the study of the mechanism of action of metoclopramide on pheochromocytoma cells. It was indeed probable that metoclopramide may have triggered catecholamine secretion from the tumor tissue, leading to severe hypertensive paroxysm. Consistent with this hypothesis, our results
showed that metoclopramide dose-dependently stimulated catecholamine production from pheochromocytoma cells. It was also physiopathologically relevant to notice that the concentration of metoclopramide usually observed in the plasma after oral intake of the drug (10^{-7} \text{ M}), was sufficient to stimulate catecholamine release by tumor cells. Moreover, culture experiments showed that metoclopramide potently stimulated the production of two granin-derived peptide release, i.e. EM66 and WE14, that have previously been detected in pheochromocytoma tissues (Guillemot et al. 2006; Montero-Hadjadje et al. 2002; Yon et al. 2003).

The observation that metoclopramide-induced catecholamine and granin-derived peptide productions were reduced by the specific 5-HT_{4} antagonist GR 113808 indicates that the stimulatory effect of metoclopramide on pheochromocytes is at least partly mediated by 5-HT_{4} receptors. The presence of functional 5-HT_{4} receptors in the patient's pheochromocytoma tissue was confirmed by the following results: cisapride, another 5-HT_{4} receptor agonist devoid of any anti-dopaminergic properties, activated catecholamine and granin-derived peptide secretion; the stimulatory effect of cisapride was completely inhibited by GR 113808. As D_{2} receptors are expressed in pheochromocytoma tissues (Wu et al. 2001), it was conceivable that metoclopramide may have also activated tumor cells through its antagonistic D_{2} receptor properties. In fact, it is well documented that metoclopramide stimulates prolactin secretion by blocking the inhibitory action of dopamine on pituitary lactotrophs (Molitch 1992). In agreement with this hypothesis, we noticed that dopamine potently decreased norepinephrine and epinephrine release, as previously reported in sympathetic nerve terminals and adrenal medulla (Mannelli et al. 1995). However, metoclopramide had no action on dopamine-evoked inhibition of catecholamine release indicating that metoclopramide does not stimulate catecholamine production by antagonizing the effect of dopamine on pheochromocytes. These results also suggest that the inhibitory action of dopamine on norepinephrine and epinephrine secretion is not mediated by D_{2} receptors. As the presence of D_{4} receptor in pheochromocytomas has been clearly established (Wu et al. 2001), it is possible that the dopaminergic regulation of catecholamine production by pheochromocytoma cells may involve this dopamine receptor type for which metoclopramide exhibits a low affinity (Rizzi et al. 1997). The observation that metoclopramide and dopamine effects on catecholamine release were independent of the D_{2} receptor
was also consistent with the lack of significant action of domperidone, a well known D₂ receptor antagonist devoid of any serotonin antagonistic properties, on norepinephrine and epinephrine secretions by the patient's tumor cells. In contrast to its inhibitory effect on norepinephrine and epinephrine release, dopamine acted as a potent stimulator of granin-derived peptides production indicating that metoclopramide could not activate granin secretion through its dopamine antagonistic properties. These data also show that dopamine can differentially regulate catecholamine and granin-derived peptide productions from pheochromocytoma cells. The processes involved in these complex regulations likely involve selective release of secretory granule contents through kiss-and-run and/or piecemeal degranulation (Crivellato et al. 2006).

The occurrence of functional 5-HT₄ receptors in the patient’s pheochromocytoma led us to investigate quantitative and qualitative expressions of 5-HT₄ receptor mRNAs in two normal adrenomedullary tissues, and benign and malignant pheochromocytomas. Using primers hybridizing to the common region of all 5-HT₄ receptor isoforms, we observed the presence of 5-HT₄ receptor mRNAs in all tissues studied, including the patient’s tumor which was one of the tissues that expressed the highest levels of the receptor. There were no statistically significant differences between the expression levels of benign vs malignant pheochromocytomas. The fact that normal adrenomedullary explants expressed 5-HT₄ receptor mRNA at levels similar to those observed in pheochromocytoma tissues was unexpected with regards to the observation that the potency of metoclopramide to stimulate catecholamine secretion *in vivo* and to elevate blood pressure is much higher in pheochromocytoma than in non-pheochromocytoma patients (Hsu et al. 1993). This apparent discrepancy may be explained by differences in the functional properties of 5-HT₄ receptors respectively expressed by the normal adrenal medulla and pheochromocytoma tissues. Indeed, although real-time RT-PCR experiments using primers specific to the common region of all 5-HT₄ receptor variants had shown that the normal adrenal medulla actually expresses the receptor, no specific cDNA band could be detected after RT-PCR amplification of mRNAs encoding the currently known isoforms of the 5-HT₄ receptor in medulla extracts. These results show that adrenomedullary cells express 5-HT₄ receptor isoforms different from the already recognized splicing variants. It is conceivable that these unidentified isoforms may be less efficiently activated by metoclopramide than
the variants that were detected in pheochromocytomas explants. In addition, recent studies have shown that 5-HT$_4$ receptors, like other G-protein-coupled receptors, can form homo- and/or heterodimers which modulate receptor function (Berthouze et al. 2005). It is thus possible that differences in the expression profiles of 5-HT$_4$ receptor splice variants among tissues could lead to a weak responsiveness to metoclopramide in normal adrenal medulla in comparison with pheochromocytomas. Conversely, we cannot exclude that metoclopramide stimulates catecholamine secretion from the normal adrenal medulla as efficiently as it does in pheochromocytoma tissues but the low mass of catecholamine-secreting tissue in non-pheochromocytoma patients prevents them from experiencing metoclopramide-evoked hypertension crisis.

