

Acetylcholine Receptor Density and Acetylcholinesterase Activity in Skeletal Muscle of Rats Following Thermal Injury

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Thermal injury causes systemic changes that result in altered sensitivity to many drugs including nondepolarizing muscle relaxants. In an effort to identify the mechanism(s) responsible for the resistance to nondepolarizing muscle relaxants, the density of acetylcholine receptors (AChR) and the activity of acetylcholinesterase (AChE) were determined in rats following a 30% total body surface thermal injury at a time when resistance to atracurium is maximum. AChR density in gastrocnemius and diaphragm was unchanged by thermal injury. Furthermore, the ratio of junctional to extrajunctional AChR in diaphragm was unaltered. Total AChE activity was unchanged in thermally injured rats compared with that in sham-injured animals. Separation of the molecular forms of AChE by sucrose gradient centrifugation also showed no changes in the relative proportions of these species. The mechanism of resistance to nondepolarizing muscle relaxants does not appear to be explained by changes in AChR number or changes in the activity of AChE. (Key words: Burns; resistance to neuromuscular relaxants. Enzymes: acetylcholinesterase. Muscle: skeletal. Neuromuscular relaxants, atracurium; resistance. Receptors: acetylcholine.)

PATIENTS with thermal injury are resistant to the effects of several nondepolarizing muscle relaxants (NDMR), including *d*-tubocurarine,¹ metocurine,^{2,3} pancuronium,⁴ and atracurium.⁵ Various authors have proposed that pharmacodynamic mechanisms, such as an increase in the density of acetylcholine receptors (AChR) on the muscle, may account for this resistance.¹⁻⁴ This hypothesis holds that an increase in the number of AChR would require more NDMR to block the action of acetylcholine (ACh) at the neuromuscular junction. An increase in AChR density has been reported following upper motor neuron lesions,⁶ as a result of denervation injury,⁷ and following burn injury in rats.⁸

A second potential mechanism for the NDMR resistance is a burn-induced decrease in acetylcholinesterase (AChE) (EC3.1.1.7) activity. This decrease would effectively raise the neuromuscular transmitter (ACh) concen-

tration and thereby require a higher concentration of NDMR to achieve the same degree of block.

We have recently established the rat as a suitable model of thermal injury in humans.⁹ The rat exhibits the distinctive time course of resistance similar to that found in burned patients: normal response to NDMR for approximately 10 days after injury, peak resistance at approximately 40 days, and a decline in resistance beyond 60 days. The rat is also suitable for mechanistic studies. We have examined the role of AChR density and AChE activity in the resistance to NDMR following thermal injury.

Materials and Methods

ANIMALS

All studies were approved by the Animal Care Committee at the University of Washington. Female Sprague-Dawley rats (200-235 g) were anesthetized with pentobarbital (43 mg/kg) and placed in a template that exposed an area on their back equal to 30% of the total body surface (TBS). This exposed area was immersed in boiling water for 10 s to cause a third-degree burn injury.⁹ Sham-injured control rats were anesthetized but not exposed to the boiling water. Because maximum resistance to atracurium was observed approximately 40 days after the injury, this time was chosen for AChR and AChE measurements. After again anesthetizing the animals with pentobarbital, the gastrocnemius muscles of burned and sham-injured rats were first tested for resistance to atracurium by measuring the gastrocnemius muscle contractile force following a 2 mg/kg bolus of atracurium. The muscles and nerve were exposed, the severed Achilles tendon fixed to a force transducer, and the sciatic nerve directly stimulated supramaximally.⁹ Following the contractile force measurements, the gastrocnemius muscle was immediately removed and stored at -80° C until analysis.

AChR number and AChE activity were also measured in denervated gastrocnemius muscles to demonstrate the capability of these measurements to detect changes of AChR number and AChE activity following a procedure that was known to alter these activities. Denervation was performed on rats anesthetized with pentobarbital by removing a 0.5-cm segment of the sciatic nerve proximal to the gastrocnemius. The rats were killed with an anes-

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thetic overdose 14 days postdenervation, a time period that was shown to produce the maximal effect of denervation-induced changes in muscle.⁷ The gastrocnemius muscles were removed and stored as above at -80°C .

MEASUREMENT OF AChR DENSITY IN THE RAT GASTROCNEMIUS MUSCLE

The method of Schmidt and Raftery¹⁰ was used to determine AChR density. Briefly, after removing the nerves, vessels, and tendons, the muscles were chopped finely and homogenized with a Brinkman Polytron at half-maximal speed for 90 s using protease inhibitor buffer** at a ratio of 7.5 ml buffer/g muscle. The homogenate was centrifuged at $100,000 \times g$ for 1 h. The pellet was resuspended in protease inhibitor buffer containing 1% triton X-100 and gently mixed for 30 min to solubilize the AChR. The supernatant obtained after recentrifugation at $100,000 \times g$ for 1 h was used as the AChR preparation. All the procedures were carried out at $0-4^{\circ}\text{C}$.

