BRIEF REPORT

Comparative Efficacy of Angiotensin II Type 1 Receptor Blockers Against Ventilator-Induced Diaphragm Dysfunction in Rats

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Mechanical ventilation (MV) is a life-saving intervention for many critically ill patients. Unfortunately, prolonged MV results in the rapid development of inspiratory muscle weakness due to diaphragmatic atrophy and contractile dysfunction (termed ventilator-induced diaphragm dysfunction (VIDD)). Although VIDD is a major risk factor for problems in weaning patients from MV, a standard therapy to prevent VIDD does not exist. However, emerging evidence suggests that pharmacological blockade of angiotensin II type 1 receptors (AT1Rs) protects against VIDD. Nonetheless, the essential characteristics of AT1R blockers (ARBs) required to protect against VIDD remain unclear. To determine the traits of ARBs that are vital for protection against VIDD, we compared the efficacy of two clinically relevant ARBs, irbesartan and olmesartan; these ARBs differ in molecular structure and effects on AT1Rs. Specifically, olmesartan blocks both angiotensin II (AngII) binding and mechanical activation of AT1Rs, whereas irbesartan prevents only AngII binding to AT1Rs. Using a well-established preclinical model of prolonged MV, we tested the hypothesis that compared with irbesartan, olmesartan provides greater protection against VIDD. Our results reveal that irbesartan does not protect against VIDD whereas olmesartan defends against both MV-induced diaphragmatic atrophy and contractile dysfunction. These findings support the hypothesis that olmesartan is superior to irbesartan in protecting against VIDD and are consistent with the concept that blockade of mechanical activation of AT1Rs is a required property of ARBs to shield against VIDD. These important findings provide a foundation for future clinical trials to evaluate ARBs as a therapy to protect against VIDD.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Prolonged mechanical ventilation results in ventilator-induced diaphragm dysfunction (VIDD). This is significant because VIDD is a major risk factor for problems in weaning patients from the ventilator. Currently, no standard treatment exists to prevent VIDD. However, emerging evidence reveals that pharmacological blockade of angiotensin II type 1 receptors (AT1Rs) protects against VIDD. Nonetheless, the specific properties of angiotensin receptor blockers (ARBs) required to protect against VIDD remain unknown.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ What characteristics of ARBs are vital for protection against VIDD?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ ARBs that prevent angiotensin II binding to AT1Rs alone do not protect against VIDD. In contrast, ARBs that block mechanical activation of AT1Rs are protective against VIDD.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ These new findings provide a foundation for future testing of ARBs in clinical trials.

Mechanical ventilation (MV) is used annually in > 13 million patients in the intensive care unit.[1] Although MV is a life-saving intervention for many critically ill patients, an unintended consequence of prolonged MV is the rapid development of inspiratory muscle (i.e., diaphragm) weakness. This MV-induced diaphragmatic weakness is due to both atrophy and contractile dysfunction of the diaphragm, collectively known as ventilator-induced diaphragm

dysfunction (VIDD).[2] VIDD is clinically important because diaphragmatic weakness is a major risk factor for problems in "weaning" patients from the ventilator.[3] This failure to wean results in extended time on the ventilator along with increased morbidity and mortality.[4] Currently, no standard therapy exists to protect against VIDD.

Recent studies into the pathogenesis of VIDD reveal that the renin angiotensin system is a potential therapeutic target to prevent VIDD. Specifically, treatment with the angiotensin II type 1 receptor (AT1R) blocker (ARB) losartan protects against VIDD.[5] Although this study reveals that ARBs are protective against VIDD, several classes of ARBs exist and the characteristics of ARBs that provide optimal protection against VIDD remain unknown. In principle, pharmacological blockade of AT1Rs via losartan can avert VIDD by one of three mechanisms.[6–8] First, losartan prevents activation of the classical RAS pathway by blocking angiotensin (AngII) binding to AT1Rs. Second, mechanical stress can activate AT1Rs and losartan blocks mechanical activation of AT1Rs. Indeed, it is feasible that mechanical activation of AT1Rs occurs during MV due to the mechanical stress placed on the receptor due to the passive, repetitive shortening/lengthening cycles of diaphragm fibers during ventilator support. Last, it is possible that losartan protects against VIDD by a combination of blocking both AngII binding and mechanical activation of the AT1R.

