JPET Fast Forward. Published on October 25, 2020 as DOI: 10.1124/jpet.120.000176
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Metoprolol Impairs Beta1-Adrenergic Receptor-Mediated Vasodilation in Rat Cerebral Arteries: Implications for Beta-Blocker Therapy

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JPET Fast Forward. Published on October 25, 2020 as DOI: 10.1124/jpet.120.000176
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Running Title: Metoprolol impairs cerebral vasodilator responses

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Tables: 0

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List of nonstandard abbreviations: β1AR, β1-adrenergic receptor; CA, cerebral artery; DOB, dobutamine; ISO, isoproterenol; MET, metoprolol; NE, norepinephrine; PSS, physiological salt solution; SD, Sprague-Dawley; SNP, sodium nitroprusside

Recommended section assignment: Cardiovascular

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ABSTRACT

The practice of prescribing beta-blockers to lower blood pressure and mitigate perioperative cardiovascular events has been questioned due to reports of an increased risk of stroke. The benefit of beta-blocker therapy primarily relies on preventing activation of cardiac β1-adrenergic receptors (β1AR). However, we reported that β1ARs also mediate vasodilator responses of rat cerebral arteries (CA), implying that beta-blockers may impair cerebral blood flow under some conditions. Here, we defined the impact of metoprolol (MET), a widely prescribed β1AR-selective antagonist, on adrenergic-elicited diameter responses of rat CA *ex vivo* and *in vivo*. MET (1-10 μmol/L) prevented β1AR-mediated increases in diameter elicited by dobutamine in cannulated rat CA. The β1AR-mediated dilation elicited by the endogenous adrenergic agonist norepinephrine (NE) was reversed to a sustained constriction by MET. Acute oral administration of MET (30 mg/kg) to rats in doses that attenuated resting heart rate and dobutamine-induced tachycardia also blunted β1AR-mediated dilation of CA. In the same animals, NE-induced dilation of CA was reversed to sustained constriction. Administration of MET for two weeks in drinking water (2 mg/mL) or subcutaneously (15 mg/kg/day) also resulted in NE-induced constriction of CA in vivo. Thus, doses of MET that protect the heart from adrenergic stimulation also prevent β1AR-mediated dilation of CA and favor anomalous adrenergic constriction. Our findings raise the possibility that the increased risk of ischemic stroke in patients on beta-blockers relates in part to adrenergic dysregulation of cerebrovascular tone.

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SIGNIFICANCE STATEMENT

Beta-blocker therapy using second-generation, cardio-selective beta-blockers is associated with an increased risk of stroke, but the responsible mechanisms are unclear. Here, we report that either acute or chronic systemic administration of a cardio-selective beta-blocker, metoprolol, mitigates adrenergic stimulation of the heart as an intended beneficial action. However, metoprolol concomitantly eliminates vasodilator responses to adrenergic stimuli of rat cerebral arteries *in vivo* as a potential cause of dysregulated cerebral blood flow predisposing to ischemic stroke.

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INTRODUCTION

Beta-adrenergic receptor (β-AR) antagonists, commonly referred to as beta-blockers, are a mainstay therapy for the treatment of hypertension, heart failure and angina (Frishman, 2003). Beta-blocker therapy mitigates sympathetic activation of the heart by antagonizing the binding of norepinephrine (NE) to β1 adrenergic receptors (β1AR) on cardiac myocytes to lower heart rate and blood pressure, and reduce ventricular workload (Frishman, 2003). The first generation of beta-blockers with propranolol as the prototype failed to discriminate between β1AR and β2AR subtypes, thereby compromising β2AR-mediated airway dilation to cause unwanted bronchoconstriction. Subsequently, metoprolol (MET) and other second-generation beta-blockers were introduced to selectively antagonize β1AR and avoid the off-target effects of nonselective beta-blockers (Frishman, 2008). In 2013, more than 83 million prescriptions were filled in the United States for MET, which is the most widely prescribed β1AR-selective antagonist (Aitken *et al.*, 2014). MET use steadily increased and accounted for 89 million prescriptions in 2017, ranking 4th in the nation for total volume of prescribed medications (IQVIA, 2018).

