Available online at www.sciencedirect.com
SCIENCE DIRECT®
Pharmacology, Biochemistry and Behavior 75 (2003) 467–471
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

The effect of stress upon hydrolysis adenine nucleotides in blood serum of rats

Ana Elisa Böhmer[a,1], Cristina Ribas Fürstenau[a,1], Iraci Lucena S. Torres[a], Leonardo Crema[a], Ana Maria O. Battastini[a], Carla Dalmaz[a], Maria Beatriz C. Ferreira[b], João J.F. Sarkis[a,*]

[a]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600 Anexo, Lab. 22, 90035-003 Porto Alegre, RS, Brazil*
[b]*Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, UFRGS, 90035-003 Porto Alegre, RS, Brazil*

Received 6 January 2003; received in revised form 24 April 2003; accepted 5 May 2003

Abstract

Alterations of enzyme activities involved in adenine nucleotide hydrolysis have been reported in spinal cord and blood serum after repeated restraint stress. On the other hand, no effect was observed in the spinal cord of rats after acute stress. In the present study, we investigated the effect of acute stress on the hydrolysis of adenine nucleotides in rat blood serum. Adult male Wistar rats were submitted to 1-h restraint stress and were sacrificed at 0, 6, 24 and 48 h. Increased ATP and ADP hydrolysis were observed in the blood serum of stressed rats 24 h after stress (58% and 54%, respectively, when compared to controls). On the other hand, the AMP hydrolysis was increased after 6 h (68% when compared to controls) and at 24 h (94% when compared to controls) after stress. The results suggest that altered activity of soluble enzymes in serum may be a biochemical marker for stress situations.

Keywords: Apyrase; 5′-Nucleotidase; Stress; Nucleotide hydrolysis; Rat; Blood serum

1. Introduction

Extracellular ATP and its breakdown products, ADP and adenosine, have been shown to present pronounced effects on a variety of biological and pathological processes (Agteresch et al., 1999; Latini and Pedata, 2001). These effects of nucleotides were initially recognized in smooth muscle contraction, neurotransmission and regulation of cardiac function and platelet aggregation. Adenosine is particularly well suited to be used as a transcellular messenger to signal metabolic imbalance. Several reports have documented an increase in the extracellular concentration of adenosine upon stressful metabolic challenges (Latini and Pedata, 2001), and it has been suggested to have neuroprotective actions (for a review on adenosine, see Cunha, 2001).

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the surface, or may be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). Soluble nucleotidases, which can breakdown ATP and other adenosine nucleotides, have also been shown to be released from sympathetic nerves (Todorov et al., 1997). Works over the past few years have demonstrated that members of several families of ectonucleotidases can contribute to the extracellular hydrolysis of nucleotides. Nucleoside 5′-triphosphates and -diphosphates (NTP and NDP) may be hydrolyzed by members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP) family and by alkaline phosphatases (Zimmermann, 2001). These ectonucleotidases, together with 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this cascade formed by ectonucleotidases and 5′-nucleotidase is an enzymatic pathway with a double function of removing a signal of ATP and generating a

* Corresponding author. Tel.: +55-51-3316-5577; fax: +55-51-3316-5540.
E-mail address: jjsarkis@plug-in.com.br (J.J.F. Sarkis).
[1] Both are first authors.

0091-3057/03/$ – see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/S0091-3057(03)00143-6

second one, adenosine. These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological conditions (Agteresch et al., 1999).

