Effects of somatostatin analog SOM230 on cell proliferation, apoptosis, and catecholamine levels in cultured pheochromocytoma cells

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Abstract

Surgery is the primary therapy for pheochromocytoma (PHEO), a catecholamine-producing tumor. Benign and malignant PHEO could develop recurrences, and the intraoperative risk of recurrent PHEO is an important unresolved issue. Non-surgical treatments of PHEO recurrence would therefore better prepare patients for reintervention as well as provide them with palliative management. We investigated the effects of the new somatostatin analog (pasireotide) SOM230 versus octreotide (OCT) in primary PHEO cell cultures (Pheo-c). Pheo-c from six benign surgical samples were set up and characterized by immunocytochemistry. Real-time PCR, using both PHEO tissues and Pheo-c, showed different levels of somatostatin receptor1–5 mRNA expression. Cells treated with various doses of OCT or SOM230 for 48 and 72 h were analyzed to assess their effects on cell proliferation and apoptosis and catecholamine levels. Even if reduction of cell viability was observed in Pheo-c treated for 48 h with either OCT or SOM230 and this effect increased after 72 h, a more significant inhibition of cell growth as well as a significantly higher induction of apoptosis was seen in Pheo-c treated with SOM230 versus OCT. In particular, apoptosis in Pheo-c was detected after 48 h and was associated with increased expression and activation of caspase-3 and cleaved poly(ADP-ribose) polymerase. OCT 10⁻⁶ M and SOM230 10⁻⁷ M significantly reduced catecholamine levels. Our results indicate that while both OCT and SOM230 modulate cell growth and apoptosis and catecholamine levels in Pheo-c through specific receptors, SOM230 is more effective. This improves our knowledge on the mechanism of SOM230 action in PHEO and supports a possible therapeutic use in benign PHEO recurrence.

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Introduction

Pheochromocytoma (PHEO) is a catecholamine-producing and -secreting tumor derived from neuroectoderm arising from the adrenal medulla or extra-adrenal neural crest. PHEO, which has an annual incidence of 2–8 per million population (Pacak et al. 2007, Scholz et al. 2007), is generally treated surgically. Both benign and malignant PHEO are at the risk of recurrence, and patients presenting with recurrent disease and repeated interventions are at high risk of perioperative complications (Plouin et al. 2001, Mannelli 2006, Chrisoulidou et al. 2007, Pacak et al. 2007, Scholz et al. 2007). There is no generally effective systemic treatment for PHEO recurrence. Although a considerable proportion of the patients respond to (131)I-meta-iodobenzylguanidine ((131)I-MIBG) radiotherapy or cytotoxic chemotherapy (Chrisoulidou et al. 2007, Pacak et al. 2007), novel agents and approaches are greatly needed to increase the efficacy of current treatment options to better prepare patients for reintervention or to provide them with palliative management. Somatostatin (SS), which acts on both endocrine and non-endocrine tissues, is a key regulator of the release of various hormones and growth factors (Patel 1999). SS exerts its effects by binding to all five SS receptor (sst) subtypes, sst₁–₅. SS analogs have been used in the detection and treatment of different neuroendocrine tumors. For example, octreotide and lanreotide are somatostatin-like oligopeptides that are widely employed in the therapy of somatotropin-secreting pituitary adenomas and neuroendocrine tumors of the gastroenteropancreatic system. In radio-labeled forms (usually 111In-pentetreotide), these analogs are also useful for somatostatin receptor imaging (SRI) (Kaltsas et al. 2001, Chrisoulidou et al. 2007); however, they bind with high affinity to only sst₂
and sst₂ subtypes (Patel 1999). Although it has been shown that PHEO tissues express more than one sst receptor (Kubota et al. 1994, Mundtschenk et al. 2003, Unger et al. 2004, Kolby et al. 2006), only a small amount of conflicting data on the role of OCT for PHEO tumor treatment exists (Invitti et al. 1993, Plouin et al. 1995, Tenenbaum et al. 1995, Kopf et al. 1997, Koriyama et al. 2000, Lamarre-Cliche et al. 2002, Zatelli et al. 2003), and knowledge of the mechanistic action of SS analogs on PHEO remains incomplete. The poor effectiveness of OCT, for example, might be due to only one (sst₂) of the several sst subtypes expressed in PHEO tissues binding with high affinity to standard somatostatin analogs. Furthermore, immunohistochemical staining has shown that greater than 60% of PHEO cells express sst₃ (Unger et al. 2004). Thus, these tumors might represent a potential target for new synthetic analogs active on sst₁–₃ and sst₅, such as SOM230 (Weckbecker et al. 2002). Whether a subgroup of SRI-positive patients will benefit from new somatostatin analogs with an improved affinity for sst₃, such as SOM230, remains to be elucidated. The purpose of this study was to define sst expression in both tissues and cells using an in vitro system of primary cultured PHEO cells derived from surgically removed PHEO tissue of patients affected by benign PHEO, and to investigate the effects of OCT and SOM230 treatment on cell growth control and apoptosis.