The widespread use of beta-blockers persists even though new guidelines in 2014 by the Eighth Joint National Committee of the U.S. Centers for Disease Control and Prevention removed beta-blockers as a first-line antihypertensive medication, citing mounting evidence for an increased risk of stroke (James *et al.*, 2014). As early as 2002, the Losartan Intervention for Endpoint Reduction trial reported a significant increased stroke risk associated with antihypertensive therapy using the beta-blocker atenolol compared to losartan, an angiotensin receptor blocker (Dahlöf *et al.*, 2002). In

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2005, the Lindholm meta-analysis reported a 16% to 26% higher risk of stroke for patients taking beta-blockers to control hypertension compared to other classes of antihypertensive drugs (Lindholm *et al.*, 2005). Finally, the results of the Perioperative Ischemic Evaluation trial reported in 2008 indicated a 2-fold higher risk of stroke in non-cardiac surgery patients treated perioperatively with MET compared to placebo (Devereaux *et al.*, 2008). The latter finding resulted in new 2009 guidelines by the American College of Cardiology Foundation and American Heart Association (ACCF/AHA) that recommended limited perioperative use of beta-blockers (Fleischmann *et al.*, 2009).

The positive correlation between antagonism of β1AR by beta-blockers and increased risk of stroke has evaded a mechanistic explanation. However, there had been indications of a potential beneficial contribution of β1AR to maintenance of cerebral blood flow and prevention of cerebral ischemia. In the peripheral vascular beds, the role of β1AR in regulating blood flow is assumed to be minimal, because sympathetic stimulation elicits α1AR–mediated vasoconstriction as the predominant adrenergic response, masking mainly β2AR-mediated dilation (Leech and Faber, 1996; Docherty, 2010). However, a different pattern of adrenergic responsiveness appears to characterize the cerebral circulation, which also is populated by sympathetic nerve endings (Edvinsson *et al.*, 1975; Cipolla *et al.*, 2004; Moore *et al.*, 2015). Furthermore, unlike many other vascular beds that are responsive to circulating catecholamines, the cerebral circulation under normal physiologic conditions is largely unaffected by levels of circulating epinephrine due to their exclusion by the blood-brain barrier (Weil-Malherbe et al., 1959; Myburgh et al., 2002). Although beta-blockers do not impair

cerebral blood flow in healthy individuals (Heinke *et al.*, 2005), beta blockade in the presence of a cardiovascular challenge such as stroke reduces cerebral blood flow (Stirling Meyer *et al.*, 1974). Indirect measurements of cerebral vasoreactivity in hypertensive or perioperative rat models indicate that β-AR may exert a tonic vasodilator influence under these challenges, as systemic administration of either propranolol or MET, a prototypic nonselective and β1AR-selective beta-blocker, respectively, attenuated cerebral blood flow (Ooboshi *et al.*, 1990; Ragoonanan *et al.*, 2009). A similar conclusion was reached by a study in nonhuman primates under conditions of induced hypertension (Aqyagi *et al.*, 1976). However, only more recently has the effect of beta-blockers on cerebral vasoreactivity *in vivo* been assessed directly using video-imaging through cranial windows. Using this method, Gorshkova *et al*. (Gorshkova *et al.*, 2011) infused norepinephrine (NE) intravenously to adrenergically activate the cerebral circulation while stabilizing blood pressure. Against a background of non-selective β-AR blockade established by propranolol, NE exerted opposing effects on different segments of pial arteries consisting of either vasodilation or vasoconstriction. Later, our laboratory (Moore *et al.*, 2015) topically suffused small-molecule inhibitors of either β1AR or β2AR into cranial windows to demonstrate that β1AR mediate the vasodilator responses of rat middle cerebral arteries to isoproterenol (ISO) and NE. Whereas these latter findings infer that β1AR can influence the adrenergic responsiveness of cerebral arteries, they do not address the question of whether systemically administered doses of clinical β1AR-selective blockers that confer intended beneficial cardiac effects, concomitantly dysregulate adrenergically driven cerebral vasodilator responses as an off-target effect.

The objective of the present study was to use cerebral window video-imaging to directly answer this question. The debate over the risks and benefits of beta-blocker therapy continues without insight into the mechanisms of increased stroke risk (Dahlöf *et al.*, 2002; Lindholm *et al.*, 2005; Devereaux *et al.*, 2008). Thus, the design of the present study included different dosing protocols intended to recapitulate the dual clinical use of MET as either a single-dose perioperative medication or a long-term therapy for cardiovascular conditions.

