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TOLERANCE TO THE SEDATIVE AND ANXIOLYTIC EFFECTS OF DIAZEPAM IS ASSOCIATED WITH DIFFERENT ALTERATIONS OF $GABA_A$ RECEPTORS IN RAT CEREBRAL CORTEX

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Abstract—The clinical use of benzodiazepines is limited by the development of tolerance to their pharmacological effects. Tolerance to each of the pharmacological actions of benzodiazepines develops at different rates. The aim of this work was to investigate the mechanism of tolerance by performing behavioral tests in combination with biochemical studies. To this end, we administered prolonged treatments of diazepam to rats for 7 or 14 days. Tolerance to the sedative effects of diazepam was detected by means of the open field test after the 7- and 14-day treatments, whereas tolerance to the anxiolytic actions of benzodiazepine manifested following only the 14-day treatment in the elevated plus maze. The cerebral cortical concentrations of diazepam did not decline after the diazepam treatments, indicating that tolerance was not due to alterations in pharmacokinetic factors. The uncoupling of GABA/benzodiazepine site interactions and an increase in the degree of phosphorylation of the $GABA_A$ receptor γ2 subunit at serine 327 in the cerebral cortex were produced by day 7 of diazepam treatment and persisted after 14 days of exposure to benzodiazepine. Thus, these alterations could be part of the mechanism of tolerance to the sedative effects of diazepam. An increase in the percentage of α1-containing $GABA_A$ receptors in the cerebral cortex was observed following the 14-day treatment with diazepam but not the 7-day treatment, suggesting that tolerance to the anxiolytic effects is associated with a change in receptor subunit composition. The understanding of the molecular bases of tolerance could be important for the development of new drugs that maintain their efficacies over long-term treatments.

Key words: benzodiazepine, GABA, $GABA_A$ receptor, tolerance, uncoupling.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; FNZ, flunitrazepam; GABA, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TBS, tris-buffered saline.

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INTRODUCTION

$GABA_A$ receptors are targets of several pharmacologically relevant drugs. In particular, benzodiazepines have been used in the clinic for more than 50 years. These drugs are used for the treatment of different disorders, such as insomnia, anxiety, epilepsy, and to facilitate muscle relaxation and anesthesia. Because benzodiazepines act by enhancing the effects of GABA instead of directly activating the $GABA_A$ receptor, they exhibit a large therapeutic index and low toxicity. Unfortunately, the use of benzodiazepines is limited by the development of tolerance to most of their pharmacological actions that occurs after prolonged administration.

Tolerance to benzodiazepines is an adaptive regulatory process that constitutes an example of neuronal plasticity. Tolerance develops over different timescales depending on the pharmacological effect of benzodiazepine. Tolerance to the sedative effects occurs rapidly, followed by tolerance to the anticonvulsant actions of benzodiazepines. Tolerance to the anxiolytic effects has been difficult to demonstrate in humans and develops after longer time periods in animals. It has been hypothesized that multiple tolerance mechanisms exist or, alternatively, that a single mechanism exists but the tolerance development depends on the $GABA_A$ receptor subtype and the brain region involved (Bateson, 2002; Vinkers and Olivier, 2012). The activation of $GABA_A$ receptors containing distinct α subunit subtypes differentially contributes to the development of tolerance to benzodiazepines. For example, the concomitant activation of α1- and α5-containing $GABA_A$ receptors is required for the manifestation of tolerance to the sedative effects of diazepam (van Rijnsoever et al., 2004; Vinkers et al., 2012).

The molecular bases of tolerance to the actions of benzodiazepines are largely unknown. Different adaptive changes in the function of $GABA_A$ receptors have been reported as a consequence of chronic benzodiazepine administration. Methodological differences, such as species, drug, dose, length of treatment, route of administration, and brain region analyzed, may account for the variable results reported in the literature. In addition, very few researchers have performed behavioral tests in combination with molecular analyses.

The decrease in benzodiazepine function associated with the development of tolerance may be the

consequence of a reduction in the allosteric coupling between GABA and benzodiazepine sites. The results from different studies indicate that prolonged benzodiazepine exposure produces uncoupling of the GABA and benzodiazepine sites (Gallager et al., 1984; Marley and Gallager, 1989; Tietz et al., 1989, 1999). The down-regulation of the number of $GABA_A$ receptors is another putative mechanism of tolerance to benzodiazepines. However, most studies suggest that chronic treatment with benzodiazepines fails to induce changes in the number of receptors. Selective alterations in the levels of $GABA_A$ receptor subunit mRNAs and peptides have been observed after prolonged benzodiazepine exposure, suggesting changes in the subunit composition of receptors (Uusi-Oukari and Korpi, 2010). As far as we know, there are no reports that have directly investigated the $GABA_A$ receptor composition after chronic administration.

The aim of this work was to investigate the mechanism of tolerance to benzodiazepines by performing behavioral experiments in combination with biochemical studies. To this end, we studied the effects of either 7 or 14 days of repeated administration of diazepam on the development of tolerance to the anxiolytic and sedative actions of diazepam in rats. Tolerance to the sedative effects of diazepam manifested following both treatments, whereas tolerance to the anxiolytic effects was detected only after the 14-day protocol, suggesting different time-courses. The 7-day diazepam treatment produced uncoupling of GABA/benzodiazepine site interactions and an increase in the degree of phosphorylation of $GABA_A$ receptors in the cerebral cortex that persisted after the 14-day protocol. On the other hand, an increase in the percentage of α1-containing $GABA_A$ receptors was observed following the 14-day but not the 7-day treatment.

EXPERIMENTAL PROCEDURES

Diazepam treatments

Male Sprague–Dawley rats weighting 250 g at the start of the study (n = 15 per group, Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica) were randomly allocated to the following groups: control (treated with vehicle), acute, 7 days and 14 days treatment with diazepam. Animals were housed in temperature (21 °C) and humidity (30–75%) controlled conditions and maintained on a 12-h light–dark cycle with free access to food and water. All the experimental procedures were in compliance with the National and International Guidelines (National Institute of Health Guide for the Care and Use of Laboratory Animals, NIH Publications No. 80–23, revised 1996) on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering. To equate handling and number of injections and to perform the behavioral tests on the same day, all the animals were treated for 14 days with single daily subcutaneous injections with vehicle (sesame oil) or diazepam (15 mg/kg, a gift from Roemmers, Buenos Aires, Argentina), as appropriate. Thus, control animals received a total of 14 injections with vehicle. Injections were given at a volume of 1 ml/kg of body weight.

