

# Burn Injury to Rat Increases Nicotinic Acetylcholine Receptors in the Diaphragm

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Thermal injury induces aberrant responses to neuromuscular (NM) blocking drugs, and it has been speculated that an increase in nicotinic acetylcholine receptors (AChR) may contribute to the altered response. Using the diaphragmatic muscle as a representative of skeletal muscle, the changes in AChR were examined. The diaphragm, rather than limb muscles, was chosen to avoid the effects of wound contracture-induced immobilization and denervation of limb, which can also increase AChR in skeletal muscle. Study of changes in diaphragm also tested the hypothesis that increase in AChR are the result of a generalized systemic effect, and not limited to area of burn. Following a 45-55% body surface area thermal injury to the trunk (not limbs) of rats, AChR changes in the diaphragm were studied at 10, 14, 21, and 28 days after injury and compared to uninjured (control) rats. The AChR changes in the diaphragmatic muscle was assayed using  $^{125}\text{I}$ -alpha-bungarotoxin as the specific ligand. At 10, 14, and 21 days after thermal injury, the animals had an arrest in weight growth and the AChR concentration was increased to (mean  $\pm$  SE)  $155 \pm 15\%$  ( $P < 0.02$ ),  $160 \pm 16\%$  ( $P < 0.003$ ), and  $141 \pm 16\%$  ( $0.05 < P < 0.1$ ), respectively, compared to control. At 28 days, probably because of wound healing and burn wound contracture, the size of thermally injured area was significantly ( $P < 0.001$ ) reduced to  $19 \pm 4\%$  of body surface area, and weight increased ( $P < 0.001$ ) compared to preburn weight. The AChR number at 28 days after burn returned to control levels ( $111 \pm 14\%$  of control,  $P < 0.6$ ). Thus major thermal injury causes significant increases in AChR at sites distant from burn, which revert to normal with decrease in size of the injured area. (Key words: Burns; receptor changes. Muscle, skeletal; acetylcholine receptors; end plate. Receptors: acetylcholine; nicotinic.

THERMAL INJURY causes increased sensitivity to the depolarizing (agonist type) neuromuscular (NM) relaxants<sup>1,2</sup> and hyposensitivity to the effects of nondepolarizing (competitive) NM relaxants, such as d-tubocura-

rine (*d*Tc).<sup>3-5</sup> Pharmacokinetic studies, specifically with *d*Tc, fail to reveal any differences in clearance or apparent volume of distribution between thermally injured patients and controls.<sup>6</sup> Neither can increased plasma protein binding of NM relaxants, due to release of acute phase reactant, orosomucoid, a component of alpha<sub>1</sub> globulin, account for all of the increased drug requirement and hyposensitivity to *d*Tc.<sup>7</sup> In the absence, therefore, of significant changes in pharmacokinetics and plasma protein binding, evidence points to an altered response of the target organ. Specifically alterations in nicotinic acetylcholine receptor (AChR) affinity and/or number at the muscle membrane may contribute to the aberrant responses to NM blocking drugs. To study the latter changes, in the rodent model<sup>8-10</sup> we examined AChR changes in diaphragm at 10, 14, 21, and 28 days after an approximate 50% body surface area scald injury. The diaphragm, rather than a limb muscle, was chosen to study the distant (systemic) effects of thermal injury, and also to eliminate the effects of immobilization and denervation resulting from direct effects of injury.  $^{125}\text{I}$ -alpha-bungarotoxin ( $^{125}\text{I}$ -a-BT) method was used to quantitate nicotinic AChR in the diaphragm.<sup>11-17</sup>

## Materials and Methods

### THERMAL INJURY

The experiments were conducted according to National Institute of Health and Shriners Burns Institute animal-care guidelines. The protocol was reviewed and approved by animal care committee. Male adult Sprague-Dawley rats (Charles River Inc., Boston, MA) (175-200 g) were anesthetized with sodium pentobarbital ip (40 mg/kg ip), and their spleens were removed under sterile conditions. The animals were re-anesthetized 10 days later in the same manner, and thermal injury was imposed to the trauma group. The back and flanks were immersed for 15 s and abdomen for 10 s in water at 75-85° C. This type of injury does not cause damage beyond the deep dermis,<sup>8,9</sup> and has been confirmed in our experiments.<sup>10</sup> Injured animals were resuscitated with crystalloid (4 ml/kg/% injured area) by ip injection. After measuring the surface area of injury, 1% Silver Sulfadiazine cream (Silvadene®) was applied to the injured area, and a bandage was applied around