The different patterns of expression of 5-HT$_4$ receptor isoforms observed respectively in the normal adrenal medulla and pheochromocytoma tissues also suggest that maturation of 5-HT$_4$ receptor primary transcripts is altered in adrenomedullary tumors in comparison with normal adrenal chromaffin tissue. Nevertheless, processing of the 5-HT$_4$ receptor mRNA does not seem to depend on the degree of differentiation of tumors since expression profiles of 5-HT$_4$ receptor isoforms were not significantly different in benign vs malignant tumors.

Pheochromocytoma crisis is a rare life-threatening emergency, with a high mortality rate, i.e. reaching 85% in some studies (Brouwers et al. 2003). Preventing paroxysmal crises and their potentially life-threatening consequences, such as acute heart failure and seizures, is a major goal of the pre-operative management of patients with pheochromocytoma. On the other hand, it is well known that hypertensive episodes may be precipitated by various drugs that potentiate the action of catecholamines or directly stimulate pheochromocytoma tissues and are therefore contraindicated in patients with pheochromocytoma (Keiser 2001; Eisenhofer et al. 2007). However, drug-induced paroxysms is often unpredictable with new compounds whose action on adrenomedullary cells is generally unknown. In this respect, several new potent 5-HT$_4$ receptor agonists, like tegaserod, mosapride and prucalopride, are currently developed or have already been commercialized in several countries as digestive tract prokinetic agents. Since decreased gastrointestinal motility is one of the non specific features of pheochromocytoma (Keiser 2001), some patients with unrecognized pheochromocytoma may potentially be treated with these compounds. Although 5-HT$_4$ receptor
agonist-evoked hypertension crisis has not yet been reported, the present study, which shows that
adrenomedullary tumors express functional 5-HT$_4$ receptors, suggests that administration of these
prokinetic agents to pheochromocytoma patients would lead to a high risk of paroxysmal crisis. This
assumption is supported by the observation that the expression rates of the 5-HT$_4$ receptor in
pheochromocytoma tissues were similar to or higher than those previously noticed in the
gastrointestinal tract and adrenal cortex, two organs that are well known to respond in vivo to the
stimulatory action of 5-HT$_4$ receptor agonists (Cartier et al. 2005; van Leyveld et al. 2007). It seems
therefore reasonable to recommend to systematically eliminate the diagnosis of pheochromocytoma by
use of urinary or plasma metanephrine measurements before prescribing 5-HT$_4$ receptor agonists when
decreased gastrointestinal motility is associated with hypertension and/or signs of catecholamine
excess.

In summary, the present in vitro study shows that metoclopramide stimulates catecholamine and
granin-derived peptide secretions from pheochromocytoma tissue by activating serotonin 5-HT$_4$
receptors rather than antagonizing D$_2$ receptors. Our data also suggest that all 5-HT$_4$ receptor agonists,
including the newly developed compounds tegaserod, mosapride and prucalopride, might better be
avoided in patients with proven or suspected pheochromocytoma.

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Declaration of interest
All authors have nothing to disclose.
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**Legends to Figures**

**Figure 1** Effect of metoclopramide on norepinephrine (NE, Panel A), epinephrine (E, Panel B), EM66 (Panel C), and WE14 (Panel D) production from pheochromocytoma cells. Graded doses of metoclopramide ($10^{-9}$ M to $10^{-5}$ M) were incubated with cultured pheochromocytoma cells in the absence (□) or presence (▲) of the 5-HT$_4$ receptor antagonist GR 113808 ($10^{-7}$ M). Each point represents the mean of at least 3 determinations, expressed as percentages of basal level (control). Bars denote SEM. *$P<0.05$ and **$P<0.01$ vs. control; #*$P<0.05$ and ##*$P<0.01$ vs. metoclopramide alone.