Behavioral tests

The behavioral tests were performed 2 h after the last injection, at the time of peak concentration of diazepam in the cerebral cortex (Fernandes et al., 1999) using rats treated with vehicle or diazepam (acute and chronic treatments). Experiments were performed in a sound-attenuated room at 21 °C with dimmed illumination (40 W).

The elevated plus-maze had two opposite open arms (40 × 10 cm) and two opposite enclosed arms of the same size with 35-cm high walls extending from a central area of 10 × 10 cm. The maze was elevated 80 cm above the ground. Rats were place in the central area facing an enclosed arm and allowed to freely explore the maze for 5 min. Sessions were recorded and analyzed with Ethovision XT 7.0 tracking software (Noldus, The Netherlands). The percentage of entries onto and time spent on the open arms, the total number of entries and distance traveled were measured. It is not possible to test the group acutely treated with diazepam in the elevated plus maze because the animals fall from the maze due to the sedative effect of benzodiazepine.

The open field was a 1 × 1 × 1-m box. A central zone of 40 × 40-cm was defined. The locomotor activity was assessed as the total distance traveled during a 20-min period. The distance traveled in the central zone was also determined.

Animals were sacrificed at the end of the experiments by decapitation and the brains were rapidly removed.

Determination of diazepam concentration in the cerebral cortex

The whole cerebral cortices from the 4 experimental groups were dissected and stored at −70 °C until used. The tissue were thawed and homogenized in 10 volumes of 0.2 N perchloric acid in a glass homogenizer and centrifuged at 9800g for 5 min. The pellets were discarded and the concentration of diazepam in the supernatants was determined by Schere laboratory (Buenos Aires, Argentina). The quantification of diazepam was performed by high-performance liquid chromatography (HPLC) using HP Agilent 1100 equipment (Santa Clara, CA, USA) equipped with a C18 column. The wavelength at 230 nm was chosen for UV detection. Clonazepam was used as the internal standard. The peak areas were determined to calculate the diazepam concentrations based on the internal standard peak ratios.

Binding assays

The whole cerebral cortices from rats treated with vehicle or diazepam were dissected and immediately homogenized in 10 volumes of 0.32 M sucrose in a Teflon homogenizer and centrifuged at 1000g for 10 min. The pellet (P1) was discarded and the supernatant was centrifuged at 27,000g for 30 min.

For osmotic shock studies, the pellet (P2) was homogenized 10 volumes of double-distilled water and centrifuged at 27,000*g* for 30 min. The final pellet was homogenized in 1 mM EDTA/1 mM phenylmethylsulfonyl fluoride and dialyzed four times against 4 l potassium phosphate buffer (pH 7.5) overnight at 4 °C.

The homogenate aliquots (75–100 μg protein) were incubated in a final volume of 0.5 ml of ice-cold phosphate-buffered saline (PBS) for 60 min at 0 °C with 0.01–20 nM [^{3}H]flunitrazepam ([^{3}H]FNZ, PerkinElmer Life Sciences, Waltham, MA, USA) in saturation binding experiments. To measure the potentiation of benzodiazepine binding by GABA, 0.5 nM [^{3}H]FNZ was used alone or in the presence of 1 mM GABA (Sigma, St. Louis, MO, USA). Nonspecific binding was determined in the presence of 100 μM diazepam and subtracted from the total binding to yield the specific binding. The reaction was stopped by the addition of 5 ml ice-cold PBS, and the aliquots were immediately vacuum filtered through glass fiber filters (GF/B Whatman, Clifton, NJ, USA). The filters were then washed 3 times with 5 ml ice-cold PBS. The radioactivity retained on the filters was quantified by liquid scintillation spectrometry. The coupling represents the GABA-potentiated [^{3}H]FNZ binding and was estimated as follows: (% potentiation$_{treated}$/% potentiation$_{control}$) × 100. The uncoupling was defined as the decrease in the GABA-potentiated [^{3}H]FNZ binding and was calculated as follows: [1 − (% potentiation$_{treated}$/% potentiation$_{control}$)] × 100. Saturation binding data were analyzed by computer-aided non-linear regression.

Real-time polymerase chain reaction (PCR)

The whole cerebral cortices from control (treated with vehicle) and chronically diazepam-treated rats were dissected and stored at −20 °C in RNA stabilization solution (RNA*later*, Ambion, Austin, TX, USA) until required. The total RNA was extracted using the RNeasy midi kit (Qiagen, Hilden, Germany). The primers (Tecnolab, Buenos Aires, Argentina) and probes (TaqMan, Applied Biosystems, Foster City, CA, USA) were designed using the Primer Express software (Applied Biosystems). The sequences of the α subunit primers were: α1, 5′-CCCCGGCTTGGCAACTAT-3′ and 5′-TGTCTCAGGCTTGACTTCTTTCG-3′; α2, 5′- GACT GGGAGACAGCATTACTGAAG-3′ and 5′- TCTGAGA CAGGGCCAAAACTG-3′; α3, 5′-CACCATGACCACCTT GAGTATCA-3′ and 5′- CCGTCGCGTATGCCACTT-3′; α5, 5′-CAACATCACAATATTCACCAGGATCT-3′ and 5′-CCCAGG CCGCAGTCTGT-3′. The sequences of α subunit probes were: α1, 5′-TAAAAGTGCGACCATA GAA-3′; α2, 5′- CTCCACCAACATCTATG-3′;α3, 5′-TGC CAGAAACTCTTTAC-3′; α5, 5′-CTCTTGGATGGCTAT GAC-3′. The ribosomal RNA probe and primers were purchased from Applied Biosystems. Quantitative one-step real-time PCR was performed in an Applied Biosystems 7500 real-time PCR system using an AgPath ID™ one-step RT PCR kit (Ambion). The standard curves for relative quantification were generated with 1 to 100 ng of total RNA isolated from control cultures (treated with vehicle). The PCR reactions were performed in triplicate in a total volume of 25 μl containing AgPath ID™ master mixture, 250 nM of the α subunit probe, 900 nM of the α subunit primers, 50 nM of the 18S rRNA probe and 50 nM of the 18S rRNA primers. The reaction conditions were 45 °C for 10 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 45 s. The relative amount of the subunit mRNAs was normalized to the relative amount of the 18S rRNA (internal control).

