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Decreased Sensitivity to Metocurine during Long-term Phenytoin Therapy May Be Attributable to Protein Binding and Acetylcholine Receptor Changes

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Long-term phenytoin therapy induces resistance to the neuromuscular blocking effects of metocurine. The hypothesis that this is attributable to increased plasma protein binding of the drug (decreased free fraction) related to increased concentrations of α_1-acid glycoprotein (AAG) or attributable to the proliferation of acetylcholine receptors (AChR) at the muscle membrane was tested in the rat. After 14 days of phenytoin 40 mg·kg^{-1}, administered intraperitoneally twice daily (n = 12), the neuromuscular pharmacodynamics were evaluated and compared with those of time-matched controls (n = 10). Protein binding was measured by equilibrium dialysis, AAG concentrations by radial immunodiffusion assay, and AChR by ^{125}I-α-bungarotoxin binding. The effective dose for 50% inhibition of baseline twitch height (ED_{50}) was significantly greater in the phenytoin group than in the control group (15.03 ± 1.65 μg·kg^{-1} *vs.* 9.98 ± 0.69 μg·kg^{-1}, respectively). The concentrations of AAG increased gradually from 133.8 ± 7.8 μg·ml^{-1} at day 0, to 343.1 ± 58.0 μg·ml^{-1} at day 7, to 1,729.5 ± 422.3 μg·ml^{-1} at day 14 in the phenytoin group. The induction of AAG concentrations in plasma was dependent on plasma phenytoin concentrations and was most prominent after 14 days of phenytoin (r = 0.77; $P < 0.01$; n = 22). The free fraction of metocurine was significantly decreased in the phenytoin group compared to the control group (67.2 ± 0.18% *vs.* 74.5 ± 2.5%). There was a significant negative correlation between increased AAG concentrations and decreased free fraction (r = 0.65). The spontaneous recovery of twitch to 75% of full recovery was significantly faster (22.7 ± 3.3 min *vs.* 36.0 ± 4.3 min) and was seen at greater total (42.6 ± 2.5 ng·ml^{-1} *vs.* 32.3 ± 2.8 ng·ml^{-1}) and greater unbound (29.9 ng·ml^{-1} ± 2.9 *vs.* 22.2 ± 1.9 ng·ml^{-1}) plasma metocurine concentrations in the phenytoin group. Concurrently, the AChR number on the muscle membrane was significantly increased in the phenytoin group (2.67 ± 0.23 fmole/mg protein *vs.* 1.84 ± 0.26 fmole/mg protein). A higher free fraction of metocurine required for a given twitch inhibition, together with an increased plasma protein binding of metocurine in the phenytoin group, suggests a pharmacodynamic and pharmacokinetic mechanism for the resistance that may include increased AChR numbers at the muscle membrane and increased plasma protein binding of metocurine related to augmented AAG concentrations. (Key words: Drug interactions: metocurine; phenytoin. Muscle, skeletal: acetylcholine receptors. Neuromuscular relaxants: metocurine. Pharmacodynamics: metocurine. Proteins: α_1-acid glycoprotein. Receptors: nicotinic; acetylcholine.)

INCREASED DOSES or plasma concentrations of nondepolarizing muscle relaxants (NDMR), including metocurine, atracurium, doxacurium, and vecuronium, are required to produce a given neuromuscular effect when patients receive phenytoin for prolonged periods.[1–4] Decreased sensitivity (or resistance) to pancuronium and pipecuronium also has been noted during long-term therapy with carbamazepine, another anticonvulsant.[5,6]

Ornstein *et al.*,[1] in an attempt to elucidate the mechanism of this interaction, studied the pharmacokinetics and pharmacodynamics of metocurine in neurosurgical patients receiving long-term phenytoin therapy. Although phenytoin is a potent inducer of hepatic microsomal enzymes,[7] no differences in pharmacokinetic parameters, including clearance, were observed.[1] These authors speculated that a pharmacodynamic cause of resistance to metocurine existed because patients receiving phenytoin recovered from a given level of paralysis at greater metocurine plasma concentrations.

