INCREASED OXIDANT ACTIVITY MEDIATES VASCULAR DYSFUNCTION IN VIBRATION INJURY

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Non-standard Abbreviations:

4-amino-5-methylamino-2', 7'-difluorofluorescein, DAF-FM DA
5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein, DCDHF
Hand-Arm Vibration Syndrome, HAVS; Nitric oxide synthase, NOS
N\textsuperscript{G}-nitro-L-arginine methyl ester, L-NAME; root mean square, rms

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ABSTRACT

Occupational exposure to hand-operated vibrating tools causes a spectrum of pathological changes in the vascular, neurological and musculoskeletal systems described as the Hand-Arm Vibration Syndrome (HAVS). Experiments were performed to determine the effects of acute vibration on the function of digital arteries. Rats paws were exposed to a vibrating platform (4 hrs, 125 Hz, constant acceleration of 49 m/s² root mean square), and digital artery function subsequently assessed in vitro using a pressure myograph system. Constriction to phenylephrine or 5-hydroxytryptamine was reduced in digital arteries from vibrated paws. However, after endothelium-denudation, constriction to the agonists was no longer impaired in vibrated arteries. Inhibition of NO synthase (NOS) with \( \text{N}^{\text{G}} \)-nitro-L-arginine methyl ester (L-NAME) increased constriction to phenylephrine or 5-hydroxytryptamine in vibrated but not control arteries, and abolished the vibration-induced depression in constrictor responses. However, NO activity, determined using the NO-sensitive probe 4-amino-5-methylamino-2’, 7’-difluorofluorescein, was reduced in vibrated compared to control arteries. Concomitantly, endogenous levels of reactive oxygen species (ROS), determined using the ROS-sensitive probe 5-(and 6)-chloromethyl-2’,7’-dichlorodihydro-fluorescein, were increased in vibrated compared to control arteries. The increased ROS levels were abolished by L-NAME or by catalase, which degrades extracellular \( \text{H}_2\text{O}_2 \). Catalase also increased constriction to phenylephrine or 5-hydroxytryptamine in vibrated but not control arteries, and abolished the vibration-induced depression in constrictor responses. The results suggest that acute vibration causes vascular dysfunction in digital arteries by increasing ROS levels, which is likely mediated by uncoupling of endothelial NOS. Therefore, therapeutic strategies to inhibit ROS or augment NO activity may be beneficial in HAVS.
INTRODUCTION

Hand-Arm Vibration Syndrome (HAVS) is a major occupational disease that causes considerable morbidity among workers exposed to vibration and is characterized by disorders of the vascular, sensorineural and musculoskeletal systems (Bernard et al., 1998; Friden, 2001; Weir and Lander, 2005). Approximately 1.45 million workers in the United States are exposed to hand-transmitted vibration, and based on data from epidemiological studies approximately 50% of those workers will develop HAVS (Bernard et al., 1998). The predominant vascular disorder is heightened constriction of digital arteries resulting in reduced cutaneous blood flow (Friden, 2001; Weir and Lander, 2005). The onset and progression of the disease is poorly understood at the cellular and molecular level. Although heightened activity of the sympathetic nervous system and endothelial injury have been proposed as important pathogenic mechanisms, clinical studies have demonstrated reduced vasoconstriction to $\alpha$-adrenergic activation and normal dilation to the endothelium-dependent agonist acetylcholine in individuals who use vibrating tools (Ekenvall and Lindblad, 1986; Chen et al., 1994; Kennedy et al., 1999; Stoyneva et al., 2003). HAVS may also be associated with medial thickening and smooth muscle hypertrophy, resulting in increased wall:lumen ratios and reduced internal diameters in small arteries and arterioles (Takeuchi et al., 1986; Kent et al., 1991; Greenstein et al., 1994; Hashiguchi et al., 1994; Littleford et al., 1997).

