Vibration Causes Acute Vascular Injury in a Two-Step Process: Vasoconstriction and Vacuole Disruption

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ABSTRACT

Hand–arm vibration syndrome is a vasospastic and neurodegenerative occupational disease. In the current study, the mechanism of vibration-induced vascular smooth muscle cell (SMC) injury was examined in a rat-tail vibration model. Tails of male Sprague Dawley rats were vibrated continuously for 4 hr at 60 Hz, 49 m/s^2 with or without general anesthesia. Ventral tail arteries were aldehyde fixed and embedded in epoxy resin to enable morphological analysis. Vibration without anesthesia caused vasoconstriction and vacuoles in the SMC. Anesthetizing rats during vibration prevented vasoconstriction and vacuole formation. Exposing tail arteries in situ to 1 mM norepinephrine (NE) for 15 min induced the greatest vasoconstriction and vacuolation. NE induced vacuoles were twice as large as those formed during vibration. When vibrated 4 hr under anesthesia after pretreatment with NE for 15 min, the SMC lacked vacuoles and exhibited a longitudinal banding pattern of dark and light staining. The extracellular matrix was filled with particulates, which were confirmed by electron microscopy to be cellular debris. The present findings demonstrate that vibration-induced vasoconstriction (SMC contraction) requires functioning central nervous system reflexes, and the physical stress of vibration damages the contracted SMC by dislodging and fragmenting SMC vacuoles. Anat Rec, 291:999–1006, 2008. © 2008 Wiley-Liss, Inc.

Key words: hand-arm vibration; rat-tail artery; smooth muscle cells; norepinephrine

Hand–arm vibration syndrome (HAVS) is an occupational disorder caused by years of exposure to vibration transmitted to the hands by powered tools. One of the major symptoms of HAVS is "vibration white finger" caused by exaggerated vasoconstriction of the arteries and skin arterioles of the hands. Many reviews implicate activation of the somatosympathetic pathway by the pacinian vibroreceptors as the reflex mechanism producing neural activation of vasoconstriction (Sakakibara and Yamada, 1995; Stoyneva et al., 2003). Finger skin biopsies from patients with HAVS reveal vascular smooth muscle cell (SMC) hypertrophy and periarterial fibrosis (Takeuchi et al., 1986).

Vasoconstriction has been demonstrated as a very early effect of vibration in the "rat-tail vibration model"

Abbreviations used: EC = endothelial cells; HAVS = hand–arm vibration syndrome; NE = norepinephrine; SMC = smooth muscle cells.

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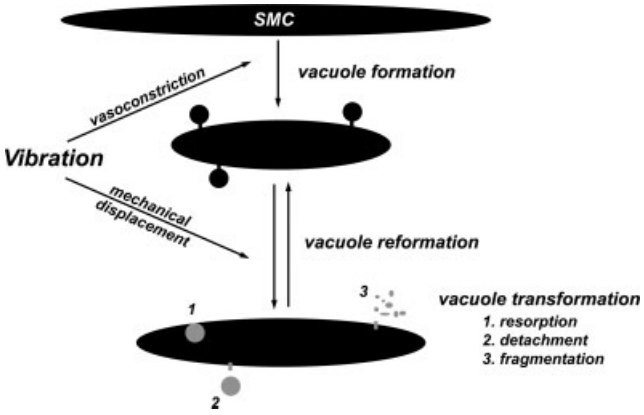

Fig. 1. Working model of acute vibration-induced smooth muscle cell (SMC) injury. Vibration has two major effects on vascular smooth muscle: stimulates vasoconstriction (contraction) and imparts mechanical stress. Vacuoles form when the SMC contracts and shortens in response to vibration. During 4-hr continuous vibration, the SMC cycles between contraction and relaxation. During relaxation, some vacuoles are resorbed by the parent cell (1). The repeated mechanical displacement of the tissue by vibration shears connections of some of the vacuoles, detaching them from the parent cell (2). Separated vacuoles eventually undergo fragmentation and represent loss of SMC membrane and cytoplasm (3). New vacuoles form during subsequent contraction cycles.

which simulates hand-transmitted vibration (Curry et al., 2002, 2005a). When exposed to vibration, rat-tail arteries exhibit morphological signs of vasoconstriction, including narrowed lumens, tightly folded internal elastic membranes with endothelial cells pinched between their folds, and vacuoles in the SMC (Govindaraju et al., 2006a,b). SMC vacuoles caused by vibration were first recognized and quantified in epoxy, semithin cross-sections of arteries at the light microscopic level (Curry et al., 2002). Electron microscopy revealed that SMC vacuoles are double membrane-limited structures, representing protrusion of the cell membrane and cytoplasm from one cell indenting the cell membrane and cytoplasm of an adjacent SMC (Curry et al., 2002; Govindaraju et al., 2006b). With our method of quantitation of vacuoles by light microscopy, it is not possible to discern which vacuoles remain attached to the parent cell. Acknowledging this caveat, we continue to call these structures vacuoles and recognize that many are cell protrusions and not true vacuoles, that is, isolated vesicles.