The requirement of a greater concentration of plasma metocurine for a given effect may be attributable to increased plasma protein binding, which results in decreased free fraction of drug to interact with the target receptor. A more intensified plasma binding of the drugs is known to occur when concentrations of the acute-phase reactant protein α_1-acid glycoprotein (AAG) are increased.[8–10] Inflammation or long-term administration of anticonvulsants increases concentrations of AAG and decreases the free fraction of drug.[8–14] However, the effects of a phenytoin-induced increase of AAG on the plasma protein binding of metocurine are unknown. An alternate explanation for the requirement of greater concentrations of plasma metocurine for a given twitch suppression may be that long-term administration of phenytoin causes a change in acetylcholine receptor (AChR) quantity or

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quality at the skeletal muscle membrane. Conditions in which proliferation of AChR numbers occur, such as burns, immobilization, and denervation, are associated with resistance to NDMR.[15–18] Denervation is associated with a qualitative change of AChR.[19]

This study in the rat tested the hypothesis that long-term phenytoin therapy causes an increased binding of metocurine to plasma protein and an increased AChR number at the skeletal muscle membrane. These changes, if induced by phenytoin, may play a role in the resistance to metocurine and other NDMR observed during prolonged treatment with phenytoin.

Materials and Methods

Animals

The experiments were conducted according to National Institutes of Health animal care guidelines and were approved by the institutional review board. Male Sprague–Dawley rats (Charles River, Wilmington, MA) weighing 225–250 g were randomized to treatment and control groups. The investigators were not blinded to the groups during the pharmacodynamic studies. The long-term phenytoin treatment group (n = 12) received 40 $mg \cdot kg^{-1}$ phenytoin sodium (Invenex Labs, Rosemont, IL) intraperitoneally twice daily for 14 days. Preliminary studies indicated that therapeutic concentrations of phenytoin (5–20 $\mu g \cdot ml^{-1}$) in humans could be achieved only with these doses. The control animals (n = 10) received the same volume of saline, at the same frequency and for the same duration. The last injection of phenytoin or saline was made 24 h before neuromuscular pharmacodynamic studies.

Pharmacodynamics of Metocurine

Neuromuscular pharmacodynamics were studied after 14 days of treatment with phenytoin or saline. The rats were anesthetized with pentobarbital sodium. The jugular vein was cannulated for intravenous drug administration and blood sampling. A tracheostomy was performed, and the animal's lungs were ventilated with room air using a Harvard ventilator that was adjusted to maintain physiologic venous blood gases sampled through the cannulated jugular vein. Rectal temperature was monitored and maintained at 36–38° C with a heat lamp. The body and left leg were restrained with tapes. The left gastrocnemius muscle was isolated, and the muscle tendon was attached to a Grass FT03 (Grass Medical Instruments, Quincy, MA) force inducer using 2-0 silk. The baseline tension was set at 50 g. The sciatic nerve exposed in the thigh was connected to a Grass S-88 stimulator, and the stimulation was performed with supramaximal pulses of 0.15 Hz and 0.2 ms duration. The twitch response of the gastrocnemius muscle was recorded on a Western Graphtec WR 7500 Instrument (Western Graphtec, Irvine, CA).

After twitch response was stable for at least 10 min, incremental doses of metocurine iodide were administered intravenously to achieve 95–100% twitch suppression. After an initial dose of 5 $\mu g \cdot kg^{-1}$ was administered, five to ten incremental doses, varying from 2 to 5 $\mu g \cdot kg^{-1}$, were administered. The neuromuscular response to each dose was allowed to stabilize before the next dose was administered. The response was considered stable when at least three of the depressed twitch responses were of the same height. The time lag between injections was approximately 40–60 s. The administration of metocurine was completed within 10 min. The twitch tension was allowed to recover spontaneously, and the time for recovery of twitch to 75% of full recovery was noted.

Blood Sampling

Before initiation and 7 days after injections of phenytoin or saline, 1 ml blood was withdrawn from the tail vein under anesthesia to assess of plasma concentrations of phenytoin or AAG. On day 14, before pharmacodynamic studies were performed, blood was drawn through the jugular vein to measure the phenytoin and AAG concentrations. In addition, during spontaneous recovery of metocurine-induced twitch suppression of the gastrocnemius muscle, three more blood samples (0.5–1 ml each) were drawn at varying points of recovery to correlate plasma metocurine concentrations with twitch recovery. When the twitch tension had recovered completely and remained stable for at least 5 min, another 3 ml blood was withdrawn to measure plasma protein binding of metocurine and concentrations of AAG. Throughout the study, for each milliliter of blood withdrawn, 3 ml saline was reinjected intravenously into the animal. All blood samples were drawn into tubes containing heparin and centrifuged at 2,000 × g for 30 min. The plasma was stored at −70° C until further analysis.

