Universidade Federal do Rio Grande do Sul

Instituto de Biociências

THYROID HORMONE UPREGULATES ECTO-5'-NUCLEOTIDASE/CD73 IN C6 GLIOMA CELLS

Alessandra Sayuri Kikuchi Tamajusuku

Orientadora: Ana Maria Oliveira Batasttini

Trabalho apresentado como um dos requisitos para obtenção do grau de Bacharel no Curso de Ciências Biológicas Ênfase Molecular

Porto Alegre, outubro de 2002.

INTRODUÇÃO

Os hormônios produzidos na tireóide, tanto a tiroxina (T_4) quanto o triiodotironina (T_3), são conhecidos, classicamente, como reguladores do metabolismo energético. Além desta importante função, estudos de pesquisa básica, através de modelos animais que simulam um estado patológico de hipo e/ou hipertireoidismo, têm demonstrado que a presença dos hormônios tireoideanos, em quantidades fisiologicamente corretas, é necessária para o bom desenvolvimento e funcionamento do Sistema Nervoso Central (SNC). A falta do hormônio durante o desenvolvimento neural causa retardo mental, assim como seu excesso estimula a expressão de proteínas e de fatores mitogênicos (Trentin e Alvarez-Silva, 1998).

Os hormônios T_4 e T_3 são produzidos a partir da tirosina e são secretados na corrente sangüínea complexados a proteínas de transporte: TBG, thyroid binding globulin (Marks *et al.*, 1996). A forma mais ativa e instável do hormônio é o T_3 e, por isso, a que recebe maior enfoque nos estudos. Ao chegar no tecido alvo, o T_4 pode sofrer deiodinação e converter-se em T_3, mas ambos são capazes de atravessar a membrana plasmática (Gonçalves *et al.*, 1990) e ligar-se aos receptores nucleares (Oppenheimer, 1983). Estes receptores contêm um sítio de ligação a cromatina e por isso sua ativação representa um sinal para o início da transcrição de genes. Diversos trabalhos confirmaram ser o hormônio tireoideo o responsável pela super expressão de proteínas em células do sistema nervoso. Portanto, a quantidade de hormônio ligado ao seu receptor torna-se importante para o mecanismo de controle da expressão de várias proteínas.

Os nucleotídeos de purina, ATP, ADP e AMP e o nucleosídeo adenosina (ADO) desempenham funções nas mais variadas situações fisiológicas, tais como agregação plaquetária, vasodilatação e vasoconstrição (Rongen *et al.*, 1997), proliferação de astrócitos (Rathbone *et al.*, 1992) e apoptose (Di Iorio *et al.*, 2002). Também em situações patológicas, como epilepsia (Bruno *et al.*, 2002), estas moléculas ligam-se em seus receptores específicos e sinalizam diferentes respostas. Suas quantidades extracelulares estão sob fino controle de uma cascata de enzimas localizadas na superfície das membranas celulares. Nesta classe de enzimas encontram-se as ecto-nucleotidases (ecto-ATPase; ecto-NTPDase;CD39 e ecto-5´nucleotidase; CD73) (Zimmermann, 2001) que hidrolisam seqüencialmente o ATP até adenosina. A adenosina assim formada pode ser captada pelas células por um sistema de transporte de nucleosídeos (Thorn e Jarvis, 1996), o que se constitui em uma importante via de salvação de purinas ou ainda ser degradada por ação da ecto-adenosina deaminase levando à formação extracelular de inosina (Franco *et al.*, 1997 e 1998).

Importantes diferenças na atividade catalítica dessas enzimas foram encontradas entre linhagens de gliomas e culturas de células astrocíticas normais (Wink *et al.*, 2002a-submetido; Wink et al., 2002b em preparação). Foi sugerido, então, que tais mudanças no comportamento enzimático poderiam ser uma estratégia desenvolvida por este tipo de tumor, a fim de garantir a sobrevivência e a proliferação desta doença. Esta idéia tem o suporte de alguns trabalhos que relacionam a atividade da ecto-5'-nucleotidase com a resistência de tumores a múltiplas drogas (Ujházy *et al.*, 1996).

O glioma C6 de rato é uma linhagem que foi induzida quimicamente a se tornar imortal (Benda *et al.*, 1969). Por causa da fácil manipulação, esta linhagem é freqüentemente estudada. Deste modo, é sabido que nela estão presentes as ecto-nucleotidases e também os receptores de T_3 de alta afinidade (Ortiz-Caro *et al.*, 1986) e que uma vez tratada, com o hormônio em questão, o padrão de expressão de algumas proteínas é modificado (Trentin and Alvarez-Silva, 1998). Além disso, existem evidências de que o hipotireoidismo parece prolongar a vida do paciente com câncer (Toms *et al.*, 1998). Dessa maneira, o objetivo deste trabalho foi a investigação de uma possível influência do estado hipertiroideo na expressão e na atividade catalítica das ecto-nucleotidases.

Para tanto, células de gliomas C6 de ratos em cultura foram tratadas com hormônio T_3 de acordo com o protocolo descrito na literatura (Trentin e Alvarez-Silva, 1998). Os resultados obtidos e apresentados na forma do manuscrito em anexo, demonstraram que a hidrólise do ATP e do ADP que é extremamente baixa nestas células, não sofreu influência do hormônio T_3 nas condições testadas, enquanto que foi observado um aumento significativo de 30% na hidrólise do AMP. Este efeito foi dose-dependente até a concentração de 0,25 nM, quando foi atingido um "plateau". Concentrações maiores de T_3 não causaram aumento no efeito sobre a atividade de hidrólise do AMP. A fim de investigar se o efeito do T_3 sobre a hidrólise do AMP era devido a um aumento na expressão da enzima ecto-5'-NT (CD73) células tratadas com 50 nM de T_3 e células controles tiveram a expressão desta proteína avaliada pela técnica do RT-PCR. Os resultados confirmaram que o aumento na hidrólise do AMP deve provavelmente ser devido a

um aumento na expressão da 5'-NT(CD73) uma vez que houve um aumento de cerca de 3 vezes na expressão do mRNA desta proteína nas células tratadas quando comparadas as células controle. É importante salientar que as análises por RT-PCR foram realizadas no laboratório da Dra. Maria Luisa Chaves do Departamento de Anatomia do Instituto de Ciências Biomédicas da USP.

REFERÊNCIAS DA INTRODUÇÃO:

Benda, P., Lightbody, J., Sato, G., Levine, L., Sweet, W. 1969. Differentiated rat glial cell strain in tissue culture. Science 161(839), 370-371.

Bruno, A.N., Oses, J.P., Bonan, C.D., Walz, R., Battastini, A.M.O., Sarkis, J.J.F. 2002. Increase of nucleotidase activities in rat blood serum after a single injection of pentylenetetrazol. Neurosci. Res. 43, 283-288.

Di Iorio, P., Kleywegt, S., Ciccarelli, R., Traversa, U., Andrew, C.M., Crocker, C.E., Werstiuk, E.S., Rathbone, M.P. 2002. Glia 38, 179-190.

Franco, R., Casadó, V., Ciruela, F., Saura, C., Mallol, J., Canela E.I., Lluis, C. 1997. Cell surface adenosine deaminase: much more than an ecto-enzyme. Prog. Neurobiol. 52, 283-294.