The goal of the present study was to examine the effects of acute in vivo vibration on the functional responses of isolated digital arteries, and to determine mechanistic responses associated with vibration injury. No previous studies have attempted this type of analysis on digital arteries. Therefore, we developed a new model of vibration injury using the rat paw. Prior to performing physiological studies, the physical (i.e., biodynamic) response of the digits
and paw to vibration was characterized. Responses of digital arteries from control and vibrated paws were then examined \textit{ex vivo} to determine vascular responses associated with acute vibration injury and to identify potential underlying mechanisms.
METHODS

Animals: Male Sprague-Dawley rats [H1a:(SD)CVF rats; 6 weeks old) were obtained from Hilltop Lab Animals (Scottdale, PA) and housed in a temperature- and humidity-controlled facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Food (Teklad 2918 rodent diet) and water were provided ad libitum. Prior to each exposure session, rats were restrained using a modified cone-shaped plastic bag (72-6414; Harvard Apparatus, Holliston, MA). The rat was placed inside the bag with the head facing the smaller opening of the bag (Figure 1). The larger opening was closed off with tape. When the rat was secured, a small opening was cut into the right side of the bag so that the rats’ right paw and forelimb could be exposed. Additional openings were cut into the bottom of the bag to allow urine to escape the bag. The rats quickly acclimated to the bag restraint and remained quite still throughout exposure sessions. All procedures were approved by the Institutional Animal Care and Use Committee.

Vibration apparatus: The restrained rat rested in a support device that was secured to a pole stand and suspended over an electromagnetic shaker (V408, Ling Dynamic Systems, UK) so that its paw rested flat on a custom-designed vibrating platform affixed to the shaker (Figure 1). The vibrating platform measured 9 cm square and 1.3 cm thick and was fabricated from a solid block of aluminum. Vibration measurements of the platform alone revealed that it was stable through a wide range of frequencies: the resonant frequency of the platform was well above 500 Hz, the highest frequency tested in the present study. Repeated vibration exposures occurred in a sound-attenuating cubicle (Med Associates, St. Albans, VT.). To measure vibration transmissibility in the paw, a scanning laser vibrometer (PSV-300, Polytec, Germany) was positioned above the vibration platform to record the distribution of vibration at specified locations on the digits,
palm, and wrist (Figure 1). Software (Version 2B, Polytec, Germany) collected and recorded velocity measurements from several locations along the dorsal surface paw.

**Vibration transmissibility:** Three rats were used to measure the vibration transmissibility in the rat paw to characterize the response of the paw to segmental vibration exposure. Prior to testing, these rats were lightly anesthetized with xylazine (5 mg/kg) and ketamine (40 mg/kg) to keep their paws completely still during operation of the scanning laser vibrometer. The anesthetized rat was placed in cone-shape plastic bag and placed over the shaker as described above. Velocity measurements were recorded from several locations along the dorsal surface of the paw, plus a reference point on the surface of the vibrating platform next to the paw. Six different vibration frequencies (32.5 Hz, 63 Hz, 125 Hz, 160 Hz, 250 Hz, and 500 Hz) were tested at three different unweighted accelerations (9.8 m/s², 49 m/s², and 100 m/s²). Measurement at each location lasted approximately 5 s – long enough for the software to record a reliable measurement.

**Vibration exposure:** A separate set of non-anesthetized rats were used for *in vitro* analyses of vascular function. Each rat was randomly assigned to one of two groups: a vibration-exposed group or a restraint-control group. Rats in the vibration group were exposed to a 4-hr period of vibration at 125 Hz and a constant acceleration of 49 m/s² root mean squared. The biodynamic response of the paw to this frequency and acceleration was fairly consistent at all measurement locations (Figure 2). In addition, exposure to tools with a dominant vibration frequency near 125 Hz is associated with the development of HAVS in humans (Bovenzi et al., 1988). Rats in the restraint-control group were similarly restrained and placed in the same exposure chamber except no vibration was applied to their paws or forelimbs. Exposure chambers were equipped with a video camera to monitor rats throughout the exposure and ensure that each rat’s paw stayed in contact with the vibrating platform at all times. After exposures, the rats were euthanatized
using pentobarbital (100 mg/kg, i.p.), and the right paw was immediately removed, immersed in
cold DMEM that was maintained at 4 °C, and transported from NIOSH to Johns Hopkins
University where the physiological analysis was performed (Krajnak et al., 2006).