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the trunk. Control animals were treated the same as the trauma group, with the exception that they were not burned. The size of injury was measured as the ratio of total injured area to total body surface (per cent body surface area burn). The total body surface was calculated using the formula  $KW^{2/3}$ , where  $W$  is the weight in grams and  $K$  is a constant equal to 10.<sup>18</sup>

#### ACETYLCHOLINE RECEPTOR ASSAY

<sup>125</sup>I-alpha-bungarotoxin (<sup>125</sup>I-a-BT), a specific ligand from snake venom for nicotinic AchR, described previously, was used to quantify AchR number at the NM junction.<sup>11-14</sup> Following thermal injury, all animals were breathing spontaneously until the day they were killed for harvesting of the diaphragmatic muscle. For reasons discussed earlier, the diaphragmatic muscle was chosen to document changes in AchR number associated with thermal injury. An additional reason for choosing the diaphragm muscle is because it is thin and has an end plate region that is easily identified and separated from the rest of the muscle.<sup>11,15</sup> The binding of <sup>125</sup>I-a-BT was performed in AchR extracted from muscle. Detergent triton X-100 was used to extract AchR from muscle.<sup>12</sup>

Randomly selected controls and thermally injured animals were reanesthetized at 10, 14, 21, or 28 days after the injury with sleep doses of sodium pentobarbital, and both hemi-diaphragms of the rat were removed. The dissected tissues were washed three times with a 50 mM phosphate buffer (pH 7.4) containing 0.1 mM benzethonium chloride and 1 mM EDTA.<sup>13</sup> A 1 × 1.5 cm muscle tissue sample was cut from the area surrounding the end of the phrenic nerve. The nerve was pulled out of the muscle, the nerve was discarded, and the muscle was weighed. The tissue samples were frozen immediately and stored until the assay.

AchR changes were quantitated using about 0.1–0.2 g (wet weight) of frozen diaphragm, which was washed once with the same buffer described above, and then shredded into fine pieces. To this sample was added 1 ml of extraction buffer, containing 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 1 mM EDTA, and 1.5% triton X-100 in 50 mM phosphate buffer, pH 7.4 (Sigma Chemical Co., St. Louis, MO). The mixture was shaken continuously at 4° C for 14 h, which was the optimal time for maximal extraction of AchR.<sup>11-13</sup> Subsequently, the sample was centrifuged at 3,000 g for 40 min at 4° C, and the supernatant was recovered. Next, 0.5 ml of the supernatant and 0.02 ml of <sup>125</sup>I-a-BT (specific activity 16.2 μCi/μg, from NEN Products, Boston, MA) were incubated for 1 h at room temperature and then frozen

until column chromatography.<sup>11,12</sup> The incubation time, 1 h, was long enough for the AchR in the extract to be completely converted to <sup>125</sup>I-a-BT bound AchR complex.<sup>11</sup>

Column chromatography was used to separate AchR bound <sup>125</sup>I-a-BT from free <sup>125</sup>I-a-BT.<sup>11-13,15</sup> Sephadex G-50 (Pharmacia Inc., Piscataway, NJ) was packed in a 1 × 25 cm column and equilibrated with eluting buffer, containing 0.1 M NaCl, 0.1 mM benzethonium chloride, 1 mM EDTA, and 1.0% triton X-100 in 20 mM phosphate, pH 7.4.<sup>11-14</sup> For each sample, 0.2 ml of the incubated mixture was applied to the column. The eluate was collected (25 drops per tube) using a Gilson fraction collector, and each tube was counted for radioactivity using the <sup>125</sup>I channel of gamma counter (Gamma 7000, Beckman Instruments Co., Irvine, CA). Total radioactivity of the first fraction in the chromatogram represented receptor bound <sup>125</sup>I-a-BT. The efficiency of <sup>125</sup>I counting was 74.5%. The protein concentration (mg/ml) of muscle extract was assayed according to the Hartree method.<sup>19</sup> The concentration of AchR in the diaphragmatic muscle was calculated as femtomoles of AchR per mg of protein in the extract.