[¹²⁵I] α -Bungarotoxin (New England Nuclear, specific activity 80–160 Ci/mmol) was used as the ligand for AChR measurement. The receptor preparation was incubated in triplicate with and without 500-fold excess of unlabeled α -bungarotoxin. In a preliminary study, incubations were carried out at various concentrations of radioactive ligand (1–10 nM) to determine the optimum time of incubation and optimum concentration of radioactive ligand. For actual AChR measurement, incubations were carried out at 32°C for 4 h with 1 nM [¹²⁵I] α -bungarotoxin. When the incubation was complete, a portion of the incubation mixture was filtered through DEAE-cellulose filters, washed three times with triton X-100 buffer,†† and the filters were counted for the ligand receptor complex in a gamma scintillation counter. Specific binding was taken as the difference between the counts in the absence and presence of excess unlabeled α -bungarotoxin. Nonspecific binding averaged 36% of the total binding (amount bound in the absence of excess unlabeled ligand) and was not different in the sham or burned animals. Protein analysis of the soluble receptor preparation was done by the method of Lowry *et al.*,¹¹ and AChR number was expressed per milligram of total protein.

MEASUREMENT OF AChR IN RAT DIAPHRAGM

Following an overdose of pentobarbital, diaphragms were dissected from burned and sham-injured rats ap-

proximately 40 days after injury and used for receptor measurements on the same day. The density of AChR was determined by the method of Berg *et al.*¹² with some modification. Both hemidiaphragms were separated from the ribs and incubated with 25 nM [¹²⁵I] α -bungarotoxin (80–160 Ci/mmol) in Krebs's solution in small petri dishes, which were shaken gently for 4 h at room temperature. At the end of incubation, the diaphragms were washed free of excess ligand by replacing the incubation mixture with fresh buffer without ligand (three washes). The petri dishes containing the diaphragms were then shaken overnight followed by three more washes. The diaphragms were then dissected into junctional and extrajunctional regions, and approximately 50 mg of wet tissue from each sample was counted in the gamma counter. AChR number was expressed per unit weight of the wet tissue.

DETERMINATION OF TOTAL AChE ACTIVITY

The gastrocnemius muscle of either sham-injured or 40 day postburn rats was chopped finely, and 0.5 g of the muscle was added to 10 ml of extraction buffer¹³ and homogenized for 1 min using a Brinkman Polytron at half-maximal speed. A sample of this homogenate was reserved for the determination of total AChE, and the remainder was then centrifuged at $36,200 \times g$ for 30 min. Comparison of total AChE in the high-speed supernatant and in the homogenate showed that 91% of the total AChE was solubilized.

Enzymatic activity was measured according to the procedure of Johnson and Russell¹⁴ by mixing 50 μl of the $36,200 \times g$ supernatant or homogenate with 100 μl of [acetyl-³H]-ACh (10 mM, 340 mCi/mol) in a total volume of 500 μl . At 30, 60, 90, and 120 min, 100 μl of the incubation mixture was removed and the [³H]-acetate released by the esterase was measured as described. The curve obtained by plotting [³H]-acetate formed *versus* time is linear, and its slope was used to calculate the AChE activity. Pretreatment with specific inhibitors of AChE and pseudocholinesterase verified that more than 95% of the activity was due to AChE.

SEPARATION AND MEASUREMENT OF DIFFERENT MOLECULAR FORMS OF AChE

Resolution of the molecular forms of AChE was carried out by the method of Groswald and Dettbarn.¹⁵ The $36,200 \times g$ supernatant described above (400 μl) was layered on 11 ml of a 5–20% linear sucrose gradient. Alkaline phosphatase, β -galactosidase, and catalase were included as sedimentation velocity standards to calibrate the gradient. The gradients were then centrifuged at $100,000 \times g$ for 19 h in a swinging bucket rotor. After separation into approximately 60 fractions, every second fraction

** NaCl 100 mM; trizma-base 50 mM; EDTA 1 mM; phenylmethylsulfonyl fluoride 0.5 mM; benzamidine 0.1 mM; benzethonium chloride 0.1 mM; soybean trypsin inhibitor 5 mg/l; bacitracin 50 mg/l; pepstatin (2 mg/l), pH 7.4.