Prior to clinical trials, additional preclinical studies are required to determine the characteristics of ARBs that are required to protect against VIDD; this forms the rationale for the current study. To delineate the properties of ARBs needed for protection against VIDD, we tested the efficacy of two US Food and Drug Administration (FDA) approved ARBs (i.e., olmesartan and irbesartan) that differ in both molecular structure and effects on the AT1R. Olmesartan blocks both AngII binding and mechanical activation of AT1Rs whereas irbesartan blocks only ligand binding to the receptor. We hypothesized that compared with irbesartan, olmesartan will provide superior protection against VIDD.

METHODS
Experimental animals
All experimental procedures were approved by the Animal Care and Use Committee of the University of Florida. Adult female (4–6 months old) Sprague-Dawley rats were housed at the University of Florida Animal Care Services Center and maintained on a 12:12-hour light:dark cycle with food and water provided *ad libitum*.

Experimental design
To determine the efficacy of irbesartan and olmesartan to protect against VIDD, animals were randomly assigned to one of four groups ($n = 8$ group):

1. 12 hours spontaneous breathing (SB); animals administered saline
2. 12 hours MV; animals administered saline
3. 12 hours mechanical ventilation; animals administered irbesartan
4. 12 hours mechanical ventilation; animals administered olmesartan

Protocol for mechanical ventilation and spontaneous breathing animals
Our protocols for spontaneous breathing and MV are described in detail elsewhere.[5,9,10] Briefly, animals were anesthetized, tracheotomized, and mechanically ventilated. The carotid artery was cannulated for monitoring of blood pressure and withdrawal of blood samples for measurement of blood gases and pH. A venous catheter in the jugular vein permitted continuous infusion of anesthesia and experimental drugs. Animals in the MV groups were exposed to 12 hours of MV; SB animals underwent identical surgical procedures, except these animals breathed spontaneously for 12 hours. After 12 hours of SB or MV, anesthetized rats were euthanized by opening of the chest cavity to induce pneumothorax. Fresh costal diaphragm tissues were separated into multiple strips for measurements of *in vitro* contractile properties, mitochondrial respiration, and immunohistochemistry. The remaining diaphragm was snap frozen in liquid nitrogen and stored at −80°C for Western blotting.

Drug administration to inhibit AT1Rs
Animals treated with irbesartan received an intraperitoneal priming dose (30 mg/kg) followed by intravenous infusion (100 μg/kg/min, infusion rate 0.30 mL/hour) during the 12-hour experiment. Animals treated with olmesartan received intraperitoneal priming doses prior to surgery (1 mg/kg) and immediately before MV (1 mg/kg); animals were then injected every 3 hours (1 mg/kg) during the MV protocol. These dosages of irbesartan and olmesartan have been shown to be effective in eliminating both hypertension and AT1R-linked metabolic disorders in rats.[11,12]

Diaphragmatic contractile function
Diaphragm muscle strips were suspended vertically between two Plexiglas clamps with one end connected to an isometric force transducer (model FT-03; Grass Instruments, Quincy, MA) within a jacketed tissue bath containing Krebs-Hensleit solution equilibrated with 95% O_2-5% CO_2 gas at constant temperature (25°C). Following determination of optimal length, the force-frequency response of the diaphragm was measured as previously described.[10]

Myofiber cross-sectional area
Transverse diaphragm muscle sections (10-μm thick) were stained for dystrophin, myosin heavy chain (MHC) type I, MHC type IIa, and MHC type IIx/B proteins to determine diaphragm fiber cross-sectional area.[13] The cross-sectional area was determined using computerized image analysis (Scion software National Institutes of Health (NIH)).

Mitochondrial respiration
Diaphragm fiber bundles were isolated and permeabilized for measurement of mitochondrial respiration, as previously described.[1] The respiratory control ratio was calculated by dividing state three respiration by state four respiration.

Western blotting
Diaphragm samples were homogenized in buffer containing 5 mM Tris-HCl, 5 mM EDTA, and a protease

inhibitor cocktail (1:20 vol:vol; Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged 1,500 *g* for 10 minutes at 4°C. Soluble proteins were separated by SDS PAGE gels and transferred to polyvinylidene difluoride membranes, which were incubated with the following primary antibodies: 4-Hydroxynoneal (ab46545; Abcam, Cambridge, MA), phospho-STAT3 (Ser727) (#9134; Cell Signaling Technology, Danvers, MA), total STAT3 (#4904; Cell Signaling Technology), and alpha II spectrin (sc-48382; Santa Cruz Biotechnology, Santa Cruz, CA). Verification of equal protein loading and transfer was accomplished by probing for α-tubulin (12G10; Developmental Studies Hybridoma Bank, Iowa City, IA).

Assessment of plasma levels of angiotensin 1-7
Arterial blood samples were collected at the conclusion of experiments and Ang1–7 levels were determined using a commercially available ELISA kit (MBS702485; MyBiosource, San Diego, CA).