The physiological response to emotional or physical stress consists of an integration of endocrine and autonomic changes. In this situation, adrenomedullary epinephrine is released and hormones such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids (GCs) are released by the hypothalamic–pituitary–adrenocortical (HPA) axis (Sapolsky, 1992). The acute secretion of GCs is critical for responding to stress. Adenosine is one of the main neuromodulators associated with cell stress (Cunha, 2001). Indeed, it is known that adenosine A1 receptors are increased by GC (Svenningsson and Fredholm, 1997). The extracellular adenosine concentrations could be increased in stressful challenges (Latini and Pedata, 2001), including exposure to inescapable shock (Minor et al., 2001). Mild stress, such as a mild foot shock, is enough to promote specific changes in the ATP and ADP hydrolysis in some tissues such as the cerebral cortex (Pereira et al., 2002). Alterations of enzyme activities involved in nucleotide hydrolysis have also been reported in spinal cord and blood serum after repeated restraint stress (Torres et al., 2002a,b). In these studies, no effect was observed in the spinal cord of rats after acute stress. On the other hand, since increased ATP release has been reported after shear stress (Bodin and Burnstock, 2001), it is possible that the effects of repeated stress on the ATP hydrolysis cascade may be a consequence of an adaptation induced by the repetition of exposure to the stressor agent. In this sense, it would be important to evaluate the effects of acute stress on these processes. Therefore, in this study, we investigated the effect of restraint stress on ATP, ADP and AMP hydrolysis in the blood serum of adult male Wistar rats in different times after stress (0, 6, 24 and 48 h).

2. Methods

2.1. Animals

The study was performed in accordance with the University Ethics Committee guidelines for experiments with animals. Adult male Wistar rats (60 days at the beginning of the treatment), weighing 150–230 g, were used. Experimentally naive animals were housed in groups of five in home cages made of Plexiglas material ($65 \times 25 \times 15$ cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at a room temperature of 22 ± 2 °C. The rats had free access to food (standard laboratory rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was always performed between 10:00 and 12:00 a.m.

2.2. Stress procedure

The animals were divided into two groups: stressed and control. Restraint was applied by placing the animals in a 25×7-cm plastic bottle and fixing it with plaster tape on the outside so that the animals were unable to move. There was a 1-cm hole at one far end for breathing (Ely et al., 1997). The animals were sacrificed immediately, 6, 24 and 48 h after 1-h stress session. Control animals were kept in their home cage.

2.3. Isolation of blood serum fraction

Trunk blood was drawn by decapitation of the animals at 0, 6, 24 and 48 h after stress session. Blood samples were centrifuged in plastic tubes for 5 min at $3000 \times g$ at room temperature. Serum was separated and used in the enzyme assay immediately.

2.4. Enzyme assays

ATP and ADP hydrolyses were performed using a modification of the method described by Yegutkin (1997). The reaction mixture containing 0.5–1.0 mg protein serum in 112.5 mM Tris–HCl, pH 8.0, was preincubated during 10 min. ADP or ATP was used as substrate, and the incubation was performed at 37 °C in a final volume of 200 μl during 40 min. The reaction was stopped by the addition of 200 μl 10% trichloroacetic acid (TCA). The amount of P_i liberated was measured by the method of Chan et al. (1986).

The reaction mixture containing AMP as substrate in 100 mM Tris–HCl, pH 7.5, was incubated with 0.5–1.0 mg protein serum at 37 °C in a final volume of 200 ml. All other procedures were the same as described above for ATP and ADP hydrolysis.

For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after addition of TCA were used to correct nonenzymatic hydrolysis of the substrates. Enzyme activities were expressed as nanomoles of phosphate released per minute per milligram of protein.

2.5. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard.

2.6. Statistical analysis

Data were expressed as mean ± S.E.M. Groups were compared using Student's *t* test. Differences between experi-

mental and control groups were considered significant for $P < .05$.

3. Results

3.1. Effect of stress on ATP-ADPase activities in blood serum of rats

The hydrolysis of ATP and ADP were evaluated in the blood serum at 0, 6, 12 and 48 h after the stress procedure. When stressed animals were compared to the control group, ATPase and ADPase activities were significantly increased 24 h (58% and 54%, respectively, when compared with controls) after exposure to stress (Fig. 1; Student's t test, $P < .001$, and Student's t test, $P < .05$, respectively).

