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Dexamethasone, but not stress, induce measurable changes of mitochondrial benzodiazepine receptor mRNA levels in rats

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Abstract

The expression of the mitochondrial benzodiazepine receptor gene was assayed by a semi-quantitative non-radioactive reverse transcriptase polymerase chain reaction (RT-PCR) assay. The level of amplified mitochondrial benzodiazepine receptor mRNA was expressed as a ratio of either glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin mRNA co-amplified in the same RT-PCR assay. The relative amounts of mitochondrial benzodiazepine receptor RNA in several rat tissues were found to be similar to the previously reported relative amount of mitochondrial benzodiazepine receptor binding sites. The level of these binding sites has also been reported to be altered by stress stimuli. In this study we specifically measured the effect of stress on the mRNA levels of the mitochondrial benzodiazepine receptor as an alternative method to the binding assay in an attempt to understand the mechanism by which stress alters binding. Sprague-Dawley male rats were either forced to swim for 15 min in 18°C water or restrained in a plastic cylinder for 45 min either once, or twice daily for 7 days. Neither the swim stress, nor acute or chronic restraint stress, caused a measurable statistically significant relative change in mitochondrial benzodiazepine receptor mRNA in the adrenal gland, kidney, testis and olfactory bulb. However, daily treatment of rats for 7 days with 4 mg/kg of dexamethasone caused a significant decrease in mitochondrial benzodiazepine receptor gene expression in adrenal glands. This finding and the measurement of the relative levels of mitochondrial benzodiazepine receptor mRNA in the various tissues indicate that mitochondrial benzodiazepine receptor density is regulated to some extent at the gene expression level. However, the lack of detectable stress-induced changes in mRNA levels for this receptor seem to indicate that either mRNA changes were below detectable levels or that other mechanisms may be involved in the previously reported stress-induced changes of mitochondrial benzodiazepine receptor density. Because the focus of this work was on the regulation of mitochondrial benzodiazepine receptor gene expression, ligand binding studies to determine changes in receptor densities were not performed. © 1997 Elsevier Science B.V.

Keywords: Peripheral-type benzodiazepine receptor; RT-PCR (reverse transcriptase-polymerase chain reaction); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); Stress; Dexamethasone

1. Introduction

The mitochondrial or peripheral-type benzodiazepine receptor is an integral membrane protein localized predominantly on the outer mitochondrial membrane (reviewed in Verma and Snyder, 1989; Weizman and Gavish, 1993; Parola et al., 1993; Krueger, 1995). The best characterized subunit of this receptor is an 18 kDa polypeptide responsible for the binding of selective benzodiazepines (e.g., Ro5-4864) and the isoquinoline derivatives PK11195 and PK14104 (Sprengel et al., 1989). The amino acid sequence of the 18 kDa subunit is highly conserved among various species, but has shown no significant homology with any other known mammalian protein (Krueger, 1995). Other polypeptides appear to be associated with the 18 kDa subunit; these are a 10 kDa (PK10) subunit photolabelled by PK14104 (Blahos et al., 1995), a 32–34 kDa subunit photolabelled by flunitrazepam (Snyder et al.,

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1987), the voltage dependent anion channel and the ATP/ADP carrier (McEnery et al., 1992). The nature of the association of these proteins with the 18 kDa subunit and their contribution to the function of the mitochondrial benzodiazepine receptor is, however, still not fully elucidated (Krueger, 1995).

Functionally, the mitochondrial benzodiazepine receptor has been associated with steroidogenesis. In particular, it has been shown that the mitochondrial benzodiazepine receptor regulates the transport of cholesterol from the outer to the inner mitochondrial membrane, which is the rate limiting step of the synthesis of pregnenolone, the precursor of all other steroids (Krueger and Papadopoulos, 1992). This role of the mitochondrial benzodiazepine receptor is reflected in its tissue distribution with the highest densities in steroidogenic tissues such as adrenal gland and testis (DeSouza et al., 1985). However, these receptors are also present in other tissues such as lung, kidney, pineal gland and lymphocytes (Weissman et al., 1984; Quirion, 1984; Drugan et al., 1988; Canat et al., 1993a). In lymphocytes, mitochondrial benzodiazepine receptors are also found on the plasma membrane reinforcing the idea that the mitochondrial benzodiazepine receptor may have multiple functions. In brain, mitochondrial benzodiazepine receptors are found at the highest density in the olfactory bulb (Benavides et al., 1983) and are predominantly expressed in glial cells (Syapin and Skolnick, 1979), which have been shown to produce neurosteroids (Jung-Testas et al., 1989). In addition to the mitochondrial benzodiazepine receptors, the steroidogenic acute regulatory protein (Lin et al., 1995) has been shown to have a similar role in steroidogenesis. The roles played by these two proteins is at the moment not fully understood.