#### STATISTICAL ANALYSIS

Data were analyzed either by a one-way analysis of variance or by Student's *t* test. Significance was assumed if  $P < 0.05$ .

#### Results

##### THERMAL INJURY

The weight of control and burned animals on days of sham burn or burn were (mean ± SE) were 292.86 ± 2.15 (n = 27) and 293.69 ± 4.14 (n = 39) g, respectively. These weights were not significantly different ( $P = 0.86$ ). The changes in burn size and body weight on the day the diaphragm was sampled are shown in table 1. The approximate size of thermal injury remained the same until 21 days. These animals, therefore, had an arrest in body weight growth compared to their preburn weight and their respective controls. At 28 days, however, the burned rats had a significant increase in body weight relative to preburn weight with a *pari passu* reduction in burn size because of wound healing and/or burn wound contracture. This group of animals at 28 days was more active than the other injured animals.

##### CHANGES OF AchR

There was no evidence of direct thermal damage to diaphragm muscle. The lungs did not show any evi-

TABLE 1. Changes in Weight, Size of Thermal Injury, and AchR After Injury

Subject	Days After Thermal Injury			
	10	14	21	28
Body weight (g)				
Control	343.4 ± 15.9†	372.2 ± 6.0†	401.0 ± 13.7†	421.1 ± 12.9†
Injured	271.1 ± 15.2	276.1 ± 7.2	277.0 ± 11.8	332.0 ± 13.9†
Size of Thermal Injury (%)*	41.6 ± 2.1	37.8 ± 2.3	43.7 ± 4.1	19.4 ± 4.3‡
AchR (fmole/mg protein)				
Control	5.63 ± 0.73	6.01 ± 0.46	5.98 ± 0.51	5.74 ± 0.60
Injured	8.71 ± 0.88§	9.63 ± 0.99§	8.43 ± 1.00¶	6.39 ± 0.84
AchR (% of control)				
Control	100 ± 13.0	100 ± 7.7	100 ± 8.5	100 ± 10.5
Injured	154.7 ± 15.6§	160.2 ± 16.5§	141.0 ± 16.7§	111.3 ± 14.6
Protein concentration (mg/ml) to wet weight (g) ratio				
Control	45.4 ± 3.5	41.7 ± 2.5	39.9 ± 2.0	40.5 ± 2.0
Injured	52.6 ± 3.8	43.4 ± 2.9	43.4 ± 2.5	38.9 ± 1.4
Number of animals				
Control	8	10	5	4
Injured	9	19	5	6

Values are given as mean ± SEM.

\* Measurement on the experimental day. Size of thermal injury was expressed as % of total body surface area.

† Significantly different from pre-injured body weight at  $P < 0.001$ .

‡ Significantly different from other injured groups at  $P < 0.001$ .

§ Significantly different from control at  $P < 0.05$ .

¶ Significantly different from control at  $0.05 < P < 0.1$ .

dence of edema or pneumonia suggestive of diaphragmatic hypoventilation. Some of the thermally injured animals, however, showed macroscopic evidence of decreases in diaphragmatic muscle mass. In order to take into account changes in the muscle mass due to weight loss or weight gain, our results of AchR changes are reported as femto moles of AchR per mg protein. The per cent change in AchR as compared to control is also shown in table 1. Although there was gross evidence in some of a decrease in muscle mass, the protein concentration to wet weight ratio was not different between control and experimental groups throughout the observation period (table 1). The AchR recovery was  $76.3 \pm 1.3\%$ , which was similar to the result of Chiu TH *et al.*<sup>12</sup> of 76–78%. The nonspecific binding of <sup>125</sup>I-a-BT was  $65.2 \pm 1.3\%$ , which is also similar to other reports.<sup>16</sup> The changes in AchR concentration in diaphragmatic muscle at 10, 14, and 21 days after injury and their respective controls are shown in table 1 and figure 1. An approximate 40–60% increase in AchR number was seen at 10, 14, and 21 days compared to controls. At 28 days after injury, however, the AchR number decreased to control level. It should be reiterated that this latter group of animals, by this time, had started gaining weight, and also had a decreased burn size.