3.2. Effect of stress on 5′-nucleotidase activity in blood serum of rats

The hydrolysis of AMP was evaluated in the blood serum at 0, 6, 24 and 48 h after stress procedure. As shown in Fig. 2, 5′-nucleotidase activity was increased at 6 h (68%, Student's t test, $P < .02$) and 24 h (95%, Student's t test, $P < .005$) in the stressed group, always when compared to the control group.

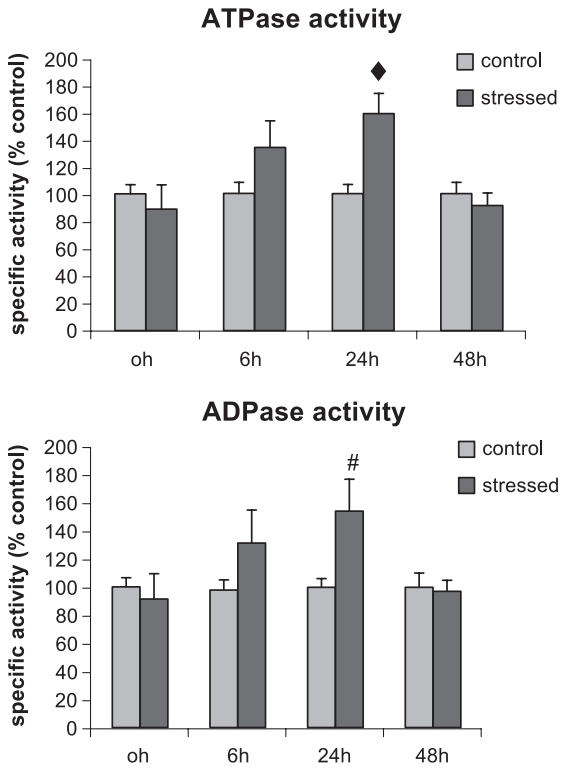

Fig. 1. Effect of acute stress on ATPase and ADPase activities in blood serum. Values are expressed as mean ± S.E.M. specific activity (nmoles of phosphate produced/mg protein picomoles), considering the values of controls as 100% (absolute value for control groups: 0.92 ± 0.06 for ATPase and 0.99 ± 0.06 for ADPase activities). Number of animals per group = 6–11. #, significantly different from control group (Student's t test, $P < .05$). ◆, significantly different from control group (Student's t test, $P < .001$).

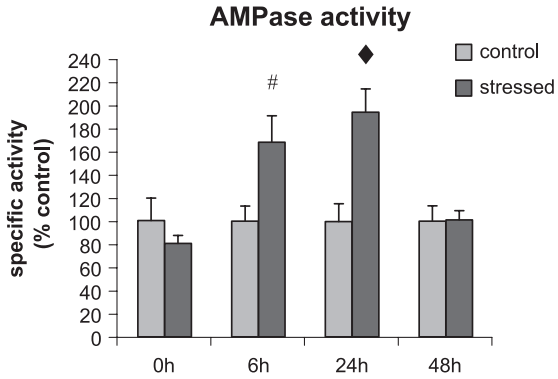

Fig. 2. Effect of acute stress on the 5′-nucleotidase activity in blood serum. Values are mean ± S.E.M. specific activity (nmoles of phosphate produced/mg protein), considering the values of controls as 100% (absolute value for control group: 0.05 ± 0.11). Number of animals per group = 5–8. #, significantly different from control group (Student's t test, $P < .02$). ◆, significant difference from the ratio in control group (Student's t test, $P < .005$).

4. Discussion

In the present study, ATPase, ADPase and 5′-nucleotidase activities in the blood serum were increased by acute restraint stress. A previous work showed that repeatedly stressed male rats present a decreased ADP hydrolysis in synaptosomes from the spinal cord (Torres et al., 2002a), with no effect when acute stress was utilized. Additionally, we also showed that only hydrolysis of ADP was altered in the blood serum of repeatedly stressed male rats (Torres et al., 2002b). Two points should be considered—that different models were used and especially that repeated restraint could lead to a process of adaptation that may cause different effects compared to those observed after acute stress. Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single stress episode do (Hashiguchi et al., 1997; Torres et al., 2001), and this phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes.

