Vascular smooth muscle desensitization in rabbit epigastric and mesenteric arteries during hemorrhagic shock

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Ratz PH, Miner AS, Huang Y, Smith CA, Barbee RW. Vascular smooth muscle desensitization in rabbit epigastric and mesenteric arteries during hemorrhagic shock. Am J Physiol Heart Circ Physiol 311: H157–H167, 2016. First published May 13, 2016; doi:10.1152/ajpheart.00926.2015.—The decompensatory phase of hemorrhage (shock) is caused by a poorly defined phenomenon termed vascular hyporeactivity (VHR). VHR may reflect an acute in vivo imbalance in levels of contractile and relaxant stimuli favoring net vascular smooth muscle (VSM) relaxation. Alternatively, VHR may be caused by intrinsic VSM desensitization of contraction resulting from prior exposure to high levels of stimuli that temporarily adjusts cell signaling systems. Net relaxation, but not desensitization, would be expected to resolve rapidly in an artery segment removed from the in vivo shock environment and examined in vitro in a fresh solution. Our aim was to 1) induce shock in rabbits and apply an in vitro mechanical analysis on muscular arteries isolated pre- and postshock to determine whether VHR involves intrinsic VSM desensitization, and 2) identify whether net VSM relaxation induced by nitric oxide and cyclic nucleotide-dependent protein kinase activation in vitro can be sustained for some time after relaxant stimulus washout. The potencies of phenylephrine- and histamine-induced contractions in in vitro epigastric artery removed from rabbits post-hemorrhage were decreased by ~0.3 log units compared with the control contralateral epigastric artery removed prehemorrhage. Moreover, a decrease in KCl-induced tonic, relative to phasic, tension of in vitro mesenteric artery correlated with the degree of shock severity as assessed by rates of lactate and K+ accumulation. VSM desensitization was also caused by tyramine in vivo and PE in vitro, but not by relaxant agents in vitro. Together, these results support the hypothesis that VHR during hemorrhagic decompensation involves contractile stimulus-induced long-lasting, intrinsic VSM desensitization.

hemorrhage; artery; downregulation; relaxant stimuli; contractile stimuli

NEW & NOTEWORTHY

Following initial homeostatic compensation, the “shock” phase of hemorrhage involves persistent vasoconstrictor-resistant vasodilatation. This study suggests that such vascular hyporeactivity is explained by intrinsic vascular muscle desensitization caused by compensatory elevations in vasoconstrictors. Further investigations into molecular targets that block desensitization may reverse vasoconstrictor insensitivity allowing reanimation of shock victims.

TO ENSURE appropriate blood flow to diverse organ systems, the degree of arterial vasoconstriction is acutely adjusted by stimuli that activate diverse signaling systems in vascular smooth muscle (VSM) cells that converge on actomyosin crossbridges to control the level of contractile tension (27, 35, 39). However, contractile stimuli not only cause immediate increases in tension but, when used at high concentrations, can also activate one or more desensitizing systems intrinsic to VSM (7, 32, 38, 40, 42, 44, 56, 58). Intrinsic VSM desensitization is revealed upon washout of the stimulus and subsequent challenge with the same or different contractile stimulus. In rabbit arteries, the weaker subsequent contractile tension involves both reductions in Ca2+ mobilization and Ca2+ sensitivity (40, 42, 44).

The shock phase of hemorrhage reflects a decompensatory hypotension that is resistant to fluid and vasoconstrictor infusion, and is caused by a poorly understood process referred to as a failure of VSM to contract (29). Other terms for this phenomenon include vasomotor paralysis (2, 5, 16, 29), vasoplegia (19, 55), and vascular hyporesponsiveness (19, 37), hyposensitivity (7), and hyporeactivity (31, 49, 50). The precise mechanism of vascular hyporeactivity (VHR) remains obscure (29), although reflex vasodilatation does not appear to play a role (10).

VHR may involve a shock-induced imbalance in levels of contractile and relaxant stimuli favoring net VSM relaxation. Mechanisms potentially activated during hemorrhagic shock that would tend to favor net relaxation include membrane hyperpolarization due to elevations in ATP-dependent K+ channel activity (29), elevations in VSM relaxant stimuli such as nitric oxide (48, 50), and reductions in VSM contractile stimuli due to adrenal insufficiency (5, 51), vasopressin depletion (29), reduced periarterial norepinephrine release due to local prostaglandin biosynthesis (9), and sympathetic withdrawal (16, 47).

Arteries are exposed to high levels of endogenous contractile stimuli during the compensatory phase preceding decompensatory hemorrhagic shock (13, 28). Thus VHR may also be due to the induction of intrinsic VSM desensitization. Mechanisms that potentially could cause hemorrhagic shock-induced intrinsic VSM desensitization include elevations in expression levels of proteins that affect vascular tone (30), depletion of NAD+ leading to failure of energy reserves (49), Ca2+-desensitization (61) and activation of VSM nitric oxide synthase activity (48).