Franco, R., Maloot, J., Casadó, V., Lluis, C., Canela, E.I., Saura, C.A., Blanco, J., Ciruela, F. 1998. Ecto-adenosine deaminase: an ecto-enzyme and a costimulatory protein acting on a variety of cell surface receptors. Drug Dev. Res. 45, 261-268.

Gonçalves, E., Lakshmanan, M., Pontecorvi, A., Robbins, J. 1990. Thyroid hormone transport in human glioma cell line. Mol. Cell Endocrinol. 69,157-165.

Marks, D.B., Marks, A.D., Smith, C.M. 1996. Basic Medical Biochemistry – A clinical approach- Ed. Lippincott Williams &Wilkins, Baltimore, United States.

Oppenheimer, J.H. 1983. The nuclear receptor-triiodothyronine complex: relationship to thyroid hormone distribution, metabolism and biological action. In: Oppenheimer, J.H., Samuels, H.H. (eds) Molecular Basis of Thyroid Hormone Action. Academic Press, New York, pp. 1-34.

Ortiz-Caro, J., Yusta, B., Montiel, F., Villa, A., Aranda, A., Pascual, A. 1986. Identification and characterization of L-triiodothyronine receptors in cells of glial and neuronal origin. Endocrinol. 119, 2163-2167.

Rathbone, M.P., Middlemiss, P.J., Gysbers, J.W., Deforge, S., Costello, P., Del Maestro, R.F. 1992. Purine nucleosides stimulate proliferation of a wide variety of cell types. In Vitro Cell Dev. Biol. 28^{A}, 529-536.

Rongen, G.A., Floras, J.S., Lnders, J.W.M., Thien, T., Smits, P., 1997. Cardiovascular pharmacology of purines. Clin. Sci. 92, 13-24.

Thorn, J.A., Jarvis, S.M. 1996. Adenosine transporters. Gen. Pharmacol. 27 (4), 613-620.

Toms, S.A., Hercbergs, A., Liu, J., Kondo, S., Barnett, G.H., Casey, G., Barna, B.P. 1998. Thyroid hormone depletion inhibits astrocytoma proliferation via a p53-independent induction of p21 (WAF/1CIP1). Anticancer Res. 18, 289-294.

Trentin, A.G., Alvarez-Silva, M., 1998. Thyroid hormone regulates protein expression in C6 glioma cells. Braz. J. Med. Biol. Res. 31, 1281-1284.

Ujházy, P., Berleth, E.S., Pietkiewicz, J.M., Kitano, H., Skaar, J.R., Ehrke, M.J., Mihich, E., 1996. Evidence for the involvement of ecto-5'-nucleotidase (CD73) in drug resistance. Int. J. Cancer 68, 493-500.

Zimmermann, H., 2001. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev. Res. 52, 44-56.

THYROID HORMONE UPREGULATES ECTO-5'-NUCLEOTIDASE/CD73 IN C6 GLIOMA CELLS

M.R. Wink[1], A.S.K. Tamajusuku[1], E. Braganhol[1], M.L.M. Barreto-Chaves[2], J.J.F. Sarkis[1] and A.M.O. Battastini[1]*

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS. Porto Alegre, RS, [2]Departamento de Anatomia, Instituto de Ciências Biomédicas, USP. São Paulo, SP, Brasil.

* Corresponding author: Dr. A.M.O. Battastini

Departamento de Bioquimica – ICBS – UFRGS

Rua Ramiro Barcelos, 2600-anexo

CEP 90035-003, Porto Alegre, RS, BRASIL

FONE: +55 51 3316 5554

FAX +55 51 3316 5535

e-mail: batas@terra.com.br

ABSTRACT

Thyroid hormone has profound effects on growth and development in Central Nervous System (CNS). Previously, it has been shown that triiodothyronine (T_3) promotes glial cells proliferation, induces cerebellar astrocytes and glioma cells to secrete mitogenic growth factors and regulates protein expression. The combined action of ecto-NTPDases and ecto-5'-nucleotidase can control the extracellular ATP/adenosine levels in the CNS. Since ATP and adenosine have been described as proliferation factors, we investigated the influence of T_3 on the enzyme cascade which catalyses interconversion of purine nucleotides in rat C6 glioma cells. Exposure of C6 cells to T_3 for 72h caused a dose-dependent increase of 30% in the AMP hydrolysis while no significant alteration was observed on ATP/ADP hydrolysis. The T_3 treatment significantly increased the expression of CD73 mRNA. These results indicate that T_3 causes an over-expression of 5´-nucleotidase with consequent enhance of adenosine levels in the extracellular medium, which could be an important proliferation signal.

Key words: Ecto-5´-nucleotidase/CD73; adenosine; Thyroid hormone; C6 gliomas.

1. Introduction

Thyroid hormones are essential to controlling fuel metabolism and others physiological functions in many tissues. In the central nervous system (CNS), they play a crucial role in brain development, which is retarded in hypothyroidism state. In adult brain, their role as neurotransmitters comes to the fore but neuronal plasticity and other growth-related processes are their continuing responsibility (Dratman and Gordon, 1996). The thyroid hormone 3-3'-5 triiodo-L-thyronine (T_3) is able to cross plasma membranes (Gonçalves *et al.*, 1990) and bind to its nuclear receptor characterized as a chromatin-associated protein leading to the regulation of specific genes (Oppenheimer, 1983). Some of these genes have been studied: a gene encoding myelin basic protein (MBP) in oligodentrocytes (Farsetti *et al.*, 1992), a gene encoding brain derived neurotrophic factor (BDNF) expressed in granular neurons (Koibuchi *et al.*, 1999), a *Pcp2* gene which is expressed in Purkinje cells (Zou *et al.*, 1994) and the gene encoding NeuroD protein in cerebellar cells (Chatoux and Francon, 2002).

Purine nucleoside adenosine (ADO) and its phosphorilated forms such as ATP, ADP and AMP play important roles in different tissues. Binding to their respective receptors they are a signal to the cell in various physiologic aspects: e.g. blood platelet aggregation, vasoconstriction or vasodilatation (Rongen *et al.*, 1997), astrocytes proliferation (Rathbone *et al.*, 1992), and apoptosis (Di Iorio *et al.*, 2002). The extracellular nucleotides and nucleosides may act as trophic factors that induce alterations in cell metabolism, structure and function of neurons and astrocytes (Rathbone *et al.*, 1999; Neary et al., 1996).

After exerting their effects, nucleotides are extracellularly hydrolyzed by the action of ecto-nucleotidases (ecto-NTPDases and 5'-nucleotidase) that convert ATP into

adenosine in the extracellular space. The molecular properties, functional roles and nomenclature of ectonucelotidases were recently reviewed by Zimmermann (2001).

Ecto-5′-nucleotidase (ecto-5′-NT, CD73), a glycosyl phosphatidylinositol-anchored cell-surface protein, is the final enzyme in the extracellular pathway for the complete hydrolysis of ATP to adenosine. Although ecto-5`NT is expressed in many different tissues (Zimmermann, 1992), only certain cell types within each tissue express the enzyme (Hansen et al., 1995). Also, an intracellular localization of 5`NT was described (Heymann et al., 1984). The main biological role of this enzyme is its participation in the adenosine formation that mediates cell signalling in physiological, as well as pathological conditions (Resta and Thompson, 1997). In addition, the involvement of ecto-5′-NT in drug resistance and tumor-promoting functions has been proposed (Ujhazy *et al.*, 1996; Spychala, 2000).