†† NaCl 100 mM; trizma-base 50 mM; 1% triton X-100, pH 7.4.

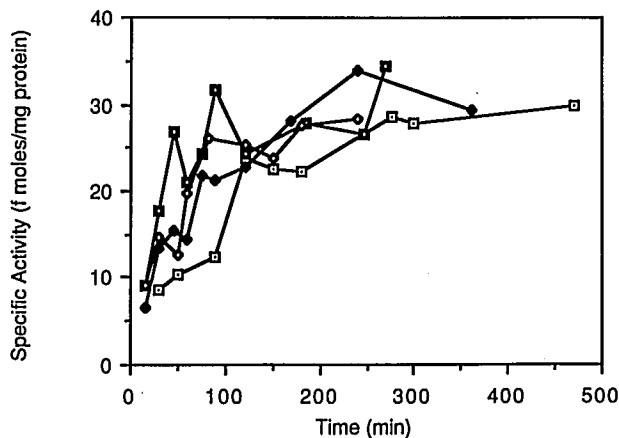


FIG. 1. Time course of specific [^{125}I] α -bungarotoxin binding with various concentrations of α -bungarotoxin. \square 1.0 nM, \blacklozenge 2.5 nM, \blacksquare 5.0 nM, \diamond 10 nM.

was analyzed for AChE activity. The sedimentation standards were assayed by colorimetric procedures as described.¹⁶⁻¹⁸ The elution pattern showed three different molecular forms corresponding to sedimentation coefficients 16S, 10S, and 4S. The area under each peak was measured and expressed as a fraction of the total area. The 16S form is the asymmetric collagen-bound A_{12} form of AChE, which is believed to be present at the postsynaptic membrane of the neuromuscular junction. Forms 10S and 4S correspond to the symmetric tetramer G_4 and monomer G_1 , respectively.¹⁹

Statistical analyses were performed using Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

Results

Figure 1 shows the specific binding at various times of incubation with all four concentrations of [^{125}I] α -bungarotoxin used. Specific binding reaches a plateau within

TABLE 1. AChR Number in Gastrocnemius Muscle and Response to Atracurium

| | Sham-injured | 40-days Postburn |
|---------------------------------------------|----------------------------|----------------------------------------|
| AChR number (fmol/mg protein) | 15.3 \pm 4.1 (n = 10) | 14.6 \pm 2.8 \dagger (n = 10) |
| % Twitch depression after atracurium bolus* | 94.5 \pm 7.1 (n = 12) | 76.7 \pm 10.2 \ddagger (n = 10) |

Values are mean \pm SD.

* Maximum depression of twitch elicited by supramaximal nerve stimulation following atracurium bolus.

$\dagger P = 0.66$ (compared with sham injury).

$\ddagger P < 0.05$ (compared with sham injury).

TABLE 2. Junctional and Extrajunctional AChR in Diaphragm

| | Sham-injured | 40-days Postburn |
|---------------------------------|-----------------------------|---------------------------------------|
| Junctional (pmol/g tissue) | 3.4 \pm 1.4 (n = 12) | 3.6 \pm 1.7* (n = 12) |
| Extrajunctional (pmol/g tissue) | 0.26 \pm 0.17 (n = 12) | 0.36 \pm 0.24 \dagger (n = 12) |
| Junctional/extrajunctional | 16.7 \pm 8.6 | 13.3 \pm 8.2 \ddagger |

Values are mean \pm SD.

* *P* = 0.70 (compared with sham injury).

$\dagger P = 0.24$ (compared with sham injury).

$\ddagger P = 0.33$ (compared with sham injury).

4 h; therefore, a 4-h incubation was chosen to measure AChR number in rat gastrocnemius in subsequent studies. The lowest concentration (1 nM) of the radiolabeled ligand was chosen to minimize nonspecific binding.

Table 1 shows the results of α -bungarotoxin binding obtained in sham-injured and 40-day postburn rats. This table also shows the response of each burned rat expressed as percentage twitch depression after atracurium was given as a 2 mg/kg iv bolus. There is a significantly smaller twitch depression following atracurium in the 40-day postburn rats, but there is no difference in the AChR density between thermally and sham-injured rats. For individual animals there is also no correlation between degree of resistance and AChR number ($r^2 = 0.0045$, NS).

Large increases in AChR density have been reported following denervation of skeletal muscle.⁷ Therefore, we included this treatment as a positive control to ascertain that the binding technique would detect changes in AChR number. Denervation results in an eightfold increase in AChR density (122 \pm 37.7 fmol/mg protein, n = 5) compared with that from the sham animals (table 1).