If VHR is due to an excess of relaxant stimuli causing net vasodilation in vivo, then in an artery removed during hemorrhage and examined in vitro, the hyporeactivity should resolve into normal reactivity because removal of the tissue from the in vivo environment and washing in fresh physiological salt solution would remove the excess relaxant stimuli. Alternatively, VHR should be retained for some time and revealed in vitro if this loss of contractile activity is due to intrinsic VSM desensitization that is not dependent on the continued presence
of stimuli. A limited number of in vitro studies support this proposal (49, 61, 62). The present investigation was designed to specifically test the hypothesis that intrinsic VSM desensitization plays a significant role in VHR caused by hemorrhagic shock in rabbit by studying muscular arteries in vitro before and after shock and in sham animals.

The superficial inferior epigastric artery (EA), a small muscular bed feed artery, and the cecal mesenteric artery (MA) branch of the ileocecal artery that feeds the colon were examined because skeletal muscle and splanchnic vascular beds display strong vasoconstriction during the compensatory phase of hemorrhage, and different degrees and potential mechanisms of VHR during decomposition (6–8). One known cause of intrinsic VSM desensitization is prior exposure of VSM to high levels of contractile stimuli (42). An alternative possibility is that prior exposure to high levels of relaxant stimuli induce a long-lasting desensitization after relaxant stimulus washout. Thus a second goal of this study was to determine whether or not exposure of rabbit arteries to high levels of relaxant stimuli induce a sustained relaxant effect (reduced contractile reactivity) for some time after relaxant stimulus washout. For this experiment, EA, MA and the larger renal artery (RA) were examined so that arteries from distinctly different peripheral vascular beds could be compared.

METHODS

Animals. All studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and conform to the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Research Council “Guide for the Care and Use of Laboratory Animals.” Specific-pathogen free, male, New Zealand White rabbits were obtained from Robinson Services (Mocksville, NC) and maintained in the vivarium at 19–22°C and a 12:12-h light/dark cycle for at least 6–7 days prior to experimentation. Animals were individually housed, provided environmental enrichment and fed a combination of pelleted high-fiber rabbit food (Harlan Teklad 2031, 1 cup/day) and hay.

Surgical procedures, blood pressure monitoring, and sampling of lactate and K+. Following a 12 h overnight fast, animals were sedated with a ketamine/xylazine cocktail (50:5 mg/kg im) supplemented if needed with isoflurane (~1–5%, balance oxygen), placed on a heating pad at 39°C, and surgical sites were prepared by hair removal and cleaning with iodine solution and alcohol. A lateral ear vein was cannulated (Jelco 23 gauge, 1-in. radiopaque IV catheter), and the animal was transitioned to Alfalax intravenously (Jurox; London, UK; 10 mg/ml; average dose ~100 μg·kg⁻¹·min⁻¹; range = 3–200 μg·kg⁻¹·min⁻¹). Tracheostomy was performed using a size 3.5 F endotracheal tube (Kendall Healthcare, Mansfield, MA). Polyethylene catheters (PE-90) were placed into the terminal aorta via the left femoral artery, terminal inferior vena cava via the left femoral vein, and ascending aorta via the right carotid artery. The positions of all catheter tips were confirmed at necropsy. Prior to catheter placement, ~6 mm of the left superficial inferior epigastric artery (EA) was removed (after tying proximal and distal ends to prevent loss of blood) for control (prehemorrhage) evaluation of contractile activity in vitro. The left femoral artery was used to monitor arterial pressure via disposable pressure transducers (Transpac IV; ICU Medical; San Clemente, CA), and transducer signals sent to an analog-digital board (MP150; Biopac Systems, Goleta, CA) were collected at 400 Hz and displayed and saved using Acknowledge software (version 4.2 Biopac Systems). The left femoral vein was used for infusion of escalating tyramine doses (0.2–8 mg·kg⁻¹·min⁻¹) until a peak pressor response was reached, or an equivalent volume of 0.9% NaCl. The right carotid artery was used for rapid blood withdrawal (hemorrhage) and the right jugular vein was used for central venous blood sampling for analyses of lactate, K⁺ and blood gases (ABL90 Flex, 65 μl; Radiometer America; Westlake, OH). A small opening was created in the abdominal wall by dissecting the linea alba and, after tying proximal and distal ends, a ~6-mm-long segment of a cecal mesenteric artery (MA) branch of the ileocecal artery was removed for control (prehemorrhage) evaluation of contractile activity in vitro. The abdomen was sutured and reopened only at the end of hemorrhage for removal of an adjacent MA for posthemorrhage in vitro analysis.