Recently we have showed a striking difference in the extracellular nucleotide degradation rates between glioma cell lines and normal astrocyte cultures. We hypothesized that changes in the ecto-nucleotidase pathway is a characteristic of this kind of tumor and possibly represent an important mechanism to facilitate high proliferation and invasion as well as low death of these cells (Wink *et al.*, 2002 – submitted).

The existence of T_3 receptors of high affinity in rat C6 glioma cells (Ortiz-Caro *et al.*, 1986) and the fact that this hormone can regulate several proteins expression (Trentin and Alvarez-Silva, 1998) became this cell line a good model for the study of T_3 influence on tumors of glial origin. Evidences are found in the literature linking thyroid hormone actions to tumorigenesis and tumor growth (Mishkin *et al.*, 1981; Hercbergs, 1996; Toms *et al.*, 1998). Considering that ATP and adenosine are implicated as trophic agents on glial cells and that ectonucleotidases play a key role in purinergic signaling regulation, we

sought to investigate the effect of thyroid hormone on the ectonucleotidase pathway. To test the hypothesis whether there is a relationship between thyroid state and extracellular nucleotides and nucleosides levels we examined the effect of T_3 treatment on ecto-nucleotidases activity and expression in C6 glioma cell line, which has been used as an *in vitro* model of tumors of glial origin.

2. Material and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fungizone were purchased from Gibco BRL. Fetal calf serum (FCS) was from Cultilab, Brazil. T_3 (Triiodo-l-thyronine), T_4 (Thyroxine), nucleotides (ATP, ADP, AMP), HEPES, Trizma base and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Treatment of cell culture

C6 glioma cell line was a generous gift from DR.A.G. Trentin. from Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, Brasil. Cells were maintained in complete medium consisting of DMEN containing 2% (w/v) L-glutamine and supplemented with 5% (v/v) fetal calf serum (FCS). The cultures were incubated at 37°C in a humidified 5% Co_2 and 95% air atmosphere. Exponentially growing cells were detached from the culture flasks with EDTA-trypsin, inoculated in complete medium into 24-well microplates and allowed to grow to confluence. After confluence, the cells were treated with 50 nM T_3 dissolved in FCS-free DMEM for 72 h, with thyroid hormones medium changes after 24 and 48 h. The cultures were then maintained 24 h without medium changes, before the experiments. For dose-response curve C6 cells were treated with 0.01, 0.05, 0.10, 0.25, 0,50, 1.0 and 10.0 nM of T_3. Control cultures were

maintained in the same way with DMEM without FCS during treatment (Trentin and Alvarez-Silva, 1998).

2.3. Ecto-nucleotidases assay

2.3.1 Ecto-NTPDase activity

To determine the ATPase and ADPase activities, the 24-well microplates containing glioma cells were washed three times with incubation medium in absence of nucleotides. The reaction was started by the addition of 200 µL the incubation medium containing 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 1 mM of ATP or ADP at 37°C. To stop the reaction, an aliquot of the incubation medium was withdrawn and transferred to an eppendorf tube containing TCA (5% w/v) previously placed on ice. The release of inorganic phosphate (Pi) was measured by Chan method (1986), using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released from nucleotide into assay medium without cells and Pi released from cells incubated without nucleotide was subtracted from the total Pi released during incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity was expressed as nmol Pi released/min/mg of protein.

2.3.2 Ecto-5´-Nucleotidase activity

The incubation medium was the same as that used for ecto-apyrase activity, except that the 2 mM $MgCl_2$ was used instead of $CaCl_2$ and 2 mM of AMP as substrate.

2.4 Ecto-Adenosine deaminase (ADA) activity

The cells were incubated the same way as described above. The reaction was started by adding adenosine (0,15 mM) in the ADA buffer (4 mM $MgCl_2$, 2 mM $CaCl_2$, 100 mM NaCl, 20 mM KCl, 5 mM glucose, 10 mM $NaHCO_3$, 15 mM Tris pH 7.4). To stop the

reaction, an aliquot of the supernatant was transferred to an eppendorf tube on ice and centrifuged at 4°C for 30 min at 16,000 *g*. Aliquots of 40 µl were applied to a reverse phase HPLC system using a C18 Shimadzu column (Shimadzu, Japan) with absorbance measured at 260 nm. The mobile phase was 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol as described (Volter *et al.*, 1980). The adenosine peak was identified by its retention time and by comparison with standards. The ectoadenosine deaminase activity was measured by the decrease in the adenosine peak and by the appearance of inosine and hypoxantine peaks. Spontaneous deamination of adenosine was not detected at any time.

2.5. Protein determination

Cells in the 24-well microplates were solubilized with 100 µL of NaOH 1N and frozen overnight. Then, an aliquot was taken and the protein was measured by the Bradford method (1976), using bovine serum albumin as standard.

2.6. RT-PCR analysis

Total RNA from C6 glioma cell line culture with or without T_3 treatment was isolated with Trizol LS reagent (Life Technologies) in accordance with the manufacturer's instructions. The cDNA species were synthesized with SuperScript II (Life Technologies) from 2µg of total RNA in a total volume of 20 µl with an oligo (dT) primer in accordance with the manufacturer's instructions. cDNA reactions were performed for 1 h at 42°C and stopped by boiling for 5 min. 2µl of cDNA was used as a template for PCR with primers specific for CD73. As a control for cDNA synthesis, β-actin-PCR was performed. 2 µl of the RT reaction mix was used for PCR in a total volume of 25µl using a concentration of

0.5μM of each primer indicated below and 50 μM of dNTP, 1 U Taq polymerase (Life Technologies) in the supplied reaction buffer.

The PCR cycling conditions were as follows: for CD73 45 s at 94 °C, 45 s at 64 °C, 1 min 30 s at 72 °C (amplification product 403bp) and the same conditions for β-actin (amplification product 200bp). All PCRs were carried out for 35 cicles and included an initial 3 min denaturation step at 94 °C and a final 10 min extension at 72°C. 10μl of the PCR reaction was analyzed on a 1.5% agarose gel. The following set of primers were used: for CD73: 5′CCC GGG GGC CAC TAG CAC CTC A3′ and 5′GCC TGG ACC ACG GGA ACC TT3′ and for β-actin: 5′TAT GCC AAC ACA GTG CTG TCT GG3′ and 5′TAC TCC TGC TTC CTG ATC CAC AT3′. Oligonucleotides were obtained from Invitrogen, Brazil.

2.7. Statistics

The data obtained for the enzyme activities are presented as mean ± SEM of at least three independent experiments done in triplicate. Statistical analysis was performed using Student's *t*-test, and the differences were considered significant at $P< 0.01$.

3. Results

3.1 T_3 stimulates ecto-5'-nucleotidase activity of C6 glioma cells

Ecto-5'-NT activity measured in control conditions in rat C6 glioma cells was 55 ± 15 nmol/min/mg protein with 2.0 mM 5'-AMP as substrate, while no detectable ATPase and ADPase activities were found. To evaluate the influence of T_3 on ecto-nucleotidases activities, we performed an enzymatic assay after hormone treatment with incubation time and substrate concentration pre-established (Wink *et al.*, 2002 - submitted). Based on data

from literature, we initially tested 50 nM of T_3 on ecto-nucelotidase activities. An increase of approximately 30% in the AMP hydrolysis were observed when the C6 cells were treated for three days in a medium containing thyroid hormone compared with control cultures. In contrast, no significant modification in ATP and ADP hydrolysis was observed (Figure 1).