Structurally similar vacuoles have been observed in SMC of arteries induced to undergo intense vasoconstriction by topical norepinephrine (NE) or epinephrine (Joris and Majno, 1977; Curry et al., 2005b; Govindaraju et al., 2006a). Joris et al., in an elegant series of electron microscopic studies, called the vacuoles herniations because the majority were continuous with the parent cell by slender necks (Joris and Majno, 1977). When isolated single SMC are stimulated in vitro to contract and shorten, they form bulbous projections called blebs (Fay and Delise, 1973). Vasorelaxants, such as calcium chelators, reverse the process by relaxing the cells and permitting resorption of the blebs (Fay et al., 1991). Joris et al. proposed that SMC herniations were not pathological, but rather "a cellular accident, resulting from an intrinsic defect, or weak point, in the fine structure of the tunica media" (Joris and Majno, 1977). However, the authors emphasized that if the herniations increased in size and detached, this was abnormal and represented cell injury.

Acute vibration injury of SMC is hypothesized as a two-step process (Fig. 1). Our previous studies of 4-hr tail vibration and the research literature suggest that the first step is SMC contraction and vacuole formation, stimulated by vibration by means of a centrally mediated, somatosympathetic neural reflex. The second step occurs when the protruding vacuoles are detached from the cell by the mechanical force of vibration. Detachment could generate persistent vacuoles on the one hand and increased fragmentation on the other. Other vacuoles may be resorbed by the parent cell during SMC relaxation. The number and size of vacuoles formed and lost will determine the severity of vascular damage induced by vibration.

The present study provides evidence supporting the two-step model of acute vibration-induced SMC injury. Inactivating the vibroreceptor somatosympathetic reflex by deeply anesthetizing the rat during vibration eliminates neural-induced vasoconstriction (SMC contraction) and blocks step one. Evidence for step two is provided by pharmacologically inducing vasoconstriction and SMC vacuole formation using norepinephrine treatment of the tail artery followed by 4-hr vibration under anesthesia to cause vacuole disruption in the absence of vibration-induced SMC contraction.

MATERIALS AND METHODS

Animal Groups

Male Sprague Dawley rats (n = 52) weighing 275–325 g were segregated into two awake and six anesthesia groups. The two awake groups of rats were either sham-vibration treated or vibrated (Awake-sham and Awake-vibration, respectively). The anesthesia groups included sham vibration treatment under anesthesia (Anesthesia-sham), vibration for 4 hr under anesthesia (Anesthesia-vibration), topical NE (NE), or vehicle application (Vehicle application) for 15 min with immediate tissue harvest, topical NE application for 15 min with tissue harvest after 4 hr of sham-vibration under anesthesia (NE + Anesthesia-sham) and topical NE application for 15 min followed by vibration for 4 hr under anesthesia (NE + Anesthesia-vibration). Sham vibration groups were treated exactly like their vibration counterparts, except the vibration motor was not activated. Rats were otherwise housed in vivarium plastic box cages in a common animal holding room at 25°C and a 12/12-light/dark cycle. Animal treatment, surgical interventions, and husbandry procedures were approved by the Medical College of Wisconsin's Animal Care Committee and complied with the Laboratory Animal Welfare Act.

Vibration Model

As previously described rats were restrained in tubular cages on a nonvibrating platform with their tails taped to a vibrating stage (Curry et al., 2002, 2005a).