Pressure-clamp hemorrhage protocols. Animals were rapidly hemorrhaged from the right carotid artery by withdrawing blood with a programmable syringe pump (PHD 2000 Series, Harvard Apparatus, Holliston MA). At no time during the surgical procedure or during the hemorrhage protocol were animals administered antiocoagulants. Heparin (~4 U/ml) was used to prevent catheters from clotting, but flush volumes were monitored to ensure that animals were not anticoagulated. The initial 3 ml·kg⁻¹·min⁻¹ rate of withdrawal was reduced to 2, then 1 ml·kg⁻¹·min⁻¹ when 20 ml of blood (~10% blood volume) was removed or when mean arterial pressure (MAP) was less than 35 mmHg. Blood withdrawal was stopped when MAP reached 30 mmHg, and restarted when MAP exceeded 35 mmHg for more than 5 s. When MAP fell to less than 30 mmHg, Ringer’s solution without added glucose (Baxter Healthcare; Deerfield, IL) was infused at 3 ml·kg⁻¹·min⁻¹ to raise MAP above 30 mmHg. In this way, MAP was maintained (clamped) between ~30 and ~35 mmHg. Samples of blood for analyses occurred immediately before, and every 10 min after the start of, hemorrhage. The hemorrhage endpoint for the protocol used to evaluate concentration-response curves of EA in vitro (see Fig. 1) was a lactate concentration ≥ 8 mM, or return of crystalloid (Lactated Ringer’s) equaling ~2-fold the blood volume withdrawn. The hemorrhage endpoint was 75 min, regardless of lactate level, for the protocol used to evaluate the effect of hemorrhage on a KCl-induced contraction in vitro (see Figs. 3 and 4). Animals given saline or tyramine infusions were not hemorrhaged, but otherwise underwent all surgical instrumentation and were followed for the same time period as hemorrhaged animals.

Artery in vitro experimental protocol. EA and MA segments were removed before the start of hemorrhage or infusion of saline or tyramine (PE) in the contralateral (EA) and an adjacent MA were removed at the end of hemorrhage. Each isolated artery segment was immediately placed in warm (37°C) physiological salt solution [PSS, composition in mM: 140 NaCl, 4.7 KCl, 1.2 NaHPO₄·7H₂O, 2.0 MOPS, 0.02 Na₂ethylenediaminetetraacetic acid to chelate heavy metals, 5.6 D-glucose, 1.6 CaCl₂ and 1.2 MgCl₂, made with high-purity (17 MΩ) deionized water and adjusted using NaOH to a pH of 7.4]. After blood was washed from the lumen and adhering tissues were removed by microdissection (Olympus SXZ12), artery segments were cut into rings ~2.5 mm wide (measured precisely using a micrometer for subsequent tension calculations that were reported as mN/mm) and each ring was secured in a tissue myograph (model 610M, Danish Myo Technology). EA rings were adjusted to the optimum length for muscle contraction (Lₒ) using an abbreviated length-tension protocol in which tissues were contracted for ~5 min with a maximum concentration of KCl (110 mM KCl substituted isosmotically for NaCl) (41). The time from artery removal to the beginning of concentration-response curve (CRC) analyses (reported in Fig. 1) was ~45 min. CRCs were constructed by exposing artery rings to phenylephrine (PE) and histamine (HA) cumulatively using 7 concentrations ranging from 30 nM to 300 μM in half-log increments. The total time for completion of each CRC was ~70 min. Data points at each concentration used for control arteries (prehemorrhage, pre-tyramine, and pre-saline) were compared statistically to corresponding data from the test arteries (respectively, posthemorrhage, post-tyramine, and post-saline; see Fig. 1). CRC data were also fit to a sigmoidal curve and the slopes of the curves identifying PE and HA potencies were analyzed statistically. MAs were used in an in vitro protocol to...
assess KCl-induced phasic/tonic active tension ratios at short and long muscle lengths pre- and post-hemorrhagic shock (see Figs. 3 and 4), as detailed in RESULTS. Prior to these experiments using tissues removed from animals before and after hemorrhage, in vitro experiments were performed on MA rings removed from other animals to determine the length-dependency of the KCl-induced phasic/tonic tension ratio (see Fig. 2). In the experiments on KCl-induced contractions in MA, 10^−6 M phentolamine was added to prevent α-adrenergic receptor activation by KCl-induced release of norepinephrine from periarterial nerves (4). For all in vitro experiments, tissues were exposed to 100 μM L-NMMA (inhibitor of nitric oxide synthesis) and 30 μM ODQ (inhibitor of soluble guanylyl cyclase).