Relatively little is known about the mechanisms regulating the expression of the mitochondrial benzodiazepine receptor. The rat mitochondrial benzodiazepine receptor gene promoter lacks a TATA or CAAT box and within the first 500 base pairs, with the exception of three CG repetitive sequences, it contains no classical regulatory transcription elements such as cyclic-AMP or glucocorticoid responsive elements (Casalotti et al., 1992). However, two positive and one negative putative transcription regulatory sites have been identified by in vitro studies (Oberto et al., 1995). It is thus possible that the level of mitochondrial benzodiazepine receptor protein may be regulated at the gene expression level. To our knowledge, mitochondrial benzodiazepine receptor mRNA expression has been investigated either only qualitatively (Sprengel et al., 1989) or quantitatively, but limited to peripheral blood cells (Canat et al., 1993a,b). These studies have indicated a good but not absolute correlation between mitochondrial benzodiazepine receptor mRNA and protein level. Conversely, there have been many studies that have shown that the level of [^{3}H]PK11194 and [^{3}H]Ro5-4864 binding to the mitochondrial benzodiazepine receptor is under the influence of various external factors. Of particular interest is the effect of acute and chronic stress on mitochondrial benzodiazepine receptor binding. Acute cold swim stress in rats was shown to rapidly increase binding to the mitochondrial benzodiazepine receptor in kidney and olfactory bulb (Novas et al., 1987), inescapable tail shock increased binding in kidney (Drugan et al., 1988), while acute noise had similar effects on adrenal gland mitochondrial benzodiazepine receptor (Ferrarese et al., 1991). The response to stress was in most cases rapid (15–30 min) and tissue specific. Whereas the acute stress paradigm tended to increase mitochondrial benzodiazepine receptor binding, the chronic stress paradigm tended to have the opposite effect by suppressing binding levels in specific tissues. Prolonged cold exposure reduced mitochondrial benzodiazepine receptor binding in brown adipose tissue and kidney (Gonzales-Solveyra et al., 1988), while repeated tail shocks caused a decrease in mitochondrial benzodiazepine receptor binding in kidney, cortex, heart and pituitary (Drugan et al., 1988). In humans, students after examinations (Karp et al., 1989), parachutists after several jumps (Dar et al., 1991) and clinically defined anxious patients (Ferrarese et al., 1990) displayed altered levels of mitochondrial benzodiazepine receptor in peripheral blood cells and platelets.

Two major pathways may be implicated in the regulation of mitochondrial benzodiazepine receptor binding level, the hypothalamus–pituitary–adrenal axis and renin/angiotensin system (Drugan and Holmes, 1991). Additionally, it remains to be established if and to what extent stress modulates mitochondrial benzodiazepine receptor gene expression. In order to investigate this problem we have developed a reverse transcriptase-polymerase chain reaction (RT-PCR) assay to measure the relative levels of mitochondrial benzodiazepine receptor mRNA. Although RT-PCR involves an exponential amplification of the mRNA of interest, it is possible to obtain quantitative or semi-quantitative values of the endogenous amounts of specific mRNA levels provided that the necessary conditions are satisfied. (Duskas et al., 1993).

Given the already extensive literature on stress-induced changes in mitochondrial benzodiazepine receptors binding level, in this work we focused exclusively on the measurement of the mRNA levels for this receptor with the aim of both introducing a new technique that could facilitate the study of regulation of the expression of the mitochondrial benzodiazepine receptor and to investigate the mechanism by which stress influences this receptor. We report here that we could not detect any changes in mitochondrial benzodiazepine receptor mRNA level induced by three paradigms of acute and chronic stress stimuli, while we observed that chronic dexamethasone injection (4 mg/kg per day for 7 days) caused a significant decrease of mitochondrial benzodiazepine receptor mRNA levels in adrenal gland of treated animals as compared to saline-injected control animals. The latter finding and our confirmation of the differential tissue distribution of mitochondrial

benzodiazepine receptor mRNA indicates that, at least in part, mitochondrial benzodiazepine receptor density is regulated at the gene expression level.

2. Materials and methods

2.1. Chemicals

Fluorescein-12-dUTP, chemiluminescence reagents, nylon membrane, blocking reagent, radiographic film and anti-fluorescein antibodies were from DuPont (Wilmington, DE, USA). Avian myeloblastosis virus (AMV) reverse transcriptase and *Taq* polymerase were from Promega (Madison, WI, USA). Sprague-Dawley rats were obtained from the National Animal Center, Mahidol University (Nakorn Pathom, Thailand). All other chemicals were obtained from regular suppliers and were of analytical grade.