Nucleotidases have been shown to act in blood serum (Kaczmarek et al., 1996; Torres et al., 2002b). NTPDase 1 is associated with the surface of endothelial cells and smooth muscles, being located in their luminal surface (for a review, see Zimmermann, 2001). The enzymes whose activity was observed to be altered by restraint stress in the present study could be the soluble ones and/or those released from membranes, since they were measured in serum in vitro. However, considering the altered hydrolysis of nucleotides in serum after stress, when both ATP and ADP hydrolysis were increased with no change of ATPase/ADPase ratio, it is possible to consider that another soluble enzyme is involved in these alterations but not the E-NTPDase 5 and E-

NTPDase-6 because these enzymes have high preference for NDPs over NTPs as substrates (Mulero et al., 1999; Zimmermann, 2001). Confirming our proposal, other studies have suggested that nucleotidases released during shear stress may have an extracellular origin (Yegutkin et al., 2000), such as the membrane of cells.

The present results reinforce the possibility of one action site for both substrates in the serum enzyme (Oses et al., submitted for publication). In addition, another nucleotide-hydrolyzing enzyme has been reported to be present in serum, the 5′-nucleotide phosphodiesterase (PDEase), which is capable of hydrolyzing both nucleotides, ATP and ADP (Sakura et al., 1998). In this work, we do not discard the possibility of the involvement of a phosphodiesterase because we did not evaluate this activity using a specific substrate for this enzyme.

Circulating nucleotides are known to be important signaling molecules, potentiating a variety of physiological responses (Brake and Julius, 1996). In blood serum, adenine nucleotides have been implicated in several functions. ATP has been suggested to play a role in vascular tone, cardiac function and renal epithelial transport (Ravelic, 2000), and adenosine has been used clinically as an antiarrhythmic agent or vasodilatator. In addition, another function of extracellular adenine nucleotides is ADP-induced platelet aggregation (Hoylaerts et al., 2000). ADP is a potent platelet-recruiting factor and induces platelet aggregation via interaction with two P2 platelet receptors. ADP induces not only platelet shape change, exposure of fibrinogen binding sites and aggregation but also the influx and intracellular mobilization of Ca^{2+}. Hydrolysis of ADP by nucleotidases present in the serum inhibits platelet aggregation by removing ADP and forming adenosine, which, besides other effects, inhibits platelet aggregation (Zimmermann, 1999). On the other hand, stress is known to trigger a hypercoagulable state, probably mediated by plasma catecholamine activity (Von Känel et al., 2002). In this sense, the response observed in the present study with serum after restraint stress may contribute to reduce these procoagulant effects, being a protective mechanism against acute coronary thrombosis and atherosclerosis development. Soluble CD39 (SolCD39), for example, improves cerebral blood flow and reduces cerebral infarct volume when given preoperatively (Pinsky et al., 2002). However, it is important to consider that repeated restraint lead to opposite effects, with decreased ADP hydrolysis in blood serum (Torres et al., 2002b). In this context, the alterations of ADP concentration in serum of stressed animals, showing increase with repeated stress and decrease with acute stress, may indicate the role of this factor in the etiology of cardiovascular diseases.

The ATPase/ADPase ratio may have an important role in the signaling properties of ATP (Chen and Guidotti, 2001). When an E-NTPDase is active, extracellular ATP is converted to AMP and then to adenosine by the action of a 5′-nucleotidase, and ADP is not an appreciable product. Because 5′-nucleotidase is also increased after restraint stress, the resulting effect is a decrease in one signal evoked by ATP and an increase in another signal evoked by adenosine. In addition, our results suggest that these enhanced adenosine levels may remain high in serum several hours after exposure to stress.