Table 2 shows the results of measurement of junctional and extrajunctional AChR in rat diaphragm. Junctional receptor density was calculated as the difference between the total and the extrajunctional receptor densities. There is no difference between the burned and sham-injured rats in either total number of junctional and extrajunctional receptors or in the ratio of junctional to extrajunctional receptors for each group.

Total AChE activity in sham-injured and 40-day postburn rats was found to be 3.67 and 3.91 pmol \cdot min⁻¹ \cdot μ g⁻¹ protein, respectively (table 3). Total AChE activity in rats 40-day postburn is thus not statistically significantly different from that of the sham-injured rats.

Resolution of AChE by sedimentation in sucrose gradients into the various molecular forms was carried out to determine whether changes in the distribution of the total enzymatic activity between the different sized molecular species occurred. Figure 2 shows a typical resolution of AChE into three molecular forms, A_{12} , G_4 , and

TABLE 3. Distribution of the Molecular Forms of AChE

| | % of Total | | | Total* (pmol · min ⁻¹ · μg protein ⁻¹) |
|--------------------------|-----------------|----------------|----------------|---------------------------------------------------------------------|
| | A ₁₂ | G ₄ | G ₁ | |
| Sham injury (n = 6) | 21.2 ± 5.2 | 49.5 ± 3.4 | 29.3 ± 2.7 | 3.67 ± 0.39 |
| 40 days postburn (n = 6) | 24.8 ± 5.0 | 49.7 ± 4.6 | 25.5 ± 3.6 | 3.91 ± 0.67 |

Values are given as mean ± SD. A₁₂ is the synaptic collagen-bound molecular form of the enzyme, which is believed to be primarily in-

involved in hydrolysis of ACh at the neuromuscular junction.
* Total AChE activity solubilized by extraction procedure.

G₁. In table 3 the proportions of the individual molecular forms in the sham-injured and 40-day postburn rats are presented. There are no significant differences in any of the forms between groups.

Denervated muscle was assayed for AChE activity to ascertain that the assay technique was sufficiently sensitive to detect changes in the enzyme activity. The total AChE activity in the denervated muscle was 0.68 pmol · min⁻¹ · μg⁻¹ protein, a decrease of nearly sixfold as compared with the activity in burned or sham-injured animals.

Discussion

We have previously demonstrated that the thermally injured rat displays resistance to NDMR, which develops and subsides over a time course similar to that observed in humans.^{5,9} Protein binding of atracurium in the plasma differs slightly between patients and rats in that the free fraction in control patients is greater than burned patients (0.498 control vs. 0.375 burned)^{‡‡} in contrast to rats in which sham and injured animals both exhibit a free fraction of 0.57.⁹ A pharmacodynamic mechanism of resistance to NDMR has been implicated in both species.^{9,20} Therefore, we have used this model to investigate the mechanism of resistance to NDMR.

An increase in skeletal muscle AChR has been suggested as the mechanism for the resistance to NDMR observed in burned patients.¹⁻⁴ Elevated numbers of either junctional or extrajunctional receptors could account for this phenomenon. An association between increased extrajunctional AChR and abnormal responses to muscle relaxants has been claimed based on indirect measures of AChR in various forms of neuromuscular injury. Avulsion or severing of a peripheral nerve results in an increase in AChR along the length of the associated muscle.⁷ Gronert showed that muscles in dog limbs totally immobilized in plaster casts for 1 mo required twice the dose of pancuronium to achieve blockade as did muscles in their normal

limbs.²¹ Several clinical studies have demonstrated the resistance to NDMR in the affected side of hemiplegic patients compared with their normal unparalyzed extremities.²²

Carter *et al.*⁶ examined the effect of spinal cord transection on neuromuscular function in the rat. They found indirect evidence for the spread of AChR over the muscle membrane.⁶ Seven days after a spinal cord lesion, depolarization of muscle membranes distal to the lesion could be effected by the ionophoretic application of ACh far from the end-plate. This coincided with the hyperkalemic response to succinylcholine administration, a well-described phenomenon in patients with spinal cord lesions.