2.2. RNA preparation

Animals were killed at the indicated times after treatments. Testes, adrenal glands, kidneys and olfactory bulbs were dissected. For the kidney and testes the RNA was extracted from a transverse section of the respective organs while for the olfactory bulb and adrenal gland the whole organs were utilized. RNA was prepared essentially according to the method of Chomczynski and Sacchi (1987). Tissue samples (up to 200 mg) were homogenized in 1 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl, 0.1 M 2-mercaptoethanol) for 30 s by Polytron. Sequentially, 2 M sodium acetate (pH 4, 100 μl), water-equilibrated phenol (1 ml) and chloroform–isomyl alcohol mixture (25:1, 200 μl) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was vortexed for 20 s and placed on ice for 30 min. Samples were centrifuged at $10{,}000 \times g$ for 20 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 1 volume of isopropanol and placed at −70°C for at least 1 h to precipitate RNA. The solution was centrifuged at $14{,}000 \times g$ for 15 min, the supernatant was removed, the RNA pellet was dissolved in 300 μl of solution D and precipitated with 1 volume of isopropanol at −70°C for 1 h. After centrifugation for 10 min at 4°C, the RNA pellet was washed with 75% ethanol and further centrifuged. The majority of the ethanol was removed and the remainder allowed to evaporate for a few minutes. The RNA pellet was resuspended in 40 μl of DEPC (diethyl pyrocarbonate) treated water and stored at −70°C. The amount and relative purity of the extracted RNA was estimated by optical density (O.D.) at $\lambda = 260$ and 280 nm, where 1 O.D. at $\lambda = 260$ nm is equivalent to 40 mg/ml RNA and an O.D. ratio of 260/280 of 1.7–1.8 was considered indicative of acceptable purity.

2.3. RT-PCR

The RT-PCR master mixture contained $1 \times$ PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 25 pmol of primers coding for mitochondrial benzodiazepine receptor, GAPDH or ACTIN (except for olfactory bulb where 12.5 pmol GAPDH primer were used), 0.2 mM of dATP, dCTP, dGTP, 0.04 mM of dTTP and 0.4 μM of fluorescein-12-dUTP, 1.25 U of Taq DNA polymerase and 4 U of AMV reverse transcriptase in a total volume of 25 μl. Control tubes contained no RNA template to check for contamination. The mixture was incubated in the thermocycler for 30 min at 42°C for RT-catalyzed first-strand cDNA synthesis, followed by 25–27 amplification cycles in a Perkin Elmer 9600 thermocycler where each cycle consisted of 10 s at 94°C, 10 s at 58°C and 30 s at 72°C with a final 10 min extension step at 72°C. Primer pairs of mitochondrial benzodiazepine receptor, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were designed using Oligo 4 software and were synthesized on an ABI 392. Purity was checked by capillary electrophoresis. The sequence of the primers used were: mitochondrial benzodiazepine receptor-upper ATCTG GGGCA CACTG TATTC: mitochondrial benzodiazepine receptor-lower AAAGC AGGAT GACCT CAACC; GAPDH upper: AGGTC GGTGT CAACG GATTT, GAPDH-lower: CAGCA TCAAA GGTGG AGGAA; actin-upper CCCAG AGCAA GAGAG GCATC and actin-lower: CTCAG GAGGA GCAAT GATCT.

2.4. Detection of RT-PCR products

RT-PCR products were electrophoresed in 1.4% agarose gels and fixed to nylon membranes by downward capillary transfer and UV-light cross-linking. Membranes were rinsed in wash buffer (0.15 M NaCl, 0.1 M Tris HCl, pH 7.5) and blocked in blocking reagent (0.5% w/v) for 1 h at room temperature. The membranes were incubated with anti-fluorescein antibodies (1:1000 in blocking reagent) and washed 4×5 min with wash buffer. The chemiluminescence reagent, was added over the whole surface of the membrane and incubated for 1 min. Excess reagent was gently blotted away and the damp membrane was placed between a plastic sheet and immediately exposed to film for 15 min to 2 h at room temperature. The film image was acquired with a flat-bed scanner using Photoshop (Adobe System) and the density of the bands analyzed by NIH program (freeware, National Institutes of Health, Bethesda, MD, USA).

2.5. Animal treatments

Cold swim stress: male Sprague-Dawley rats (approx. 250 g) were forced to swim in an 18°C water tank for 15

min and either killed immediately or following a 30 min recovery period. Tissues were dissected and stored at −70°C until used. Control animals were removed from cages and immediately killed. All animals were previously handled daily for at least three days. The handling consisted in transporting the animals from the animal room to the experimental room, holding the rats for about 1 min and returning them to the cage and to the animal room.

Restrain stress: male Sprague-Dawley rats (approx. 250 g) which had been pre-handled as described above were placed in a perforated cylindrical clear plastic tube for 45 min for either one session (acute) or for 2 daily sessions (8 a.m. and 6 p.m.) for 7 days (chronic). Animals were killed immediately after the acute session (8.45 a.m.) or 14 h (8.45 a.m.) after the last chronic treatment. Tissues were dissected and stored at −70°C. Control animals were handled daily for 7 days and killed.