The reaction catalyzed by 5′-nucleotidase is the rate-limiting step in this extracellular pathway from ATP to adenosine (for a review, see Cunha and Ribeiro, 2000). It is important to observe that this enzyme is inhibited by ATP and/or ADP (Cunha and Sebastião, 1991). Therefore, only when ATP and ADP levels decrease below the threshold of inhibition of 5′-nucleotidase will an important amount of adenosine be formed. In this context, it is important to consider that in the present study, the hydrolysis of ATP and ADP were also increased after stress, giving the possibility to 5′-nucleotidase to act, producing adenosine.

Several biological and mechanical stressors may induce endogenous self-protective mechanisms to avoid cellular injury. For example, adenosine may act as an endogenous cardioprotective substance in pathophysiological conditions of the heart, such as ischemia (for a review, see Kitazake et al., 1999). Adenosine release during ischemia is beneficial by proving receptor-mediated vasodilatory protection and has also been shown to mediate ischemic preconditioning (Downey et al., 1993). Both ecto-5′-nucleotidase activity and adenosine levels are increased in blood and in myocardium in patients with chronic heart failure (for a review, see Kitazake et al., 1999). These reports suggest adenosine as a protective factor after stress situations.

In conclusion, ATPase, ADPase and 5′-nucleotidase activities in blood serum were altered in stressed male rats. These effects may represent a protective mechanism against some of the stress effects. It is tempting to propose that the altered ectonucleotidase activities in serum may be a biochemical marker for stress situations.

Acknowledgements

This work was supported by PRONEX, CAPES and National Research Council of Brazil (CNPq).

References

Agteresch HJ, Dagnelie PC, van den Berg JW, Wilson JL. Adenosine triphosphate: established and potential clinical applications. Drugs 1999;58:211–32.

Bodin P, Burnstock G. Evidence that of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. J Cardiovasc Pharmacol 2001;38:900–8.

Bradford MMA. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:218–51.

Brake AJ, Julius D. Signaling by extracellular nucleotides. Annu Rev Cell Dev Biol 1996;12:519–41.

Chan K, Delfert D, Junger KD. A direct colorimetric assay for Ca^{2+}-ATPase activity. Anal Biochem 1986;157:375–80.

Chen W, Guidotti G. Soluble apyrases release ADP during ATP hydrolysis. Biochem Biophys Res Commun 2001;282:90–5.

Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in nervous system: different roles, different sources and different receptors. Neurochem Int 2001;38:107–25.

Cunha RA, Ribeiro JA. ATP as a presynaptic modulator. Life Sci 2000; 68:119–37.

Cunha RA, Sebastião AM. Extracellular metabolism of adenosine nucleotides and adenosine in the innervated skeletal muscle of the frog. Eur J Pharmacol 1991;197:83–92.

Downey JM, Liu GS, Thorton JD. Adenosine and the anti-infart effects of preconditioning. Cardiovasc Res 1993;27:3–8.

Ely DR, Dapper V, Marasca J, Correa JB, Gamaro GD, Xavier MH, et al. Effects of restraint stress on feeding behavior of rats. Physiol Behav 1997;61:395–8.

Hashiguchi H, Ye HSH, Morris M, Alexander N. Single and repeated environmental stress: effect on plasma oxytocin, corticosterone, catecholamines and behavior. Physiol Behav 1997;61:731–6.

Hoylaerts MF, Oury C, Toth-Zsamboki E, Vermylen J. ADP receptors in platelet activation and aggregation. Platelets 2000;11:307–9.

Kaczmarek E, Koziak K, Sévigny J, Siegel JB, Anrather J, Beaudoin AR, et al. Identification and characterization of CD39/vascular ATP diphosphohydrolase. J Biol Chem 1996;271:33116–22.

Kitazake M, Minamino T, Node K, Takashima S, Funaya H, Kuzuya T, et al. Adenosine and cardioprotection in diseased heart. Jpn Circ J 1999;63: 231–43.

Latini S, Pedata F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. J Neurochem 2001;79:463–84.