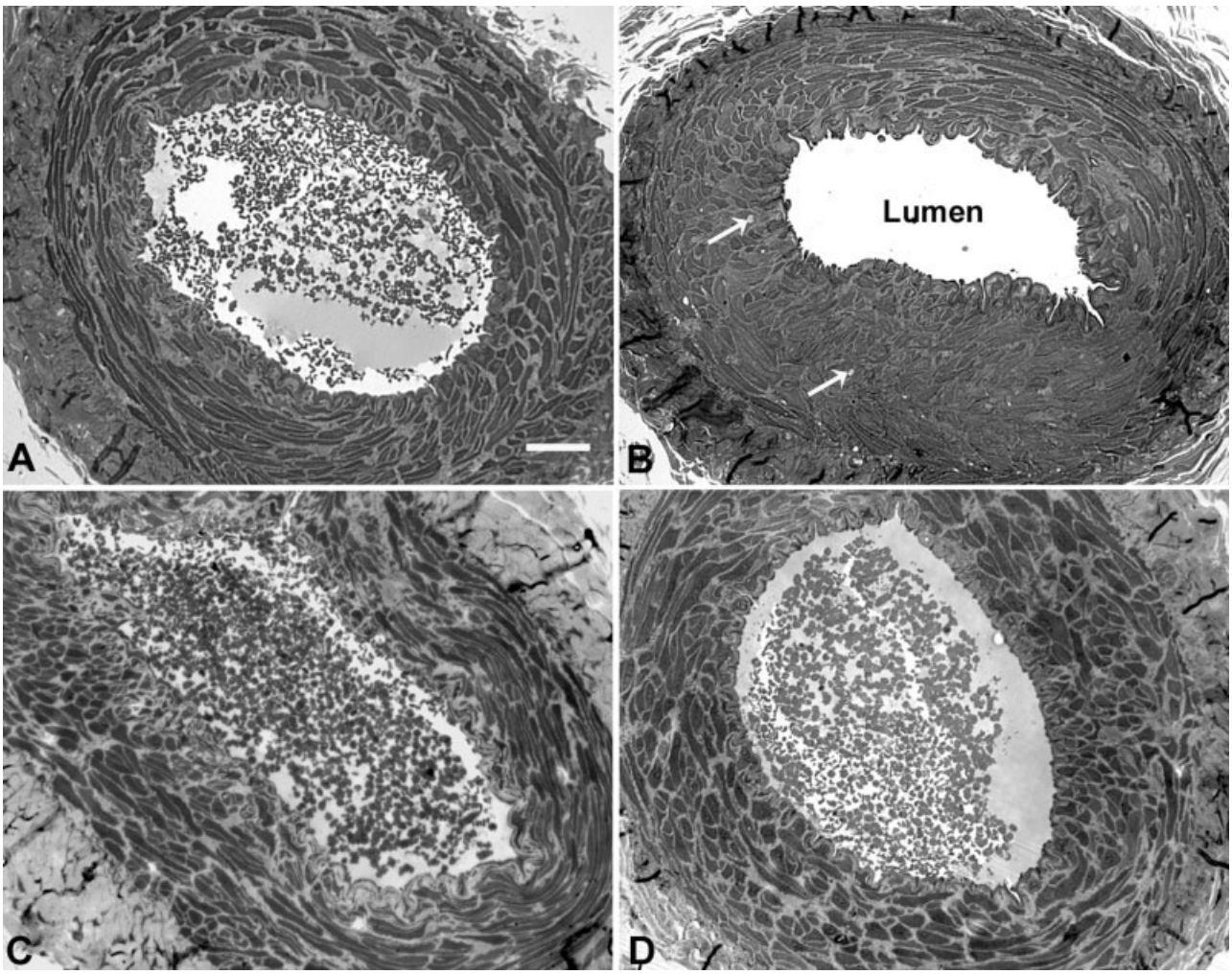

Fig. 2. **A–D**: Semithin cross-sections of tail arteries stained with toluidine blue from the (A) Awake-sham, (B) Awake-vibration, (C) Anesthesia-sham and (D) Anesthesia-vibration groups. Compared with the Awake-sham group, the lumen of the artery from the Awake-vibration group is smaller in size indicating vasoconstriction, and smooth muscle cells contain vacuoles (arrows). Vasoconstriction and vacuoles are not present in the Anesthesia-sham and Anesthesia-vibration groups. Scale bar = 50 μm in A (applies in all panels).

In most instances they slept during vibration. A 1-cm gap separated the vibrating and nonvibrating platforms, restricting direct vibration exposure to the tail. A Simpson 420 function generator produced 60 Hz sine waves which were amplified by a B&K Power Amplifier type 2706 to activate a B&K motor type 4809. Vibration consisted of vertical oscillations at an acceleration of 49 m/s^2 with a calculated peak-to-peak amplitude of 0.98 mm.

In Situ Tail Artery Preparation

A skin incision was made ventrally in the proximal tail segments of deeply anesthetized rats to expose ~5 cm of the ventral artery. The artery was bathed in 1 mM NE in sterile Hanks' Balanced Salt Solution (Gibco) or vehicle alone for 15 min and then flushed out with Hanks solution. The concentration of NE was based on a previously published study addressing the efficacy of

TABLE 1. Comparison of lumen size and vacuole numbers in rat-tail arteries

Treatment	N	Lumen size	SMC vacuoles
Awake-sham	7	52.3 ± 3.8	3.6 ± 2.3
Awake-vibration	7	42.1 ± 3.6[a]	44.8 ± 6.1[b]
Anesthesia-sham	6	54.5 ± 3.3	2.0 ± 1.0
Anesthesia-vibration	6	53.4 ± 3.1	4.0 ± 1.0
Vehicle application	5	59.8 ± 3.2	0.0 ± 0.0
NE	7	29.4 ± 2.0[a]	106.6 ± 16.3[a]
NE +Anesthesia-sham	6	61.0 ± 2.7	44.5 ± 8.3[b]
NE + Anesthesia-vibration	8	52.0 ± 4.2	0.4 ± 0.2

Lumen size is defined as the percentage ratio of the lumen circumference to the internal elastic membrane length. Vacuoles are total numbers per section. N, number of rats per group; NE, norepinephrine.
[a]Significantly different ($P < 0.05$) from all other groups.
[b]Significantly different ($P < 0.05$) from all other groups, except each other, when tested using Newman-Keuls pairwise comparison test.