Plasma Metocurine Assay

Plasma concentrations of metocurine were analyzed using the high-performance liquid chromatography method with our recently described modifications.[20] The high-performance liquid chromatograph (HPLC) was equipped with a guard column, 4.6 mm × 20 mm, packed with 10-μm Polar Bonded phase CN on silica (Vydac, Hesperia, CA) and a Beckman 5-μm Ultrasphere Cyano (Beckman Institutes, Irvine, CA) column, 4.6 mm × 250 mm, to which a mobile phase, a mixture of acetonitrile: methanol:water:1 M dibutylamine phosphate (40:10:10:3, volume/volume, pH 2.5), was pumped at a flow rate of 1 $ml \cdot min^{-1}$ at room temperature. Metocurine was detected by ultraviolet absorption at 204 nm using a variable

wavelength detector (model 481, Waters, Milford, MA). Plasma samples and standards of metocurine were prepared before injection into the HPLC. Plasma (0.1 ml) and 10 μl of an internal standard, *d*-tubocurarine chloride (1 $\mu g \cdot ml^{-1}$), were applied to C18 1-ml cartridges and prewashed (with tetrahydrofuran, methanol, and water) supported on a vacuum manifold. The cartridge was rinsed with 1 ml water and eluted with 0.5 ml of the mobile phase. The eluate was evaporated under nitrogen gas. The dried sample was reconstituted with 50 μl of the mobile phase, and 25 μl of the solution was injected into the HPLC. The standard curve was constructed as the peak area ratios of metocurine to internal standard against a known amount of metocurine, 0.025–5 $\mu g \cdot ml^{-1}$. The coefficient of variation of the assay was 5.7% at 0.025 $\mu g \cdot ml^{-1}$, 3.3% at 0.25 $\mu g \cdot ml^{-1}$, and 4.7% at 5 $\mu g \cdot ml^{-1}$.

α_1-Acid Glycoprotein Assay

The plasma concentration of AAG was assayed using radial immunodiffusion plates.[21,22] The plates consisted of 1% Sea-Kem ME agarose (FMC Bioproducts, Rockland, ME) in 7 mM Na_2HPO_4, 2 mM NaH_2PO_4, 145 mM NaCl, 5 mM EDTA, *p*H 7.0 with 0.1% sodium azide as a preservative. The gel contained 20% (volume/volume) of the rabbit AAG antiserum prepared as described previously.[23] Plates were prepared by combining equal volumes of diluted antiserum and 2% agarose gel solution. The temperature of the antiserum solution was maintained at 52° C before preparation. The 2% agarose gel was heated at 100° C until the solution was clear, and it was allowed to cool to 52–55° C before mixing with the antiserum solution. The solutions were mixed slowly to avoid the formation of air bubbles. The gel containing the antibody was poured slowly onto radial immunodiffusion plates, which were cooled at room temperature until the gel solidified. The plates were stored at 4° C until needed. Each plate contained 9 ml of antibody-containing agarose gel with ten wells punched. Each well accommodated 5 μl plasma. After 5 μl plasma was applied to each well, the plates were allowed to stand for 24 h at room temperature to yield a fully developed precipitin ring. From the standard curve of 100–2,000 $\mu g \cdot ml^{-1}$ of AAG, the quantity of the AAG in plasma was calculated. The radial immunodiffusion developed (by F. J. Arnold) to measure rat AAG has been shown to be highly specific and accurate, yielding correlation coefficients of 0.98 with standards.[23] There is no cross-reactivity with other proteins, including albumin, hemopexin, or α_2-macroglobulin.[23]