Minor TR, Rowe MK, Soames Job RF, Ferguson EC. Escape deficits induced by inescapable shock an metabolic stress are reversed by adenosine receptor antagonist. Behav Brain Res 2001;200–12.

Mulero JJ, Yeung G, Nelken ST, Ford JE. CD39-L4 is a secreted human apyrase, specific for the hydrolysis of nucleoside diphosphates. J Biol Chem 1999;274:20064–7.

Oses JP, Cardoso CM, Germano RA, Kirst IB, Rücker B, Fürstenau CR, Wink MR, Bonan CD, Battastini AMO, Sarkis JJF. Soluble NTPDase: an additional system of nucleotide hydrolysis in rat blood serum. Life Sci [submitted for publication].

Pereira GS, Mello e Souza T, Battastini AMO, Izquierdo I, Sarkis JJF, Bonan CD. Effects of inhibitory avoidance training and /or isolated foot-shock on ectonucleotidase activities in synaptosomes of the anterior and prosterior cingulated cortex and the medial precentral area of adult rats. Behav Brain Res 2002;128:121–7.

Pinsky DJ, Broekman MJ, Peschon JJ, Stocking KL, Fujita T, Ramasamy R, et al. Elucidation of the thromboregulatory role of CD39/ectoapyrase in the ischemic brain. J Clin Invest 2002;109:1031–40.

Ravelic V. P2 receptors in the central and peripheral nervous systems moduling sympathetic vasomotor tone. J Auton Nerv Syst 2000;81: 205–11.

Sakura H, Nagashima S, Nagashima A, Maeda M. Characterization of fetal serum 5′-nucleotide phosphodiesterase: a novel function as a platelet aggregation inhibitor in fetal circulation. Thromb Res 1998; 91:83–9.

Sapolsky RM. An introduction to the adrenocortical axis. In: Sapolsky RM, editor. Stress, the aging brain, and the mechanisms of neuron death. Cambridge: Bradford; 1992. p. 11–27.

Svenningsson P, Fredholm BB. Glucocorticoids regulate the expression of A1 but not A (2A) receptors in rat brain. J Pharmacol Exp Ther 1997; 280:1094–101.

Todorov LD, Mihaylova-Todorov S, Westfall TD, Sneddon O, Kennedy C, Bjur RA, et al. Nature 1997;387:76–9.

Torres ILS, Gamaro GD, Silveira-Cucco SN, Michalowski MB, Corrêa JB, Perry MLS, et al. Effect of acute and repeated restraint stress on glucose oxidation to CO_2 in hippocampal and cerebral cortex slices. Bras J Med Biol Res 2001;34:111–6.

Torres ILS, Buffon A, Silveira PP, Duarte MSZ, Bassani MG, Oliveira SS, et al. Effect of chronic and acute stress on ecto-nucleotidase activities in spinal cord. Physiol Behav 2002a;75:1–5.

Torres ILS, Buffon A, Dantas G, Fürstenau CR, Böhmer AE, Battastini AMO, et al. Chronic stress effects on adenine nucleotides hydrolysis in blood serum and brain structures of rats. Pharmacol Biochem Behav 2002b;74:181–6.

Von Känel R, Mills PJ, Ziegler MG, Dimsdale JE. Effect of [beta]2-adrenergic receptor functioning and increased norepinephrine on the hypercoagulable state with mental stress. Am Heart J 2002;144:68–72.

Yegutkin GG. Kinetic analysis of enzymatic hydrolysis of ATP in human and rat blood serum. Biochemistry (Moscow) 1997;62:619–22.

Yegutkin GG, Bodin P, Burnstock G. Effect of shear stress on the release of soluble ecto-enzymes ATPase and 5′-nucleotidase along with endogenous ATP from vascular endothelial cells. Br J Pharmacol 2000;129: 921–6.

Zimmermann H. Nucleotides and CD39: principal modulatory players and thrombosis. Nat Med 1999;5:987–8.

Zimmermann H. Ectonucleotidases: some recent developments and note on nomenclature. Drug Dev Res 2001;52:44–56.