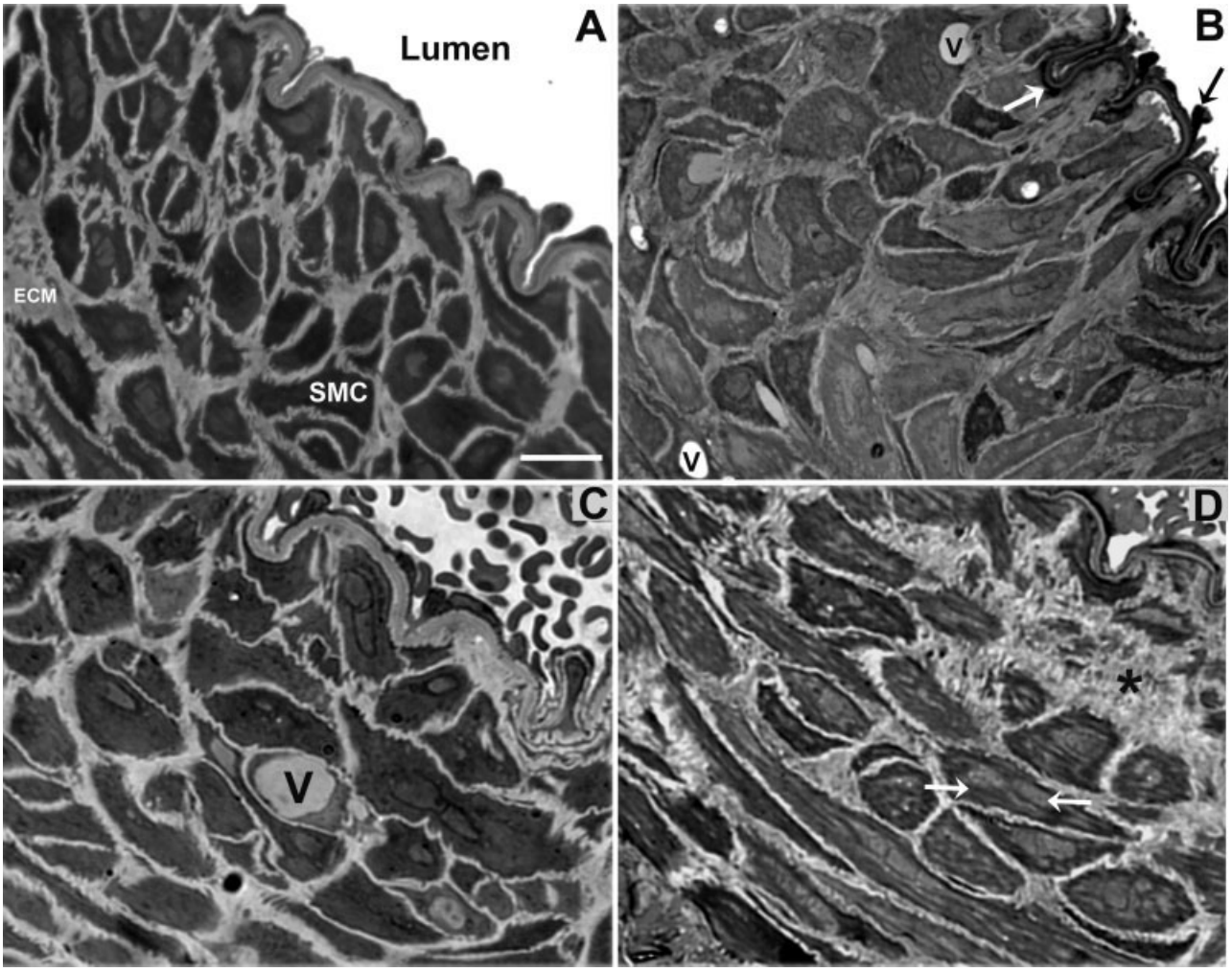

Fig. 3. A–D: Semithin cross-sections of tail arteries stained with toluidine blue from the (A) Awake-sham, (B) Awake-vibration (C) NE + Anesthesia-sham and (D) NE + Anesthesia-vibration groups. The SMC cells exhibit uniform staining of the cytoplasm in the Awake-sham and NE + Anesthesia-sham arteries. Arteries from Awake-vibration group exhibit signs of intense vasoconstriction, including tightly-folded internal elastic membrane (white arrow), endothelial cells pinched between the folds of IEM, with their nuclei protruding into the lumen (black arrow), and non-uniformly stained smooth muscle cell (SMC) with vacuoles (V). The artery in C from the NE + Anesthesia-sham group is not vasoconstricted but contains large vacuoles (V). The NE + Anesthesia-vibration artery in D. is not vasoconstricted and lacks vacuoles, the SMC show dark and light bands (arrows) and the extracellular space is filled with particulate material (*). Scale bar = 20 μm in A.

exogenous epinephrine and NE to induce vacuolation in the rat-tail artery (Curry et al., 2005b). The ventral artery resides in a recess bordered by tendons and skeletal muscles which prevent direct mechanical trauma during vibration. The recess formed a natural well for NE to continuously bathe the artery.