In order to do a complete investigation of all enzymes involved in the extracellular nucleotide degradation, we performed adenosine deaminase assay. Stefanovic *et al.* (1976) demonstrated that no substantial ecto-adenosine deaminase activity was measurable in C6 glioma cell line. In contrast, in our assay conditions, this enzymatic reaction occurs, releasing inosine as product of adenosine deamination (data not shown). The presence of different T_3 concentrations did not affect this enzyme action. Therefore, ecto-5'-nucleotidase appears to be the unique thyroid hormone responsive in the ecto-nucleotidase chain in these cells.

3.2 Dose-response curve for activation of ecto-5'-nucleotidase by T_3

To investigate in more detail how thyroid hormone affects the ecto-5'-NT activity, rat C6 cells were treated with 0.01; 0.05; 0.10; 0.25; 0,50; 1.0 and 10 nM of T_3 in the conditions described in Material and Methods session. In the presence of T_3 a dose-response curve was observed (Figure 2). The increased responsiveness to different T_3 concentration was observed up to 0,25 nM; after that, a plateau was reached and the AMPase activity did not change with higher T_3 concentration. The L-thyroxine hormone (T_4) was also tested in C6 cells cultures, but it did not alter all nucleotides hydrolysis (data not shown).

3.3 T_3 increases CD73 mRNA expression in C6 cells

Considering that thyroid hormone regulates protein expression in C6 glioma cells (Trentin and Alvarez-Silva, 1998) and that this hormone promoted a significative increase on ecto-5'-NT activity we evaluated whether the enhancement of ecto-5'-NT activity by T_3 resulted from increased CD73 mRNA levels. Two important things can be observed from the

results of these experiments: first, it was showed that CD73 is present in control cells by RT-PCR using synthesized oligonucleotide primers specific to this protein. Second, and more importantly, Figure 3 also shows that in the thyroid hormone treated C6 cells, CD73 mRNA expression was found 2.5-fold higher compared with control conditions, supporting the proposal that the increasing in 5'-NT activity is related to an over-expression of the mRNA and suggesting an increase on protein synthesis.

4. Discussion

Extracellular adenine nucleotides and nucleosides have been related with diverse physiological as well pathological conditions in the central nervous system. ATP and adenosine are known to produce potent effects when are released from neurons and astrocytes. Ectonucleotidases constitute a highly organized pathway for the regulation of the homeostasis of nucleotide-mediated signaling, controlling the ATP degradation and adenosine formation. The hydrolysis of ATP to AMP is catalyzed by NTPDases (1 to 6), a group of enzymes that with different specificities catalyze the dephosphorylation of commonly occurring tri- and diphosphonucleosides. The AMP formed by these enzymes is hydrolyzed to adenosine by the action of an ecto-5′-nucleotidase (CD73). In addition to the protective effects of adenosine against ischemia- and trauma associated neuronal cell death, it has been postulated that adenosine may accumulate to high concentrations in solid tumors being an important factor in stimulating angiogenesis, cancer growth and inhibition of immune response toward the malignant tissue (Spychala *et al.*, 2000). Therefore, the observation that both adenosine as well as ecto-5'-NT expression may be related with transformation of different malignant tissues has significant biological implications.

In contrast with intact astrocyte cultures from newborn rat cerebral cortex, that present a rapid dephosphorylation of ATP and ADP but a slow degradation of AMP (Lai and Wong, 1991), the C6 cells, a cell line that was cloned from a N-nitrosomethylurea induced rat brain tumor (Benda *et al.*, 1968), had an exactly inverted behavior. Results from our laboratory showed that other human glioma cell lines also present an extremely low ATP and ADP degradation. In contrast, ecto-5'-nucleotidase activity was increased in all glioma cell lines studied when compared to astrocytes (Wink *et al.*, 2002 - submitted). Considering the similarity among four human glioma cell lines and one rat cell line examined, we hypothesized that changes in the ecto-nucleotidase pathway is a characteristic of this kind of tumor and possibly represent an important mechanism to facilitate high proliferation and invasion as well as low death of these cells.

Recently, T3 was shown to induce astrocytes and C6 glioma cells to secrete mitogenic growth factors that could be in part, responsible to the proliferative effect of the hormone (Trentin et al., 2001). Clinical hypothyroidism has been associated with prolonged survival in several types of malignancies. Toms *et al.* (1998) showed that thyroid hormone depletion inhibited astrocytoma proliferation via of the p21 induction. The results presented here indicate that thyroid state may influence ecto-5'-nucleotidase activity. In the presence of this hormone, this enzyme has its potential of degradation enhanced by about 30%. The RT-PCR technique showed that the higher AMPase activity observed in the enzymatic assays is related to the ecto-5'-nucleotidase/CD73 over expression caused by addition of T_3 in the medium culture.

In this work, we also could confirm that T_3 hormone is transported into the cell and acts on ecto-nucleotidases expression binding at its nuclear receptors (Oppenheimer, 1983) and, consequently, triggering protein transcription. Like a broad variety of hormones, the

triiodothironine exerts its effects at very low concentrations. The T_3 dose-dependent response of C6 was observed up to 0,25 nM and higher concentrations did not affect the AMP catabolism. This probably is due to the receptor sensitivity threshold. These findings are consistent with the physiological values found in the organisms (Yen, 2001).

Increased ecto-5'-nucleotidase protein expression was also observed in several multidrug-resistance (MDR) cell lines. Ujházy *et al.* (1996) demonstrated that this nucleotidase activity is acquired in the drug resistance induction, suggesting that this salvage pathway may be a process to compensate the ATP losses. Ecto-5'-nucleotidase enhanced produces more adenosine quantities that could be used to promote proliferation (Rathbone et al., 1992) and also to be uptaken forming ATP which is used by the Pgp170 protein in the drug extrusion (Abraham *et al.*, 1993) or used as energy source for the cell metabolism. So, we hypothesize that the hyperthyroidism may accelerate the process of drug resistance acquired by some tumors, through an enhanced 5'-nucleotidase activity.

Thyroid hormone appears to have influence just on ecto-5'-nucleotidase activity. No significant difference is observed in the other enzymes participants of extracellular nucleotides cascade degradation: ecto-ATPases, ecto-ATPDase and ecto-adenosine deaminase verified in ours experiments. It is important to note that no difference in the ATPase and ADPase activities could be observed even with prolonged time (five days) of treatment with T_3 (data not shown). So, these results suggest that the ecto-5'-nucleotidase gene is under some kind of regulation by T_3. Interestingly, the ecto-5'-NT promoter has several binding sites for Sp1, a transcription factor that interacts with thyroid hormone receptor binding protein associated factors (TAFs) and is proposed to stabilize the transcription machinery and to activate transcription (Hansen et al., 1995; Kim et al., 1999: Spychala et al., 1999).

In contrast to cell of neuronal origin, glioma cells transported L-T_4 almost as effectively as L-T_3 by the same carrier system (Gonçalves *et al.*, 1990). Those authors suggested that an active transport system for T_4 may help to explain why these cells are more resistant to hypothyroidism and also to strengthen the hypothesis that glial cell may have a protective role for optimal neuronal function, maintaining a more constant hormonal environment. Although T_4 transport into the cell has been reported in human glioma cells, when C6 cells were treated with the thyroxine hormone (T_4), no significant alteration in the extracellular nucleotides levels occur compared with the control cultures. This poor response to T_4 may be explained as a difference in the affinity of the T_3 receptors for this compound. T_4 has been reported to bind to nuclear receptor with a lower affinity than for T_3. (Yusta *et al*, 1988). Thus, the conversion of T_4 to T_3, via 5'-deiodination, found in brain is needed to the thyroid effect.