**Vasoconstrictor responses:** Vasoconstrictor responses of arteries from control and vibrated rat
paws were recorded simultaneously (Krajnak et al., 2006). Briefly, digital arteries from control
and vibrated paws were dissected out and placed in cold Krebs-Ringer bicarbonate solution (in
mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, and 11.1 glucose
(control solution). Arteries were cannulated at both ends with glass micropipettes, secured using
12-0 nylon monofilament suture, and placed in a microvascular chamber (Living Systems,
Burlington, VT). The chamber was superfused with control solution, maintained at 37°C, pH 7.4
(gassed with 16% O2-5% CO2-balance N2), and the artery segment pressurized to a constant
transmural pressure (PTM) of 60 mm Hg (Krajnak et al., 2006). The chamber was placed on the
stage of an inverted microscope (X10, Nikon Eclipse TS100, Japan) and connected to a video
camera (Sony, CCTV camera, Japan). The vessel image was projected onto a video monitor, and
the internal diameter continuously determined by a video dimension analyzer (Living Systems
Instrumentation, Burlington VT) and recorded using a BIOPAC data acquisition system (Santa
Barbara, CA). For experiments involving endothelium-denuded arteries, a 1-ml air bubble was
passed through the vessel lumen after cannulation and the luminal surface was gently rubbed
with a piece of fine wire. Endothelial denudation was confirmed by lack of relaxation to the
endothelium-dependent dilator, acetylcholine (0.001-1 µM).

Cumulative concentration-response curves to the α1-adrenoceptor (α1-AR) agonist
phenylephrine (0.001 to 0.3 µM), 5-hydroxytryptamine (5-HT)(1 to 30 nM) or to the α2-AR
agonist UK-14, 304 (1 to 100 nM) [5-Bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine] were
generated by increasing the concentration of the agonists in half-log increments, once the
constriction to the previous concentration had stabilized. After each concentration-effect curve
was completed, the agonist was removed from the superfusate and the artery was allowed to
return to its baseline level for 30 minutes before beginning the next curve. Cumulative
concentration-response curves to acetylcholine (0.001-1 µM), substance P (0.001-1 µM) or
sodium nitroprusside (SNP) (0.001-1 µM) were obtained following constriction of the arteries to
~50% of the stable baseline diameter with phenylephrine.

Concentration-effect curves to constrictor agonists were determined in the absence and
presence of the nitric oxide synthase (NOS) inhibitor, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-
NAME; 100 µM), or catalase (1,000 units/ml), which degrades extracellular hydrogen peroxide
(H\textsubscript{2}O\textsubscript{2}). When inhibitors were used, the preparations were incubated with the drugs for 30 min
before and then during exposure of the arteries to the agonist. Only one inhibitor was assessed in
each blood vessel. Time control experiments, in which the arteries were incubated for a similar
time without inhibitors, demonstrated that sequential concentration-effect curves to
phenylephrine and to 5-HT were similar (data not shown).

After all vasoconstriction curves were recorded, the perfusion solution was switched to
calcium-free control solution (containing 3 mM EGTA) and passive diameter at 60 mmHg was
recorded.

Nitric Oxide (NO): NO activity of digital arteries from control and vibrated paws was
determined using the NO-sensitive fluorescent probe 4-amino-5-methylamino-2’, 7’-
difluorofluorescein diacetate (DAF-FM DA) (Molecular Probes, Eugene, Oregon) (Santhanam et
al., 2007; Ryoo et al., 2008). Digital arteries were pinned in a Silastic-coated dish in Hepes
buffered saline solution (HBS) (in mmol/L): 10.0 Heps, 134.0 NaCl, 6.0 KCl, 1.0 MgCl\textsubscript{2}, 10.0
glucose and 2.0 mM CaCl₂. Arteries were incubated with DAF-FM DA (5 µM in HBS), for 45 minutes at 37°C. The probe was then removed and the arteries allowed to stabilize for 10 min (in HBS) before commencing imaging. Arteries were imaged using a fluorescent microscope (Nikon Eclipse 80i), x10 objective (NA 0.3), and 10 ms exposure times. The number of exposures was limited to prevent bleaching. To determine the influence of NOS inhibition, arteries were treated with L-NAME (100 µM) before, during and after the incubation with DAF-FM DA. The average change in fluorescent intensity from L-NAME treated background was calculated for each group.