Free Fraction of Metocurine in Plasma

An equilibrium dialysis method using dialysis cells was used to measure total bound and free (unbound) fractions of metocurine in plasma.[8,9,24] One milliliter of plasma was spiked with ^{14}C-dimethyl-*d*-tubocurarine iodide (specific activity 94.7 mCi/mmole; Amersham Co., Arlington Heights, IL) and placed on one side of the cell, which was separated from another cell containing 1 ml buffer by a protein impermeable membrane. After 24 h of dialysis at room temperature, buffer and plasma were recovered, and each was mixed with 15 ml of scintillation cocktail solution, Hydrofluor (National Diagnostics, Manville, NJ), and their radioactivity was counted on a Beckman LS7500 scintillation counter (Beckman Institutes). The free fraction or percent unbound was calculated as the ratio of counts per minute in buffer to counts per minute in plasma. The concentrations of free metocurine in plasma was calculated by multiplying the free fraction (percent) by total concentration of drug in plasma (micrograms per milliliter) as analyzed by the HPLC method.

Phenytoin Assay

Fifty microliters of plasma was used to analyze the concentration of phenytoin with a TDx assay kit (Abbot Laboratories, Chicago, IL).[25] The lower limit of sensitivity of the assay is 1.0 $\mu g \cdot ml^{-1}$ with a coefficient of variation of 2–4%.

Nicotinic Acetylcholine Receptor Assay

At the end of the neuromuscular pharmacodynamic studies, the left gastrocnemius was dissected and washed with 20 mM phosphate buffer (*p*H 7.4) containing 1 mM EDTA, 2 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide. The tissue was stored at −70° C until assay for AChR number. ^{125}I-α-bungarotoxin (^{125}-I-α-BT) was used as the specific ligand. The binding affinities of the toxin to junctional and extrajunctional AChR was not different.[19,26,27] The whole muscle mass was used for the AChR assay with no distinction made between junctional and extrajunctional areas.

On the day of the AChR assay, the frozen gastrocnemius was thawed, finely chopped, and homogenized for 1 min in 4 volumes of 0.01 M potassium phosphate buffer, *p*H 7.4, containing 1 mM EDTA, 2 mM benzamidine hydrochloride, 0.1 M benzethonium chloride, 0.1 mM phenylmethylsulfonyl fluoride, bacitracin, and 0.02% (weight/volume) sodium azide at 4° C. The homogenate was centrifuged at 20,000 × g for 30 min at 4° C. The supernatant was discarded, and the precipitants were resuspended and homogenized for 1 min in the same buffer containing additional 2% (volume/volume) Triton X-100 (Sigma Chemical Co., St. Louis, MO). This extraction was shaken overnight in a cold room and was centrifuged at 20,000 × g for 45 min at 4° C. The supernatant was recovered and stored at −70° C. Triplicate samples of crude muscle extract were incubated with 2.5 nM ^{125}I-α-

BT (specific activity 14.2 μCi/μg; New England Nuclear Products, Boston, MA) in the Triton buffer for 90 min at room temperature. Excess α-BT was separated from the α-BT–AChR complex using polyethylenimine, pretreated Whatman 2.5 cm, GF/B glass fiber filters by vacuum filteration on a Millipore filter unit. Specific binding of ^{125}I-α-BT was blocked in parallel incubations using 1 μM unlabeled α-BT. Each filter was counted for radioactivity using a TM analytic 1191 γ counter (Tracor Analytic, Elk Grove Village, IL). The protein concentration in the Triton-extracted muscle protein was assayed using the method described by Hartree.[28] The number of AChR in the muscle extract was expressed as femtomoles AChR per milligram protein.

Statistical Analysis

The dose–response curves of metocurine were plotted on a log-probit scale (log dose *vs.* log 1 − y/y, where y is the maximal neuromuscular suppression for each cumulative dose). Effective doses that produced 50% (ED_{50}) and 95% (ED_{95}) twitch paralysis were calculated by least-squares regression analysis of the log dose–response curves. Similarly, least-squares regression analysis was used to construct plasma metocurine concentration *versus* effect curves for each animal and to calculate plasma metocurine concentrations at which the twitch recovered to 75% of complete recovery (25% twitch suppression). Pearson's correlation coefficient (r) or, when data were not normally distributed; Spearman's rank correlation coefficient (ρ) tested the relationship between variables such as phenytoin concentrations, AAG concentrations, and free fractions of metocurine. The statistical significance of the data was tested by Student's t-test and analysis of variance, with a value of $P < 0.05$ considered significant. The results are reported as the means ± SEM.