In conclusion, here we have demonstrated that T_3 hormone up regulates the expression of CD73 protein with subsequent alterations in the extracellular purine catabolism. The over expression of 5'-NT supports the hypothesis that adenosine production is enhanced when C6 glioma cells are treated with T3 hormone, and can be involved in the effects of this hormone on proliferation of the tumors of glial origin. Whether the same effect is observed in human glioma cell lines in culture and if thyroid hormone affects the evolution of glioma *in vivo* remains to be investigated.

Acknowledgements

This work was supported by the Brazilian funding agencies: Conselho de Desenvolvimento Cientifico e Tecnologico (CNPq) and Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS). M. R. Wink and E. Braganhol were recipient of CNPq-Brasil fellowship. A.S.K. Tamajusuku was recipient of FAPERGS fellowship. We are grateful to Dr. A. G. Trentin for C6 gliomas.

REFERENCES

Abraham E.H., Prat, A.G., Gerweck, L., Seneveratne, T. Arceci, R.J., Kramer, R., Guidotti, G., Cantiello, H.F., 1993. The multidrug resistance (mdr1) gene product functions as an ATP channel. Proc. Nat. Acad. Sci. (Wash.) 90, 312-316.

Benda P., Lightbody J., Sato G., Levine L., Sweet W. 1969. Differentiated rat glial cell strain in tissue culture. Science 161(839), 370-371.

Bradford, M. M. A., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 218-251.

Chan, K. M., Delfert, D., Junger, K. D., 1986. A direct colorimetric assay for Ca^{+2}-ATPase activity. Anal. Biochem. 157, 375-380.

Chantoux F., Francon J., 2002. Thyroid hormone regulates the expression of NeuroD/BHF1 during the development of rat cerebellum. Mol. Cell Endocrinol. In press.

Di Iorio, P., Kleywegt, S., Ciccarelli, R., Traversa, U., Andrew, C.M., Crocker, C.E., Werstiuk, E.S., Rathbone, M.P. 2002. Glia 38, 179-190.

Dratman, M.B., J.T. Gordon. 1996. Thyroid Hormones as neurotransmitters. Thyroid. 6 (6), 639-647.

Farsetti, A., Desvergne, B., Hallenbeck, P., Robbins, J., Nikodem, V.M., 1992. Characterization of myelin basic protein thyroid hormone response element and its functions in the context of native and heterologous promoter. J. Biol. Chem. 267, 15784-15788.

Gonçalves, E., Lakshmanan, M., Pontecorvi, A., Robbins, J. 1990. Thyroid hormone transport in human glioma cell line. Mol. Cell Endocrinol. 69,157-165.

Hansen, K.R., Resta, R., Webb, C.F., Thompson, L.F. 1995. Isolation and characterization of the promoter of the human 5`-nucleotidase (CD73)-encoding gene. Gene 167, 307-312.

Hercbergs A. 1996. The thyroid gland as an intrinsic biologic response-modifier in advanced neoplasia- a novel paradigm. In Vivo 10(2),245-247.

Heymann, D., Reddington, M., Kreutzberg, G.W. 1984. Subcellular localization of 5`-nucleotidase in rat brain. J. Neurochem. 43, 971-978.

Lai, K.M., Wong, P.C.L. 1991. Metabolism extracellular adenine nucleotides by cultured rat brain astrocytes. J. Neurochem. 57, (5), 1510-1515.

Kin, M.K., Lee, J.S., Chung, J.H. 1999. In vivo transcription factor recruitment during thyroid hormone receptor-mediated activation. Proc. Natl. Acad. Sci. 96, 10092-10097.

Koibuchi N., Fukuda H., Chin W.W. 1999. Promoter-specific regulation of the brain-derived neurotropic factor gene by thyroid hormone in the developing rat cerebellum. Endocrinol. 140 (9), 3955-3961.

Mishkin SY, Pollack R, Yalovsky MA, Morris HP, Mishkin S. 1981. Inhibiion of local and metastatic hepatoma growth and prolongation of survival after induction of hypothyroidism. Cancer Res. 41(8), 3040-3045.

Neary, J.T., Rathbone, M.P., Cattabeni, F., Abbracchio, M.P., Burnstock, G. 1996. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci. 19, 13-18.

Oppenheimer, J.H. 1983. The nuclear receptor-triiodothyronine complex: relationship to thyroid hormone distribution, metabolism and biological action. In: Oppenheimer, J.H.,

Samuels, H.H. (eds) Molecular Basis of Thyroid Hormone Action. Academic Press, New York, pp. 1-34.

Ortiz-Caro, J., Yusta, B., Montiel, F., Villa, A., Aranda, A., Pascual, A. 1986. Identification and characterization of L-triiodothyronine receptors in cells of glial and neuronal origin. Endocrinol. 119, 2163-2167.

Rathbone, M.P., Middlemiss, P.J., Gysbers, J.W., Deforge, S., Costello, P., Del Maestro, R.F. 1992. Purine nucleosides stimulate proliferation of a wide variety of cell types. In Vitro Cell Dev. Biol. 28^{A}, 529-536.

Rathbone, M.P., Middlemiss, P.J., Gysbers, J.W., Andrew, A., Herman, M., Reed, J.K., Ciccarelli, R., Di Iorio, P., Caciagli, F. 1999. Trophic effects of purines in neurons and glial cells. Prog. Neurobiol. 59, 663-690.

Resta, R., Thompson, L.F. 1997. T cell signalling through CD73. Cell. Signal. 9, 131-139.

Rongen, G.A., Floras, J.S., Lnders, J.W.M., Thien, T., Smits, P., 1997. Cardiovascular pharmacology of purines. Clin. Sci. 92, 13-24.

Spychala, J., Zimmermann, A.G., Mitchell, B.S. 1999. Tissue-specific regulation of the ecto-5`-nucleotidase promoter. J. Biol. Chem. 274, 22705-22712.

Spychala, J., 2000. Tumor-promoting functions of adenosine. Pharmacol. Ther. 87, 161-173.

Stefanovic, V., Mandel, P., Rosenberg, A. 1976. Ecto-5'-nucleotidase of intact cultured C6 rat glioma cells. J. Biol. Chem. 251, (13), 3900-3905.

Toms, S.A., Hercbergs, A., Liu, J., Kondo, S., Barnett, G.H., Casey, G., Barna, B.P. 1998. Thyroid hormone depletion inhibits astrocytoma proliferation via a p53-independent induction of p21 (WAF/1CIP1). Anticancer Res. 18, 289-294.

Trentin, A.G., Alvarez-Silva, M., 1998. Thyroid hormone regulates protein expression in C6 glioma cells. Braz. J. Med. Biol. Res. 31, 1281-1284.

Trentin, A.G., Alvarez-Silva, M., Moura Neto, V. 2001. Thyroid hormone induces cerebellar astrocytes and C6 glioma cells to secrete mitogenic growth factors. Am. J. Physiol. 281, E1088-E1094.

