CHARACTERIZATION OF SKELETAL MUSCLE EFFECTS ASSOCIATED WITH DAPTOMYCIN IN RATS

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ABSTRACT: Daptomycin is a lipopeptide antibiotic with strong bactericidal effects against Gram-positive bacteria and minor side effects on skeletal muscles. The type and magnitude of the early effect of daptomycin on skeletal muscles of rats was quantified by histopathology, examination of contractile properties, Evans Blue Dye uptake, and effect on the patch repair process. A single dose of daptomycin of up to 200 mg/kg had no effect on muscle fibers. A dose of 150 mg/kg of daptomycin, twice per day for 3 days, produced a small number of myofibers ($\leq$0.22%) with loss of plasma membrane integrity and/or infiltration by neutrophils and/or macrophages. Multiple doses of daptomycin are required for a quantifiable effect on skeletal muscles of rats. Some fibers were Evans Blue Dye–positive but were not yet infiltrated by neutrophils. This suggests that the sarcolemma may be the primary target for the observed effects.

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Daptomycin is a *Streptomyces roseosporus*–derived lipopeptide antibiotic that has a strong and prolonged bactericidal effect against Gram-positive bacteria,[1] and it is especially effective against vancomycin- and methicillin-resistant bacteria.[2] Daptomycin is usually well tolerated, but some patients ($\backsim$1.5%) develop side effects in skeletal muscles,[1,3] although smooth and cardiac muscles are not affected. The side effects on skeletal muscles are not unique and have been reported previously for other antibiotics.[4]

Currently, no conclusive data exist that clarify the exact mechanisms responsible for any possible effects or their magnitude that daptomycin might have on skeletal muscles, and no factors have been identified that might predispose some patients to develop daptomycin-related side effects. Studies in dogs have shown that multiple doses of daptomycin may have a very mild effect on skeletal muscle fibers.[5] Although no direct data exist, the lipopeptide structure of daptomycin and its mechanisms of action on bacteria[6] suggest that the mechanisms of action in mammalian cells might involve an effect on the plasma membrane.

The objectives of this study were to characterize: early effects of intravenous administration of daptomycin on the skeletal muscle of healthy Sprague-Dawley rats; potential effects of daptomycin on structural and functional properties of selected skeletal muscles; and potential effects of daptomycin on the plasma membrane repair mechanisms involved in the patching of small sarcolemmal breaches associated with normal muscle activity. We hypothesized that the sarcolemma is the primary target of daptomycin's effect on skeletal muscle.

METHODS

Animals. Adult male Sprague-Dawley rats (4–6 months of age), obtained from Harlan Sprague-Dawley (Indianapolis, Indiana), were used for the experiments. For investigation of multiple doses per day, rats were obtained from the vendor with their carotid artery cannulas in place. Animal housing, operations, and subsequent animal care were carried out in accordance with the guidelines of the Unit for Laboratory Animal Medicine at the University of Michigan.

Daptomycin Administration. Prior to dosing, daptomycin was dissolved in saline (sterile 0.9% NaCl solution) at a final concentration of 50 mg/ml. For the single-dose experiments, non-cannulated rats were used. Each of the rats (8–10 rats/group) in both the daptomycin- and saline-treated groups received a single dose through the tail vein and were euthanized 72 hours later. Daptomycin was administered at 100, 150, and 200 mg/kg. The saline-treated group received a volume of saline that was equal to the volume administered to the daptomycin-injected rats. Gastrocnemius (GTN), tibialis anterior (ATB), extensor digitorum longus (EDL), plantaris, and biceps muscles were analyzed in all rats by microscopic evaluation of the tissue sections. A single dose was utilized initially to assess the earliest changes in skeletal muscles associated with daptomycin exposure.

In subsequent experiments, daptomycin was administered twice daily to rats for 4 days to increase the potential of an early effect on skeletal muscle. A previous study demonstrated that skeletal muscle changes associated with daptomycin treatment in dogs are greatest for

fractionated (multiple doses) daily dosing as compared with once-daily dosing.[5]

For the multiple-dose investigations, rats in both the daptomycin- and the saline-treated groups had in-dwelling catheters in the carotid artery. Eleven saline-treated and 11 daptomycin-treated rats were used in the protocol for the twice-daily dosing at 150 mg/kg per dose for 3 days (a total of six doses). During each of the treatment days, either daptomycin or saline was given through the carotid artery catheters twice per day at approximately 8:00 A.M. and 4:00 P.M. On day 3, prior to the second daptomycin dose, or for the control rats prior to the saline administration, a 1-ml blood sample was drawn from the carotid cannula for analysis of serum creatine kinase (CK). CK values (IU/liter) were determined by the Animal Diagnostic Laboratory at the University of Michigan using an automated chemistry analyzer (Vettest 8008; IDEXX, Westbrook, Maine). The rats were observed for any clinical signs of effects throughout the study.

For the multiple-dose study, half of the rats were injected with a 1% solution of Evans Blue Dye (1% of body mass) following the last dose of daptomycin (or saline) on day 3. In research studies on skeletal muscle, Evans Blue Dye is frequently used as an early in vivo marker of myofiber damage and, specifically, loss of cell membrane integrity.[7,8] On the morning of day 4, when the study ended, rats were anesthetized with an intraperitoneal dose of sodium pentobarbital. EDL and soleus muscles were dissected from the right hind-limb of each rat and contractile properties were evaluated. GTN, ATB, EDL, soleus, plantaris, and biceps muscles from left hind-limb and triceps muscle from the left upper limb of each rat were preserved for histological analysis by embedding in Triangel Biomedical Sciences (TBS) medium (Triangle Biomedical Sciences, Durham, North Carolina). After removal of the muscles, the animals were euthanized with an overdose of the anesthetic.