Materials and methods

Cell culture

Tissue explants were obtained from six patients (aged 25–57 years) after the achievement of presurgical diagnosis of sporadic benign PHEOs by clinical, hormonal, and morpho-functional evaluation. Patients who underwent surgical removal of the adrenal mass gave consent for surgery and for genetic and research studies on tissue specimens. Tissue samples were minced and placed in the lid of a small culture dish with 3 ml dissociation solution: 2.6 mg/ml collagenase type IV, 3 mg/ml BSA, 0.15 mg/ml DNase I, and 0.15 mg/ml hyaluronidase I-S in Hanks’ Balanced Salt Solution (HBSS) at room temperature. Medulla chunks and the solution were recovered and transferred into a Falcon tube using a glass Pasteur pipette. The tube was placed in a thermostatic bath at 37 °C for 30 min. The solution was gently resuspended every 10 min with a Pasteur pipette. Over the last 5 min, the solution was continuously resuspended until the medullary tissue became not visible by eye, then stopped to avoid damage of the cells. To stop the enzymatic reaction, cold HBSS was added. The tube containing the digested tissue was placed in the rotor of a pre-cooled (4 °C) refrigerated centrifuge and spun at 313 g for 10 min. The pellet was resuspended in pre-warmed Nutrient Mixture F12 Ham/-Dulbecco’s modified essential medium (dMEM) (vol/vol) supplemented with 1-glutamine, 10% fetal bovine serum (FBS), and antibiotics (GibcoBrl, Life Technologies). To assess the quality of chromaffin cells cultures, the cells were stained with trypan blue solution (0-2%) and counted in a hemocytometer chamber to estimate both the yield and the cellular viability. The purity in chromaffin cells of our cultures was estimated by staining the cells with neutral red, a dye that selectively reacts with monoamine-containing cells. Pheo-c was used for experimental protocols at the first and second culture passages, and the experiments were repeated at least thrice.

Immunocytochemistry of Pheo-c

Cells were grown on four-chamber tissue-cultured glass slides (Falcon Becton Dickinson, Labware, NJ, USA) pre-coated with poly-L-lysine to enhance cell attachment at a density of 5000 cells per well. Cultured cells were rinsed with PBS, and finally fixed in alcohol.

Immunostaining was performed using the linked streptavidin–biotin horseradish peroxidase technique (LSAB-HRP). Briefly, after hydration, the slides were treated with 0.3% H₂O₂ for 15 min to quench endogenous peroxidase. Antigen retrieval was performed using microwave heating of the slides immersed in 10 mM citrate buffer (pH 6), firstly for 3 min at 650 W, and a second and third time for 3 min at 350 W. The slides were then blocked for 60 min with 1-5% bovine serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS buffer before incubating with primary antibody (Ab). Mouse monoclonal Ab against chromogranin A (AB356M – Biogenex, San Ramon, CA, USA) and mouse monoclonal Ab against Neuron Specific Enolase (Dako, Dako, Denmark) were diluted 1:100 with 0.05 M Tris–HCl buffer (pH 7.4) containing 1% BSA and were incubated with samples for 120 min at 24 °C. Negative control slides without primary antibody were included for each staining condition. After two washes with PBS, the slides were treated with biotinylated species-specific secondary antibodies and streptavidin horseradish peroxidase reagent (LSAB-HRP, Dako, Denmark), which was followed by color development in 3,3’-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer’s hematoxylin, and mounted using xylene-based mounting medium. Evaluations of the immunocytochemical staining were performed separately by two observers with at least 10 high power fields using optical microscopy.
Real-time quantitative RT-PCR