Ujházy, P., Berleth, E.S., Pietkiewicz, J.M., Kitano, H., Skaar, J.R., Ehrke, M.J., Mihich, E., 1996. Evidence for the involvement of ecto-5'-nucleotidase (CD73) in drug resistance. Int. J. Cancer 68, 493-500.

Voelter W., Zech K., Arnold P., Ludwig G., 1980. Determination of selected pyrimidines, purines and their metabolites in serum and urine by reversed-phase ionpair chromatography. J. Chromatogr. 199, 345-354.

Wink MR, Lenz G, Braganhol E, Tamajususku ASK, Schwartsmann G, Sarkis JJF and Battastini AMO., 2002 Implications of altered extracellular ATP, ADP and AMP catabolism in pathology of glioma cell lines. Submitted to Cancer Letters.

Yen, P.M. 2001. Physiological and Molecular basis of thyroid hormone action. Physiol. Rev. 81 (3): 1097-1142.

Yusta, B., Ortiz-Caro, J., Pascual, A., Aranda, A. 1988. Mechanism of L-triiodothyronine (T3) uptake by glial C6 cells: regulation by butyrate. Mol. Cell. Endocrinol. 58, 191-198.

Zimmermann, H., 1992. 5'-nucleotidase: molecular structure and functional aspects. Biochem. J. 285, 345-365.

Zimmermann, H., 2001. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev. Res. 52, 44-56.

Zou, L., Hagen, S.G., Strait, K.A., Oppenheimer, J.H. 1994. Identification of thyroid hormone response elements in rodent Pcp-2, a developmentally regulated gene of cerebellar Purkinje cells. J. Biol. Chem. 269. 13346-13352.

LEGENDS TO FIGURES

Figure 1. **Effect of T_3 on the extracellular hydrolysis of adenine nucleotides in C6 glioma cells.** After confluence, the cells were cultured in FCS-free DMEM (white bars) or treated with 50 nM T_3 dissolved in FCS-free DMEM for 72 h (hatched bars), without medium changes 24 h before the nucleotidase activities measurements. The treated and control cells were incubated with 1 mM ATP or ADP for 30 minutes, and with 2mM AMP for 10 minutes as described in Material and Methods. Specific activity values are expressed as nmol Pi/min/mg protein. Bars represent mean ± SEM. of three independent experiments done in triplicate. * AMP hydrolysis in treated-group significantly different from control group ($P < 0,01$, Student's *t*-test).

Figure 2. **Dose-response curve for activation of ecto-5'-nucleotidase by T_3.** After confluence, C6 cells were treated with 0.01, 0.05, 0.10, 0.25, 0,50 and 1.0 nM of T_3 for 72 h and incubated with 2 mM of AMP for 10 min as described in Materials and Methods. Specific activities values are expressed as nmol Pi/min/mg protein. Data represents mean ± SEM obtained from three independent experiments with triplicate determinations. The data were fitted in a sigmoid dose-response curve by PRISM V 2.0 (GraphPad).

Figure 3. **Effect of T_3 treatment on expression of ecto-nucleotidases in C6 glioma cells analyzed by RT-PCR.** Monolayers of C6 cells were maintained in culture media without serum (control) or were exposed to T_3 50 nM for 3 days without medium changes 24 h before total RNA extraction. Total RNA was extracted with Trizol and analyzed using primers for

CD39 (ATPDase) and CD73 (5'-nucleotidase). The PCR products were separated on a 1,5% agarose gel. As a control for cDNA synthesis, β-actin-PCR was performed.

FIGURES

Figure 1

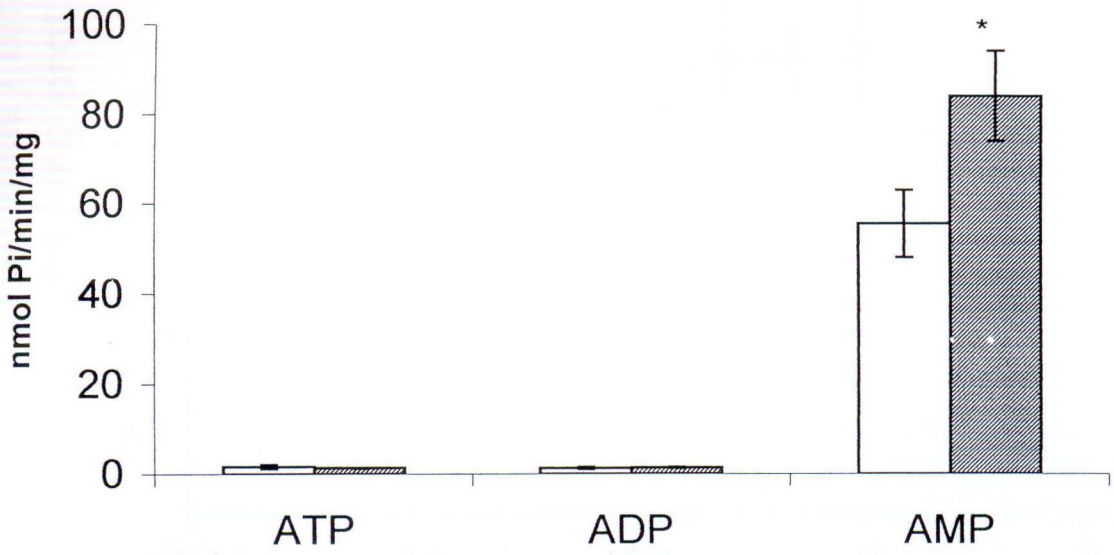


Figure 2.