Measurements of Contractile Properties. On the morning of day 4 of the experimental protocol, both soleus (predominantly slow type 1 fibers) and EDL (predominantly fast type 2a and 2b fibers) muscles were dissected from each of the daptomycin-treated and saline-treated rats. The muscles were mounted in an experimental apparatus designed for the measurement of contractile properties of intact muscles in vitro. The bath was filled with an aerated Krebs–Henseleit buffer solution. The solution was maintained at 25°C, and pH was maintained at approximately 7.4 by buffering with a gas mixture of 95% O_2 and 5% CO_2. At comple-

tion of the measurements of contractile properties, muscle length and mass were determined, and the specific force was calculated.[9] The force deficit was measured as the maximum force generated 1 minute after two 30% stretches of the maximally activated muscle expressed as a percentage of the initial maximum force. The stretch protocol was designed to detect substantial differences in susceptibility to contraction-induced injury.[10] A protocol of two lengthening contractions was chosen to prevent the confounding effects of fatigue that may accompany multiple muscle contractions.

Histochemical and Immunohistochemical Analysis. Muscles embedded in the TBS medium and stored at −80°C were subsequently sectioned on a cryostat (12 μm) at the mid-belly region. Muscle cross-sections were stained with hematoxylin and eosin (H&E), as previously described.[11] Muscle cross-sections from the rats injected with Evans Blue Dye were fixed in ice-cold acetone for 1 minute, dried, and coverslipped with Permount (Fisher Scientific, Fair Lawn, New Jersey). The number of affected fibers was quantified by microscopic observations of the two or three adjacent cross-sections for each of the muscles. Muscle cross-sections were photographed at low magnification, and cross-sectional areas were estimated by outlining two or three adjacent cross-sections using Image J software (NIH, Bethesda, Maryland; http://rsb.info.nih.gov/ij/). Cross-sectional areas of individual muscle fibers were estimated by outlining 100–200 fibers in three to five sections of muscles from different rats. Mean fiber areas were calculated separately for each of the muscles analyzed. To determine the approximate number of total muscle fibers per section, the cross-sectional area of a particular muscle section was divided by the mean of the cross-sectional areas of muscle fibers in a specific muscle.

For immunostaining to assess effects in type I versus type II muscle fibers and for immmunostaining of the patch repair proteins, sections were incubated in a blocking buffer [20% calf serum in phosphate-buffered saline with Tween 20 (PBST)] for 1 hour and then in a solution of primary antibody overnight at 4°C. The following primary antibodies were used: mouse anti–slow myosin (type I myosin; clone A4.84); mouse anti–fast IIa myosin (clone A4.74), developed by Dr. Helen M. Blau and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Iowa City, Iowa); rabbit anti-laminin (Chemicon International, Temecula, California); mouse anti-dysferlin (Lab Vision Corp., Fremont, California); rabbit anti-dysferlin (Affinity

BioReagents, Rockford, Illinois); mouse anti–caveolin-3 (BD Biosciences, San Jose, California); mouse anti–LAMP-1 (Stressgen, Victoria, British Columbia, Canada); rabbit anti–LAMP-2 (Abcam, Cambridge, Massachusetts); mouse anti-macrophage (Abcam); and rabbit anti-neutrophil elastase (Abcam). Depending on the source of primary antibody, 1-hour of room-temperature incubation with Cy3-conjugated (red) anti-mouse or anti-rabbit secondary antibodies or Cy2-conjugated (green) anti-rabbit secondary antibodies (all from Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) was done for visualization. Nuclei were stained by 5-minute incubation with a 4′-6-diamidino-2-phenylindole (DAPI) solution (Sigma Co., St. Louis, Missouri) in PBST. The sections were examined and photographed with a Leica microscope.

Adjustments in Data Analysis. The CK value for one saline-treated rat and one daptomycin-treated rat fell outside the range of 2 standard deviations beyond the mean. These two data points were excluded from the final figure in the Results section. No difference was observed in the contractile properties of either EDL or soleus muscles of rats injected with Evans Blue Dye when compared with those not injected. Based on these observations, the data for the EDL and soleus muscles were combined for rats injected with saline only and those with saline/Evans Blue Dye. The data for the rats given daptomycin only and daptomyocin/Evans Blue Dye were also combined. One daptomycin-treated rat had a much larger than usual number of affected fibers in the triceps muscle, so the data from this muscle were not used in this study. One saline-injected rat also had an uncharacteristically large number of affected fibers in two of the muscles (GTN and ATB). These muscles were also excluded from the study. The deletion of these three muscles from our study was justified, because they did not represent the general pattern found in the other 81 muscles analyzed with twice-daily dosing at 150 mg/kg per dose. We excluded two daptomycin-treated rats and one saline-injected rat from the analysis of Evans Blue Dye fluorescence, because the low levels of fluorescence with Evans Blue Dye in some, or all, of the muscles did not allow the number of affected muscle fibers to be assessed.

Statistical Analysis. Data are expressed as mean ± standard error of the mean (SEM). A t-test analysis was performed to compare the differences between the saline- and daptomycin-treated groups of rats. Differences were considered significant at $P \leq 0.05$.