Total RNA was isolated from tissues and primary cell cultures at early passages (first to second) using TRIZOL (Invitrogen). Before RNA extraction, we washed tissues twice in PBS and macroscopic fibrosis were cut off to avoid contamination by blood cells and to reduce stromal cells. Residual DNA was removed by RNase-free DNase I treatment (Promega). RNAs were reverse transcribed with 5 μg total RNA and reverse transcriptase (Superscript-BRL-200 U) as previously described (Sinisi et al. 1997, Pasquali et al. 2000, 2004). We include a total RNA sample that was not reversed transcribed and a no-cDNA control to monitor for genomic DNA contamination or exogenous contamination respectively. The expression of ssts in Pheo-c and PHEO was quantified using the SYBR green reagent (2× SYBR Green Supermix, Bio-Rad Laboratories) following the manufacturer’s instructions on a Bio-Rad iCycler. PCR was performed in multiplicate under optimized conditions: 95°C denaturation for 3 min, followed by 40 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C using the following primers: sst1 (5’-tgatgctcgtcggctcatc-3’ (sense) and 5’-tgagtcagctgtcggtcatc-3’ (antisense)); sst2 (5’-ctttgtggtggtcctcacct-3’ (sense) and 5’-gcagagcacattgtggaagc-3’ (antisense)); sst3 (5’-taggccctactcc-tcaagg-3’ (sense) and 5’-tggccgacatatgcatcaacag-3’ (antisense)); sst4 (5’-egctaggagaagaatac-3’ (sense) and 5’-ctccgacagagcacaaga-3’ (antisense)); sst5 (5’-cggccgagcttcatccactca-3’ (sense) and 5’-agcaggtcaggcagcatg-3’ (antisense)). In these experiments, the amount of specific PCR products from each gene was measured over 40 PCR cycles. The cycle number was recorded when the signals crossed a threshold set to an internal control. The following primers used were: sense, 5’-ccacagagaaatggtcgc-3’ and antisense, 5’-gatgctc gtgctgctcgc-3’. No other products were amplified because melting curves showed only one peak in each primer pair and only one specific product was observed on agarose ethidium bromide gel for each primer pair. The identity of PCR products was confirmed by comparing the size of the product with the size expected from the gene sequence. Fluorescence signals were measured over 40 PCR cycles. The cycle number was recorded when the signals crossed a threshold set within the logarithmic phase. Data were expressed as the amount of specific PCR products from each gene in cells and in tissues, and were normalized based on the housekeeping gene product β2-microglobulin (which showed no significant differences between samples). The efficiency of amplification of each pair of primers was determined by serial dilutions of cDNA templates and all were larger than 0.9. Experiments were repeated at least thrice and represented as folds of expression.

Cell proliferation assay

Cell proliferation was evaluated with the tetrazolium salt (MTT) method as previously described (Pasquali et al. 2000). The MTT Cell Proliferation Assay is a quantitative colorimetric method used to determine cell proliferation. It utilizes the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium-bromide), which is metabolized by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple formazan reaction product. The MTT assay is an alternative to the thymidine incorporation test, which measures cell proliferation by determining the amounts of incorporated tritiated thymidine into freshly synthesized DNA. Conversely, when metabolic events lead to apoptosis or necrosis, MTT measures the reduction in cell viability. Briefly, cells were plated in 96-well plates at a density of 2000 cells per well, starved for 24 h in MEM without FBS, then they were incubated in 1% FBS-supplemented medium with OCT or SOM230 at 10⁻⁶ M, 10⁻⁷ M, and 10⁻⁸ M or solvent (control cells) for 48 and 72 h, and then processed according to the manufacturer’s instructions. Absorbance was read at 550 nm using a microtitre plate reader.