Western blotting (Figs. 6A, B and 7)

The whole cerebral cortices from rats chronically treated with vehicle or diazepam were dissected and stored at −70 °C until used. The tissues were thawed and homogenized in 10 volumes of 0.32 M sucrose to prepare the P2 pellet fractions as previously described. The P2 pellets were homogenized in ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 3% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and incubated on a rotating shaker for 20 min at 4 °C. For phosphorylation studies, the lysis buffer also contained a phosphatase inhibitor cocktail (Sigma). The lysates were centrifuged at 27,000*g* for 30 min. The protein concentration was determined in the supernatants and then the samples (40 μg of protein) were resuspended in 30 μl denaturing sample buffer and boiled for 5 min. The proteins were separated on 10% acrylamide gels and transferred to nitrocellulose membranes. The blots were blocked for 2 h with 5% nonfat dry milk in 20 mM of Tris-buffered saline (TBS) buffer containing 0.1% Tween-20. The blots were incubated with goat anti-GABA$_A$ receptor α1 subunit (1:250 dilution, Santa Cruz, Dallas, TX, USA) or rabbit anti-GABA$_A$ receptor γ2 subunit (phospho serine 327) (1:250, Abcam, Cambridge, UK) overnight at 4 °C with shaking. The proteins were detected by enhanced quimioluminescence (ECL detection kit, Pierce, Rockford, IL, USA) using an anti-goat or anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1.5 h at room temperature, 1:1000 dilution, Santa Cruz). The blots were stripped and reprobed by incubating with a rabbit antibody against actin (1:500 dilution, Sigma) or total GABA$_A$ receptor γ2 subunit (phosphorylated and non-phosphorylated forms) (1:250 dilution, Millipore, Billerica, MA) overnight at 4 °C, followed by incubation with an anti-rabbit HRP-conjugated antibody (1.5 h at room temperature, 1:2000 dilution, Santa Cruz). To control for the variability in loading amounts, the signals of the receptor α1 subunit were normalized to the signals of actin, whereas the signals of phospho receptor γ2 subunit were normalized to the signals of the total receptor γ2 subunit.

Receptor immunoprecipitation and western blotting (Fig. 6C, D)

The whole cerebral cortices from rats chronically treated with vehicle or diazepam were dissected and stored at −70 °C until required. The tissues were thawed and

homogenized in 10 volumes of 0.32 M sucrose to prepare the P2 membrane fraction as described above. To extract the $GABA_A$ receptors, the P2 pellet was homogenized as previously described (Jechlinger et al., 1998) in deoxycholate buffer containing 10 mM Tris–HCl pH 8.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.05% phosphatidylcholine, 1 mM EDTA, and a protease inhibitor cocktail (Roche) and incubated on a rotating shaker for 20 min at 4 °C. The lysate was centrifuged at 27,000*g* for 30 min and the supernatants were diluted to 1 mg/ml protein with PBS buffer. The cell lysate was pre-cleared by the addition of 20 μl protein A agarose beads (Santa Cruz) to 500 μl of the cell lysate. The samples were then incubated at 4 °C for 20 min with rotation. The lysates were centrifuged for 5 min at 2000*g* and the supernatants were incubated with 2 μg rabbit anti-γ2 antibody (Alpha Diagnostic, San Antonio, TX, USA) overnight at 4 °C on a rotating shaker. Negative controls were produced by incubating the reaction mixture without the specific antibody. Immunocomplexes were captured by addition of 20 μl of protein Aagarose beads followed by incubation overnight at 4 °C on the shaker. The agarose beads were collected by centrifugation (5 min at 2000*g*) and the pellets were washed 3 times by resuspension in ice-cold deoxycholate buffer followed by centrifugation. The final pellets were resuspended in 30 μl of denaturing sample buffer. The proteins were analyzed by western blot analysis as described in the previous section. The blots were incubated with a goat antibody against $GABA_A$ receptor α1 subunits (1:200 dilution, Santa Cruz) overnight at 4℃. The protein subunit was detected by incubation with anti-goat HRP-conjugated antibody (1:2000 dilution, Santa Cruz,) for 1.5 h at room temperature. The blots were stripped and reprobed by incubating with an anti-rabbit HRP-conjugated antibody (1:2000 dilution, Santa Cruz) for 1.5 h at room temperature to measure the amount of IgG, the antibody used to immunoprecipitate the $GABA_A$ receptors. The subunits signals were normalized to the signal of the rabbit IgG to control for the variability in loading amounts.

RESULTS

Tolerance to the anxiolytic and sedative effects of diazepam

To test the effect of repeated benzodiazepine treatment on the anxiolytic and sedative actions of diazepam, rats received daily injections of vehicle or diazepam (15 mg/kg) for 1 (acute treatment), 7 or 14 days. These diazepam treatment regimes are based on previous studies by other groups (Holt et al., 1996; Fernandes et al., 1999; Allison and Pratt, 2006). Diazepam was administered subcutaneously in order to produce relative more stable plasma levels of benzodiazepine than the intraperitoneal treatment regimen. Behavioral tests were performed 2 h after the last injection, at the time of peak diazepam concentration (Fernandes et al., 1999).

The anxiolytic effect of diazepam was estimated in the elevated plus-maze as an increase in the percentage of entries made onto and the time spent on the open arms (Fig. 1A, B). Unfortunately, the acute anxiolytic effect of diazepam was not possible to measure in the maze because the animals fall from the apparatus due to the sedative actions of diazepam. The total number of arm entries and distance traveled in the maze (Fig. 1C, D) did not change following both diazepam treatments compared to control animals, suggesting the absence of sedative effects probably due to the development of tolerance. A significant anxiolytic effect was observed following 7 days of treatment with diazepam, though this effect was absent after 14 days of administration, suggesting the occurrence of tolerance.