Reactive oxygen species (ROS): Digital arteries were pinned in a Silastic-coated dish in HBS and incubated with the ROS-sensitive fluorescent probe, 5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (DCDHF) (Molecular Probes, Eugene, Oregon), 5 µM, for 30 minutes (37°C) (Santhanam et al., 2007; Ryoo et al., 2008). The probe was then removed and the arteries allowed to stabilize for 10 min (in HBS) before commencing imaging. Arteries were imaged using a fluorescent microscope (Nikon Eclipse 80i), x10 air objective (NA 0.3), and 10 ms exposure times. Only 3 images were captured from each DCDHF-loaded artery to prevent generation of ROS or bleaching of the DCDHF signal, and the average of these three images was used for all calculations. To determine the effects of inhibition of NOS or of extracellular H₂O₂, arteries were treated with L-NAME (100 µM) or catalase (1,000 U/ml) before, during and after the incubation with DCDHF. In control and vibrated arteries, DCDHF fluorescence was virtually abolished by treatment with the antioxidant N-acetylcysteine (20 mM)(data not shown). The average fluorescent intensity was calculated for each group.

Reagents: Unless stated otherwise, all reagents were from Sigma (St. Louis, MO). Stock solutions of drugs were prepared fresh each day and stored at 4°C during the experiment. Drugs
were dissolved in distilled water, with the exception of DAF-FM DA and DCDHF, which were dissolved in DMSO. All drug concentrations are expressed as final molar concentration.

**Calculations and statistics:** All data are expressed as means ± SEM, where ‘n’ refers to the number of animals in each group. Transmissibility was calculated for each location by dividing the velocity at that location by the velocity at the reference point. Transmissibility values greater than 1.0 indicate vibration amplification, and values less than 1.0 indicate attenuation. These values were averaged across rats for each location and plotted as a function of frequency and magnitude.

Myogenic tone was calculated as the percent difference in diameter at a transmural pressure of 60 mmHg when arteries were superfused with Ca\(^{2+}\)-free versus Ca\(^{2+}\)-replete control solution. Vasomotor responses were expressed as a percentage change in the baseline diameter before administering vasoconstrictor agonists. Concentration-response curves were compared by analyzing areas under the curves. Statistical evaluation was performed using Student t test for either paired or unpaired observations. When more than 2 means were compared, analysis of variance was used followed by Dunnett or Bonferroni multiple comparison tests (GraphPad Software, San Diego, CA). A probability of P < 0.05 was accepted as statistically significant for all comparisons.
RESULTS

BIODYNAMIC ANALYSES

Vibration transmissibility from 32 Hz to 250 Hz was at or slightly above 1.0 in the digits and palm at 9.8 m/s² rms and 49 m/s² rms (Figure 2). At 100 m/s² rms and at the higher frequencies, transmissibility in the palm and wrist was less than 1.0.

FUNCTIONAL ANALYSES

For the functional studies, rats in the vibrated group were exposed to a 4-hr period of vibration at 125 Hz and constant acceleration of 49 m/s² root mean squared. At a transmural pressure of 60 mmHg, the baseline diameters of control and vibrated digital arteries were similar (211 ± 6 and 196 ± 6 µm, respectively, n = 18; P = not significant or NS). In the absence of constrictor agonists, these arteries did not display significant basal tone.