**Figure 2** Effects of the 5-HT$_4$ receptor agonist cisapride and the dopamine D$_2$ receptor agonist domperidone on norepinephrine (NE), epinephrine (E), EM66, and WE14 production from pheochromocytoma cells. Cisapride ($10^{-7}$ M) was incubated with cultured pheochromocytoma cells in the absence (black bar) or presence (dark grey bar) of GR 113808 ($10^{-7}$ M). Domperidone was incubated with pheochromocytoma cells at the concentration of $10^{-6}$ M (light grey bar). Each point represents the mean of at least 3 determinations, expressed as percentages of basal level (control, open
bar). Bars denote SEM. \( *P<0.05, **P<0.01, \) and \( ***P<0.001 \) vs. control; \( \#P<0.05, \#\#P<0.01 \) and \( \###P<0.001 \) vs. cisapride alone.

**Figure 3** Effect of dopamine on norepinephrine (NE, Panel A), epinephrine (E, Panel B), EM66 (Panel C), and WE14 (Panel D) production from pheochromocytoma cells. Graded concentrations of dopamine (\( 10^{-9} \) M to \( 10^{-6} \) M) were incubated with cultured pheochromocytoma cells in the absence (■) or presence (▲) of metoclopramide (\( 10^{-6} \) M). Each point represents the mean of at least 3 determinations, expressed as percentages of basal level (control). Bars denote SEM. \( *P<0.05, \) \( **P<0.01 \) and \( ***P<0.001 \) vs. control.

**Figure 4** Expression of 5-HT\(_4\) receptor mRNA in pheochromocytoma tissues. 5-HT\(_4\) receptor mRNA level was quantified in normal adrenal medulla (■), the patient’s pheochromocytoma (●), benign (▲) and malignant (▼) pheochromocytomas by use of real-time RT-PCR. The horizontal bars represent the median values observed in the groups of benign and malignant pheochromocytomas. The level of expression in each sample was normalized by dividing copies/ng total RNA of 5-HT\(_4\) receptor gene by copies/ng total RNA of porphobilinogen deaminase gene, and expressed as a percentage.
Table 1. Expression of 5-HT$_4$ receptor isoforms in normal adrenal medulla and pheochromocytomas.

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N1-2, normal adrenal medulla; PP, patient’s pheochromocytoma; 1-9, benign pheochromocytomas; 10-17, malignant pheochromocytomas; +, expression; -, no detectable expression.
Effect of metoclopramide on norepinephrine (NE, Panel A), epinephrine (E, Panel B), EM66 (Panel C), and WE14 (Panel D) production from pheochromocytoma cells. Graded doses of metoclopramide (10^{-9} M to 10^{-5} M) were incubated with cultured pheochromocytoma cells in the absence (■) or presence (? of the 5-HT4 receptor antagonist GR 113808 (10^{-7} M). Each point represents the mean of at least 3 determinations, expressed as percentages of basal level (control). Bars denote SEM. *P<0.05 and **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. metoclopramide alone.
Effects of the 5-HT4 receptor agonist cisapride and the dopamine D2 receptor agonist domperidone on norepinephrine (NE), epinephrine (E), EM66, and WE14 production from pheochromocytoma cells. Cisapride (10^{-7} M) was incubated with cultured pheochromocytoma cells in the absence (black bar) or presence (dark grey bar) of GR 113808 (10^{-7} M). Domperidone was incubated with pheochromocytoma cells at the concentration of 10^{-6} M (light grey bar). Each point represents the mean of at least 3 determinations, expressed as percentages of basal level (control, open bar). Bars denote SEM. *P<0.05, **P<0.01, and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. cisapride alone.
Effect of dopamine on norepinephrine (NE, Panel A), epinephrine (E, Panel B), EM66 (Panel C), and WE14 (Panel D) production from pheochromocytoma cells. Graded concentrations of dopamine (10⁻⁹ M to 10⁻⁶ M) were incubated with cultured pheochromocytoma cells in the absence (■) or presence (?) of metoclopramide (10⁻⁶ M). Each point represents the mean of at least 3 determinations, expressed as percentages of basal level (control). Bars denote SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. control.
Expression of 5-HT4 receptor mRNA in pheochromocytoma tissues. 5-HT4 receptor mRNA level was quantified in normal adrenal medulla (n), the patient’s pheochromocytoma (?), benign (?) and malignant (?) pheochromocytomas by use of real-time RT-PCR. The horizontal bars represent the median values observed in the groups of benign and malignant pheochromocytomas. The level of expression in each sample was normalized by dividing copies/ng total RNA of 5-HT4 receptor gene by copies/ng total RNA of porphobilinogen deaminase gene, and expressed as a percentage.