Statistical analysis
The data were analyzed using a one-way analysis of variance (ANOVA). A Tukey honestly significant difference test was performed *post hoc* when appropriate. Significance was established at $P < 0.05$. Values are expressed as mean ± SEM.

RESULTS
Systemic responses to MV
No differences existed between the experimental groups in animal body weight, heart rate, arterial blood gases, and arterial pH following 12 hours of MV (**Table S1**). However, compared with SB and MV animals, mean arterial blood pressure was significantly lower in animals treated with irbesartan and olmesartan; this decrease in blood pressure provides evidence that both irbesartan and olmesartan were provided at therapeutically effective doses.

Olmesartan protects against VIDD
To determine the pharmacological properties of ARBs required to protect against VIDD, we studied two FDA approved drugs that differ in their pharmacological effects on AT1Rs. Our results reveal that olmesartan provided significant protection against both MV-induced contractile dysfunction and diaphragm fiber atrophy (**Figure 1**). By comparison, irbesartan did not protect against MV-induced contractile dysfunction and provided limited defense against diaphragm fiber atrophy as only type IIa fibers were protected (**Figure 1**).

Treatment with ARBs increase plasma levels of Ang1–7
Ang1–7 is a metabolite of AngII that opposes classical RAS signaling via activation of the MAS receptor.[14] This is significant because infusion of Ang1–7 during prolonged MV provides partial protection against VIDD.[15] Because ARBs increase circulating Ang1–7, we measured the plasma concentrations of Ang1–7 at the conclusion of each experiment. Compared with both MV and SB animals, plasma Ang1–7 levels were significantly higher in animals treated with irbesartan. No differences existed in plasma Ang1–7 levels between the irbesartan-treated and olmesartan-treated groups (**Figure 2a**).

Olmesartan protects the diaphragm against MV-induced mitochondrial dysfunction, oxidative stress, and protease activation
Prolonged MV promotes mitochondrial dysfunction (lower respiratory control ratio), oxidative damage (elevated 4-Hydroxynoneal levels), and protease activation (calpain and caspase-3) in the diaphragm; importantly, treatment with olmesartan prevented these MV-induced changes (**Figure 2**). In contrast, treatment with irbesartan did not protect the diaphragm against mitochondrial dysfunction, oxidative damage, or protease activation (**Figure 2**).

Because activation of JAK/STAT3 signaling is associated with mitochondrial dysfunction and VIDD, we measured the abundance of phosphorylated (i.e., active) STAT3 (pSTAT3) in the diaphragm. The p-STAT3 was increased in the diaphragm of animals exposed to MV and MV with irbesartan. Importantly, treatment with olmesartan protected against MV-induced activation of STAT3 (**Figure 2**).
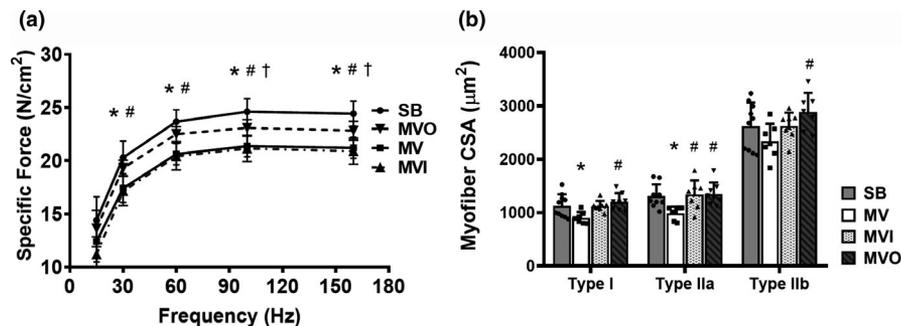