Tissue preparation for in vitro relaxant stimuli experiments. EA, MA and renal arteries (RA) were isolated from adult New Zealand white rabbits euthanized by ketamine/xylazine/euthasol overdose and prepared as described previously with minor modifications (41). Immediately after death, each artery segment was rapidly removed and placed in cold (2.6°C) PSS. After blood was removed from the lumen and adhering tissues were removed by microdissection (Olympus SZX12), artery segments were stored overnight in PSS at 2.6°C (cold-stored). The following day, artery segments were cut into rings ~2.5 mm wide, and each ring was secured in a tissue myograph (model 610M, Danish Myo Technology), adjusted to L0 as described above and subjected to a PE-induced CRC, also as described above. For comparison, 3 rabbits were euthanized by ketamine/xylazine/euthasol overdose, placed in PSS at 37°C, cleaned, cut into rings and secured in a tissue myograph so that in vitro experiments were performed on the same day without subjecting the tissues to a reduction in temperature. Also for comparison, 2 rabbits were prepared exactly as in the hemorrhage experiments (sedated with a ketamine/xylazine cocktail then transitioned to Alfaxan iv) and re-

Fig. 1. Intrinsic vascular smooth muscle desensitization induced by hemorrhage and in vivo tyramine infusion. Concentration-response curves (CRCs) constructed by adding phenylephrine (PE; A, C, and E) and histamine (HA; B, D, and F) cumulatively in an in vitro experiment performed on epigastric artery removed before and after hemorrhage (A, B), and before and after tyramine infusion (C, D) compared with controls (sham/saline infusion, E, F). Data are mean values ± SE; n values are shown in each panel. *P < 0.05; NS, not significantly different. Each panel includes the EC50 values (mean ± SE) representing potencies of the two curves.

Fig. 2. Differential length-dependencies of phasic- and tonic-phase contractions induced in mesenteric artery by KCl. Example of an in vitro protocol for imposed length changes (A) and resulting tension changes (B), and the phasic and tonic phases (B, inset) of KCl-induced contractions at each muscle length (B, solid bars indicate duration of exposure to KCl) used to examine the length-dependency of the phasic/tonic tension ratio for KCl-induced contractions in mesenteric artery in vitro. Active and passive tension responses (C) reveal that tonic active tension (T_t-Tonic) relative to phasic active tension (T_p-Phasic) was nearly 4-fold greater at short muscle lengths, and approached unity by ~0.6 mm (D). T_p = passive tension derived during unloading when tissues were in Ca2+-free PSS (A) (45). Data in C and D are mean values ± SE; n = 3. *P < 0.05 comparing T_p-Phasic to T_t-Tonic.
tained in the anesthetized condition for ~1 h before the EA was removed and prepared as described above to replicate the prehemorrhage control tissue conditions. In the experiment shown in Figs. 5–7, the strength of active contractions was normalized to the maximum active tension produced at L₀ by KCl (T/T₀). In the experiment shown in Fig. 8, contractions to 0.56 μM PE in the presence, and after washout, of a relaxant stimulus were normalized to the control 0.56 μM PE-induced contraction obtained prior to exposure to the relaxant stimulus (T/Tₜₐₓₜₑᵢₓₑᵢ₋ᵢₓₑᵢₑₑᵢₑ). Full active and passive length-tension curves were constructed for MA as described previously (45) to assess the relative length-dependent contractile strengths of KCl-induced phasic- and tonic-phase contractions (see Fig. 2).

**Data analysis and statistics.** All data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA) and are presented as example tracings over time (see Figs. 2, A and B, and 3, A and C), actual data (see Fig. 4), and as the mean ± standard error of the mean (SE). CRC data for each tissue and each contractile stimulus were fitted to a sigmoidal curve (25). When comparing 2 groups for statistical analyses, data were evaluated by the Student’s t-test, and the null hypothesis was rejected at P < 0.05. When comparing a group more than once, ANOVA with Dunnett’s post hoc test (comparisons of test values to control values only), or the Student’s t-test with the Bonferroni method were used. In the latter case, if one group was compared twice or three times, then the null hypothesis was rejected at, respectively, P < 0.025 or P < 0.0125.

**RESULTS**

**Effect of hemorrhagic shock and in vivo tyramine on in vitro PE- and HA-induced CRC.** Both PE- (Fig. 1A) and HA-induced (Fig. 1B) CRCs analyzed in vitro ~45 min after artery removal from rabbits posthemorrhage displayed reduced potencies (CRCs were significantly right-shifted) compared with the contralateral EA removed before hemorrhage (Prehemorrhage) and analyzed in vitro 45 min after artery removal. Also, the maximum contraction induced at 30 μM HA was significantly reduced posthemorrhage compared with prehemorrhage (reduced maximum efficacy, Fig. 1B). Although the mean tension value induced by 30 μM PE was also reduced in EA from the post- compared with prehemorrhage group (Fig. 1A), this decrease was not statistically significant.