The sedative properties of diazepam were measured as a decrease in the motor activity by means of the open field test (Fig. 2A). The acute diazepam administration reduced the locomotor activity of rats, quantified as the total distance moved, which indicated a sedative effect of diazepam. In contrast, the sedative action of diazepam was abolished in animals treated with benzodiazepine for either 7 or 14 days due to the development of tolerance. The open field test is usually used to measure animal's ''emotionality''. In fact, an increase in the distance traveled in the central zone was detected after the acute and 7-day treatments compared to control animals, suggesting an anxiolytic effect (Fig. 2B).

Cortical concentrations of diazepam

Diazepam tolerance may be due to alterations in pharmacokinetic factors that result in a decrease in the benzodiazepine concentration at the action sites. To test this hypothesis, we determined the concentration of diazepam in the cerebral cortices of rats 2 h after injections in the acute and prolonged treatments (Fig. 3).The cortical concentration of diazepam was similar in rats treated for 7 days compared to rats receiving an acute injection of benzodiazepine. A significant increase in the cortical concentration of diazepam was detected in rats following 14 days of administrations compared with the acute group, indicating drug accumulation. These results suggest that changes in the pharmacokinetic parameters are not responsible for diazepam tolerance.

Prolonged diazepam administration produces uncoupling of GABA and benzodiazepine sites

Though numerous reports indicate that prolonged benzodiazepine exposure produces a reduction in the allosteric interactions between GABA and benzodiazepine sites, the relevance of uncoupling to the tolerance mechanism has not been determined. To investigate whether our protocols of repeated diazepam administration induced uncoupling, we measured the stimulation of [^{3}H]FNZ binding by GABA in membrane homogenates of cerebral cortex (Fig. 4).

The 7-day diazepam treatment induced a significant reduction in the potentiation of benzodiazepine binding by GABA (equivalent to approximately 60% uncoupling) that persisted after 14 days of treatment. Acute treatment with benzodiazepine failed to produce a significant uncoupling, suggesting that this regulatory process is the result of a prolonged treatment. The

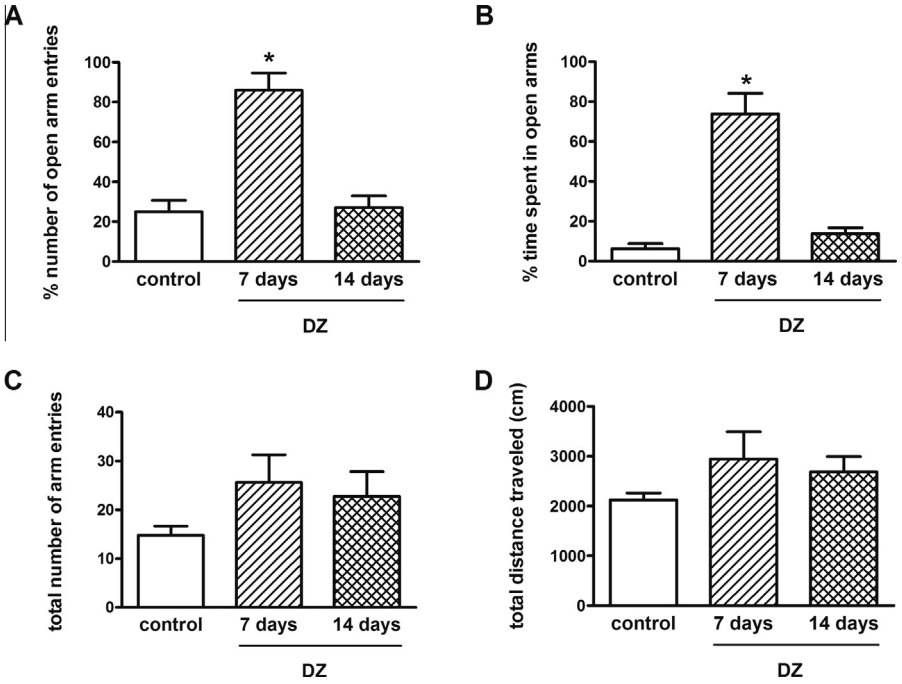

Fig. 1. Tolerance to the anxiolytic effects of diazepam. Rats were treated with vehicle (control) or diazepam (DZ) for 7 or 14 days. To determine anti-anxiety behavior, the percentage of entries onto (A) and the percentage of time spent on (B) the open arms of an elevated plus maze were measured. Spontaneous motor activity was measured as total number of arm entries (C) and total distance traveled (D). The results are expressed as mean ± SEM (n = 15 per experimental group). *Significantly different from control (A: $F_{(2,42)}$ = 16.03, p = 0.0001; B: $F_{(2,42)}$ = 58.72, p < 0.0001, one-way ANOVA and Tukey's post hoc test).

uncoupling process occurs in the absence of changes in the affinity or number of [^{3}H]FNZ binding sites (Table 1). Because uncoupling manifested before the development of tolerance to the anxiolytic effects of diazepam, this regulatory process seems to mediate the development of tolerance to the sedative but not the anxiolytic actions of benzodiazepine.

Experiments performed in a cell line suggest that prolonged exposure to diazepam induces an increased internalization of $GABA_A$ receptors in intracellular vesicles (Ali and Olsen, 2001). Since these intracellular compartments, which are present in our membrane preparations, are permeable to benzodiazepines but not to GABA, it is possible that the reduction in the potentiation of benzodiazepine binding by GABA we observed after the prolonged treatments with diazepam is the result of an increase in receptor endocytosis. In order to test this hypothesis, we investigate the effect of an osmotic shock treatment to lyse the intracellular compartments (Table 2). These results indicate that this treatment fails to inhibit uncoupling, suggesting that this effect is not the result of a receptor internalization process.

Prolonged diazepam treatment produces a change in the subunit composition of $GABA_A$ receptors

The action of benzodiazepines is influenced by the subtype of α subunits in the $GABA_A$ receptor (Puia et al., 1991; Wafford et al., 1993; Smith et al., 2001). As a first step to test whether tolerance to diazepam is associated with a switch of α subunits in the $GABA_A$ receptor, we studied the effects of the repeated treatments on the mRNA levels of α1, α2, α3 and α5 subunits in the cerebral cortex by means of quantitative real-time PCR assays (Fig. 5).