Dexamethasone treatment: male Sprague-Dawley rats (approx. 250 g) were handled daily for a week and injected subcutaneously daily for 7 days with either saline or dexamethasone (4 mg/kg). Animals were killed 24 h after the last dexamethasone treatment, tissues were dissected and stored at −70°C.

All animal treatments were carried out in accordance to the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, National Institutes of Health, Bethesda, MD, USA).

2.6. Statistical analysis

Data were analyzed by Student's *t*-test for independent means. Results are expressed as means ± S.E.M. throughout the study.

3. Results

3.1. Evaluation of the non-radioactive single-tube RT-PCR technique

The non-radioactive single-tube RT-PCR technique used was designed to yield quantification of mitochondrial benzodiazepine receptor RNA from small tissue samples relative to an internal standard. The reaction parameters were adjusted to obtain a linear relationship between number of PCR cycles and RT-PCR products (not shown) and between initial amount of RNA and RT-PCR products (Fig. 1). It can be seen that for both mitochondrial benzodiazepine receptor and GAPDH a linear increase in RT-PCR products was measured by increasing the initial RNA content from 0.05 to 0.5 mg, while the ratio between the two amplified products remained nearly constant (Fig. 1). The same was observed for the amplification of β-actin mRNAs (not shown). Variation within triplicate measurements was not greater than 15%.

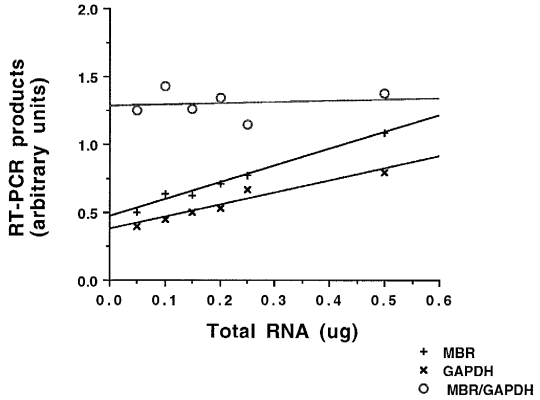

Fig. 1. Mitochondrial benzodiazepine receptor (+) and GAPDH (×) RT-PCR products and their ratio (○) co-amplified from kidney RNA. Values are expressed in arbitrary units of optical density of the bands on the photographic film following chemiluminescence detection of the RT-PCR products. Each point is the average of triplicate measurements, where the maximum variation observed for each point was less than 15% (error bars are omitted for clarity). The lines were drawn by linear regression of the scatter points. The *r* values for mitochondrial benzodiazepine receptor and GAPDH linear regressions were 0.98 and 0.93, respectively.

3.2. Relative expression of mitochondrial benzodiazepine receptor gene in different organs

Amplification from an equivalent amount of mRNA, as measured by optical density at $\lambda = 260$, from different tissues of control animals indicated that the mitochondrial benzodiazepine receptor gene is expressed at different levels in various tissues (Fig. 2) and that the relative amounts of mitochondrial benzodiazepine receptor mRNA is in good correlation to known amounts of mitochondrial benzodiazepine receptor binding sites in the same tissues (Anholt, 1986). For this experiment, the mitochondrial benzodiazepine receptor mRNA values, expressed as per-

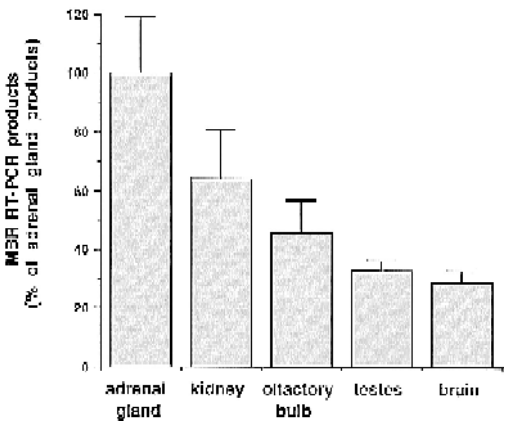

Fig. 2. RT-PCR products of mitochondrial benzodiazepine receptor mRNA. Values are expressed as percentage of adrenal gland RT-PCR products. Tissues were dissected as described, brain was an aliquot of a total brain homogenate. Values are averages of 2 measurements from each tissue repeated for 5 different animals. Equal amounts of RNA (0.3 μg) was used for each sample. Error bars indicate S.E.M.