Vibration Does Not Affect Vasodilator Agonist Responses

Vasodilator responses to the endothelial agonists, acetylcholine or substance P (0.001-1 µM) were analyzed during constriction of arteries to phenylephrine (by ~ 50% of baseline diameter). There was no difference in vasodilation concentration-effect curves between control and vibrated arteries in response to either agonist (P = NS), whether assessed in the absence or presence of the NO synthase (NOS) inhibitor L-NAME (100 µM) (Figure 3). L-NAME (100 µM) inhibited (Figure 3) whereas endothelial denudation abolished vasodilator responses to each agonist (data not shown). The endothelium-independent dilator sodium nitroprusside (0.001-1 µM) caused concentration dependent relaxation of digital arteries that was similar between control and vibrated paws (Figure 3) (P = NS).
Vibration Depresses Vasoconstrictor Agonist Responses

Vasoconstrictor responses to the $\alpha_1$-AR agonist phenylephrine (0.001-0.3 µM) or 5-HT (0.001 to 0.03 µM) were significantly inhibited in vibrated compared to control digital arteries (Figure 4) (P < 0.001). $\alpha_2$-AR activation by UK 14,304 did not cause significant constriction of these arteries (data not shown).

Role of the Endothelium: Endothelial-denudation decreased constriction to phenylephrine or 5-HT in control (P < 0.05) but not vibrated arteries, and eliminated the vibration-induced decrease in agonist responses (figure 5).

Role of NO: The NOS inhibitor L-NAME (100 µM) increased vasoconstriction to phenylephrine or 5-HT in vibrated (P < 0.05) but not control arteries, and abolished the vibration-induced depression in vasoconstrictor responses (Figure 6).

NO activity, determined using the NO-sensitive fluorescent probe DAF-FM DA, was significantly reduced in arteries from vibrated compared to control paws (P < 0.05) (Figure 7A).

Role of ROS: Endogenous levels of ROS, which were determined using the ROS-sensitive fluorescent probe DCDHF, were significantly increased in vibrated compared to control digital arteries (P < 0.01) (Figure 7B). Degradation of extracellular H$_2$O$_2$ by catalase (1,000 units/ml) did not influence ROS levels in control arteries but significantly reduced the increased oxidant levels in arteries from vibrated paws (P < 0.01) (Figure 7B). Inhibition of NOS by L-NAME also significantly reduced oxidant activity in arteries from vibrated (P < 0.01) but not control paws (Figure 7B).

Catalase (1,000 units/ml) increased vasoconstrictor responses to phenylephrine or 5-HT in vibrated but not control arteries (P < 0.01) (figure 8), and abolished the vibration-induced depression in vasoconstrictor responses (Figure 8).
DISCUSSION

The present study analyzed the effects of vibration on the function of digital arteries using a new model of vibration injury. Exposure of rat paws to a single period of vibration caused a pronounced depression in arterial reactivity to vasoconstrictor stimuli, which was prevented by endothelial denudation or inhibition of NOS suggesting that it might be mediated by increased activity of endothelium-derived NO. However, NO activity, assessed using the fluorescent probe DAF-FM, was actually reduced in vibrated arteries whereas the endogenous levels of ROS, assessed using the ROS-sensitive probe DCDHF, were increased in vibrated arteries. The vibration-induced increase in levels of ROS was abolished by inhibition of NOS or by inactivation of H₂O₂ with catalase. Catalase also abolished the vibration-induced depression in vasoconstrictor responses. The results suggest that after vibration, endothelial NOS generates ROS leading to increased levels of H₂O₂ and depressed vasoconstrictor responses. Therefore, oxidative stress, which may result from uncoupling of endothelial NOS, appears to be an early response to vibration injury and could be an important early event in the pathogenesis of HAVS.

Vibration-induced soft tissue damage is believed to be dependent on the amount of tissue stress and strain, which can be estimated by calculating the transmissibility of the vibration stimulus to the tissue (Dong et al., 2005; Wu et al., 2006). The transmission of vibration to the paw was frequency and location dependent as it is in the hand-arm system. In humans, exposures to lower vibration frequencies (32 Hz to 63 Hz) affect the whole hand-arm system, whereas exposures to higher vibration frequencies (100 Hz to 350 Hz) preferentially affect the fingers and hands (Sorensson and Lundstrom, 1992; Dong et al., 2004). The responses of the fingers to vibration are also frequency dependent, with transmissibility at the higher frequencies usually being greater than one (Sorensson and Lundstrom, 1992; Dong et al., 2004). In the rat
paw and digits transmissibility was also at or slightly above 1.0 between 63 and 250 Hz. Transmissibility in the rat paw was less than that observed in human fingers (Dong et al., 2005) or rat tails (Welcome, et al., in press), which may be explained by their differences in mass and stiffness. However, the results demonstrate that the paw model can be used to study the effects of vibration and provide mechanistic insight into how vibration causes pathological changes in the hand-arm system.