Anesthesia and Tissue Processing

Rats were deeply anesthetized by intramuscular injection of a mixture of ketamine (72 mg/kg), xylazine (12 mg/kg), and acepromazine (0.09 mg/kg). For tissue harvesting, the tail skin was removed. Caudal segment C7 was excised by cutting through the intervertebral joints and immersion fixed in situ in 4% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer (pH 7.4) for 2.5 hr. The arteries were dissected from the segments, post-fixed in 1.3% osmium tetroxide and conventionally embedded in epoxy resin for semithin (0.5 μm) and ultrathin (~70 nm) sectioning for light and electron microscopy.

Arterial Lumen Size and Vacuole Number

Version 1.28v ImageJ software (National Institutes of Health, Bethesda, MD) was used to count total vacuole number, measure lumen circumference, and determine the internal elastic membrane (IEM) length in toluidine blue-stained, semithin cross-sections of arteries. Vacuoles counted were round to oval shaped, intracellular inclusions containing clear or homogeneously dense material and 2–20 μm in the longest dimension. The degree of vasoconstriction (lumen size) was defined as the lumen circumference divided by the path length of

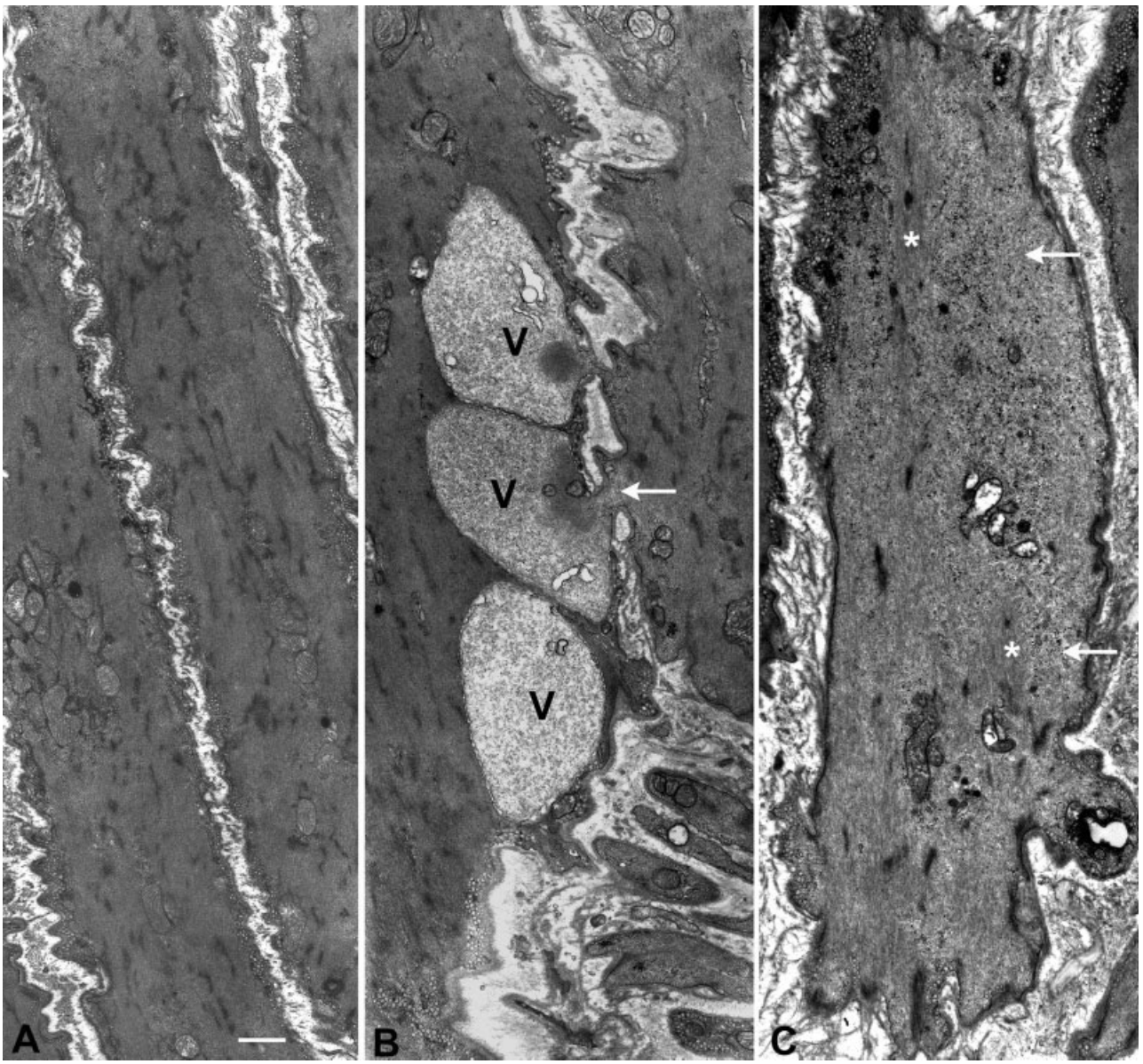

Fig. 4. **A–C**: Electron micrographs of smooth muscle cells, oriented longitudinally, in the tail arteries from the (A) Awake-sham, (B) Awake-vibration and (C) NE + Anesthesia-vibration groups. Vascular smooth muscle cells in the Awake-sham group are closely packed and interdigitated, and the cytoplasm is filled with dense bodies and actin microfilaments. In the Awake-vibration group, vacuoles (V) project into the neighboring SMC while remaining in continuity with the parent cell by narrow necks (arrow). In the arteries of NE + Anesthesia-vibration group, SMC exhibit regions of polymerized (astericks) and depolymerized actin (arrows). Scale bar = 1 μm in A; 0.7 μm in B,C.

the IEM times 100. A fully dilated vessel would have a lumen size of 100.