A significant increase in the levels of α1 subunit mRNA was observed after 14 days but not after 7 days of diazepam treatment. The mRNA levels of the α2, α3 and α5 subunits remained constant after both diazepam treatments. The results of the western blot experiments (Fig. 6A, B) showed that the increase in the mRNA levels of the α1 subunit was associated with a corresponding enhancement of the α1 subunit peptide following the 14-day treatment. The 7-day treatment did not induce significant alterations in the α1 peptide level.

We next investigated whether these changes in α1 subunits resulted in alterations in the subunit composition of the $GABA_A$ receptors. To this end, we immunoprecipitated $GABA_A$ receptors with an antibody against γ2 subunits, which are present in most of the receptors, and then we monitored the α subunit composition by western blot assays (Fig. 6C, D). Chronic treatment with diazepam for 14 days induced a small but significant increase in the percentage of α1-containing $GABA_A$ receptors. The 7-day treatment did not produce alterations in the percentage of α1-containing receptors. These results suggest that the manifestation of tolerance to the anxiolytic effects of diazepam is accompanied by a change in the combination of receptor subunits.

Prolonged diazepam treatment induces an increase in the phosphorylation state of $GABA_A$ receptors

We have previously reported that the uncoupling of GABA and benzodiazepine sites induced by persistent activation

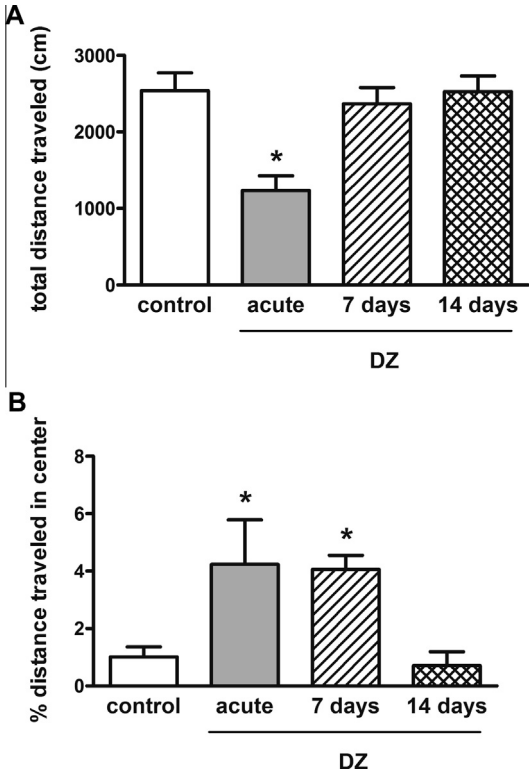

Fig. 2. Effect of diazepam treatments on the open field test. Rats treated with vehicle (control) or diazepam (DZ) for 1 (acute), 7 or 14 days were tested in an open field. (A) The total distance traveled was measured. The data represent mean ± SEM (n = 15 per experimental group). *Significantly different from control ($F_{(3,56)}$ = 3.95, p = 0.0159, one-way ANOVA and Tukey's post hoc test). (B) The percentage of distance traveled in the central zone was determined. The data represent mean ± SEM (n = 15 per experimental group). *Significantly different from control ($F_{(3,56)}$ = 7.034, p = 0.025, one-way ANOVA and Tukey's post hoc test).

of $GABA_A$ receptors by GABA in rat cortical neurons is associated with an increase in the degree of phosphorylation of the receptor γ2 subunit at serine residues (Gutiérrez et al., 2014). To investigate whether uncoupling induced by prolonged diazepam treatment is accompanied by alterations in the phosphorylation state of receptors, we performed western blot assays using an antibody specific for the $GABA_A$ receptor γ2 subunit phosphorylated at serine 327 (Fig. 7). The results showed that repeated diazepam administrations for 7 days produced an increase in the levels of the phosphorylated form of the receptor γ2 subunit that was sustained after 14 days of treatment with diazepam. Thus, the occurrence of tolerance to the sedative effects of diazepam is associated with a change in the phosphorylation state of $GABA_A$ receptors. However, this alteration is not temporally correlated with the manifestation of tolerance to the anxiolytic effects of diazepam, suggesting that is not responsible for the development of tolerance to this pharmacological action.

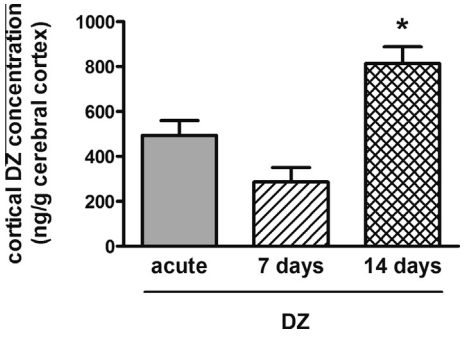

Fig. 3. Cortical concentrations of diazepam. The concentration of diazepam (DZ) in the cerebral cortex (ng DZ per g of cortex) of rats that received 1 (acute), 7 or 14 injections of the benzodiazepine was determined by HPLC. The results are expressed as mean ± SEM of 4 independent determinations. *Significantly different from acute group ($F_{(2,9)}$ = 13.04, p = 0.0104, one-way ANOVA and Tukey's post hoc test).

DISCUSSION

Long-term consumption of benzodiazepines produces several side effects, such as motor incoordination and memory problems. The most commonly reported problem of chronic usage is the development of tolerance and dependence. The understanding of the mechanism of tolerance to benzodiazepines is very important from a clinical perspective because these drugs, which possess a high therapeutic index, could be prescribed for long-term use. Benzodiazepines allosterically stimulate GABA actions by binding to $GABA_A$ receptors containing α1, α2, α3 or α5 subunits in combination with a β and a γ2 subunit (Sieghart and Sperk, 2002). Therefore, the $GABA_A$ receptor constitutes a good candidate to mediate adaptive alterations induced by prolonged benzodiazepine consumption.

Here, we investigated the mechanism of tolerance to benzodiazepines following prolonged diazepam administration for 7 or 14 days in rats. Our results demonstrated that tolerance to the sedative actions of diazepam occurred following the 7-day and 14-day treatments but the tolerance to the anxiolytic effects was detected only after the 14-day treatment (Figs. 1 and 2).