Hemorrhage causes strong sympathetic nerve activation and release of norepinephrine during the compensatory phase (13). To determine whether release of norepinephrine can cause, like hemorrhage, a long-lasting reduction in the ability of adrenergic and histaminergic stimuli to cause contraction in artery segments in vitro, tyramine was employed because tyramine infusion in vivo causes release of norepinephrine from sympathetic vasoconstrictor nerves (17). One EA was removed before tyramine infusion (Pre-Tyramine) and analyzed in vitro ~45 min later, and the contralateral EA was removed after tyramine infusion (iv) and analyzed in vitro ~45 min later. As occurred following hemorrhage, tyramine infusion in vivo caused VSM desensitization, revealed by in vitro CRC analyses showing reduced potencies and maximum efficacies to PE (Fig. 1C) and HA (Fig. 1D).

By contrast, the CRCs produced by PE (Fig. 1E, Pre-Saline) and HA (Fig. 1F, Pre-Saline) in the EAs removed from sham/saline rabbits and analyzed in vitro were not different from the CRCs produced in the contralateral EA removed at the end of the saline infusion period and also analyzed in vitro (respectively, Fig. 1, E and F, Post-Saline). By serving as a control, data from the sham/saline group revealed that animal surgery, time and infusion of saline (the vehicle for tyramine) had no effect on the ability of PE and HA to produce strong contractions in arteries removed and examined in vitro. These data together indicate that both hemorrhage and tyramine infusion can induce intrinsic VSM desensitization lasting at least ~2 h (~45 min for artery removal, cleaning, mounting in

**Fig. 3.** Tension tracings (A and C) and KCl-induced phasic/tonic active tension (T₉) ratios (B and D) from one MA ring removed before (Pre-Hemorrhage; A and B) and after hemorrhage (Post-Hemorrhage; C and D) and analyzed in vitro. Phasic active tension (A, “P”) and tonic active tension (A, “T”) were measured and examined at 0.2 mm and 0.6 mm (arrowheads indicate when the tissue was stretched to the indicated length). Solid bars in B indicate duration of exposure to KCl.
myograph and length adjusting to L₀, plus ~70 min for the CRC duration.

Effect of hemorrhage on the in vitro phasic/tonic tension ratio of MA. KCl-induced contractions are often biphasic, producing a rapid peak (phasic) response followed by steady-state (tonic) tone (Fig. 2B, inset). In the rabbit MA, the KCl induced phasic/tonic active tension ratio, calculated from a length-tension analysis (Figs. 2, A–C), is dependent on muscle length (Fig. 2D). Specifically, the phasic contraction is ~4-fold stronger than the tonic contraction at short muscle lengths (e.g., Fig. 2D, 0.2 mm), and at longer muscle lengths, the phasic/tonic contraction ratio approaches 1 (e.g., Fig. 2D, 0.6 mm). There is evidence that phasic and tonic phases of contraction are regulated by different mechanisms (14, 52). We therefore examined the effect of hemorrhage on the phasic/tonic tension ratio in the MA of 3 rabbits.

In one hemorrhaged rabbit that displayed a relatively large increase in lactate and K⁺, we observed that the phasic/tonic tension ratios at artery lengths of 0.2 and 0.6 mm were increased posthemorrhage (Figs. 3, C and D) compared with prehemorrhage (Fig. 3, A and B), and that the increase at 0.2 mm was large (~3-fold). Thus, for each of 3 rabbits, the phasic/tonic tension ratios posthemorrhage were normalized to the prehemorrhage ratios, and plotted as a function of the rates of increase in arterial lactate (Fig. 4A) and K⁺ (Fig. 4B). A positive correlation was evident at muscle lengths of 0.2 mm and 0.6 mm, and the slopes of the relationships were robustly large at 0.2 mm (Fig. 4, circles). These data suggest that the depression in KCl-induced tonic relative to phasic contraction of in vitro MA is as reliable an indicator of the degree of hemorrhagic shock-induced insult as is the well-established indexes of increases in blood lactate and K⁺ levels.

Effect of washout of activators of cAMP- and cGMP-dependent protein kinases, and of nitric oxide (NO), on PE-induced CRC. In vitro experiments were performed to test the hypothesis that VSM relaxation can be retained for some time after washout of a relaxing stimulus. EA rings were exposed for 30 min to 100 μM 8-bromo-cGMP (8b-cGMP) to activate PKG, 10 μM forskolin (FSK) to activate PKA (38), and 30 μM dipropyleneritramine NONOate (DPTA-NONOate) to cause a sustained increase in the level of NO (half-life of NO release = 3 h) (33), then all tissues were challenged with PE to construct CRCs. Afterward, tissues were washed several times over a period of 60 min to remove PE, 8b-cGMP, FSK and DPTA NONOate, and again challenged with PE to construct a second set of CRCs. Control tissues were not exposed to the relaxant stimulus, but only to the drug’s vehicle. VSM relaxation sensitization would be revealed if the reduced potencies and/or efficacies induced by acute exposure of relaxant agents compared with control were retained 60 min after relaxant stimulus washout. For a comparison, tissues were either 1) cold-stored overnight or 2) never placed in a cold solution and 2a) tested on the same day as the animal was euthanized or 2b) removed from an anesthetized animal and tested on the same day.