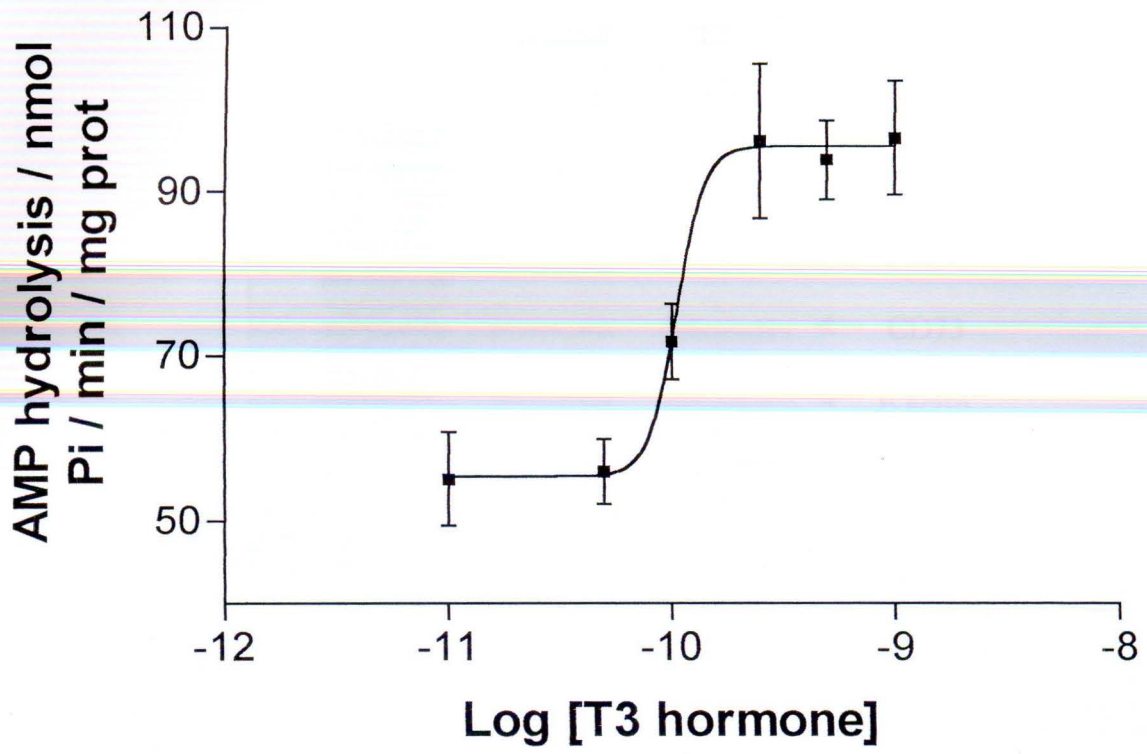
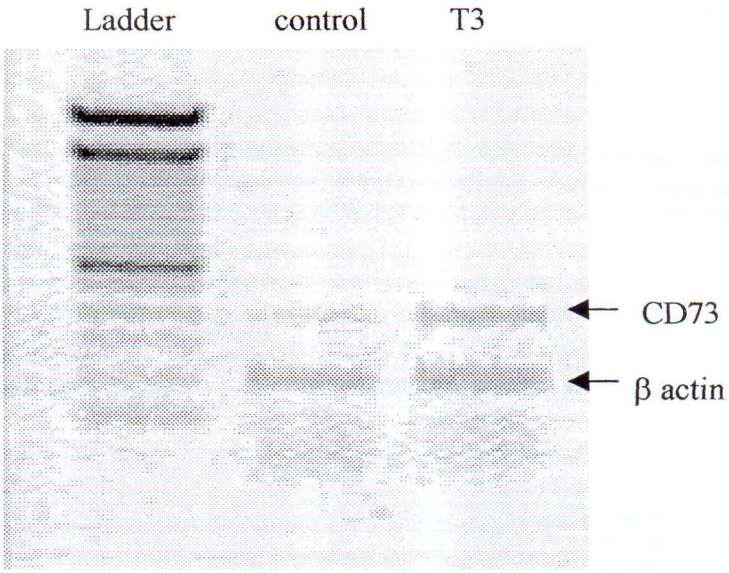

AMP hydrolysis / nmol
Pi / min / mg prot
110
90
70
50
-12
-11
-10
-9
-8
Log [T3 hormone]

Figure 3



authorGATEWAY for Elsevier Science Journals

Molecular and Cellular Endocrinology

print page close wind

Guide for Authors

Types of papers
(a) **Full-length original research papers.**
(b) **Rapid Papers.** These articles will be short, complete and essentially final reports. The Managing Editors will rapidly evaluate the submission on a yes/no basis and, if appropriate, immediately pass on to the Publisher. The overriding criterion for inclusion in this section is that a paper must be of sufficient immediate importance to the work of other investigators to justify urgent publication.
(c) **At the Cutting Edge.** These articles (2000 word equivalents maximum) will be brief essays on rapidly developing areas in contemporary endocrinology. Inferences from data presented in abstract form, or as personal communications, are welcome as pointers to the directions in which a particular field is evolving. Preferably, the essay includes at least one explanatory figure.
(d) **Critical, short review articles.** These contributions should preferably be sponsored by a member of the Editorial Board and will be published with the shortest possible delay at the discretion of the Editors.
(e) **Book reviews.**

Submission of manuscripts
The acceptance of articles for publication is on the understanding that they are subject to editorial revision, and on the condition that they are contributed solely to this journal, and have not been and will not be published in whole or in part in any other journal or book. The preferred medium of final submission to the accepting Editor is on disk with the accompanying reviewed and revised manuscript (see `Electronic manuscripts' below). Full-length papers should be submitted to:

Prof. I.T. Huhtaniemi
Department of Physiology
Institute of Biomedicine
University of Turku
Kiinamyllynkatu 10
FIN-20520 Turku
Finland
Tel: +358-2 3337579
Fax: +358-2 250 2610
E-mail: ilpo.huhtaniemi@utu.fi

Dr. R.J. Rodgers (Managing Editor for Asia and the Pacific Region)
Reproductive Medicine Unit
Obstetrics and Gynaecology
Adelaide University
Adelaide, SA 5005
Australia
Tel. 61.8.8303 3932 / 8222 6788
Fax. 61.8.8303 4099 / 8222 7521
E-mail: ray.rodgers@adelaide.edu.au

Dr. W.E. Rainey (Managing Editor for the Americas)
Dept. of Obstetrics & Gynecology
Division of Reproductive Endocrinology
Univ. of Texas Southwestern Medical Center of Dallas
5323 Harry Hines Boulevard, Room J6.114
Dallas, TX 75390-9032, USA
Telnr. +1.214.648 4747
Faxnr +1.214.648 8066
E-mail: braine@mednet.swmed.edu

Rapid Papers will usually be maximally 4 printed pages long (including figures, tables and references) and can be submitted to any of the Managing Editors. A page of the journal that contains nothing but text has about 750-800 words, but adequate allowance must be made for the space taken up by tables, illustrations, etc. Please note that, because of the speed of publication, the letter you send to the Managing Editor that accompanies your submitted manuscript should:

(a) provide information on reprints and address of receipt for these. For all articles 25 reprints will be made available free of charge but extra copies may be ordered (minimum order 100) in which case the address where the invoice should be sent should be supplied;

(b) provide a statement that the copyright of the article is transferred to the Publisher in the event of its acceptance to ensure the widest possible dissemination of the information. Should this transfer not be possible, could you please state the reasons;

At the **Cutting Edge** and **Review articles** should be addressed to:

Review Editor
Prof. J.A. Grootegoed
Department of Endocrinology and Reproduction
Faculty of Medicine and Health Sciences
Erasmus University Rotterdam
P.O. Box 1738
3000 DR Rotterdam
The Netherlands
Tel. +31.20.4087345
Fax +31.20.4366832
E-mail: grootegoed@endov.fgg.eur.nl

At the Cutting Edge essays will be published 6-8 weeks after acceptance. Because of the speed of publication, the letter you send to the Review Editor together with the submitted manuscript should be accompanied by the same information and statement as required for Rapid Papers. Contributions in the form of short review articles will be reviewed by the Review Editor and one or two members of the Editorial Board. The speed of publication will be enhanced when the review is sponsored by a member of the Editorial Board. All other forms of communication: book reviews, announcements and comments about topics relevant to the aims and scope of the Journal should be directed to one of the Managing Editors.