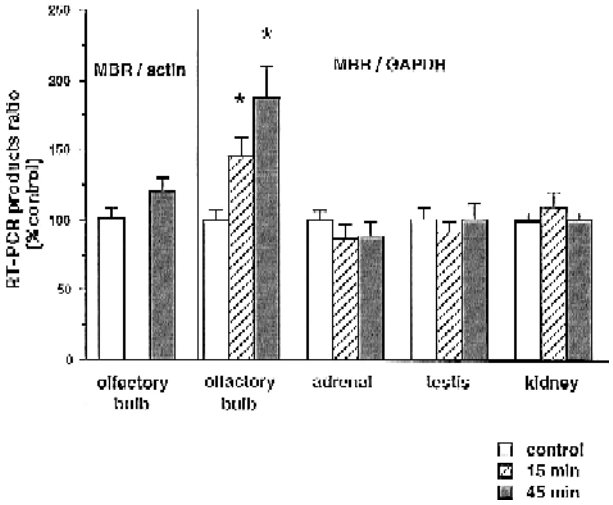

Fig. 3. RT-PCR products of mitochondrial benzodiazepine receptor mRNA from tissues of rats subjected to forced swim stress expressed as ratio of actin or GAPDH RT-PCR products. Measurements from rats killed 15 min (hatched columns) or 45 min (shaded columns) after initiation of the 15 min swim stress are expressed as percentage of control animals (open columns). * Statistically different from controls of the same tissue, Student's *t*-test, $P < 0.05$, $n = 5–8$.

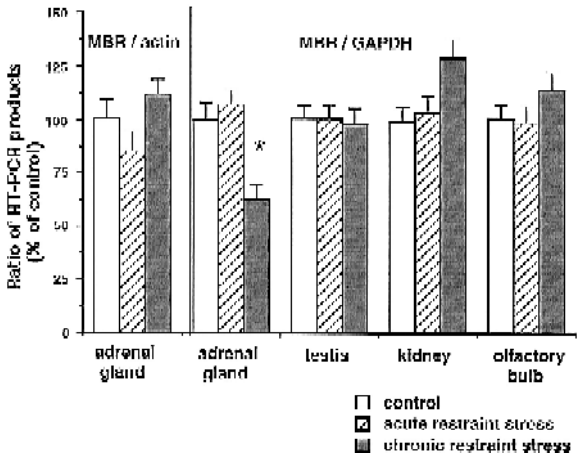

Fig. 4. RT-PCR products of mitochondrial benzodiazepine receptor RNA from tissues of rats subjected to restraint stress expressed as a ratio of actin or GAPDH RT-PCR products. Measurements from rats sacrificed immediately after acute treatment (hatched columns) or 14 h after chronic treatment (shaded columns) are expressed as percentage of control animals of each group (open columns). * Statistically different from control of the same tissue, Student *t*-test, $P < 0.05$, $n = 5–8$.

centage of adrenal gland levels, were calculated from the absolute values of RT-PCR products as opposed to as a ratio of an internal standard because both standards, actin and GAPDH, are differentially expressed in the various tissues.

3.3. Effect of experimentally induced stress on mitochondrial benzodiazepine receptor gene expression

When rats were subjected to swim stress and compared to control animals no significant difference in the level of mitochondrial benzodiazepine receptor mRNA was observed in any of the tissues tested with the apparent exception of the olfactory bulb when mitochondrial benzodiazepine receptor mRNA was expressed as mitochondrial benzodiazepine receptor/GAPDH RT-PCR products (Fig. 3). However, analysis of the absolute values of mitochondrial benzodiazepine receptor and GAPDH RT-PCR products (Table 1) for these tissues indicate that a change in expression had occurred in GAPDH rather than mitochondrial benzodiazepine receptor mRNA levels. This was confirmed by measurement of actin mRNA where neither the values of actin, mitochondrial benzodiazepine receptor or mitochondrial benzodiazepine receptor/actin were sig-

Table 1
Measurement of RNA levels for GAPDH, actin and mitochondrial benzodiazepine receptor by RT-PCR

Stress paradigm [a] (tissue) [b]	RT-PCR products (% of control animals) [c]					
	GAPDH	Actin	MBR	MBR [e]	MBR GAPDH	MBR actin
Swim stress, 15 min [f] (olfactory bulb)	85.3 ± 17.8 [i]	ND	105.3 ± 11.7	ND	145.6 ± 13.8 [i]	ND
Swim stress, 45 min [f] (olfactory bulb)	49.5 ± 10.3 [i]	100.3 ± 1.2	91.1 ± 15.2	120.4 ± 8.1	187.3 ± 22.9 [i]	120.5 ± 10.1
Restraint stress, acute [g] (adrenal gland)	81.6 ± 5.54	98.6 ± 2.8	96.2 ± 3.1	82.3 ± 4.3	107.3 ± 6.5	85.2 ± 9.5
Restraint stress, chronic [g] (adrenal gland)	135.4 ± 10.4 [i]	88.6 ± 6.4	102.6 ± 2.7	93.9 ± 9.2	62.9 ± 6.5 [i]	111.6 ± 7.5
Dexamethasone injection [h] (adrenal gland)	83.5 ± 12.6	85.6 ± 13	61.8 ± 13.2 [i]	67.9 ± 12.6 [i]	63.7 ± 5.5 [i]	72.8 ± 11.5 [i]