Apoptosis detection

From among the many ways of detecting apoptosis at different stages, we used the most common method called TUNEL (terminal deoxynucleotidyl transferase biotin d-UTP nick end labelling), which identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to strand breaks of cleaved DNA after the activation of Ca/Mg-dependent endonucleases in individual apoptotic cells. Cells were plated at 2×10⁶ cells/100 mm Petri dish. The cell monolayers were grown directly on sterilized slides (Superfrost; Carlo Erba, Milan, Italy), starved for 24 h in dMEM without FBS, and then incubated in 1% FBS-supplemented medium with OCT or SOM230 at 10⁻⁷ M, or solvent (control cells) for 48–72 h. Using the in situ Cell Death Detection Kit (La Roche, Italy), samples were processed and analyzed as described previously (Pasquali et al. 2000).

Caspase-3 activity

Caspase-3 enzymatic activity was measured using ApoONE Homogenous Caspase-3 Assay (Promega). Briefly, Pheo cells were seeded in 96-well plates (50 000 cells/well) and treated with or without octreotide, and SOM230 (at 10⁻⁸, 10⁻⁷, 10⁻⁶ M) for 48 h at 37°C, in DMEM (Sigma Chemical) supplemented with 10% FCS. The timing of treatment was determined on the basis of preliminary experiments carried out at different time intervals showing the maximal stimulation at 48 h. Experiments were repeated at least twice and each determination was done in quintuple.
Electrophoresis and western blot analysis

Lysates of Pheo-c treated with $10^{-7}$ M OCT or SOM230 for 48 h were subjected to gel electrophoresis followed by immunoblotting using polyclonal antibody anti-caspase-3 (H-277), and mouse monoclonal IgG 200 μg/ml anti-poly ADP-ribose polymerase (PARP)-1 (F-2) (Santa Cruz Biotechnology, Inc.) at working concentrations of 1:200 and 1:500 respectively. Western blot analysis was done as reported previously (Pasquali et al. 2000).

Determination of intracellular catecholamine levels

Catecholamine levels were determined in medium collected from cells, starved for 24 h in dMEM without FBS, and in the absence (control) or presence of OCT and SOM230 $10^{-8}$ M, $10^{-7}$ M, $10^{-6}$ M for 24, by high-pressure liquid chromatography (HPLC) with electrochemical detection. A 12.5 cm long, 5 μm C-18 reverse phase column (Waters Chromatography Division, USA) was connected to a carbon electrode set at a potential of +0.75 V relative to the Ag/AgCl reference electrode, in turn connected to an LC-4 amperometric detector (Bioanalytic System Inc., West Lafayette, IN, USA) in the HPLC system. The detection limit for catecholamines, defined as a peak height to noise ratio greater than 2, was 40 pg. The mobile phase consisted of 1.75 g heptanesulfonic acid, 0.1 g disodium EDTA, 3.5 ml triethylamine, 4 ml phosphoric acid, and 40 ml acetonitrile made up to 1 l in distilled water and was filtered and degassed just prior to use. External standards of dopamine and norepinephrine were dissolved in 0.1 M perchloric acid and run simultaneously with each experiment. The protein content of the tissue pellets solubilized in 0.5 M NaOH was assayed by the method of Lowry.

Statistical analysis

The data were reported as mean ± S.E.M. from at least three separate experiments performed in triplicate. The means were compared using ANOVA. Differences in expression of sst1–5 in PHEO tissues or Pheo cells evaluated by real-time PCR were determined using one-way ANOVA followed by a Neuman–Keuls post hoc analysis.

Results

Immunocytochemical identification of PHEO cells in primary culture

Ultrastructural analysis of PHEO shows that the cells contain numerous neurosecretory-type dense-core granules containing norepinephrine and epinephrine. Immunohistochemical analyses of PHEO tissues always show reactivity for chromogranin, neuron-specific enolase, as well as catecholamines, catecholamine-synthesizing enzymes, synaptophysin, and opioid peptides. We therefore used specific monoclonal antibodies designed for the immuno-localization of chromogranin A and neuron-specific enolase (NSE). PHEO cells in primary culture (Pheo-c), which were processed for immunocytochemical analysis, stained positively in a granular pattern with the antibody against chromogranin A (Fig. 1A), and NSE (Fig. 1B). Morphological assessments by pathologists and the strong immunoreactivities of the specific neuroendocrine markers have convinced us that the cells cultured are selected chromaffin cells, and we thus excluded the possibility of contaminating material.