Figure 1 Olmesartan prevents diaphragm contractile dysfunction and fiber atrophy induced by prolonged mechanical ventilation. (**a**) Diaphragm specific force production as a function of the stimulation frequency (i.e., force-frequency curve) measured *in vitro* in costal diaphragm strips following 12 hours of mechanical ventilation (MV) or spontaneous breathing (SB). Values are means ± SD. *SB significantly different ($P < 0.05$) from MV. #Mechanical ventilation with olmesartan (MVO) significantly different from MV. †MVO significantly different ($P < 0.05$) from SB. (**b**) Diaphragm muscle fiber cross-sectional area in: (1) type I fibers, (2) type IIa fibers, and (3) type IIb/x fibers. Values are mean ± SD. *Significantly different ($P < 0.05$) from SB. #Significantly different from MV. CSA, cross-sectional area; MVI, mechanical ventilation with irbesartan.
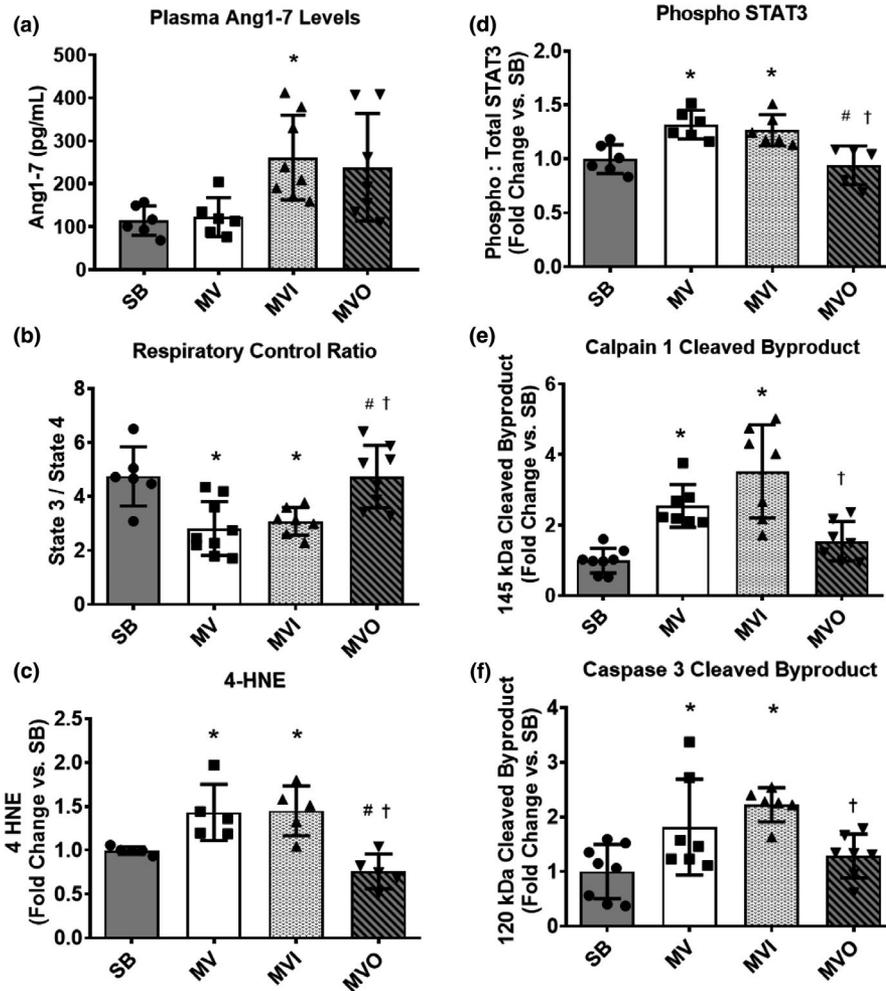
Figure 2 Olmesartan prevents mitochondrial dysfunction, oxidative stress, and proteolysis in VIDD. (**a**) Plasma angiotensin 1–7 levels at completion of experiment. Values are means ± SD. *Significantly different ($P < 0.05$) from spontaneous breathing (SB) and mechanical ventilation (MV). (**b**) Mitochondrial respiratory control ratio (state 3/state4) measured using permeabilized diaphragm muscle fibers. Values are means ± SD. *Significantly different ($P < 0.05$) from SB. #Significantly different from MV. †Significantly different ($P < 0.05$) from mechanical ventilation with irbesartan (MVI). (**c**) The relative abundance of 4-hydroxynonenal (4-HNE)-modified proteins (index of lipid peroxidation) in the diaphragm as determined by Western blot and normalized to α-tubulin. Values are means ± SD. *Significantly different ($P < 0.05$) from SB. #Significantly different from MV. †Significantly different ($P < 0.05$) from MVI. (**d**) The ratio of phosphorylated (active) pSTAT to total STAT in the diaphragm. Values are means ± SD. *Significantly different ($P < 0.05$) from SB. #Significantly different from MV. †Significantly different ($P < 0.05$) from MVI. (**e**) Calpain-mediated cleavage of alpha II spectrin releases a specific breakdown product at 145 kDa that can be quantified by western analysis; abundance of product is surrogate biomarker of calpain activity. Values are means ± SD. *Significantly different (P < 0.05) from SB. †Significantly different ($P < 0.05$) from MVI. (**f**) Caspase-3 mediated cleavage of alpha II spectrin releases a specific breakdown product at 120kDa that can be quantified by western analysis; abundance of product is surrogate biomarker of calpain activity. Values are means ± SD. *Significantly different ($P < 0.05$) from SB. †Significantly different ($P < 0.05$) from MVI. MVO, mechanical ventilation with olmesartan.