RESULTS

Effect of a Single Dose of Daptomycin on Muscles of Rats In Vivo. After a single intravenous dose of daptomycin none of the supratherapeutic doses given (100, 150, and 200 mg/kg) inflicted any muscle damage, and no affected muscle fibers were detected at 72 hours postinjection when analyzed in sections stained with H&E or Evans Blue Dye (data not shown). The exception was one GTN muscle in a rat treated with 150 mg/kg of daptomycin and killed 72 hours postinjection. This muscle had one small area infiltrated with macrophages and/or neutrophils (not shown). No differences were observed for the maximum or specific isometric tetanic forces developed by the EDL and soleus muscles of rats treated with a single injection of daptomycin (data not shown).

Effect of Twice-Daily Dosing of Daptomycin on Muscles of Rats In Vivo. To study the early effects of the continuous presence of daptomycin in tissue fluids for several days we evaluated the effect after multiple intravenous doses. In our initial studies we investigated the effect of twice-daily supratherapuetic doses of 100 mg/kg or 150 mg/kg daptomycin. Skeletal muscles were evaluated 48, 72, or 96 hours postinjection. With a twice-daily dose of 100 mg/kg for 2 days, no significant damage was detected in any of the muscles analyzed. Only one macrophage-infiltrated muscle fiber in one of the cross-sections of a plantaris muscle was detected (data not shown). At twice-daily doses of 150 mg/kg for 4 days (a total of eight doses), multiple muscle fibers infiltrated with macrophages were clearly identified (Fig. 1) on H&E-stained muscle sections. Some of the affected areas already showed signs of ongoing satellite-cell activation and muscle fiber regeneration (not shown).

For detailed analysis of the early effect of multiple intravenous doses of daptomycin on rat skeletal muscles in vivo and for the quantification of the affected muscle fibers, a dose regimen of 150 mg/kg twice daily for 3 days (total daily dose of 300 mg/kg) was selected. At this dose of daptomycin, rats did not have any general toxicity and appeared healthy, although they had a small number of easily detectable macrophage-infiltrated muscle fibers. The damaged muscle fibers were grouped into two or three selected areas per muscle, whereas the remainder of the muscle was unaffected. Rats were euthanized, and muscles were dissected on the morning of day 4. For saline-treated and daptomycin-treated rats, the total body masses were not different (Fig. 2), although small differences in mass for several of the muscles were observed between daptomycin- and saline-treated rats (Fig. 2).
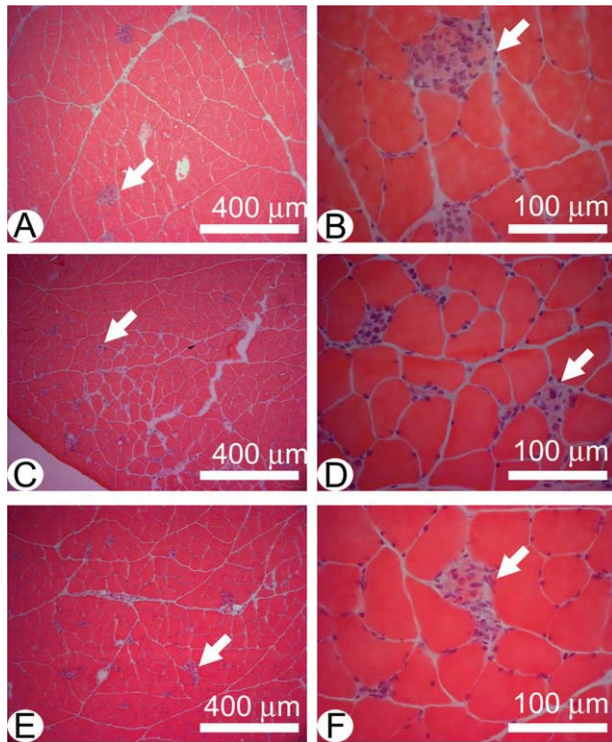
FIGURE 1. H&E staining of muscle cross-sections of rats injected twice per day for 4 days with 150 mg/kg daptomycin and killed on day 5. Representative images of ATB **(A, B)**, EDL **(C, D)**, and plantaris **(E, F)** muscles of rats at two different magnifications are shown. All muscles in daptomycin-treated rats had signs of structural muscle fiber damage, although the number of damaged fibers varied in different muscles. Arrows indicate damaged fibers infiltrated with macrophages. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
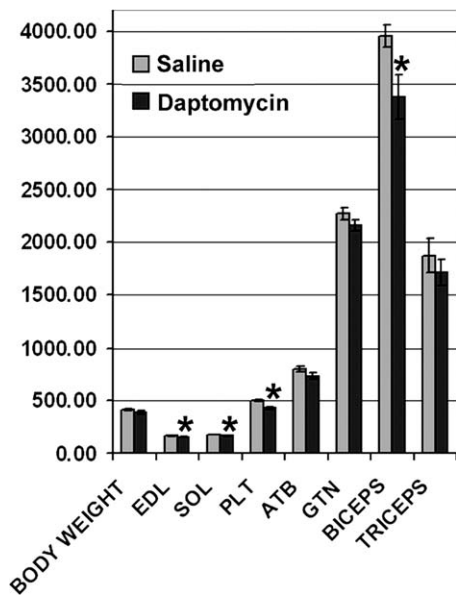
Daptomycin treatment was not associated with any detectable functional effect on skeletal muscle up to the highest dose tested. No differences were observed for the maximum isometric tetanic force (P_o) developed by the EDL or soleus muscles (data not shown). The specific maximum force (sP_o, in kN per m^2) was used to evaluate any subtleties in the individual differences in the response of individual rats to this dosage of daptomycin (Fig. 3). No significant difference was observed for sP_o between rats that received Evans Blue Dye and those that did not. Therefore, the data points for Evans Blue Dye–injected and non-injected rats in Figure 3 were combined for both the saline- and daptomycin-treated groups. For saline- and daptomycin-treated rats, sP_o was not different for the EDL and soleus muscles (Fig. 3A, B). The force deficit was measured in soleus muscle after two 30% stretches of the maximally activated muscle (Fig. 3C). No differences were observed for force
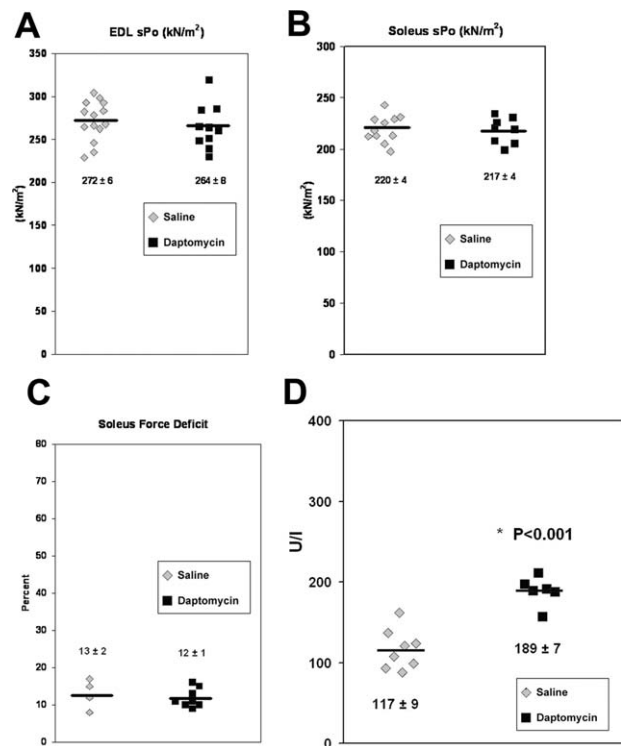