Statistical Analysis

Group means for lumen size and vacuole number were compared by analysis of variance and Newman-Keuls multiple comparison testing. Differences are considered significant at $P < 0.05$. Values are presented as mean ± SEM.

RESULTS

Anesthesia Prevents Vibration-Induced Vasoconstriction and Vacuole Formation

Arterial lumen size was reduced by 20% in the Awake-vibration group compared with the Awake-sham group (Fig. 2A,B; Table 1). Arteries in the Awake-vibration group manifested morphological signs of vasoconstriction, including tight folding of the IEM, pinching of endothelial cells between the folds of the IEM and closely-packed SMC (Fig. 3B). SMC vacuoles, some still con-

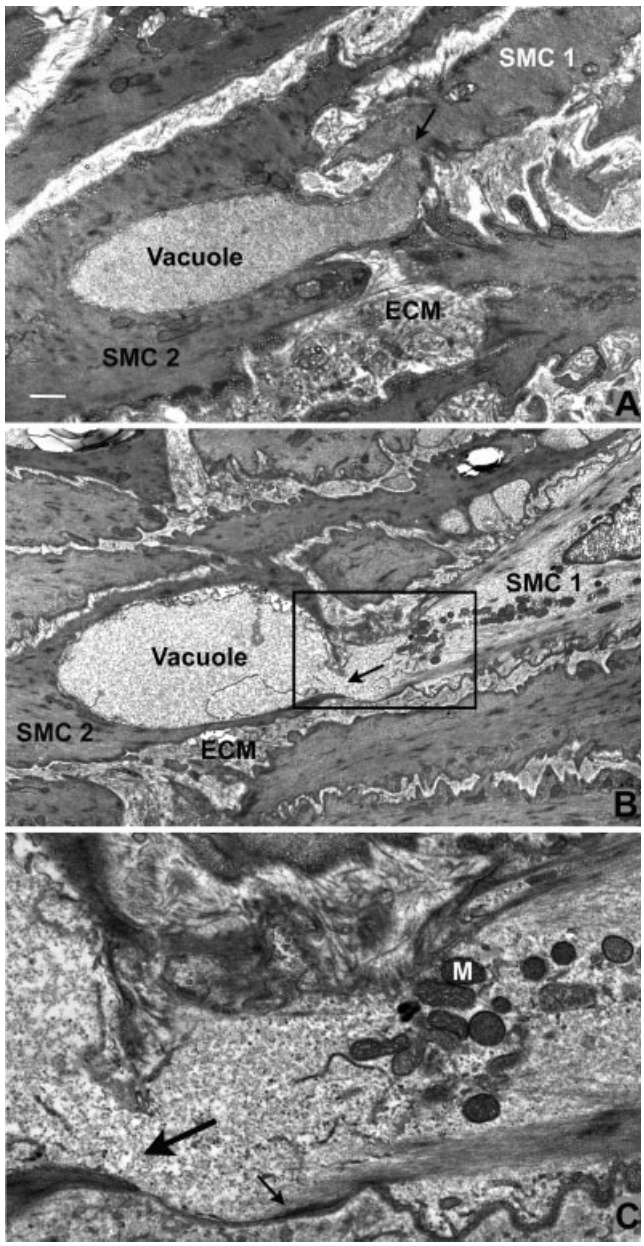

Fig. 5. **A,B:** Electron micrographs showing structural similarities of SMC vacuoles induced by vibration (A) and after norepinephrine treatment (B). The smooth muscle cells are closely packed in both groups, and the process from smooth muscle cell 1 (SMC1) invaginates into smooth muscle cell 2 (SMC 2) to form a vacuole. The cytoplasm of SMC1 in B. is filamentous at the periphery of the cell and depolymerized in the region from which the protrusion emanates. Arrows point to the narrow necks at the presumed sites of origin of vacuoles from the parent cells in A. and B. **C:** A higher magnification view of the boxed region in B. The large arrow points to the neck of the vacuole and the small arrow points to actin microfilaments attached to a dense adhesion plaque. A cluster of mitochondria (M) occupy the region lacking filaments. Scale bar = 0.7 μm in A;, 0.5 μm in B; 0.2 μm in C.

nected to the parent cells, were present in the Awake-vibration group (Fig. 4B). There was no reduction in lumen size or vacuoles present in SMC when the tails of anesthetized rats were vibrated (Fig. 2C,D; Table 1). Lumen sizes of arteries in the Anesthesia-sham and Anesthesia-vibration groups were similar, and contained no vacuoles (Table 1).