[a] Male Sprague-Dawley's were subject to treatments that either induce or mimic stress, as described in Section 2.
[b] For each treatment, the adrenal gland, kidney, testes and olfactory bulb were tested, only selected results are reported here. All other tissues showed non-statistically significant changes (Student *t*-test, $P > 0.05$).
[c] Data are expressed as percentage of the value obtained in the control group of each experiment as described in Section 2 (Average ± S.E.M., $n = 5–8$).
[d] Relative mitochondrial benzodiazepine receptor (MBR) values obtained from experiments of co-amplification with GAPDH.
[e] Relative mitochondrial benzodiazepine receptor (MBR) values obtained from experiments of co-amplification with actin.
[f] Time animals were killed following initiation of swim stress. All animals were forced to swim for 15 min.
[g] Restraint was carried out as described, once (acute) and the animals were immediately killed, or twice daily for 7 days (chronic) and the animals were killed 14 h after the last restraint.
[h] Dexamethasone (4 mg/kg) was injected subcutaneously daily for 7 days and the animals were killed 24 h after the last injection.
[i] Statistically different from the control animals of the respective treatment (Student *t*-test, $P < 0.05$).
ND, not determined.

nificantly changed in olfactory bulb of stressed animals as compared to control animals (Fig. 3, Table 1).

Both acute and chronic restraint stress also failed to produce measurable changes in relative amounts of mitochondrial benzodiazepine receptor mRNA (Fig. 4) Again, we observed an unexpected change in GAPDH expression in the adrenal gland of restraint-stressed rats and amplification of actin mRNA confirmed that no real changes had occurred in mitochondrial benzodiazepine receptor gene expression in any of the tissues studied. Analysis of absolute values of mitochondrial benzodiazepine receptor, actin and GAPDH and their ratios confirm this observation (Table 1).

3.4. Effect of dexamethasone injections on mitochondrial benzodiazepine receptor gene expression

To approximate the effect of stress rats were injected with dexamethasone (4 mg/kg) daily for 7 days. This treatment had an effect on the body weight of the animals. The weight of saline injected rats increased from an average of 220 ± 7.1 to 246 ± 5.8 g over the 7 days, in contrast, the weight of dexamethasone-treated rats decreased from 216 ± 6.1 g to 176 ± 2.9 g. Chronic injection of dexamethasone caused a significant decrease of relative levels of mitochondrial benzodiazepine receptor mRNA in the adrenal gland of treated rats as compared to saline injected animals (Fig. 5). To confirm this result we also amplified actin mRNA from adrenal glands of dexamethasone treated rats and we observed a relative decrease of mitochondrial benzodiazepine receptor/actin (Fig. 5) while the absolute values of neither GAPDH nor actin were changed (Table 1).

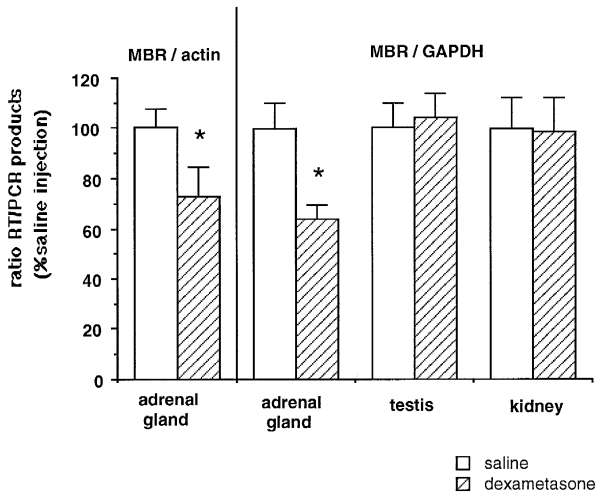

Fig. 5. RT-PCR products of mitochondrial benzodiazepine receptor RNA from tissues of dexamethasone-injected rats (4 mg/kg) expressed as a ratio actin or GAPDH RT-PCR products. Measurements from rats sacrificed 24 h after the last dexamethasone injection (hatch columns) are expressed as percentage of saline injected animals (open columns). * Statistically different from control of same tissue, Student *t*-test, $P < 0.05$, $n = 5–8$.