Figure 1 Strong cytoplasmic immunoreactivity for (A) chromogranin A and (B) neuron-specific enolase in cultured PHEO cells. These cells show nuclear pleomorphism and hyperchromatism, and mimic neuronal and ganglion cells with cytoplasmic extensions similar to neuritic (¶) and dendritic (asterisk) processes. The immunoreaction with the specific antibodies, and the morphological characteristics demonstrate the nature of pheochromocytoma cells (LSAB-HRP, original magnification (A) × 100 and (B) × 400; nuclear counterstaining with hematoxylin; for further details see Materials and methods).
PHEO tissue and primary cultured cell expression of sst genes

In order to further characterize our various tissue samples and the in vitro cell system used, we studied the expression pattern of sst, which would be useful for explaining potential differences of SOM230 and OCT action on Pheo-c. Transcripts of the ssts family were differentially expressed in PHEO tissues and Pheo-c cells. In all tissues and cultures studied, the sst2 and sst5 mRNAs were found (Table 1). However, sst1, sst3, and sst4 were absent in PHEO1, PHEO3, and PHEO6 respectively and their corresponding cultured cells, demonstrating the heterogeneity which can exist in these neuroendocrine cells (Table 1). Furthermore, real-time PCR analysis using PHEO tissue samples and Pheo-c cultures showed that sst2 was the dominant receptor expressed in these samples (Fig. 2).

In vitro effects of octreotide and SOM230 on cell viability

Cells were seeded at 2000 cells per well, and treated with 10^{-8} M, 10^{-7} M, and 10^{-6} M OCT or SOM230 for 48 and 72 h. Pheo-c cell growth was inhibited after 48-h treatment with 10^{-7} and 10^{-6} M SOM230 (P<0.05 and P<0.001 respectively), while only a significant reduction in cell viability was seen using 10^{-6} M OCT (Fig. 3A). This effect was even greater after 72-h exposure to either compound. As shown in Fig. 3B, OCT and SOM230 at 10^{-7} M increased the TUNEL-positive process in cultured cells after 48-h treatment (8% and 16%, respectively). Induction of the apoptotic-related event was significantly higher in Pheo-c treated with SOM230 versus OCT (P<0.01).

Caspase-3 and poly (ADP-ribose) polymerase levels

We studied the effect of OCT and SOM230 on the cellular apoptotic protein markers caspase-3 and proteolytic cleavage products of PARP-1. Western blot analysis of cells that were treated with 10^{-7} M OCT or SOM230 for 48 h revealed a significant increase in the expression of the 32 kDa caspase-3 protein (Fig. 4A) and activity (Fig. 5). In cultured cells obtained from all PHEO tissues, OCT and SOM230 induced a dose-dependent increase in caspase-3 activity that was detected at concentrations higher than 10^{-8} M (data not shown) and maximal at 10^{-7} M (178±20% and 180±20% respectively versus basal) (Fig. 5). PARP-1 is a nuclear enzyme involved in DNA repair and is specifically digested into 89 and 24 kDa fragments by caspase-3 during the apoptotic process (Soldani & Scovassi 2002). After treatment of Pheo-c with 10^{-7} M OCT or SOM230 for 48 h, an increase was seen in 113 kDa PARP-1 protein compared with control cells, and in addition, 89 kDa fragment was present (Fig. 4B).

Table 1

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PHEO 1, 2, 3, 4, 5, and 6, pheochromocytoma tissues; Pheo-c 1, 2, 3, 4, 5, and 6, pheochromocytoma cell cultures. +, mRNA present; -, mRNA absent.
Thus, the apparent enzymatic digestion of PARP-1 shown here is a further evidence for the caspase-dependent apoptotic induction by these compounds.

**Effects of octreotide and SOM230 on intracellular catecholamine levels**

PHEO cells were treated with octreotide and SOM230 at different doses for 24 h to examine the intracellular catecholamine levels by HPLC. As shown in Table 2, $10^{-8}$ M octreotide and SOM230 had no effect on intracellular catecholamine levels. At $10^{-7}$ M and over, SOM230 significantly inhibited the intracellular dopamine and norepinephrine levels ($P<0.05$ and $P<0.01$ respectively). Octreotide induced a significant reduction of catecholamine levels only at the higher doses.