Instructions to contributors:
Author Checklist can be found in this PDF file: Author Checklist

Manuscripts should be submitted in triplicate to enable prompt forwarding to referees and to hasten selection and processing of papers. The following order should be maintained;
The title page - should contain the title of the article (not exceeding 85 characters, including spaces), author's initials, names, hospital and academic affiliations. Also, as a footnote, the corresponding author's *full* address (including telephone, fax, and e-mail) must be given.
A summary - not exceeding 150 words should be included at the beginning of the article. This summary should mention the essential information contained in this article, plus the author's conclusions.
Keywords - A list of 3-6 key (indexing) words should appear above the abstract. Terms indicating the origin of the material studied should usually be used as sub-keywords, i.e. within parentheses - e.g. (Rat testis), (Sertoli cell). A keyword consisting of more than one separate work will appear in the index under the first work unless specifically requested to be otherwise.
The text - should be typewritten, with double or triple spacing, and with liberal margins on all four sides. If a dot-matrix printer is used to generate the manuscript, the typeface must be of a near-letter quality. The journal will essentially follow the rules defined in `Information for Contributors' for Biochimica et Biophysica Acta (available from BBA Editorial Office, P.O. Box 1345, 1000 BH Amsterdam). Unfamiliar terms, arbitrary abbreviations, and trade names should be defined when first used. Unnecessary abbreviations and symbols should be avoided. For drugs, generic names should be used; trade names may be mentioned in parentheses the first time the name of the drug appears in the text. All items that should appear in italics (e.g. species and gene names) should be clearly identified by underlining. Also, super- and subscripts must clearly be above or below the typeline.
The tables - should be given a short informative title and should be numbered consecutively in Arabic numerals. Each table plus its caption should be submitted on a separate sheet.
The figures - should be submitted as sharp original drawings, or as well-contrasting, unmounted photographs on glossy paper. The lettering on the figures must be proportional to the size of the figure, to ensure legibility after reduction. The following symbols can be typeset and should be used in preference to non-standard forms:
▲▼♦

- ■△▽◊⊗⊕⊙ * + x

Figures should be prepared suitably for either one column width (82 mm) or the entire page width (173 mm). The maximum height is 232 mm. Each figure must have the name of the (first) author written on the back in pencil, as well as an arrow indicating the top. All figures should be numbered consecutively in Arabic numerals. Illustrations should be submitted in black and white unless color reproduction is requested. Color prints should be submitted in actual size and authors will be responsible for the additional costs.
References In the text, references should be cited by author and year (Harvard System). More than one paper from the same author in the same year must be identified separately, by the letters a, b, c, etc., placed after the year of publication. In the text, when referring to a work by more than two authors, the name of the first author should be given followed by et al. In the list, references should be assembled in alphabetical order on a separate sheet. Unpublished data, personal communications and papers in preparation or `submitted' should not be listed in the references (but may be incorporated at the appropriate place in the text); work `in press' may be listed only if it has been accepted for publication. Personal communications must be accompanied by a letter from the named person(s) giving permission to quote such information. Abstracts (whether published or not), theses and similar material

are not to be quoted in the text. If necessary, they can be referred to in the text in parentheses and without serial number, or be presented in footnotes. Periodicals, books and edited books should accord with the following examples:

Petty, K.J., 1995. Tissue- and cell- specific distribution of proteins that interact with the human thyroid hormone receptor-ß. Mol. Cell. Endocrinol. 108, 131-142.

Berry, M.N., Barritt, G.J., Edwards, A.M., 1991. Isolated Hepatocytes: Preparation, Properties and Applications (Laboratory Techniques in Biochemistry and Molecular Biology, vol 21), pp. 201-214, Elsevier, Amsterdam.

Koesling, D., Bohme, E., Schultz, G., 1993. Guanylyl cyclases as effectors of hormone and neurotransmitter receptors. In: Hucho, F. (ed.), Neurotransmitter Receptors. Elsevier, Amsterdam, pp. 325-328.

If a publication is in press, the reference should be made as complete as possible, stating the name of the journal and adding `in press'. Abbreviations of journal titles should conform to those adopted by the List of Serial Title World Abbreviations, ISDS International Centre, 20, rue Bachaumont, 75002 Paris, France (ISBN 2-904938-02-8).

Acknowledgements should be given at the end of the text part, on a separate sheet.

ProofsPage proofs will be sent to the corresponding author for checking. Corrections to the proofs must be restricted too printers errors. Any substantial alterations, other than these may be charged to the author. **Corrected proofs must be returned within 48 hours of receipt**, otherwise the article may appear without the author's corrections to avoid delaying publication of the journal.

Please note that after the date of acceptance no further changes may be made to the manuscript.

Page charge and reprints There will be no page charge and no manuscript processing fee. Upon acceptance of their papers, the authors will receive an order form which may be used to order reprints of their articles. Of each article, 25 reprints will be made available free of charge.

Electronic manuscripts Electronic manuscripts have the advantage that there is no need for the rekeying of text, thereby avoiding the possibility of introducing errors and resulting in reliable and fast delivery of proofs. For the initial submission of manuscripts for consideration, hardcopies are sufficient. **For the rapid processing of papers electronic versions are essential**. After final acceptance your disk plus two, final and exactly matching printed versions should be submitted **together**. Double density (DD) or high (HD) diskettes (3 1/2 or 5 1/4 inch) are acceptable. It is important that the file saved is in the native format of the wordprocessor program used. Label the disk with the name of the computer and wordprocessor package used, your name, and the name of the file on the disk. Further information may be obtained from the Publisher.

Reproducibility of results and statistical tests Authors should state how many times experiments were repeated and whether mean or representative results are shown. Authors should be aware of the fact that, for example, triplicate samples from a single homogenate or from one cell culture do not represent three independent experiments, and that, for the purpose of statistical analysis, they must be assigned an n value of 1, not 3. Variability should be indicated statistically wherever possible as part of, but not in place of, a proper statistical analysis. If results are expressed as percentages, the absolute value corresponding to 100% must be stated. Avoid values with unjustified numbers of significant figures; in most cases three significant figures is consistent with the accuracy attained in experiments. The term `significant' should not be used unless statistical analysis supporting it was performed. Results of statistical tests should be presented wherever possible as evidence for conclusions reached. Such information must be presented concisely to illuminate the results, but not to dominate them. The test used should be briefly described in the Materials and Methods section. Details of the diagnostic checks made for the assumptions of the statistical tests and for the validity of any transformation used should be stated clearly.

Ethical and related aspects The Managing Editors expect that all new and variant propagative material such as cell lines, DNA clones, hybridomas etc. described in the journal will be made available, upon written request and for their own use, to all qualified members of the scientific community who may wish to duplicate the published experiments. If delays in strain or vector distribution are anticipated or if they are available from sources other than the authors, this should be indicated. Authors should deposit important strains and material in publicly accessible culture collections or depositories and refer to the collections and accession numbers in the text. In the case of materials that have been distributed by individuals, authors should indicate the laboratory strain designations and the name and address of the donor as well as the original culture collection identification number, if any. Papers describing experimental work with humans must include a statement that the Ethical Committee of the institution in which the work was done has approved it, and that the subjects gave informed consent to the work. Experiments with animals must have been done in accordance with the legal requirements of the relevant local or national authority. Procedures should be such that experimental animals do not suffer unnecessarily. Submission of a manuscript implies that the work described is complete and can be reproduced, that it has not been published before (except in the form of an abstract or as part of a published lecture, review or academic thesis), that it is not under consideration for publication elsewhere, that the corresponding author has ensured that its publication has been approved by all the authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out, that all persons entitled to authorship have been so named, and that, if accepted, it will not be published elsewhere in the same form in either the same or another language, without the consent of the Editors and the Publisher. References should be made to previously published abstracts, etc. at the end of the Introduction or in footnotes.

The articles published in *Molecular and Cellular Endocrinology* represent the scientific findings and opinions of the authors. Whilst the Editors and the Publishers make every effort to ensure the accuracy of all published matter, they can accept no responsibility or liability, collectively or individually, for any erroneous, misleading or unintentionally damaging statements which may appear in the journal. Authors must draw attention to chemical or biological hazards that may be involved in materials and methods used in experiments.

authorGATEWAY journals

print page close window