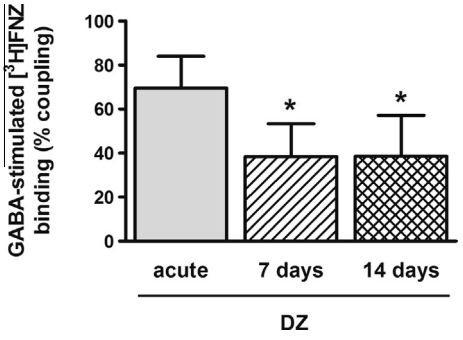

Fig. 4. Uncoupling of GABA/benzodiazepine site interactions is induced by prolonged treatments with diazepam. Rats were treated with vehicle (control) or diazepam (DZ) for 1 (acute), 7 or 14 days. The coupling between GABA and benzodiazepines was estimated as the potentiation of [^{3}H]FNZ binding by GABA in the cerebral cortex. Data are expressed as percentages of control values (defined as 100%) and represent mean ± SEM of 3–6 independent determinations. *Significantly different from control (p < 0.05, one-sample Student's t test).

Table 1. Exposure of prolonged administration with diazepam has no effect on [^{3}H]FNZ binding

	[^{3}H]FNZ binding	
	K_d (nM)	B_{max} (pmol/mg prot)
Control	6.78 ± 0.50	3.68 ± 0.09
7 days DZ	6.18 ± 0.70	3.54 ± 0.20
14 days DZ	5.63 ± 0.76	3.05 ± 0.13

Rats were treated with vehicle (control) or diazepam (DZ) for 7 or 14 days. Membrane homogenates were prepared from cerebral cortexes. Data represent mean ± S.E.M. of 3 independent experiments performed in triplicate.

These results support the notion that tolerance to benzodiazepines develops with different rates depending on the pharmacological action (Bateson, 2002; Vinkers and Olivier, 2012).

Tolerance to benzodiazepines may be induced by changes in the absorption, distribution, metabolism or excretion of the drug, which result in a decrease in the concentration at the action sites. To test this possibility, we determined the concentration of diazepam in the cerebral cortex of rats that received acute and repeated diazepam treatments at the time of peak diazepam concentration (2 h after injection) (Fernandes et al., 1999). The cortical diazepam concentration following the 7-day treatment with benzodiazepine was similar to that of the acute group. In contrast, the diazepam concentration in the cerebral cortex of rats treated for 14 days with benzodiazepine was significantly higher compared to the acute group (Fig. 3). These data indicate that drug accumulation occurs after 14 days of treatment with benzodiazepine. This may be due to saturation of metabolic pathways, as was previously reported following the administration of high concentrations of diazepam (St-Pierre and Pang, 1995). The cortical concentration values of diazepam reported in this work are very close to those shown in previous studies in which similar benzodiazepine treatments were performed (Fernandes et al., 1999). In agreement with our results, other groups have demonstrated that the plasma and cortical concentrations of different benzodiazepines do not decrease over time during chronic treatments (Miller et al., 1988; Cowley et al., 1995; Fernandes et al., 1996). In conclusion, our results demonstrate that tolerance to diazepam is not the result of changes in pharmacokinetic factors.

The decrease in the efficacy of benzodiazepines induced by chronic exposure may be mediated by a reduction in the allosteric coupling between GABA and benzodiazepine sites. A number of reports have demonstrated that prolonged administration of benzodiazepine *in vivo* produces uncoupling of the GABA/benzodiazepine site interactions. Uncoupling has been detected using different methodological approaches, including a decrease in the ability of zolpidem to prolong the decay of miniature inhibitory postsynaptic currents in the rat hippocampus (Tietz et al., 1999), a reduction in the potentiation of GABA-stimulated chloride influx by benzodiazepines in vesicles from the rat cerebral cortex (Marley and Gallager, 1989) and a decrease in the stimulation of benzodiazepine binding by GABA in the rat cerebral cortical membranes (Gallager et al., 1984; Tietz et al., 1989). A study from Hernandez et al. (Hernandez et al., 1989) has shown that chronic administration of flumazenil in rats does not produce uncoupling, whereas the degree of uncoupling induced by long-term treatments with other benzodiazepine site ligands in the rat cerebral cortex is correlated with their efficacies. Benzodiazepine-induced uncoupling has also been demonstrated in primary cultured neurons and cell lines expressing recombinant $GABA_A$ receptors. Chronic treatment of chick brain neurons with flurazepam for 48 h induces uncoupling with a $t½$ of 18 h (Roca et al., 1990). In cell lines benzodiazepines induce uncoupling with a variable $t½$ ranging from 32 min in LTK$^-$ cells (Klein et al., 1994) to 3 h in WSS-1 cells (Wong et al., 1994).

The results presented here demonstrate that prolonged treatments with diazepam for 7 or 14 days resulted in the uncoupling of GABA/benzodiazepine site interactions, which was detected as a decrease in the potentiation of benzodiazepine binding by GABA in the cerebral cortex (Fig. 4). Uncoupling occurs without a change in receptor number (Table 1). Therefore, tolerance to the sedative effect of diazepam could result

Table 2. Effects of osmotic shock on diazepam-induced uncoupling

Treatment	GABA stimulated [^{3}H]FNZ binding (% coupling)
Control	100
DZ	
7 days	44 ± 11[a]
14 days	38 ± 10[a]
Control, osmotic shock	100
DZ, osmotic shock	
7 days	42 ± 10[a]
14 days	53 ± 9[a]

Rats were treated with vehicle (control) or diazepam (DZ) for 7 or 14 days. Membrane homogenates were prepared from cerebral cortexes. An osmotic shock treatment was performed in some of the membrane preparations as indicated. The coupling between GABA and benzodiazepines was estimated as the potentiation of [^{3}H]FNZ binding by GABA. Data represent the mean ± S.E.M. of 3–4 independent experiments performed in triplicate. Significantly different from control: [a]$p < 0.05$ (Student's t test).