FIGURE 3. (A) Specific forces of EDL muscles of saline- and daptomycin-treated rats. **(B)** Specific forces of soleus muscles of saline- and daptomycin-treated rats. **(C)** The force deficit of soleus muscle for saline- and daptomycin-treated rats. The force deficit was measured as a percent of initial force 1 minute after two 30% stretches to the muscle. **(A–C)** Data points represent individual muscles, and black lines represent the mean for that group. No significant differences were observed between rats that received saline and those that received daptomycin. **(D)** Serum CK levels for saline- and daptomycin-treated rats. Data points represent individual rats, and black lines represent the group average. There was a statistically significant difference between the saline- and daptomycin-injected groups ($P < 0.001$).

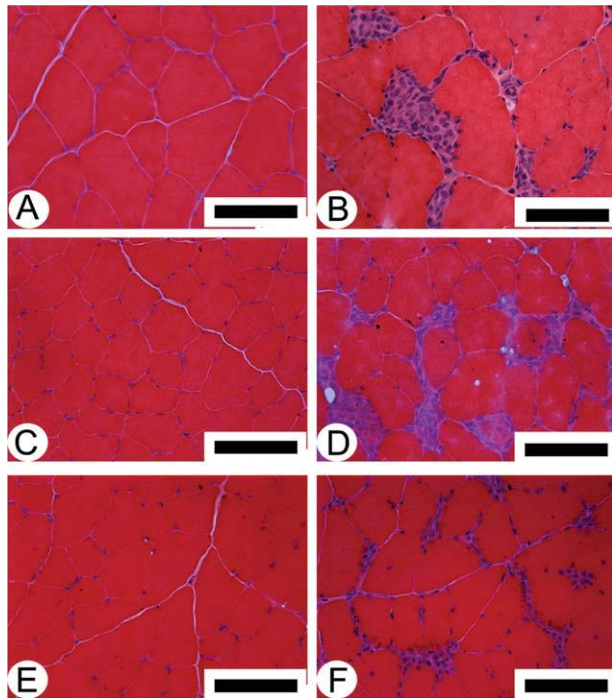

FIGURE 2. Comparison of total body weight (grams) and muscle mass (milligrams) for saline- and daptomycin-treated rats. Asterisk represents statistically significant difference between the saline- and daptomycin-injected groups ($P < 0.05$).

FIGURE 4. H&E staining of skeletal muscles from saline-treated rats [**(A)**, **(C)**, and **(E)** 3 days/twice per day] and daptomycin-treated rats [**(B)**, **(D)**, and **(F)** 3 days/twice- daily treatment with 150 mg/kg]. Representative images of GTN **(A, B)**, ATB **(C, D)**, and triceps **(E, F)** muscles of rats are shown. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

deficits between daptomycin- and saline-treated groups. The daptomycin-treated group had a small, but statistically significant increase in CK values when compared with the saline-treated group (Fig. 3D).
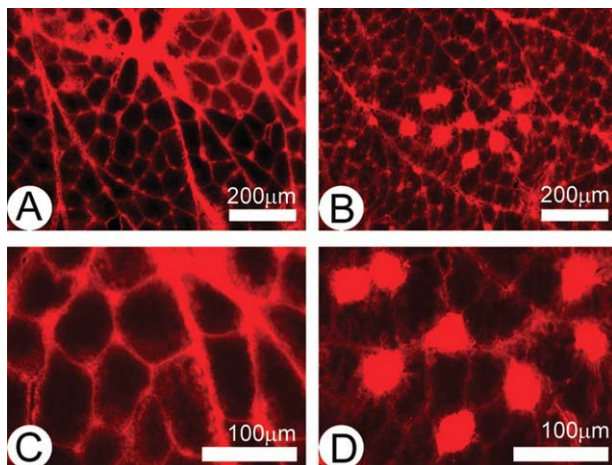