**Discussion**

In order to gain further mechanistic insights into the responses of PHEO to compounds which could interact via somatostatin receptors (sst), this study was aimed at...
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van der Harst et al. 2001, Scholz et al. 2007). For endocrine tumors, somatostatin (SS) and its receptors are classical tools for diagnosis, long-term treatment with no radioactive analogs, and for radiotherapy using, for example, 90YDOTA-d-Phe-Tyr-octreotide (Bruns et al. 1996). Pharmacological therapy with SS analogs has dramatically improved symptom control, proving helpful to stabilizing neuroendocrine tumor progression by inhibiting specific growth factors such as IGF-I and angiogenesis (Patel 1999). SS binds to the five known SS receptor subtypes, sst<sub>1</sub>, sst<sub>5</sub>, with high affinity, whereas the SS analogs used clinically (OCT and lanreotide), bind preferentially and with high affinity to only sst<sub>2</sub> (Patel 1999, Hannon et al. 2002). Recently, SOM230, a new synthetic SS analog, has been shown to bind four out of five sst receptors, with high affinity for sst<sub>1</sub>, sst<sub>2</sub>, and in particular for sst<sub>5</sub>, which is 40 times greater than OCT, and even improved over SS. This peculiar capacity of SOM230 may be crucial for its inhibitory effects (Weckbecker et al. 2002). Our results indicate that both PHEO tissues and Pheo-c express more than one of the genes coding for known SS receptor subtypes with high (sst<sub>2</sub>) or intermediate (sst<sub>5</sub>) affinity for the analog OCT. Moreover, we also detected mRNA for sst<sub>1</sub>, which has high affinity for SOM230, and is not always found in PHEO (Unger et al. 2004). Interestingly, sst<sub>3</sub> mRNA, which is reported to be very frequently expressed in PHEO (Zatelli et al. 2003, Unger et al. 2004), was absent in PHEO 1 and its cell culture Pheo-c 1. The observations of both inhibition of cell growth and programmed cell death induction suggest that intracellular signal transduction pathways, activated by SS analog interactions with sst<sub>2</sub> and sst<sub>5</sub>, are functional in Pheo-c. Different cell death mechanisms control many physiological and pathological processes in humans. Caspases are a family of cysteine-aspartate proteases that are synthesized as inactive proenzymes. Activated caspase-3 contributes to apoptosis by cleaving substrate proteins (Hong et al. 2004). Poly(ADP-ribose)polymerase-1 (PARP-1)-mediated release of apoptosis-inducing factor appears to be an important, recently identified, cell death program. We found increased levels of caspase-3 and PARP-1 protein expression after 48-h treatment with either 10<sup>-6</sup> M OCT or SOM230, showing that SS analogs can induce the activation of both apoptotic pathways. Moreover, the duration and the efficacy of SOM230 on cell growth inhibition, apoptotic induction, and reduction of catecholamine levels were markedly stronger than the effects elicited by OCT. SOM230 at 10<sup>-7</sup> M was able to inhibit Pheo-c cell growth significantly after 48-h treatment, and by even greater levels after 72 h. This short term as well as the chronic efficacy of SOM230 is probably due to both its improved pharmacodynamic and pharmacokinetic properties.

In conclusion, our results confirm the remarkable heterogeneity of sst expression in neuroendocrine cells. They also demonstrate that OCT and SOM230 are both capable of reducing cell viability and inducing programmed cell death via caspase-3 and PARP-1 apoptotic pathways; however, SOM230 appears to be more effective than OCT. Data in this study add further to the mechanistic insights of SOM230 in PHEO, suggesting a potential role in the diagnostic procedures (as radiolabeled form) and management of the recurrence of these tumors especially in patients at high risk for reintervention.

Acknowledgements

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References

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Table 2 Effects of octreotide (OCT) and SOM230 on intracellular catecholamines content

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Significantly different from control: *P<0.01, †P<0.05.