Results

Plasma α_1-Acid Glycoprotein Concentrations and Protein Binding of Metocurine

Concentrations of phenytoin in plasma 7 and 14 days after injection were 5.25 ± 1.42 $\mu g \cdot ml^{-1}$ and 11.5 ± 3.05 $\mu g \cdot ml^{-1}$, respectively. The AAG concentrations before injections of saline or phenytoin did not differ between groups (table 1). After 7 and 14 days of phenytoin injections, the AAG concentrations were significantly increased, approximately 3-fold and 10-fold respectively, compared to baseline values or time-matched controls. The AAG concentrations in controls did not change during the study period. The correlation coefficient between plasma phenytoin concentrations (which was 0 in controls) and induction of AAG concentrations at day 14 was significant ($R^2 = 0.60$, r = 0.77, $P < 0.01$, n = 22; fig. 1). Even with the extreme AAG value of 6,000 $\mu g \cdot ml^{-1}$ removed from the regression, the correlation between phenytoin concentrations and induction of AAG was significant ($R^2 = 0.37$, r = 0.61, $P < 0.01$). This positive correlation also was observed when the AAG concentrations and phenytoin concentrations at 0, 7, and 14 days for both groups were pooled ($\rho = 0.7$, n = 66, $P < 0.01$). To test the effect of multiple blood samplings and repeated injections of saline during the pharmacodynamic studies, AAG concentrations were measured at the end of the pharmacodynamic studies and were compared with concentrations measured before initiation of the pharmacodynamic studies. The values did not differ within the groups (table 1). The unbound (free) fraction of metocurine in plasma was significantly decreased at 14 days in the phenytoin group compared to time-matched controls (table 1).

Metocurine Dose-Response and Recovery

There was no observable difference in the mobility or activity of the animals in the phenytoin group compared with those in the control group. Rats receiving phenytoin had gained less weight than controls by day 14 (table 2). The ED_{50} and ED_{95} of metocurine for twitch inhibition were increased approximately 50% and 100%, respectively, in the phenytoin group compared with the control group (table 2). The completely recovered twitch heights at the end of the experiment relative to twitch heights before administration of metocurine were 90.3 ± 4.3% and 79.2 ± 5.8% in the control and phenytoin groups, respectively, and were not significantly different between

TABLE 1. Phenytoin Effects on Plasma AAG Levels and Plasma Protein Binding to MTC

	Plasma AAG Levels (μg/ml)				MTC Free Fraction (%)
Group	Day 0	Day 7	Day 14 (Prestudy)	Day 14 (Poststudy)	Day 14 (Poststudy)
Control	149.6 ± 10.3	198.5 ± 33.3	162.0 ± 20.5	146.6 ± 12.3	74.5 ± 2.5
Phenytoin	133.8 ± 7.8	343.1 ± 58.0*†	1729.5 ± 422.3*†	1639.2 ± 595.9*†	67.2 ± 1.8‡

Values are mean ± SEM.
* $P < 0.001$ from baseline (day 0).
† $P < 0.002$ compared to controls.
‡ $P < 0.05$ compared to controls.

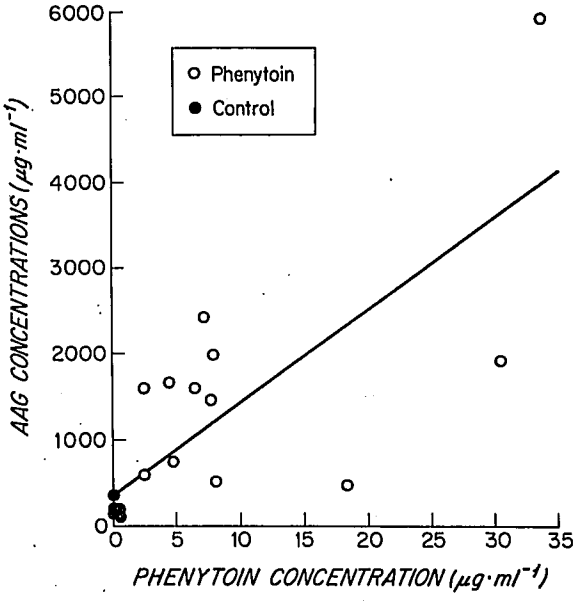

FIG. 1. The relationship of AAG concentrations to phenytoin concentration in controls (n = 10) and the experimental group (n = 12) after 14 days of saline or phenytoin, respectively. Open circles = phenytoin group; filled circles = control. There is a significant positive correlation between phenytoin concentrations and AAG levels in plasma ($R^2 = 0.60$, $r = 0.77$, $P < 0.01$).