FIGURE 5. Evans Blue Dye fluorescence of gastrocnemius muscles from saline-treated [**(A)** and **(C)** 3 days/twice per day] and daptomycin-treated [**(B)** and **(D)** 3 days/twice-daily treatment with 150 mg/kg] rats. **(C, D)** Higher magnifications of **(A)** and **(B)**, respectively. Note grouping of the daptomycin-affected fibers in a small area of the muscle **(B, D)** [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.].
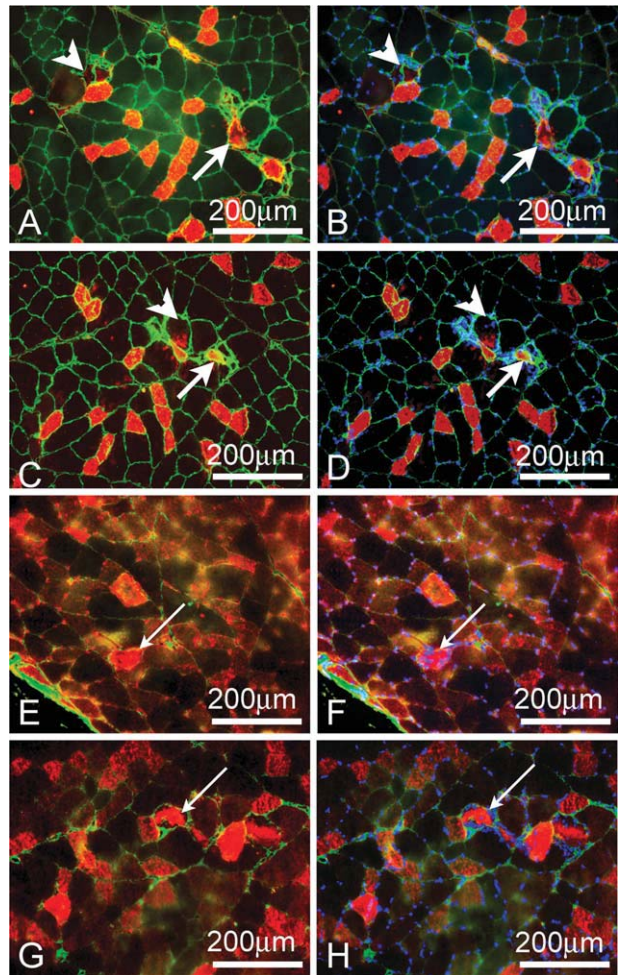

FIGURE 6. Fluorescent microscopy of GTN muscle cross-sections of a rat injected with daptomycin and with Evans Blue Dye for identification of affected fibers. Cross-sections were labeled with antibody against laminin (green in all pictures) to visualize muscle fibers and stained with DAPI [**(B)**, **(D)**, **(F)**, and **(H)**] to visualize nuclei. Antibody against slow myosin heavy chain [myosin type I; bright red staining in **(A)**–**(D)**] and antibody against fast IIa myosin heavy chain [myosin type IIa; bright red staining in **(E)**–**(H)**] were used for fiber typing. Arrows in **(A)**–**(D)** show affected fibers labeled with antibody against myosin type I. Arrowheads in **(A)**–**(D)** show Evans Blue Dye–positive (dark red staining) affected fibers not labeled with antibody against myosin type I, which are either type IIb or type IIa. Arrows in **(E)**–**(H)** show affected fibers stained with antibody against myosin type IIa. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Results of histological evaluations demonstrate that only a small number of myofibers were affected by daptomycin treatment (Figs. 4–7, and Figs. S1 and S2 in the Supplementary Material). Although muscles of the saline-treated rats had essentially no fibers affected, the seven muscles analyzed in each of the daptomycin-treated rats displayed various degrees of macrophage infiltration and/or number of plasma membrane-breached muscle fibers (Figs. 4–7). Affected muscle fibers displayed characteristic grouping in one to three
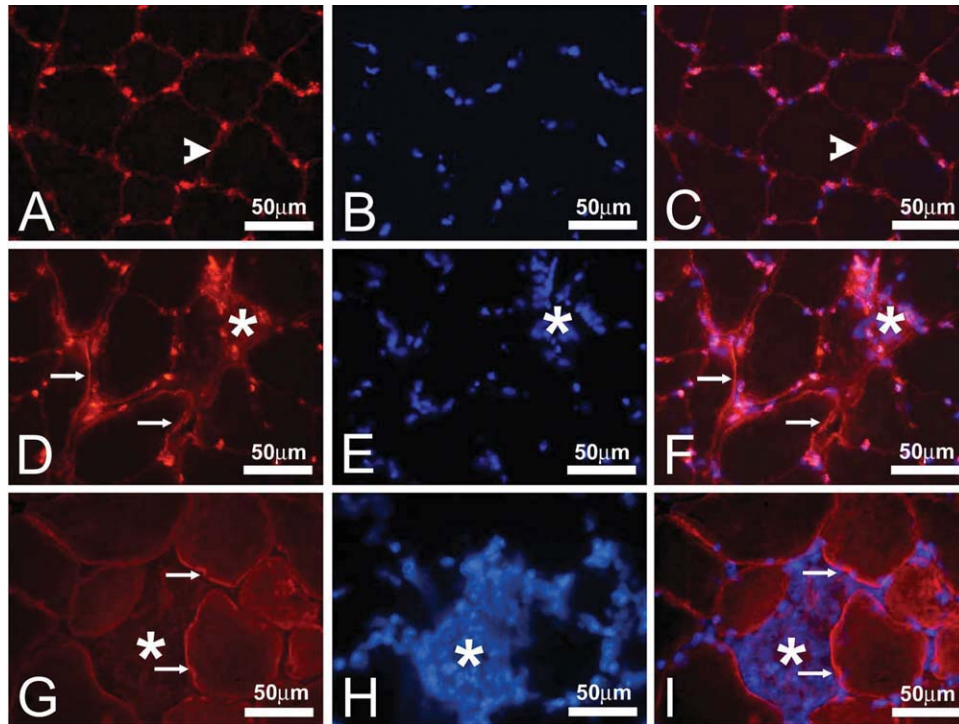
FIGURE 7. Representative images of the immunostaining of triceps muscle from saline-treated **(A–C)** and daptomycin-treated **(D–I)** rats with rabbit anti-dysferlin [red in **(A)**, **(C)**, **(D)**, and **(F)**] and mouse anti-dysferlin [red in **(G)** and **(I)**] antibodies. Nuclei were stained with DAPI [blue in **(B)**, **(C)**, **(E)**, **(F)**, **(H)**, and **(I)**]. Combined dysferlin/DAPI images are shown in **(C)**, **(F)**, and **(I)**. Asterisks indicate neutrophils and macrophages in the areas of damage. Arrows indicate areas of intact muscle fibers in a daptomycin-treated rat, with bright sarcolemmal immunostaining for dysferlin. Arrowheads indicate faint sarcolemmal immunostaining for dysferlin in muscle fibers from saline-treated rats. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