Exposure of rat paws to a single period of vibration caused a long-lasting depression in vasoconstrictor responses of digital arteries to activation of $\alpha_1$-ARs or serotonergic receptors. This generalized decrease in contractile activity would be consistent with a proposed vibration-induced disruption of smooth muscle cell integrity (Curry et al., 2002). However, the vibration-induced depression in constriction was prevented by endothelial-denudation indicating that it resulted from altered activity of endothelial rather than smooth muscle cells. Although endothelial cells can express serotonergic receptors, functional $\alpha_1$-ARs are not present on arterial endothelium. Therefore, the vibration-induced, endothelium-dependent depression in constrictor responses would be consistent with basal rather than stimulated activity (by phenylephrine or 5-HT) of an endothelial mediator. Indeed, vibration did not alter the magnitude of endothelium-dependent relaxations evoked by the endothelial activators, acetylcholine or Substance P. The vibration-induced depression in vasoconstriction was also prevented by inhibition of NOS with L-NAME, which would be consistent with a vibration-induced increase in basal NO activity. Surprisingly, the basal activity of NO, assessed using the fluorescent probe DAF-FM, was actually reduced in vibrated arteries. In contrast, the basal levels of ROS, assessed using the ROS-sensitive probe DCDHF, was dramatically increased in vibrated arteries. The vibration-induced increase in levels of ROS was abolished by L-NAME suggesting that in vibrated
arteries, NOS was actually associated with the generation of ROS. Active NOS is a homodimer that generates NO and l-citrulline from l-arginine. When deprived of its cofactor tetrahydrobiopterin (BH₄) or substrate l-arginine, or exposed to oxidant stress including peroxynitrite, NOS can uncouple to the monomeric form, which acts as a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and generates superoxide rather than NO (Heinzel et al., 1992; Xia et al., 1996; Rosen et al., 2002; Kuzkaya et al., 2003). The vibration-induced increase in DCDHF fluorescence was abolished by catalase, which inactivates H₂O₂. DCDHF is more sensitive at detecting H₂O₂ compared to superoxide (Su et al., 2001). Catalase, which inactivates H₂O₂ in the extracellular environment, abolished the vibration-induced increase in ROS and the vibration-induced depression in vasoconstrictor responses. The results therefore suggest that in vibrated arteries, H₂O₂ is generated as a result of endothelial NOS activity and is the active metabolite that diffuses through the blood vessel wall to cause depression in vasoconstrictor responsiveness.

Although endothelial denudation or inhibition of NOS each prevented the vibration-induced depression in vasoconstrictor responses, they had distinct effects on the concentration-effect curves to vasoconstrictor agonists in control and vibrated arteries. Inhibition of NOS had no effect on vasoconstrictor responses in control arteries, but amplified responses in vibrated arteries. This indicates that the basal activity of NOS-derived mediators causes relaxation in vibrated but not control arteries. Endothelial denudation inhibited vasoconstrictor responses in control arteries, but had no effect on constriction in vibrated arteries. This indicates that the net basal effect of the endothelium in control arteries is amplification of vasoconstriction, consistent with the generation of an endothelium-derived contractile mediator, whereas there is no net basal effect in vibrated arteries. In vibrated arteries, inhibition of NOS with L-NAME increased
constrictor responses in endothelium-containing arteries, but had no effect in endothelium-denuded arteries (data not shown), thereby revealing an endothelium-dependent constrictor influence (cf. Figures 5 and 6). Therefore, although there appears to be no net basal effect of the endothelium in vibrated arteries, this actually reflects a balanced activity of endothelium-derived contracting and dilating mediators. The major difference between control and vibrated arteries and the reason for the interesting divergent shifts in the concentration-effect curves appears to be the dramatic increase in endothelial (and NOS-dependent) dilator activity in vibrated arteries and maintained activity of endothelium-derived contractile factors in control and vibrated arteries. The endothelium-derived dilator in vibrated arteries appears to be H₂O₂, whereas the nature of the contracting factor was not pursued.