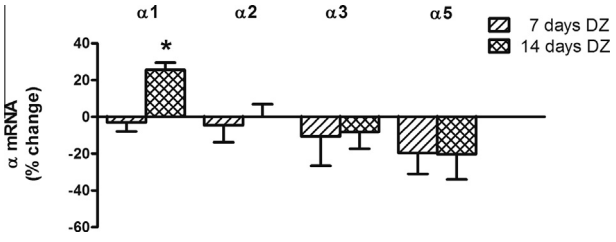

Fig. 5. Effect of prolonged treatments with diazepam on the mRNA levels of α1, α2, α3, and α5 $GABA_A$ receptor subunits. Quantitative real-time PCR experiments using the total RNA from the cerebral cortices of rats treated with vehicle (control) or diazepam (DZ) for 7 or 14 days were performed. 18 S RNA was used as an internal control to normalize the results. The results are expressed as the percentage of change compared to the control experiments (defined as 0%) and represent mean ± SEM of 6 independent determinations. *Significantly different from control ($p < 0.05$, one-sample Student's t test).

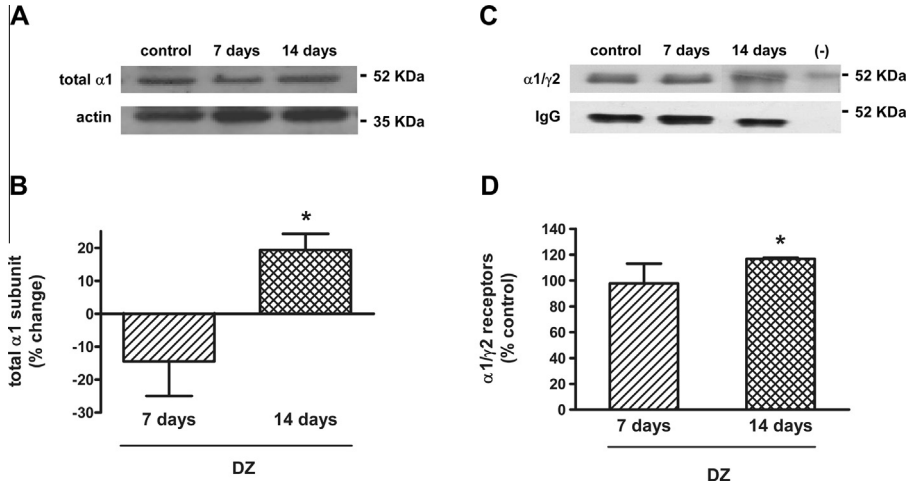

Fig. 6. Effect of repeated treatments with diazepam on the $GABA_A$ receptor subunit composition. Rats were chronically treated with vehicle (control) or diazepam (DZ) for 7 or 14 days. (A) Representative western blots of the total protein homogenates from rat cerebral cortices to measure the total α1 $GABA_A$ receptor subunit. Size of molecular weight markers is indicated on the right. (B) Densitometry analysis of α1 subunit levels normalized to actin expression. Data are expressed as the percentage of change relative to the control values (defined as 0%) and represent mean ± SEM of 4 independent determinations. *Significantly different from control ($p < 0.05$, one sample Student's t test). (C) Representative western blots of the protein homogenates obtained by immunoprecipitation of cortical $GABA_A$ receptors with a rabbit antibody against the γ2 receptor subunit. The abundance of α1 subunits was measured with a specific goat antibody. Negative control experiments (−) were performed in the absence of the anti-γ2 receptor antibody during the immunoprecipitation step. (D) Densitometry analysis of α1 subunit levels normalized to the rabbit IgG signal to control for loading variability. Results are expressed as the percentage of control values (defined as 100%) and represent mean ± SEM of 4–5 independent determinations. *Significantly different from control ($p < 0.05$, one sample Student's t test).

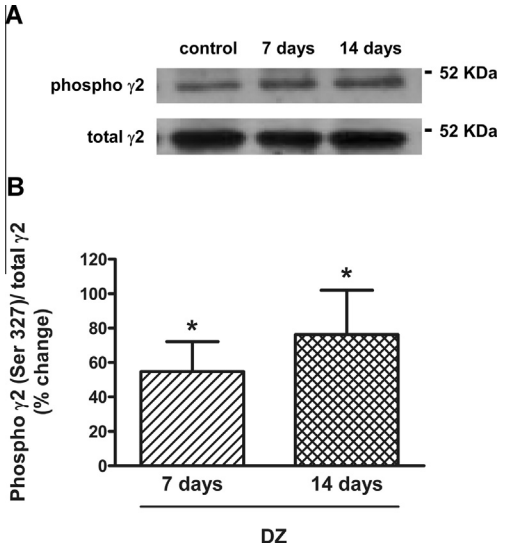

Fig. 7. Repeated diazepam administration increases the degree of phosphorylation of the γ2 $GABA_A$ receptor subunit. Rats were chronically treated with vehicle (control) or diazepam (DZ) for 7 or 14 days. (A) Representative Western blots of the protein homogenates from cerebral cortices to detect phosphorylated γ2 $GABA_A$ receptor subunits (serine 327). Size of molecular weight markers is indicated on the right. (B) Densitometry analysis of the phospho γ2 receptor subunit levels normalized to the signal of the total γ2 receptor subunit levels. Data are expressed as the percentage of control values (defined as 100%) and represent mean ± SEM of 4 independent determinations. *Significantly different from control ($p < 0.05$, one-sample Student's t test).

from a decrease in the allosteric coupling between GABA and benzodiazepine sites. Because the occurrence of uncoupling precedes the manifestation of tolerance to the anxiolytic effect of diazepam, tolerance to this pharmacological effect seems to be mediated by a different mechanism. The results produced by Holt et al. (1999) showed that a single injection of diazepam induces uncoupling in the rat cerebral cortex and cerebellum, suggesting that this phenomenon is not part of the tolerance mechanism. However, the relevance of uncoupling to the development of tolerance to some of the pharmacological actions of benzodiazepines has been supported by different reports. It has been shown that the degree of uncoupling produced in the rat cerebral cortex by chronic treatments with different benzodiazepine ligands correlates with the magnitude of tolerance to the anticonvulsant actions induced by these compounds (Hernandez et al., 1989). Moreover, Tietz et al. (1989) have demonstrated that long-term but not acute treatment with benzodiazepines induces uncoupling in rat cerebral cortical membranes, suggesting that this phenomenon is produced by a chronic exposure. Similar results were presented here, as we did not detect uncoupling after the acute treatment with diazepam.