areas of the muscle, whereas the remainder of the muscle was not affected (Figs. 4 and 5). Tables 1 and 2 show the results of quantification of number of affected muscle fibers in seven different muscles of saline- and daptomycin-treated rats.

The average cross-sectional area of muscle fibers was 2800 μm^2 in GTN muscle, 2450 μm^2 in ATB, 2318 μm^2 in EDL, 3789 μm^2 in soleus, 2684 μm^2 in plantaris, and 2788 in μm^2 triceps. The average number of muscle fibers per analyzed section was 2860 for EDL, 2473 for soleus, 17,423 for

ATB, 5443 for plantaris, 16,561 for GTN, 10,835 for biceps, and 26,302 for triceps. Triceps muscle cross-sections were the largest among all of the muscles analyzed, and they showed the largest absolute number of affected muscle fibers ($\sim$40 fibers; Table 1). Nevertheless, the percentage of affected muscle fibers was similar for soleus, GTN, plantaris, and triceps muscles, with each averaging $\sim$0.11–0.22% (Table 2). The EDL, ATB, and biceps muscles displayed a much lower percentage of affected muscle fibers, $\sim$0.01–0.13% (Table 2).

Table 1. Number of affected muscle fibers in saline- and daptomycin-treated rats.

| | | | Muscle | | | | | | |
| | | | EDL | Sol | ATB | PLT | GTN | Biceps | Triceps |
Group	Type of analysis		(n)	(n)	(n)	(n)	(n)	(n)	(n)
Saline-treated	H&E	Average	0.00	4.40	0.25	1.00	0.25	0.00	0.00
		SEM	0.00	2.49	0.20	0.91	0.20	0.00	0.00
Daptomycin-treated	H&E	Average	0.29	5.43	11.14	7.71*	19.14*	0.71	40.00*
		SEM	0.31	0.70	8.64	1.94	6.29	0.51	5.28
Saline-treated	EBD	Average	0.00	2.00	1.75	0.25	1.00	0.00	3.67
		SEM	0.00	1.41	1.43	0.20	0.47	0.00	1.65
Daptomycin-treated	EBD	Average	3.50	4.80	10.86	8.17	32.00*	4.57	34.50*
		SEM	2.94	1.06	4.51	4.90	13.09	4.58	13.95

H&E, hematoxylin and eosin stain; EBD, Evans Blue Dye fluorescence; EDL, extensor digitorum longus; Sol, soleus; ATB, tibialis anterior; PLT, plantaris; GTN, gastrocnemius.
*Statistically significant difference (P < 0.05).

Table 2. Percentage of affected muscle fibers in saline- and daptomycin-treated rats.

| Group | Type of analysis | | No. of fibers | | | | | | |
			EDL	Sol	ATB	PLT	GTN	Biceps	Triceps
Saline-treated rats	H&E	Average	0.00	0.16	0.00	0.04	0.00	0.00	0.00
		SEM	0.00	0.09	0.00	0.04	0.00	0.00	0.00
Daptomycin-treated rats	H&E	Average	0.02	0.22	0.06	0.15	0.11*	0.01	0.17*
		SEM	0.02	0.03	0.05	0.05	0.03	0.00	0.04
Saline-treated rats	EBD	Average	0.00	0.07	0.01	0.00	0.00	0.00	0.01
		SEM	0.00	0.05	0.01	0.00	0.00	0.00	0.01
Daptomycin-treated rats	EBD	Average	0.13	0.19	0.06	0.19	0.22*	0.04	0.18
		SEM	0.10	0.04	0.02	0.13	0.09	0.04	0.11

Statistically significant difference (P < 0.05).