Although all major proteolytic pathways are activated in the diaphragm during MV,[16] the proteases calpain and caspase-3 play key roles in promotion of VIDD.[9] Prolonged MV activated calpain and caspase-3 in the diaphragm and, importantly, olmesartan attenuated this activation. By contrast, irbesartan did not prevent MV-induced activation of calpain and caspase-3 (**Figure 2**).

DISCUSSION
Overview of major findings
Our findings demonstrate that pharmacological of both ligand binding and mechanical activation of AT1Rs

(olmesartan) protects against VIDD whereas solitary blockade of AngII binding to AT1R (irbesartan) is not effective in preventing VIDD. Together, these results reveal that a key property of ARB-mediated protection against VIDD is the prevention of mechanical activation of AT1Rs. A brief discussion of the mechanisms responsible for olmesartan-mediated protection against VIDD follows.

Mechanisms responsible for olmesartan-mediated protection against VIDD
Our experiments support the concept that preventing mechanical activation of AT1Rs protects the diaphragm

against VIDD. The key question arises, "why does MV promote mechanical activation of AT1Rs on the sarcolemma of diaphragm fibers?" Although a definitive answer to this query is not available, a likely explanation is that MV imposes greater stress on the sarcolemma of diaphragm fibers during MV-induced passive shortening compared with the active muscle fiber shortening that occurs during spontaneous breathing.[17–19] Specifically, at any tidal volume, the amount of diaphragm fiber shortening is greater during MV compared with SB.[19] Therefore, it is feasible that the AT1Rs on the sarcolemma of diaphragm muscle fibers are activated during ventilator support by the high mechanical forces applied during MV-induced passive shortening of diaphragm fibers. A brief summary of the cellular events linking AT1R activation with VIDD follows.

Activation of AT1Rs results in G-protein coupled signaling, including the Janus kinase/signal transducers/activators of transcription (JAK/STAT) pathway.[20] A required element that contributes to VIDD is the increased production of reactive oxygen species in diaphragm muscle fibers.[21,22] Patients who underwent MV exhibit a significant decrease in antioxidant glutathione,[23] suggesting a significant role of oxidative stress on VIDD. Consistent with these findings, our MV rats also exhibited increased lipid peroxidation (**Figure 2c**), which was abrogated by olmesartan treatment. The MV-induced increase in reactive oxygen species production in diaphragm fibers is a required upstream trigger to activate proteolytic systems, including activation of calpain and caspase-3[21,22]; activation of calpain and caspase-3 are key events leading to VIDD.[9,24] Olmesartan treatment downregulated the increased caspase-3 activity elicited by MV.

Our results reveal that pharmacological blockade of mechanical activation of AT1Rs by olmesartan protects the diaphragm against key AT1R-mediated signaling events that promote VIDD, consistent with our previous report using losartan.[5] Specifically, olmesartan prevented the MV-induced increase in STAT3 signaling along with a reduction in both MV-induced mitochondrial uncoupling and oxidative damage to diaphragmatic proteins (**Figure 2**). Further, olmesartan prevented the activation of both calpain and caspase-3 in the diaphragm and defended against VIDD (**Figure 2**).

SUMMARY AND CONCLUSIONS

To define the characteristics of ARBs required to shield against VIDD, we investigated the efficacy of two ARBs that differ in their effects on AT1Rs. Although olmesartan prevents both AngII binding and mechanical activation of AT1Rs, irbesartan blocks only AngII binding to AT1Rs. Our results reveal that irbesartan is not protective against VIDD; by contrast, olmesartan provides significant protection against VIDD. Note that treatment with irbesartan and olmesartan resulted in similar levels of circulating Ang1–7, therefore, the olmesartan-mediated protection against VIDD was not due to elevated plasma levels of Ang1–7. Therefore, we conclude that blockade of mechanical activation of AT1Rs contributes to the protection against VIDD;

this important finding provides the groundwork for future testing of ARBs in clinical trials.

Author Contributions. S.E.H., B.A., and S.K.P. wrote the manuscript. S.E.H., B.A., and S.K.P. designed research. S.E.H., B.A., A.J.S., A.B.M., J.M.H., M.P.W., and K.J.S. performed the research. S.E.H., B.A., and H.H. analyzed the data.

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