### Discussion

These observations confirm the hypothesis that thermal injury causes increases in nicotinic acetylcholine re-

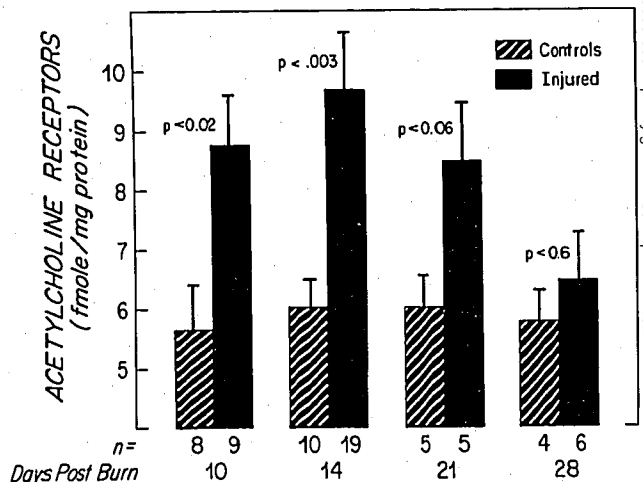


FIG. 1. AchR changes with time. At 10, 14, and 21 days, increases in AchR concentrations were seen in the diaphragm of burned rats. At 28 days, the burned rats started to gain weight, had decreases in burn size, and the AchR concentrations in the diaphragm returned to control levels.

ceptors at sites distant from area of trauma. These changes, therefore, may in part account for the hypersensitivity to agonist drugs (succinylcholine)<sup>1,2</sup> and, also, hyposensitivity to antagonist drugs (d-tubocurarine) after thermal injury.<sup>3-5</sup> This study does not establish a cause and effect relationship between *d*Tc hyposensitivity and increase in AchR, but preliminary studies in the same rat model have documented that increase in AchR in the gastrocnemius to be associated with hyposensitivity to *d*Tc.<sup>20</sup> The present study does not differentiate between changes in junctional and extrajunctional receptors, since the muscle tissue immediately beneath and around the NM junction (nerve) was used for AchR analysis. Future studies using cholinesterase stains to differentiate end-plate and non end-plate regions morphologically can better address this question.

We selected a rodent model to characterize changes in the NM junction. Numerous studies have documented rodents to be a useful model for thermal injury in terms of studying hypermetabolism, humoral response, and immune suppression,<sup>8-10,21</sup> but the usefulness of this model for studying NM changes has not been confirmed. Our present study on AchR changes complements the pharmacodynamic studies with d-tubocurarine and atracurium,<sup>10,20,22</sup> wherein the rodent is shown to replicate the clinically observed hyposensitivity to nondepolarizing NM relaxants following thermal injury. Studies evaluating NM blocking drugs in burned patients describe a critical requirement in terms of magnitude of thermal injury and a critical time post-trauma when NM changes are observed. Generally, not always, an injury involving greater than 25-30% of body surface area and a time after injury of at least 7 days is required.<sup>2-5,10,23</sup> Thus, in our model, an injury of an approximate surface area of 45% was inflicted and studies performed at 10, 14, 21, and 28 days after trauma. As documented by us and other authors,<sup>8,10</sup> all animals initially did not gain weight up to 21 days, probably because of the hypermetabolic and catabolic state. During this time, the AchR number in the diaphragm was increased. At 28 days, the size of thermal injury decreased, the animals gained weight, and the AchR reverted to normal. We cannot, therefore, exclude a possible association between a hypercatabolic state in muscle and AchR changes. This speculation is consistent with a previous report where resistance to nondepolarizing NM blockers, in immobilized muscle, is associated with wasting (catabolism) of the same muscle.<sup>24</sup>

One might also pose the question as to whether the increase in AchR is only a relative increase due to a decrease in muscle mass. Although the possibility should be entertained, this seems unlikely for the following reasons. In the control animals, as the animals

gain weight (day 0 through 28 days), which would also include an increase in muscle mass, one would see a decrease in AchR with time when reporting AchR number relative to protein concentration. If this were true at 28 days, for example, a 44% increase in body weight observed in the control animals compared to pre-(sham) burn weights, should result in some proportionate decrease in AchR concentration relative to protein concentration. Our results shown in table 1 are not consistent with this thesis. In the burned group, it is important to note that the animals' total body weight did not decrease, and that not all diaphragmatic muscles in burned animals showed macroscopic evidence of a decrease in muscle mass. Furthermore, if the protein mass in muscle decreased 40-60% (to account for the increase in AchR concentration), this would result in cellular edema which would be reflected in a change in protein concentration to wet weight ratio of the muscle sample assayed. A change in the ratio of protein concentration to wet weight ratio was not observed throughout the experimental period (table 1). Therefore, muscle mass loss, though perhaps present, cannot explain the 40-60% increase in AchR. Finally, preliminary studies in our laboratory§ by northern blot analysis using cDNA clones specific for alpha subunit of AchR<sup>25</sup> indicate that the rates of transcription of alpha subunit of AchR (mRNA) levels are increased following burns, which suggests increased synthesis of AchR.