For four of the seven muscles, soleus, ATB, plantaris, and triceps, the two different methods for counting the number of affected muscle fibers (Evans Blue Dye fluorescence and H&E staining) showed good correlation with regard to the number of affected fibers (Table 1). However, for the other three muscles analyzed, EDL, GTN, and biceps, Evans Blue Dye appeared to be more sensitive for detecting an early effect. This observation could reflect the fact that Evans Blue Dye detects the early changes in plasma membrane integrity (minutes–hours), whereas H&E only detects muscle fibers that have been structurally altered. This usually takes a longer time to occur (2–3 days).

The fiber type analysis of the affected fibers using myosin heavy chain–specific antibodies and fluorescently labeled secondary antibodies revealed that both slow (type I) and fast (type II) muscle fibers were affected (Fig. 6). The majority of muscles analyzed (except for the soleus) were fast muscles. Consequently, a larger portion of the affected fibers in muscles analyzed were fast fibers (Fig. 6).

In order to determine whether patch repair proteins were involved in the response to the plasma membrane breach in daptomycin-treated rats, immunostaining with antibodies against dysferlin (Fig. 7), caveolin-3 (Fig. S1), LAMP-1 (Fig. S2), and LAMP-2 (not shown) was used. Each of these proteins may be involved in the response to rupture of the plasma membrane. The most informative data were obtained with two different antibodies against dysferlin (Fig. 7). Dysferlin immunostaining showed that, in daptomycin-treated rats, the part of the sarcolemma of intact muscle fibers that was in direct contact with the macrophage-infiltrated muscle areas showed the highest intensity of dysferlin immunostaining (arrows in Fig. 7D, F, G, I). Muscle fibers that did not have direct contact with the affected areas displayed weak sarcolemmal immunostaining for dysferlin. Intact muscle fibers from saline-treated rats also showed weak sarcolemmal immunostaining for dys-

ferlin (arrowheads in Fig. 7A, C). The two anti-dysferlin antibodies showed similar results. The only difference was that anti-dysferlin antibody produced in rabbits labeled nuclei in muscle sections from both saline-treated (Fig. 7A, C) and daptomycin-treated (Fig. 7D, F) rats, whereas antibody produced in mice did not label the nuclei (Fig. 7G, I). Immunostaining with caveolin-3 antibodies was less distinct, because the sarcolemma of all muscle fibers showed very strong immunostaining with this antibody. Consequently, small variations in immunostaining for caveolin-3 were more difficult to detect (Fig. S1). Although severely damaged fibers lost caveolin-3 immunostaining (asterisks in Fig. S1), some of the intact fibers located close to the affected areas appeared to display stronger caveolin-3 immunostaining (arrows in Fig. S1A) than the remaining muscle fibers of the section. The brightest immunostaining with anti–LAMP-1 (Fig. S2) and anti–LAMP-2 (not shown) antibodies was localized to the affected areas. Anti–LAMP-1 and anti–LAMP-2 antibodies showed similar results. Neutrophils and macrophages in the area of damage displayed the most prominent immunostaining due to the large accumulation of lysosomes in these cells (asterisks in Fig. S2). There was faint punctate immunostaining in the sarcoplasm and in the sarcolemma and/or endomysium of both intact and damaged muscle fibers with both anti–LAMP-1 (Fig. S2) and anti–LAMP-2 antibodies (not shown).

DISCUSSION

This study was designed to investigate the early functional and histological effect of daptomycin on skeletal muscles of young healthy rats. The effects were assessed by: (1) quantifying the type and magnitude of effect on the different types of muscle fibers; and (2) investigating plasma membrane patch repair mechanisms. Our experiments in adult rats after one dose of daptomycin show that a single injection did not have any detectable adverse effect on skeletal muscles even when high

doses were administered. In our experiments, multiple doses of daptomycin were required to produce a measurable effect on skeletal muscles of adult rats. Twice-daily administration at a supratherapeutic dose of 150 mg/kg over 3 days showed morphologically detectable changes in ∽0.2% of muscle fibers in several different muscles, but there were no clinical signs associated with this finding. The dose levels and dosing regimen were specifically selected to evaluate early effects of daptomycin on the sarcolemma and to avoid massive damage to a large number of muscle fibers, which would make it difficult to study the specific effects on individual muscle fibers.

The mechanisms behind the adverse effects of daptomycin on skeletal muscles have not been delineated. The presence of the amino acid head group and lipopeptide structure of daptomycin make it most likely that daptomycin is inserted into the outer leaflet of the plasma membrane without translocation through the membrane. In bacteria, daptomycin is inserted into the cytoplasmic membrane through calcium-dependent mechanisms that cause membrane depolarization and death of the bacteria.[6] Although no direct data exist for skeletal muscles, the most likely mechanism by which daptomycin has an effect involves breaching of plasma membrane integrity through gaps in the outer lipid-based leaflet of the sarcolemma, leakage of calcium from extracellular compartments into the myofibers, and activation of downstream mechanisms that lead to cell death.