4. Discussion

Our study indicates that dexamethasone can alter mitochondrial benzodiazepine receptor gene expression while three stress paradigms did not produce statistically significant changes. The RT-PCR technique described here was designed to measure relative amounts of specific mRNA from small tissue samples without the use radioactive materials. The quantitation of the RT-PCR products via antibody overlay and chemiluminescence reaction does involve additional steps as compared to radioactive nucleotide incorporation or ethidium bromide staining. However, radioactive nucleotides are more hazardous, labile and expensive, while ethidium bromide staining is not as sensitive. We routinely stained all gels with ethidium bromide and for some experiments RT-PCR products were not visible by ethidium bromide but yielded measurable bands by chemiluminescence detection. A major advantage of the described RT-PCR protocol is that it allows use of small amount of RNA and a low number of PCR cycles. This ensures a linear relationship between initial template RNA and final RT-PCR product over a 10 fold range of mRNA concentration (Fig. 1). During the course of this study Kell et al. (1995) described a similar technique which also demonstrated the feasibility of chemiluminescence-RT-PCR for measuring relative amounts of mRNAs. A common limitation of both the technique described here and that of Kell et al. (1995), is that we did not observe a 1:1 relationship between increase of RNA and increase of RT-PCR product (Fig. 1). This is probably due to both limits of PCR amplification efficiency and the sensitivity of the photographic films. The implication of this problem is that it limits the sensitivity of the technique for detecting small changes of mRNA levels. Because a doubling in initial amount of RNA resulted in only a 30–40% increase in RT-PCR products, it follows that less than a 50% change would not be reliably detected as we observed experimental variation in sample measurements up to 15% (data not shown). In this respect although RT-PCR allows work with much smaller amounts of RNA it is not more sensitive than other techniques such as Northern blot or RNAse protection assay in detecting small quantitative changes in specific mRNA species. However, the fact that we observed only up to 15% variation among triplicate measurements indicates that the technique is suitable for semi-quantitative studies.

The unexpected observed changes in the house-keeping gene GAPDH in olfactory bulbs of swim-stressed animals and adrenal glands of restraint-stressed animals reinforces the importance of critically analyzing any apparent observed changes of a mRNA species expressed as a ratio of an internal control. GAPDH is widely used as an internal control for gene expression studies (Apanakis, 1993) as its expression is unaffected by many treatments including glucocorticoids (Fukumoto et al., 1992). However, insulin has been reported to alter GAPDH expression (Alexander-

Bridges et al., 1992). In this work we have observed GAPDH mRNA changes in GAPDH only in two tissues, olfactory bulb and adrenal under different experimental conditions, and it would be difficult to attribute the change to the action of insulin. There is an unpredictable risk in using internal standards for any biological study. Accordingly, in this work we have examined the absolute values of each amplification product to test whether they were affected by the treatment given and additionally expressed mitochondrial benzodiazepine receptor mRNA level both as a ratio of GAPDH and of actin mRNA.

Measurement of mitochondrial benzodiazepine receptor RNA in various tissues indicated that the adrenal gland contained the highest amount, followed by kidney, olfactory bulb, testis and brain. With the exception of the lower than expected level of testis mitochondrial benzodiazepine receptor mRNA, the relative distribution of mitochondrial benzodiazepine receptor mRNA correlates well with the known distribution of mitochondrial benzodiazepine receptor binding sites (Anholt, 1986; DeSouza et al., 1985). It must be realized that because both actin and GAPDH are differentially expressed in the tissues studied the reported values for mitochondrial benzodiazepine receptor mRNA are not corrected for an internal standard and thus could be subject to error in the calculation of the initial amount of RNA.

Neither the acute nor the chronic stressors employed here were able to induce measurable changes in mitochondrial benzodiazepine receptor mRNA in the tissues studied. This is in apparent contrast to several studies on ligand binding to mitochondrial benzodiazepine receptor where acute stress caused an increase and chronic stress a decrease in binding to mitochondrial benzodiazepine receptor in specific tissues. Of particular relevance is the report of Novas et al. (1987) which described a decrease in mitochondrial benzodiazepine receptor binding in rat olfactory bulb and kidney following cold swim stress. In the work of Novas et al. (1987) the rats were not previously handled like in our study. The rationale of habituating the animals to handling is to reduce stress response induced by the handling on the day of the experiment. Biggio et al. (1981) for example, have reported a difference in gamma aminobutyric acid binding in some rat brain areas following chronic prehandling and concluded that the differences in receptor density was caused by the stress of the handling at the time of killing in the non-handling-habituated rats, which argues in favor of prehandling the rats. Incidentally in a previous experiment in which we had not prehandled the rat we also did not observe a swim-stress induced change in mitochondrial benzodiazepine receptor mRNA (results not shown). It would however be of interest to determine whether handling itself alters the mRNA expression and/or mRNA levels of this receptor. Post-transcriptional mechanisms of receptor regulation, such as conformational changes or phosphorylation could be proposed to explain changes in receptor density, especially in the case of acute stress where changes in binding were reported to occur within 15–30 min (Novas et al., 1987, Ferrarese et al., 1991). It has been reported that mitochondrial benzodiazepine receptors undergo affinity changes within seconds of hormone stimulation (Boujard et al., 1994). The mitochondrial benzodiazepine receptor has been shown to contain phosphorylation sites and to undergo phosphorylation in vitro, however the role of phosphorylation in regulation of mitochondrial benzodiazepine receptor function has not yet been clarified (Whalin et al., 1994). Additionally, it could be speculated that chronic stress alters gene regulation of the other putative subunits of the mitochondrial benzodiazepine receptor complex (Krueger, 1995) affecting mitochondrial benzodiazepine receptor binding in this manner. We have not performed binding studies to establish whether the receptor protein had changed following the stress paradigms. Such measurements would have been useful to confirm and expand the present volume of published data on the stress-induced changes in mitochondrial benzodiazepine receptor binding. However, given that the experimental paradigms used are known to induce stress, performing receptor binding was not in line with our main point of interest, namely whether mitochondrial benzodiazepine receptor gene expression studies can be readily used for assessing stress responses. It can be concluded, that at least in the tissues studied, measurements of stress-induced changes in mitochondrial benzodiazepine receptor gene expression is not a suitable approach for understanding the role of this protein in the response to stress. The data however, should not be interpreted as a lack of stress-induced change in mitochondrial benzodiazepine receptor function.