Vibration increased endothelial dilator activity under basal conditions, but did not change the magnitude of endothelium-dependent relaxation to endothelial agonists, acetylcholine and Substance P. Although NOS uncoupling can reduce endothelium-dependent responses to these agonists, the NOS-dependent generation of H₂O₂ can also effectively substitute for NOS-derived NO enabling maintained relaxation (Cosentino and Katusic, 1995). The selective vibration-induced increase in endothelial dilator activity under basal conditions could result from persistent basal activation of the endothelium in vibrated arteries or from different subcellular compartments of NOS.

A vibration-induced diminution in constrictor activity maybe a somewhat surprising finding because HAVS is characterized by exaggerated vasoconstriction (Weir and Lander, 2005). However, clinical and in vitro studies have also demonstrated diminished responses to vasoconstrictor stimuli in individuals or in isolated blood vessels exposed to vibration (Ekervall and Lindblad, 1986; Lindblad et al., 1986; Chen et al., 1994). Although low levels of H₂O₂ may
act as a temporary compensatory mechanism to maintain vasodilatation and decrease vasoconstriction, sustained exposure to high levels of oxidants could contribute to disease progression. NO is vasoprotective and acts to inhibit platelet aggregation and adhesion, and to vascular remodeling and inflammation, whereas H₂O₂ and other oxidants can initiate pathological changes in the vasculature, stimulating inflammatory genes, vascular remodeling and enhanced vasoconstriction (Boulden et al., 2006; Forstermann, 2006; Forstermann and Munzel, 2006). Indeed, NOS uncoupling and subsequent oxidative stress are thought to contribute to many cardiovascular disorders including hypertension, atherosclerosis and diabetes (Cosentino et al., 1998; Oelze et al., 2000; Hink et al., 2001; Landmesser et al., 2003; Forstermann, 2006; Forstermann and Munzel, 2006) and may be an important early step in the vasculopathy of HAVS. Chronic exposure to vibration injury, by causing persistent oxidant stress and loss of NO activity, could then contribute to structural and functional pathological changes in these blood vessels and disease progression. Therapy targeted towards preserving NO activity may be beneficial in this disease process.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Illustration of vibration apparatus showing the restrained rat and its paw in contact with the vibrating platform. The scanning laser vibrometer was used for initial biodynamic testing of vibration transmissibility in different regions of the paw.

Figure 2: Transmissibility of the vibration stimulus (ratio of the measured velocity on the paw to the measured velocity on the platform) plotted for different areas of the rat paw as a function of vibration frequency and amplitude. Transmissibility in the digits and palm was at or somewhat greater than 1.0 relative to the platform across most frequencies and amplitudes. At 100 m/s² rms and the higher frequencies, transmissibility in the wrist was less than 1.0 (dampened) compared with the digits and palm. A vibration frequency of 125 Hz and an amplitude of 49 m/s² rms was chosen for exposures in this study because transmissibility was at or slightly above 1.0 in the palm and digits and because this frequency has been associated with the greatest risk of HAVS in humans. Results are expressed as means ± SEM for n = 3.

Figure 3: Effects of vibration on dilation to the endothelium-dependent agonists acetylcholine (panel A & B), and substance P (panel C & D) or the endothelium-independent agonist sodium nitroprusside (SNP) (panel E) in untreated (panels A, C, E) and L-NAME (100 μM)–treated (panels B, D) digital arteries. Arterial constriction was titrated to approximately 50% of baseline diameter (B) by the α₁-AR agonist phenylephrine (PE) before administering vasodilator agonists. Because of decreased reactivity of arteries from vibrated paws, higher concentrations of phenylephrine were required to constrict vibrated compared to control arteries. Vasomotor
responses were expressed as percent constriction from baseline diameters (before administering phenylephrine) and are presented as means ± SEM. (n = 4-11).