Our experiments show that multiple doses of daptomycin at supratherapeutic levels have small, but measurable effects on selected skeletal muscle fibers of healthy adult Sprague-Dawley male rats, ranging from 0.01% to 0.22% of the total number of fibers analyzed. In three of the seven muscles analyzed in this study, EDL, GTN, and biceps, it appears that Evans Blue Dye fluorescence is more sensitive than H&E staining. This may suggest that, in these muscles, the initial effect of daptomycin is on the sarcolemma. The affected muscle fibers were usually grouped in two or three distinct areas within a given muscle. It is not clear exactly what predisposes specific groups of fibers so that they display a measurable response to the daptomycin. Naturally occurring damage to the sarcolemma, or deficiency in the plasma membrane repair mechanisms in the small number of myofibers, could individually, or collectively, play a role. These might be the groups of fibers in specific motor units that are recruited most frequently on a daily basis in rats and therefore undergo most of the normal membrane "wear and tear" and high membrane stress.

Skeletal muscle fibers have active plasma membrane repair mechanisms that are involved in the patching of small sarcolemmal breaches developing during normal everyday muscle activity. Dysferlin, caveolin-3, and LAMP have been reported previously to be important in the processes of regeneration and repair of small-membrane damage and tears in skeletal muscle fibers.[12–15] The resealing of damaged sarcolemma in muscle fibers is associated with enrichment of dysferlin, caveolin-3, and LAMP in the repaired membrane patches.[12–15] It was hypothesized that daptomycin administration could interfere with repair of the breaches of the sarcolemma by inhibiting the fusion of vesicles containing dysferlin, LAMP, and caveolin-3 with the membrane. Our immunostaining experiments show that dysferlin was highly enriched in the sarcolemma of the unaffected myofibers located in close proximity to macrophage-infiltrated fibers. This observation suggests that there were ongoing highly effective plasma membrane repairs in the undamaged fibers. Therefore, the plasma membrane repair mechanisms that engage dysferlin-containing vesicles most probably are not impaired in the skeletal muscle fibers of daptomycin-treated rats. Although we cannot exclude the possibility that, in the small number of daptomycin-affected, macrophage-infiltrated myofibers detected in our experiments, some of the naturally occurring defects in the plasma membrane repair mechanisms predisposed them to the daptomycin effect and subsequent neutrophil/macrophage infiltration.

In conclusion, our findings show that the early effect of daptomycin was localized to a small subpopulation of skeletal muscle fibers. It appears that the early effects of daptomycin on skeletal muscle may be due to loss of sarcolemmal integrity. The factors that predispose myofibers to be responsive to an effect when they are treated with daptomycin are uncertain, but could involve: (1) the level of previous contractile activity of selected motor units; and/or (2) the presence of small sarcolemmal breaches that developed during normal everyday muscle activity.

REFERENCES

1. Livermore DM. Future directions with daptomycin. Antimicrob Agents Chemother 2008;62:iii41–49.
2. Mortin LI, Li T, Van Praagh AD, Zhang S, Alder JD. Rapid bactericidal activity of daptomycin against methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* peritonitis in mice as measured with bioluminescent bacteria. Antimicrob Agents Chemother 2007; 51:1787–1794.

3. Rybak MJ. The efficacy and safety of daptomycin: first in a new class of antibiotics for Gram-positive bacteria. Clin Microbiol Infect 2006; 12(suppl 1):24–32.

4. Steenbergen JN, Alder J, Thorne GM, Tally FP. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J Antimicrob Chemother 2005;55:283–288.

5. Oleson FB, Berman CL, Kirkpatrick JB, Regan KS, Lai JJ, Tally FP. Once-daily dosing in dogs optimizes daptomycin safety. Antimicrob Agents Chemother 2000;44:2948–2953.

6. Silverman JA, Perlmutter NG, Shapiro HM. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother 2003;47:2538–2544.

7. Hamer PW, McGeachie JM, Davies MJ, Grounds MD. Evans Blue Dye as an in vivo marker of myofibre damage: optimizing parameters for detecting initial myofibre membrane permeability. J Anat 2002;200: 69–79.

8. Lovering RM, De Deyne PG. Contractile function, sarcolemma integrity, and the loss of dystrophin after skeletal muscle eccentric contraction-induced injury. Am J Physiol Cell Physiol 2004;286: C230–C238.

9. Lynch GS, Hinkle RT, Faulkner JA. Power output of fast and slow skeletal muscles of mdx (dystrophic) and control mice after clenbuterol treatment. Exp Physiol 2000;85:295–299.

10. Han R, Kanagawa M, Yoshida-Moriguchi T, Rader EP, Ng RA, Michele DE, et al. Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of alpha-dystroglycan. Proc Natl Acad Sci USA 2009;106:12573–12579.

11. Luna LG. Manual of histologic staining methods of the Armed Forces Institute of Pathology. New York: McGraw-Hill; 1968. p 94–95.

12. Galbiati F, Engelman JS, Volonte D, Zhang Xl, Minetti C, Li M, et al. Caveolin-3 null mice show a loss of caveolae, changes in the microdomain distribution of the dystrophin–glycoprotein complex, and t-tubule abnormalities. J Biol Chem 2001;276:21425–21433.

13. Bansal D. Campbell KP. Dysferlin and the plasma membrane repair in muscular dystrophy. Trends Cell Biol 2004;14:206–213.

14. Doherty KR, McNally EM. Repairing the tears: dysferlin in muscle membrane repair. Trends Mol Med 2003;9:327–330.

15. Reddy A, Caler EV, Andrews NW. Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell 2001;106:157–169.