NE Causes Bigger and Larger Numbers of Vacuoles Than Vibration

When rat-tail arteries were stimulated to vasoconstrict in situ with topical application of 1 mM NE treatment for 15 min, lumen size narrowed by 48% compared with vehicle application, and extensive vacuolation was present in SMC (Table 1). At the light microscopic level, the vacuoles caused by NE were significantly ($P < 0.001$) larger with a mean diameter of 6.07 ± 0.65 μm, compared with those generated by vibration, whose mean diameter averaged 3.61 ± 0.28 μm (Fig. 3B,C). Ultrastructurally, both Awake-vibration and NE groups showed SMC in close approximation, with little intervening matrix, characteristic of vasoconstriction (Fig. 5A,B). In each condition, the SMC appeared to have herniated into an adjacent SMC to form a vacuole (Fig. 5). The herniations were double membrane-limited, with the inner membrane continuous with the plasma membrane of SMC1 and the outer membrane continuous with SMC2. The cytoplasm of SMC1 contained putative actin microfilaments at the periphery of the cell, but the actin was depolymerized at the vacuole origin and within the vacuole (Figs. 4B, 5A–C). Four hours after NE application (NE + Anesthesia-sham), vasoconstriction was completely reversed, and the SMC vacuole number was reduced, presumably by resorption, to 42% of that present in the NE group sampled 15 min after application (Fig. 3C; Table 1).

Vibration Causes SMC Vacuole Disruption

At the light microscopic level, SMC in the NE + Anesthesia-vibration group lacked detectable SMC vacuoles, and the SMC exhibited pronounced dark and light intracellular banding in toluidine-blue stained, semithin cross-sections (Fig. 3D; Table 1). The banding pattern was striking in longitudinally oriented cells, and the pattern suggested cytoskeletal rearrangement. Electron microscopy revealed that the banding was due in part to alternating regions of intact and depolymerized actin microfilaments (Fig. 4C). In the arteries from Awake-sham and NE + Anesthesia-sham groups, toluidine blue staining was uniform and actin microfilaments were homogeneously distributed. The Awake-vibration group also demonstrated uneven staining of SMC with toluidine blue but less striking than that in the NE + Anesthesia-vibration group (Figs. 3, 4).

In semithin sections, the extracellular matrix in the NE + Anesthesia-vibration group contained more particulate debris compared with the other groups (Fig. 3D). Electron microscopy showed that the extracellular matrix in the Awake-sham group contained collagen filaments and proteoglycan strand-like molecules in most regions (Fig. 6A). In the wider extracellular regions of the Awake-sham arteries, the matrix contained small amounts of scattered membrane vesicles that ranged in