groups. The time for spontaneous recovery of twitch from maximal depression to 75% of complete recovery (25% suppression) was significantly faster in the phenytoin group (table 3) despite the greater effective doses administered.

The recovery index, calculated as the time between 25% and 75% recovery, also was significantly shorter in the phenytoin group (table 3). The twitch tensions recovered to 75% of complete recovery at greater total plasma metocurine concentrations in the phenytoin group than in the control group (table 3). The free fraction of metocurine in plasma was decreased in the phenytoin group compared to time-matched controls (table 1). The pharmacologically active concentrations of free metocurine concentrations at 75% recovery were calculated from values of unbound fraction and total metocurine concentrations for that recovery; they are shown in table 3. Despite the increased binding, the phenytoin group had a greater concentration of free metocurine in plasma at 75% recovery of twitch.

CHANGES IN ACETHYLCHOLINE RECEPTORS

The nonspecific binding of α-BT was 39.8 ± 2.03% and was not significantly different between the two groups ($P > 0.45$). The numbers of AChR in the treated group (2.67 ± 0.23 fmole/mg protein) were significantly greater ($P < 0.05$) than those of the control group (1.84 ± 0.26 fmole/mg protein).

Discussion

Several studies in humans receiving long-term phenytoin or carbamazepine therapy have documented an interaction with NDMR in which a rightward shift in dose– or concentration–response curves or faster recovery of twitch was observed.[1–6] Our study in rats receiving phenytoin replicates all of these aspects relative to metocurine: increased effective doses, a greater plasma total metocurine concentration requirement for a given twitch inhibition, and a faster recovery of twitch despite a significantly greater effective dose. In the current study, we document that long-term phenytoin administration causes increased protein binding of metocurine and modest elevations of AChR at the muscle membrane.

Phenytoin is a potent inducer of hepatic microsomal enzymes, including cytochrome P-450 isozymes, and results in enhanced clearance of many drugs.[7] However, a pharmacokinetic study of metocurine in humans found no enhanced metabolic clearance, which may be related to metocurine's minimal hepatic and predominant renal elimination.[29] Another hepatic effect of phenytoin is that it induces the release of acute-phase reactant protein AAG, which binds to cationic drugs.[11–14] Although this effect has been seen in humans and dogs, our study confirms this effect in rats and documents that AAG causes increases in plasma protein binding of metocurine. The increase in AAG concentrations was dependent on plasma phenytoin concentrations and was most prominent at 14 days of phenytoin therapy ($r = 0.77$). Although the AAG concentrations were increased 10-fold at 14 days of phenytoin administration, the protein binding of metocurine did not parallel this increase, which suggests that the ca-

TABLE 2. Systemic and Neuromuscular Effects of Phenytoin

Group	Body Weight (g)	ED_{50} of MTC ($\mu g \cdot kg^{-1}$)	ED_{95} of MTC ($\mu g \cdot kg^{-1}$)	Acetylcholine Receptor Number (fmol/mg protein)
Control	330.91 ± 5.85	9.98 ± 0.69	20.49 ± 1.5	1.84 ± 0.26
Phenytoin	286.17 ± 10.85*	15.03 ± 1.65†	39.94 ± 5.81†	2.64 ± 0.23†

Values are mean ± SEM.
* $P < 0.005$ *versus* controls.
† $P < 0.05$ *versus* controls.