In EA that had been cold-stored overnight, exposure to 8b-cGMP, FSK and DPTA NONOate for 30 min reduced the potency of PE compared with control-1 (Fig. 5A). FSK and DPTA NONOate also reduced the maximum efficacy. A similar response was produced using tissues that had never been exposed to a cold solution and had been removed from animals euthanized the same day as the experiment (Fig. 5C). Likewise, a similar response was produced using anesthetized animals (Fig. 5E) to ensure that conditions were similar to those used for the experiment shown in Fig. 1. After washout of 8b-cGMP, FSK and DPTA NONOate for 60 min, the potencies and maximum efficacies of each PE-induced contraction returned to the control-2 level (Fig. 5, B, D, and F). In another experiment, EA was exposed to these relaxant agents for 60 min rather than 30 min (Fig. 6, A and D) followed by a 60 min washout using tissues that had been cold-stored overnight (Fig. 6, A–C) and tissues that had not been cold-stored and used the day the animals were euthanized (Fig. 6, D and E). In one tissue, 300 μM NONOate was used (Fig. 6, A and C). In all cases, despite the very strong relaxation responses induced by NONOate and FSK, each PE-induced contraction returned to the control-2 level after a 60 min washout period (Fig. 6, B, C, and E).

When exposed for 30 min to 8b-cGMP and FSK, MA (Fig. 7A) and RA (Fig. 7D) behaved much like EA (see Fig. 5A). However, FSK did not reduce the maximum efficacy of RA (Fig. 7D) as it did in MA (Fig. 7A) and EA (Fig. 5A). As in EA, a 60 min washout restored the PE CRC curves induced in MA (Fig. 7B) and RA (Fig. 7E) at least to the control levels. Interestingly, the strengths of PE contractions after FSK wash-
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Tissues were contracted with 0.56 μM PE for ~5 min and the maximum response was recorded. Then tissues were exposed to 10 μM FSK and 100 μM 8b-cGMP (or vehicle for control tissues) for 30 min and contracted again with 0.56 μM PE for ~5 min to measure the effect of the relaxant stimulus (Fig. 8, t = 0). Three sets of tissues were then washed for, respectively, 10, 30, and 60 min and contracted a third time with 0.56 μM PE to assess the ability of tension to return to the control level. Although FSK abolished the ability of 0.56 μM PE to produce a contraction (Fig. 8, triangles, t = 0), a washout duration of only 10 min was sufficient to fully reverse FSK’s relaxing effect in both EA (Fig. 8, triangles, 10 min) and MA (Fig. 8B, triangles, 10 min). A longer washout duration of 30 min was required for full reversal of 8b-cGMP’s relaxing effect (Fig. 8, squares). Without performing diffusion calculations and considering inactivation kinetics, the time required for abolition of the direct effect exerted by relaxant stimuli in an artery ring is difficult to predict. However, based on a Ca2+ diffusion calculation for artery (43), relaxant agent washout to levels below that necessary to cause relaxation may be expected to take several minutes. Thus these data suggest that the relaxant stimulus effect dissipates relatively rapidly after relaxant stimulus washout in small, muscular, peripheral rabbit arteries.

DISCUSSION

Arteries removed from hemorrhaged rabbits and stimulated in vitro with PE and HA from ~45 min to 2 h later displayed weaker contractions than control tissues. Moreover, the degree of reduction in the KCl-induced tonic, relative to phasic, tension of arteries removed from hemorrhaged animals correlated with the degree of shock severity as assessed by rates of lactate and K+ accumulation. Infusion of tyramine in vivo, which releases periarterial norepinephrine, mimicked this effect of hemorrhage when examined using tissues in vitro. Last, exposure of arteries to relaxant agents in vitro failed to induce a long-lasting attenuation of contraction. Together, these data support the hypothesis that long-lasting intrinsic VSM desensitization caused by contractile stimuli (7, 32, 38, 40, 42, 44, 56, 58) plays a critical role in the VHR responsible for the decompenatory phase of hemorrhage (hemorrhagic shock) in...
This result supports the results obtained by others who have shown that arteries removed from hemorrhaged animals (7, 30, 49, 61) and rat aorta removed from splanchnic artery occlusion (48), an experimental model of circulatory shock, retain a desensitized state. Although our data cannot rule out the potential additional contribution of an imbalance in relaxant over contractile stimuli in causing VHR (9, 47, 48, 50), it emphasizes the need to further understand mechanisms causing intrinsic VSM desensitization when considering therapy for hypotensive, vasodilatory shock.