Figure 4: Effects of vibration on vasoconstrictor responses to phenylephrine (panel A) or 5-HT (panel B) in endothelium-intact digital arteries. Vasoconstrictor responses are expressed as a percentage of the baseline internal diameter and are presented as means ± SEM (n = 5).

Vasoconstrictor responses were significantly different between control and vibrated arteries for each agonist. *** P < 0.001.

Figure 5: Effects of endothelium-denudation on vasoconstrictor responses to phenylephrine (panel A) or 5-HT (panel B) in digital arteries from control and vibrated rat paws.

Vasoconstrictor responses are expressed as a percentage of the baseline internal diameter and are presented as means ± SEM, n = 5 all groups. Endothelium-intact, vibrated arteries demonstrated blunted responses to both vasoconstrictor agonists when compared to controls (P < 0.001).

Endothelium-denudation decreased vasoconstriction to phenylephrine or to 5-HT in control (P < 0.05) but not vibrated arteries. After endothelium denudation vasoconstrictor responses were similar in digital arteries from control and vibrated paws. *** P<0.001, * P < 0.05, NS not statistically significant, with brackets and symbols indicating comparisons.

Figure 6: Effects of NOS inhibition with L-NAME (100 μM) on vasoconstrictor responses to phenylephrine (panel A) or 5-HT (panel B) in digital arteries from control and vibrated rat paws.

Vasoconstrictor responses are expressed as a percentage of the baseline internal diameter and are presented as means ± SEM, n = 5 all groups. Vibrated arteries demonstrated blunted responses to
both vasoconstrictor agonists when compared to controls (P < 0.001). L-NAME increased vasoconstriction to phenylephrine or to 5-HT in vibrated (P < 0.05) but not control arteries. After L-NAME, vasoconstrictor responses were similar in digital arteries from control and vibrated paws. *** P<0.001, * P < 0.05, NS not statistically significant, with brackets and symbols indicating comparisons.

Figure 7: Fluorometric analysis of NO (panel A) and ROS (panel B) activity in control and vibrated digital arteries. Fluorescence is expressed as arbitrary fluorescent units. A: DAF-FM DA fluorescence was significantly reduced in vibrated paws compared to controls (*, P < 0.05), n = 3 all groups. B: Conversely, DCDHF fluorescence was significantly increased in vibrated paws when compared to control (** P < 0.01). The vibration-induced increase in DCDHF fluorescence was abolished by the NOS inhibitor L-NAME (100 μM) or by catalase (1,000 U/ml), which catalyzes the degradation of H2O2 (# # P < 0.01, untreated vibrated arteries versus treated vibrated arteries). Data is expressed as means ± SEM for n = 7 (untreated control and untreated vibrated arteries) or n = 4 (other groups).

Figure 8: Effects of catalase (1,000 U/ml), which degrades extracellular H2O2, on vasoconstrictor responses to phenylephrine (panel A) or 5-HT (panel B) in digital arteries from control and vibrated rat paws. Vasoconstrictor responses are expressed as a percentage of the baseline internal diameter and are presented as means ± SEM (n = 6). Endothelium-intact, vibrated arteries demonstrated blunted responses to the vasoconstrictor agonists compared to control arteries (P < 0.05). Catalase increased vasoconstriction to phenylephrine or 5-HT in vibrated (P < 0.01) but not control arteries. After catalase, vasoconstrictor responses were similar in digital
arteries from control and vibrated arteries. *** P<0.001, ** P < 0.01, * P < 0.05, NS not statistically significant, with brackets and symbols indicating comparisons.
Figure 1
Figure 2
Figure 3
Figure 4

A

Constriction, %

Phenylephrine, log M

Control

Vibrated

***

B

Constriction, %

5-HT, log M

Control

Vibrated

***
Figure 5

A

B

Constriction, %

Phenylephrine, log M

Constriction, %

5-HT, log M

Control, EC
Vibrated, EC
Control, no EC
Vibrated, noEC

Control, EC
Vibrated, EC
Control, no EC
Vibrated, noEC

***

NS

***

NS

-8.5 -8 -7.5 -7 -6.5

-9 -8.5 -8 -7.5 -7 -6.5
Figure 6
Figure 7