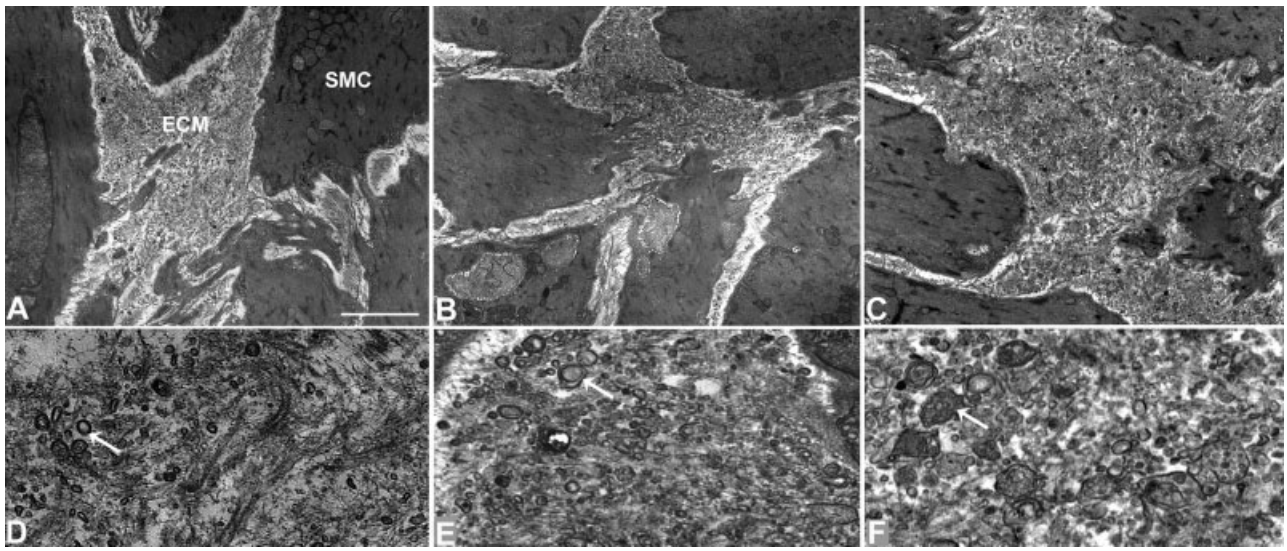

Fig. 6. **A–F:** Electron micrographs of the extracellular matrix of the Awake-sham (A,D), Awake-vibration (B,E) and NE + Anesthesia-vibration (C,F) groups. Compared with the Awake-sham group, the NE + Anesthesia-vibration group has larger areas of dense filamentous matrix (ECM). Compared with Awake-sham, the Awake-vibration and NE-Anesthesia-vibration groups have a higher content of cellular membranous and particulate debris (arrows in D,E,F). The cellular debris in NE + Anesthesia-vibration group is more abundant and larger in size than that in the Awake-vibration group. Scale bar = 3.6 μm in A (applies in A–C); 440 nm in D–F.

size from one half to three times the width of caveolae (Fig. 6D). The composition of the extracellular matrix in the Awake-vibration group was similar to Awake-sham except for increased cellular debris (Fig. 6B,E). The NE + Anesthesia-vibration group, however, had larger areas of dense filamentous matrix and extensive amounts of cellular debris, including vesicles of different sizes and shapes, stripes of membrane, and occasional mitochondrial profiles (Fig. 6C,F). The matrix composition in the Anesthesia-sham and NE + Anesthesia-sham groups was similar to the Awake-sham group.

DISCUSSION

This study was designed to test a model of vibration injury of SMC injury in which vibration induces vasoconstriction and imparts disruptive mechanical forces. General anesthesia was used to prevent vibration-induced somatosympathetic reflex activation of vasoconstriction and vacuole formation. The physical stress component of vibration was studied in anesthetized rats by producing SMC vacuoles in the tail artery with exogenous NE application and then attempting to disrupt these vacuoles by tail vibration. The results indicate that SMC vacuole formation is dependent on a functioning somatosympathetic reflex-induced vasoconstriction, and, furthermore, the physical stress of vibration dislodges vacuoles and transforms vacuolation, a normal physiological consequence of smooth muscle cell contraction, into cell injury.

SMC vacuoles caused by both NE and vibration were similar in morphology. However, the degree of NE-induced vasoconstriction was twice that caused by vibration, and the number and size of vacuoles formed in the SMC were greater. This signifies that vasoconstriction (SMC contraction) is necessary for vacuole formation and the degree of vasoconstriction dictates the number and size of vacuoles.

The morphological finding of depolymerized actin in the SMC cytoplasm at the sites of vacuoles (blebs) provides a clue into the mechanism of vacuole formation. In cell culture, contraction of a cell causes compression of the cytoskeletal network and local increases in hydrostatic pressure, which leads to blebbing at regions weak in membrane-cytoskeletal attachment (Charras et al., 2005). In SMC, depolymerization of actin microfilaments and breakdown of focal adhesions at the base of the forming protrusion may facilitate cytoplasm and membrane addition to vacuoles. The cell membrane contains proteins involved in cellular trafficking, lipid homeostasis, and signal transduction. If the vacuoles are dislodged during vibration, the loss of cell membrane and constituent proteins may injure the cell and alter function.

We have previously reported that nearly half the number of SMC vacuoles induced by 4-hr vibration persist 24 hr later (Govindaraju et al., 2006a). Repeated mechanical displacement in vibrated arteries may shear connections of vacuoles and dislodge them from the parent cell. Dislodged vacuoles cannot be reabsorbed into the cell and may account for persistence. The total absence of vacuoles and the extensive appearance of a particulate matrix suggest that the vacuoles induced by NE before vibration were sheared from the cells during vibration. The cellular debris in the extracellular matrix most likely represents the remnants of vacuoles fragmented by vibration stress or osmotically disrupted once the vacuole is detached from the cell. Vacuoles caused by vibration alone were significantly smaller in size and fewer in number, compared with those generated by NE treatment. Fragmentation of fewer and smaller dislodged vacuoles is consistent with less debris in the

extracellular matrix of the Awake-vibration group compared with that after NE +Anesthesia-vibration group. Reduced vacuole number without elevated extracellular debris in the NE + Anesthesia-sham group indicates that vacuoles formed by NE induction are resorbed under nonvibrated conditions. The absence of vacuoles and prominence of extracellular debris in arteries from NE + Anesthesia-vibration group argues for detachment of vacuoles from parent cells due to repeated mechanical displacement.

CONCLUSION

The present study supports our two-step model of acute vibration-induced SMC injury: (1) vasoconstriction (SMC contraction) causing vacuole formation and (2) mechanical displacement detaching the vacuoles and causing their disruption. Cell injury occurs when vibration mechanically detaches these vacuoles because vacuoles are vulnerable and break off during vibration stress. The loss of SMC membrane, receptors and ion channels are likely to alter the function of the cell.

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