One conclusion from the results is that the large increases in the levels of contractile stimuli produced during the compensation phase of hemorrhage led to strong VSM receptor activation which, over time, caused intrinsic VSM desensitization. This desensitization was revealed by in vitro CRC analyses of EA and MA isolated from rabbits posthemorrhage. An alternative hypothesis is that VSM relaxation was retained by the excised arteries because of an intrinsic adaptation to high levels of relaxant stimuli in vivo, leading to a long-term VSM relaxation sensitization (i.e., intrinsic VSM desensitization is caused by intrinsic VSM relaxation sensitization). VSM relaxation is largely controlled by cAMP- and cGMP-dependent protein kinases (respectively, PKA and PKG) (36, 39). In addition, high levels of NO released during hemorrhage (50) could potentially cause long-term VSM relaxation sensitization by non-cGMP-dependent pathways (54), including those involving additional reactive nitrogen species (1), regeneration of NO (53), and nitrosylation (3, 22) and tyrosine nitration reactions (57).

Whereas our data reveal that VSM desensitization was readily activated in rabbit arteries in vivo both by hemorrhage and tyramine, and in vitro by PE, we did not find evidence that the relaxant agents PSE, 8b-cGMP and DPTA NONOate could induce a long-lasting sensitization of relaxation. Moreover, in vitro experiments were conducted in the presence of inhibitors of nitric oxide production and soluble guanylyl cyclase activation, so VSM desensitization witnessed in the present study was likely not due to in vivo activation of VSM nitric oxide synthase that was retained in the in vitro arteries, as occurs in rat aorta during splanchnic artery occlusive vasodilatory shock (48). Thus VSM desensitization induced by hemorrhage in rabbit arteries appears...
to be the result of VSM cell signaling adaptation caused by high levels of contractile, not relaxant, stimuli.

There is strong evidence that the sensitivity of VSM to contractile stimuli such as α-adrenergic agonists is not fixed, but is a parameter that can be relatively rapidly adjusted (7) in a negative feedback manner by the level of α-adrenoceptor activation (15, 18, 23, 26, 34). Notably, VSM sensitivity to α-adrenergic stimuli is inversely related to the degree of peripheral sympathetic vasoconstrictor nerve output in humans (11). Moreover, angiotensin II desensitization occurs relatively rapidly in the mesenteric vascular bed during hemorrhage in rat (37). The current study revealed that EA from hemorrhaged rabbits was desensitized to PE, HA and the non-receptor contractile agent, KCl. The finding that the phasic/tonic tension ratio of a KC1-induced contraction was affected by hemorrhage suggests that VSM desensitization was a generalized phenomenon because KCl bypasses receptors to activate contraction by causing membrane depolarization (4). Histamine potency was reduced by both hemorrhage and tyramine, and although histamine is generally not considered a sympathetic neurotransmitter, there is limited evidence from one laboratory supporting the hypothesis that histamine is coreleased with norepinephrine in several species, including rabbit (12, 24). Thus whether reduced sensitivity to HA was due to a generalized VSM desensitization, or to sympathetically released histamine activation of VSM histaminergic receptors followed by subsequent HA receptor desensitization, remains to be determined. A limitation in the study was that other stimuli, such as angiotensin II and vasopressin, were not tested. As a result, we can only tentatively conclude that the VSM desensitization induced by hemorrhage was a generalized effect that would limit VSM responsiveness to all contractile stimuli.

The clinical relevance of our finding is that current treatments for hemorrhagic shock during the hypotensive, compensatory phase include infusion of multiple types of vasoconstrictors in an attempt to increase vascular tone, total peripheral resistance, and blood pressure (21). If VSM becomes desensitized during hemorrhage because of the compensatory release of high levels of endogenous vasoconstrictors, then infusion of any exogenous contractile stimulus will be an ineffective vasocon-

**Fig. 7.** Concentration-response curves (CRCs) constructed by adding phenylephrine (PE) cumulatively in an in vitro experiment performed on mesenteric (A–C) and renal (D–F) arteries to assess whether a 30 min exposure to a relaxant stimulus can induce long-lasting vascular smooth muscle desensitization. Responses were assessed using tissues that had been removed from animals euthanized with a ketamine/xylene/euthasol cocktail, and had been cold-stored overnight. Sixty minutes prior to, and throughout, the CRC in control-1 and to control-2.