Long-term administration of benzodiazepines induces uncoupling in a regionally specific manner. The results from Marley and Gallager (1989) indicate that a decrease in the sensitivity of GABA-stimulated chloride influx to flunitrazepam is observed in the cerebral cortex but not in the cerebellum of rats receiving chronic diazepam treatments. Likewise, chronic flurazepam treatment in rats produces a reduction in the ability of GABA to stimulate flunitrazepam binding in the cerebral cortex, whereas the coupling between GABA and benzodiazepine sites remains unaltered in the cerebellum, medulla, striatum, hypothalamus, midbrain, hippocampus and olfactory bulb (Tietz et al., 1989). These data could indicate that the tolerance mechanism depends on the brain region involved.

The mechanism of benzodiazepine tolerance may involve the expression of aberrant $GABA_A$ receptors due to alterations in the composition of subunits. Numerous studies have reported changes in the expression of $GABA_A$ receptor subunits that vary depending on the treatment paradigm and the brain region examined (Uusi-Oukari and Korpi, 2010). For example, different administration protocols with diazepam in rats have been reported to produce a decrease (Pesold et al., 1997; Chen et al., 1999), an increase (Pratt et al., 1998) or no change (Wu et al., 1994) in the α1 subunit expression in the cerebral cortex. On the other hand, the subcutaneous administration of diazepam to rats for 21 days in rats results in a decrease in α1 subunit levels in the hippocampus, whereas the same treatment does not produce alterations in the levels of this subunit in the cerebral cortex (Wu et al., 1994). The exposure of cultured rat hippocampal neurons to flurazepam for 24 h has been shown to decrease the surface and total levels of α2-containing $GABA_A$ receptors. This reduction is mediated by a selective increase in the degradation of α2-containing receptors that leads to a reduction in the efficacy of synaptic GABAergic inhibition (Jacob et al., 2012). This subtype-selective down-regulation of $GABA_A$ receptors could induce alterations in the subunit combination of receptors at the cell surface.

Benzodiazepine actions depend on the particular subtype of α subunit present in the $GABA_A$ receptor (Puia et al., 1991; Wafford et al., 1993; Smith et al., 2001), suggesting that alterations in the subtype of this subunit underlie the tolerance phenomenon. Our data demonstrated that the tolerance to the sedative effects of diazepam induced by a 7-day treatment with diazepam occurs in the absence of changes in the expression of benzodiazepine-sensitive $GABA_A$ receptor α subunits in the cerebral cortex. Conversely, we showed that a prolonged treatment with diazepam for 14 days results in an increase in the $GABA_A$ receptor α1 subunit mRNA and peptide levels that is associated with an increase in the percentage of α1-containing receptors (Figs. 5 and 6). These data suggest that a change in the subunit composition of $GABA_A$ receptors in the cerebral cortex contributes to the mechanism of tolerance to the anxiolytic effects of diazepam.

Chronic exposure to another drug of abuse like nicotine produces upregulation of nicotinic acetylcholine receptors at the cellular plasma membrane that is mediated by a chaperoning mechanism (Corringer et al., 2006; Srinivasan et al., 2014). The chaperoning of nicotinic acetylcholine receptors by nicotine involves changes at several steps of intracellular trafficking such as assembly at the endoplasmic reticulum, export of assembled receptors from the endoplasmic reticulum, vesicle transport and insertion in the plasma membrane. Because benzodiazepines, like nicotine, can permeate the cellular plasma membrane, it is tempting to speculate that the selective increase in the expression of α1-contaning $GABA_A$ receptors induced by prolonged benzodiazepine administration might be mediated by a similar chaperoning mechanism.

The function and trafficking of $GABA_A$ receptors are controlled by phosphorylation of residues within the major intracellular loop between transmembrane domains 3 and 4 of receptor subunits (Kittler and Moss, 2003; Song and Messing, 2005; Comenencia-Ortiz et al., 2014). Phosphorylation can potentiate or inhibit $GABA_A$ receptor function depending on the receptor subtype. Phosphorylation can also regulate the action of allosteric modulators of $GABA_A$ receptors. Protein kinase C (PKC) activation induces a decrease in the potency of benzodiazepines to stimulate GABA currents in neuron-like NT2-N cells (Gao and Greenfield, 2005). Experiments performed in mutant mice lacking PKCε showed that this kinase inhibits the behavioral effects of barbiturates, benzodiazepine and neurosteroids as well as the ability of neurosteroids to potentiate muscimol-induced chloride influx (Hodge et al., 1999, 2002). Uncoupling of GABA/benzodiazepine site interactions induced by chronic benzodiazepine exposure may be mediated by a change in the phosphorylation state of $GABA_A$ receptors. We have previously demonstrated that the persistent activation of $GABA_A$ receptors by GABA in cultured neurons of the rat cerebral cortex produces uncoupling of GABA and benzodiazepine sites that is associated with an increase in the phosphorylation of receptor γ2 subunits at serine residues by PKC (Gravielle et al., 2005; Gutiérrez et al., 2014). The results presented here indicate that the prolonged treatments with diazepam for 7 days produce an increase in the degree of phosphorylation at serine 327 of the receptor γ2 subunits in the cerebral cortex. This phosphorylation is sustained after 14 days of treatment with benzodiazepine (Fig. 7). Therefore, tolerance to the sedative effects of diazepam may be mediated by changes in the phosphorylation state of $GABA_A$ receptors that could lead to uncoupling of GABA and the benzodiazepine sites.

CONCLUSIONS

In this study, we demonstrate that tolerance to the sedative effects of diazepam is associated with the development of uncoupling of GABA/benzodiazepine site interactions that may result from an increase in the degree of phosphorylation of the receptor γ2 subunits at serine 327. In contrast, tolerance to the anxiolytic effects of diazepam is associated with a change in the percentage of α1-contaning $GABA_A$ receptors. However, it is possible that the uncoupling and/or the increase in the phosphorylation of $GABA_A$ receptors observed following the 14-day treatment with diazepam also contributed to the development of tolerance to the anxiolytic effects of diazepam. Thus, benzodiazepine tolerance may be mediated by multiple, simultaneously coexisting adaptive mechanisms.

DISCLOSURES

There are no known conflicts of interest.

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