TABLE 3. Pharmacodynamics of Twitch Recovery

Group	Time to 75% of Complete Recovery (min)	Recovery Index (min)	Total MTC Concentration at 75% Recovery ($ng \cdot ml^{-1}$)	Unbound MTC Concentration at 75% Recovery ($ng \cdot ml^{-1}$)
Control	36.03 ± 4.26	23.45 ± 3.31	32.3 ± 2.8	22.2 ± 1.9
Phenytoin	22.71 ± 3.33*	13.88 ± 2.38*	42.6 ± 2.5*	29.9 ± 2.9*

Values are mean ± SEM.

* $P < 0.05$ *versus* controls.

pacity of AAG to bind metocurine, though significant, is limited. Thus, the 50–100% shift in the ED_{50} and ED_{95} is not totally explained by the approximately 30% increase in protein binding of metocurine (bound fraction changed from 25.5% in the control group to 32.8% in the phenytoin group). In addition, if the increased binding was the sole cause of resistance, the free (unbound) concentration of metocurine for a given level of twitch (*e.g.*, 75% recovery) would have been similar between the groups. Thus, our findings do not support the thesis that binding alone is the cause for the resistance.

Despite the increased fraction bound to plasma, greater free (and total) concentrations of metocurine were required for a given twitch inhibition (or recovery) in the phenytoin group. The greater concentrations of free metocurine required for a given twitch inhibition suggests a contributing pharmacodynamic mechanism. During upregulation (increase) of AChR, as is seen in burns, immobilization, long-term NDMR infusion, or denervation, an increased requirement for NDMR has been observed.[14–18] It is unclear whether this increased requirement or resistance to NDMR is attributable to the increase in receptors or to qualitative changes in receptor function, such as change in affinity.[30–32] The current study documents a modest, but significant, increase in AChR in the same muscle in which pharmacodynamic studies were performed, and therefore is consistent with previous observations of resistance to NDMR with upregulation of AChR.[14–18,30,31] The total body weight gain in the phenytoin-treated animals was less than that of control animals. It could not be determined whether the increase in AChR is an artifact attributable to a phenytoin-induced decrease in muscle mass or it is real. If the AChR number were unaffected by phenytoin treatment but the phenytoin group had a smaller muscle mass (protein), the AChR number per unit of muscle protein could be artifactually increased. It also is possible that the phenytoin-induced resistance is attributable to qualitative changes in AChR. For example, after motor nerve denervation, the adult (mature) AChR is converted to denervation (immature)-type AChR, which results in altered sensitivity to agonist (*e.g.*, acetylcholine) and antagonists (*e.g.*, NDMR).[30,31] Such a change, if present after long-term phenytoin, would result in greater dose or concentration requirements of NDMR for a given effect.[30,31]

The molecular mechanisms that induce quantitative and qualitative changes in AChR during long-term phenytoin are unknown, but they may be attributable to several factors operating concurrently. The acute neuromuscular blocking effects of phenytoin are similar to those of small doses of NDMR[33,34] and can be reversed by edrophonium.[33] The mechanism of this postjunctional neuromuscular blocking effect may be attributable to allosteric inhibition or phosphorylation of AChR by phenytoin.[35] Alternately, this potentiation seen acutely may be related to its prejunctional effects, by which it decreases the release of acetylcholine.[36,37] However, long-term prejunctional and postjunctional inhibition of neuromuscular function can cause upregulation of AChR numbers.[17,31,38,39] Resistance to NDMR and the proliferation of AChR can be seen with even subparalytic doses of NDMR administered for long periods.[17] This form of long-term, subclinical, chemical-denervation of AChR may have occurred in the phenytoin group. Other long-term effects of phenytoin therapy include peripheral neuropathies and neuromuscular disorders,[40,41] the manifestations of which include lower extremity aflexia, sensory deficits, and reduced conduction velocities. These neuromuscular deficits may have contributed to the denervationlike syndrome that is associated with the proliferation of AChR and resistance to NDMR.[14–18] Although there were no obvious changes in mobility between study groups, the association between decreased weight gain in the phenytoin group and neuromuscular changes observed in the phenytoin-treated animals is unclear.

In the current study, a fixed dose of phenytoin was used and its effects on the neuromuscular junction at a defined time period of 2 weeks were studied. This protocol resulted in marked increases in AAG related to phenytoin concentrations and modest increases in AChR. It is not known whether greater doses or a longer duration of phenytoin therapy would result in more pronounced changes.

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