The increase in AchR following thermal injury is reminiscent of changes following denervation.<sup>15,16,26</sup> Thermally injured and denervated patients have in common a hypersensitivity to succinylcholine and acetylcholine<sup>2,26</sup> and hyposensitivity to curare-like drugs.<sup>3-5,27</sup> Recently, Mills *et al.*<sup>28</sup> using electromyography have confirmed denervation-like changes, including fibrillation potentials and positive sharp waves in some muscles of burned patients. However, quite unlike the denervated state, the increase in AchR in the present study was seen in a muscle remote from thermal injury and in a muscle which was active because all these animals were breathing spontaneously until the excision of diaphragm. As indicated previously, there was no evidence of direct thermal damage to, or hypo-activity of, the diaphragm. The duration of the scald exposure was such that even the abdominal muscles did not show evidence of thermal injury. In the diaphragm, therefore, factors such as immobilization and denervation

§ Kim C, Ouellette A, Martyn J: Burn injury in splenectomized rat increases acetylcholine receptors and mRNA levels of its alpha subunit. Abstract presented at the Massachusetts General Hospital Annual Research Symposium, January, 1987.

probably do not play a role in the increased AchR numbers. As a result of the systemic effects of thermal injury, one might also anticipate changes in AchR in other skeletal muscles. As indicated previously, a study of limb muscles (gastrocnemius) in fact confirms an increase in AchR associated with hyposensitivity to *d*Tc in the same muscle.<sup>20</sup>

Denervation causes a 10–20-fold increase in AchR number in the denervated muscle to account for the altered sensitivity to NM blockers, and changes occurring in the unaffected muscles are less dramatic.<sup>29</sup> We, however, observed only a 1.4–1.6-fold increase in AchR in the diaphragm after thermal injury. Although smaller in magnitude compared to denervation, there seems to be a modest but generalized increase in nicotinic AchR. It is, therefore, possible that the absolute numbers of AchR available throughout the body which can combine to agonist or antagonist drug may be similar in both denervation and burns, and, therefore, could explain the similar responses to NM blockers. It should also be pointed out, however, that changes in AchR do not necessarily need to be present to explain all of the altered NM pharmacodynamics. For example, mice with dystrophic muscles *in vitro* have twice ED<sub>50</sub> for *d*Tc compared to controls, but no changes in junctional or extrajunctional receptors could be documented.<sup>30</sup> Similarly, following immobilization, there is only a modest increase in AchR compared to denervation,<sup>29,31</sup> but the magnitude of hyposensitivity to nondepolarizing relaxants in immobilization far exceeds<sup>24</sup> that seen with denervation.<sup>27,32</sup> Thus, other changes, such as an increase in quantal content of evoked end-plate potentials,<sup>30</sup> altered receptor affinity, and circulating mediators, such as cyclic nucleotides and prostaglandins,<sup>10,33,34</sup> may contribute to the hyposensitivity.

This study, therefore, documents a modest increase in AchR at sites distant from burn. The magnitude of the change in receptor in the diaphragm is not as extensive as seen following denervation, but the changes seen in the diaphragm (and gastrocnemius)<sup>20</sup> suggest a generalized increase in AchR. Although this study has not established a correlation between increase in AchR in the diaphragm and *d*Tc hyposensitivity, another study in the same model, but in the gastrocnemius muscle, has established such a relationship between *d*Tc hyposensitivity and AchR changes.<sup>20</sup> Additional factors, including increases in quantal content of end-plate potentials, altered affinity for receptor, and circulating mediators, may contribute to altered NM sensitivity. Because of the generalized nature of the AchR changes, it appears that neural and/or humoral mediators play a role. The mediators and the mechanisms by which these are brought about are currently under investigation.

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