A number of clinical studies have documented resistance to a variety of NDMR in burned patients.¹⁻⁵ An increase in AChR has been proposed as a mechanism based on observations made following injuries sustained by the affected muscle or its associated neurons in the studies described above. However, thermal injury differs from these injuries in that neither the muscle that demonstrates resistance nor its nerve are directly injured. In

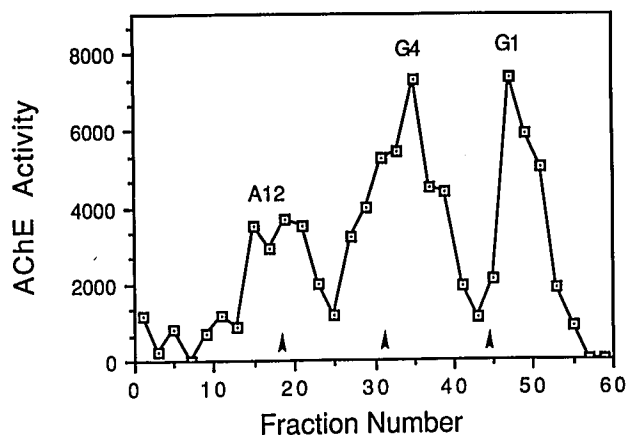


FIG. 2. Separation of the molecular forms of AChE by sucrose gradient centrifugation. Arrows indicate positions of marker enzymes used to determine the size of the molecular forms. Fraction 18, β-galactosidase (16S); fraction 31, catalase (11S); and fraction 44, alkaline phosphatase (6S).

‡‡ Marathe PH: Effect of thermal injury on the pharmacokinetics and pharmacodynamics of atracurium in patients. ANESTHESIOLOGY (in press)

our studies in rats, the cutaneous injury is sustained on the back while resistance is observed in leg muscle.

In this study, we have confirmed earlier observations that 40 days after sustaining a 30% TBS thermal injury, the gastrocnemius muscle in rats shows significant resistance to atracurium (table 1). However, there was no increase in AChR in these resistant muscles nor in the diaphragm. Furthermore, we specifically measured the concentrations of both junctional and extrajunctional AChR in the diaphragm, a skeletal muscle in which they may easily be separated. No difference between thermally injured and sham control animals could be found. The suitability of the technique of measuring AChR was demonstrated by the observation of an eightfold increase in AChR of gastrocnemius from rats in which the sciatic nerve had been severed 14 days earlier.

In a recent study, Kim *et al.*⁸ demonstrated an increase of approximately 50% in AChR in the diaphragm of burn-injured rats. An explanation for the differences between these results and ours is not readily apparent but may be related to several notable differences in the experimental model. We used a 30% body surface area burn injury as compared with 45–55% in their study. Our animals demonstrated maximal resistance to atracurium at approximately 40 days postburn with no significant resistance at the 10–21 day period,⁹ when Kim *et al.*⁸ reported the AChR increases. All of the animals we studied for AChR number were first tested and found to be resistant to atracurium. No resistance measurements were reported for the animals in the work by Kim *et al.*⁸ Their rats had been splenectomized prior to the burn injury, which might result in a decreased ability to resist sepsis or alter the recovery from the burn in other ways.

AChE terminates the transmitter–receptor interaction at the neuromuscular junction by hydrolyzing ACh. Decreased enzymatic activity, as a result of treatment with neostigmine or edrophonium, results in an increased requirement for NDMR. These observations suggest that a decrease in AChE activity at the neuromuscular junction following burn injury could result in an increased ACh concentration and account for the resistance to NDMR. However, we observed no change in total AChE activity following burn injury.

Because a measurement of total AChE in muscle may mask a deficiency of a specific molecular form, and because it is believed that the A₁₂ form exists at the neuromuscular junction and is primarily responsible for ACh hydrolysis to terminate the neurotransmitter action,^{23,24} we examined the relative proportions of the molecular forms.¹⁹ The forms differ in size as a result of the enzyme having several complex quaternary structures, which can be separated by sedimentation velocity in a sucrose density gradient.¹⁵ There was no deficiency of the A₁₂ form of

the enzyme, which is believed to be the synaptic collagen-bound form primarily involved in hydrolysis of ACh at the neuromuscular junction.

We conclude, therefore, that the mechanism of resistance to NDMR following a 30% TBS burn injury does not include changes in the density of AChR, as measured by α -bungarotoxin binding, or in the activity of AChE. Previously, we showed that at the time when burn-injured rats were maximally resistant to atracurium the Cp50 determined at steady state drug concentration in plasma was approximately 1.7-fold higher compared with sham controls.⁹ This would suggest that the required concentration of atracurium at the neuromuscular junction necessary for 50% inhibition of contraction also was elevated. These data implicate an altered affinity of the AChR for agonist (ACh) or antagonist (NDMR) as the basis for the burn-induced resistance. Techniques to measure skeletal muscle AChR affinity for agonist and antagonist must therefore be developed. Although affinity has been measured in the specialized electric organs and brain, the low density of AChR in skeletal muscle has thus far precluded affinity measurements.

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