In this work we observed that chronic injection of dexamethasone (4 mg/kg) caused a significant decrease in adrenal gland mitochondrial benzodiazepine receptor mRNA. This chronic dose of dexamethasone is known to decrease the number of corticosteroid receptors in the brain in a way similar to that of chronic stress, thus affecting feedback inhibition via the hypothalamus pituitary axis (Sapolsky and McEwen, 1985). It is therefore difficult to explain why in this work we observed a dexamethasone-induced change in mitochondrial benzodiazepine receptor mRNA, but not a corresponding effect induced by chronic restraint stress, which is known to elevate glucocorticoids levels (Delbende et al., 1991). Possibly the restraint stress did not elevate glucocorticoids to a level equivalent to that of the injected dexamethasone. Alternatively, it maybe that certain effects of glucocorticoids are normally inhibited or counteracted by other stress-induced factors such as CRF (corticotropin releasing factor) or ACTH (adrenocortocotropin hormone) Therefore, some glucocorticoid-induced effects, including down regulation of mitochondrial benzodiazepine receptor gene expression, would be detected in the dexamethasone-treated rats but not in the stress-stimulated animals. It has also been reported that chronic injection of dexamethasone can

lead to a reduction in the weight of the adrenal gland (Calogero et al., 1990). We did not perform a detailed analysis of morphological changes of the adrenal gland, although we have noticed a decrease in total body weight of the dexamethasone-treated animals. As we used the whole adrenal gland for RNA preparation, and expressed mitochondrial benzodiazepine receptor level as ratio of the internal standards, the observed reduction of mitochondrial benzodiazepine receptor mRNA in dexamethasone-treated animals could also be explained by specific degeneration of adrenal cortex cells which, within the adrenal gland, preferentially express mitochondrial benzodiazepine receptor. A detailed in situ hybridization study would be required to fully address this possibility. It is interesting to note that certain steroids inhibit ligand binding to the mitochondrial benzodiazepine receptor in vitro (Deckert and Marangos, 1986). If this occurred also in vivo it may work in parallel to the possible reduction of mitochondrial benzodiazepine receptor density due to the here reported decrease in mRNA. It has also been reported that food deprivation causes a reduction in mitochondrial benzodiazepine receptor binding (Weizman et al., 1990). It may be speculated that dexamethasone, which caused a reduction in body weight in the injected animals, elicited a starvation-like effect that could have been responsible for the reduction in mitochondrial benzodiazepine receptor mRNA.

The in vitro work of Oberto et al. (1995) has clearly indicated that the mitochondrial benzodiazepine receptor gene promoter contains both positive-acting and negative-acting regulatory sites and the present work supports that regulation of this gene occurs also in vivo as indicated by the fact that the distribution of the mitochondrial benzodiazepine receptor mRNA correlates with the reported differential expression of mitochondrial benzodiazepine receptor binding sites and that dexamethasone appears to alter mitochondrial benzodiazepine receptor gene expression in vivo. With respect to the involvement of the mitochondrial benzodiazepine receptor in stress, in this work no stress-induced mitochondrial benzodiazepine receptor changes were detected suggesting that the previously reported stress-induced receptor density changes may have occurred via mechanisms other than alteration of gene expression. However, since we have not measured ligand binding in our stress paradigms, we can only comment that stress does not seem to induce wide-spread changes of mitochondrial benzodiazepine gene expression even in tissues where these receptors are most enriched. The administration of other hormones, steroids, receptor antagonists and other stress paradigms followed by both RNA measurement and binding studies are required to elucidate the complex regulation of the mitochondrial benzodiazepine receptor.

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