**Fig. 8.** The time course for reversal of relaxation induced by relaxant stimuli 8b-cGMP and forskolin (FSK) in an in vitro experiment in which epigastic (A) and mesenteric (B) arteries were first contracted with 0.56 μM phenylephrine (PE) for ~5 min, relaxed by PE-washout, then exposed to vehicle for drugs (Control), 100 μM 8b-cGMP and 10 μM forskolin (FSK). One set of tissues was contracted with 0.56 μM PE to determine the extent of acute relaxation induced by 8b-cGMP and FSK (t = 0). Three other sets of tissues were each washed 3-times, set-1 for 10 min, set-2 for 30 min and set-3 for 60 min, and contracted with 0.56 μM PE to determine the time-course of relaxation-reversal. Data are mean values ± SE; N = 3; *P < 0.025 compared with control.
striction during the decompensation phase. Moreover, certain vasoconstrictors added exogenously in an attempt to increase peripheral vascular resistance during decompensation may actually enhance the VSM desensitization initiated by compensatory release of endogenous vasoconstrictors, further reducing the effectiveness of vasoconstrictor therapy. Based on the present and other studies (7, 30, 49, 61), additional work on VSM desensitization mechanisms and the relative abilities of different stimuli to induce VSM desensitization seems warranted.

A characteristic of vasodilatory shock is poor responsiveness to vasoconstrictor stimuli, and one proposed cause is an imbalance between contractile and relaxant stimuli, due to excess relaxant stimuli production and withdrawal or depletion of contractile stimuli, that favors net VSM relaxation (5, 16, 29, 47, 48, 50, 51). Our study cannot rule out this possibility. In fact, our in vitro data obtained using relaxant stimuli and the contractile stimulus PE provide support for the notion that endogenous relaxant stimuli in vivo could lead to a contractile-relaxant stimulus imbalance favoring net VSM relaxation. Moreover, during the decompensation phase of hemorrhage in vivo, there is evidence of withdrawal of sympathetic tone and a decrease in vasopressin levels, so the combination of high levels of relaxant stimuli in the face of reducing levels of contractile stimuli would be expected to favor net VSM relaxation. This effect would be heightened if VSM desensitization was also present. Interestingly, our data show that activation of PKA concomitantly with PE reduced the degree of PE-induced desensitization. Thus, if VSM desensitization is the primary cause of VHR during hemorrhagic decompensation, then our data support the hypothesis that the degree of VSM desensitization may be elevated if VSM PKA activity is concomitantly reduced. This novel finding may motivate further research on unique signaling interactions activated by contractile and relaxant stimuli in the context of vasodilatory shock. In support of this concept, the activity of the vascular PKA-activator adrenomedullin is attenuated during hemorrhage, and experimental reactivation reduces mortality (60).

An unexpected finding was the ability of the phasic/tonic tension ratio of a KCl-induced contraction to reflect the severity of hemorrhagic shock. Whereas this measure would not improve the ability of monitoring shock severity, given that blood analyses of other indexes such as K+ and lactate are accurate and accessible, it supports the notion that the vasculature acts as a highly sensitive monitor of certain compensatory homeostatic mechanisms, and relatively rapidly adapts to changes in stimulation levels over time. The data also provide some insight into potential desensitization mechanisms. Actin is in a highly dynamic state of polymerization-depolymerization in smooth muscle (20), and there is strong evidence that the tonic phase of a KCl-induced contraction is highly dependent on increased actin polymerization (46, 59). Thus it is possible that hemorrhage-induced desensitization attenuates the ability of contractile agents to enhance actin polymerization sufficiently to maintain strong tonic tension. The tonic phase of a KCl-induced contraction is also highly dependent on Ca2+ entry and rho kinase activity (52) and, as proposed by Xu and Liu (61), these systems may be desensitized in VSM during hemorrhage.

In summary, compared with control EAs removed from rabbits prior to hemorrhagic shock and tyramine infusion, those removed after these interventions and assessed by in vitro CRC analysis displayed reductions in PE and HA contractile potentials. Moreover, when normalized to the phasic phase, the strength of the tonic phase of a KCl-induced contraction in MA correlated inversely with the degree of lactate and K+ accumulation during hemorrhage, identifying contractile strength as an index of hemorrhage severity. The attenuation in PE-, HA- and KCl-induced contractile strength for a given stimulus concentration was apparent ~2 h after arteries were removed from the hemorrhaged animals, indicating that a long-lived, intrinsic VSM desensitization mechanism was activated during hemorrhage. Stimuli used to cause VSM relaxation in vitro did not induce a long-lasting VSM desensitization. In conclusion, these results support the hypothesis that high levels of contractile stimuli not only caused acute VSM contraction during the compensatory phase of hemorrhage, but also activated a response modulator causing prolonged VSM desensitization. Moreover, our data support the hypothesis that VSM desensitization is a cause of VHR.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