MATERIALS AND METHODS

Animals

All experiments in this study were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Male Sprague-Dawley (SD) rats (Harlan/Envigo Labs, Indianapolis, IN, USA) were used at 10 to 12 weeks of age for all experiments.

Diameter measurements

Measurements of diameter in isolated superior cerebellar arteries (CA) were performed as described earlier (Moore *et al.*, 2014). Briefly, CA were isolated, cannulated and pressurized at an intraluminal pressure of 80 mm Hg, then equilibrated at 37°C for 1 hour during superfusion with physiological salt solution (PSS) bubbled with a 7% CO_2 - 93% O_2 gas mixture to maintain a pH of ~7.4. CA that failed to develop spontaneous tone after 1-hour equilibration or failed to constrict to 60 mmol/L KCl were omitted from studies. External diameters were continuously measured and recorded by DMT

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software (Danish Myo Technology, Aarhus, Denmark). Although the internal diameter is a more accurate determinant of resistance, internal and external diameters correlate closely with each other in an isolated vessel (**Supplemental Fig. 1**). At the end of each study, arteries were superfused with Ca^{2+}-free PSS solution to obtain maximal diameter. In preliminary studies, we identified the concentration of MET required to fully block β1AR-mediated dilations in isolated CA. Vasodilation was elicited either by the β1AR agonist dobutamine (DOB) or the mixed β1/α1AR agonist NE prior to and then during incubation of the vessel with increasing concentrations of MET.

Acute and chronic administration of metoprolol (MET)

Acute doses of (±) metoprolol (+)-tartrate (MET, 10 or 30 mg/kg) were dissolved in water (10 or 30 mg/mL) and administered to rats 30 minutes before the start of cranial window surgery. For chronic treatment, rats either were provided *ad libitum* access for two weeks to MET in drinking water (0.3 or 2.0 mg/mL to provide an average dose of 35 or 130 mg/kg/day, respectively) or were implanted with MET-filled subcutaneous osmotic mini-pumps (Model 2002, Alzet, Cupertino, CA, USA) to administer 5 or 15 mg/kg/day. Water bottles containing MET were light protected to prevent photocatalytic degradation.

Dobutamine (DOB) challenge

DOB was administered intravenously to anesthetized rats to confirm effective antagonism of β1AR by systemic doses of MET. A DOB dosing regimen was chosen that would increase heart rate in control animals to approximately 85% of maximal heart rate (Bolter and Atkinson, 1988; Plante *et al.*, 2005; Hazari *et al.*, 2012), similar to DOB stress tests in patients (Henzlova *et al.*, 2016). Rats were anesthetized with 1.5-2.0%

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isoflurane and the heart rate was monitored by a three-lead electrocardiogram (ADInstruments, Colorado Springs, CO, USA). Body temperature was monitored with a rectal probe and maintained at 37°C with a direct feedback heating pad (Physitemp). Control or DOB-containing normal saline solution was administered by a syringe pump (Harvard Apparatus, Cambridge, MA) via an intravenous line (SV-S25BL, Terumo, Tokyo, Japan) into the lateral tail vein. DOB was prepared as a 33 µg/mL solution per 0.3 kg body weight. An initial 2-minute infusion of normal saline was used to establish a baseline heart rate. Subsequently, DOB was administered by three progressive infusion rates (3.6, 11, and 18 µg/kg/min). Heart rate was averaged for two minutes before DOB infusion to obtain a baseline value, and then measured during the final 40 seconds of DOB infusion to obtain average values for analysis. Experiments were completed within the 4-hour window after the dark period of the rats' light-dark cycle to match the time of day of the cranial window experiments.

Craniectomy and *in vivo* imaging

Craniectomy surgeries and fitting of a custom-made cranial window containing a drug port were performed as described earlier (Moore *et al.*, 2014). The tissue surface underneath the cranial window was suffused with 37°C PSS bubbled with a 7% CO_2-93% O_2 gas mixture to maintain pH of ~7.4. Branches of the middle cerebral artery were imaged using an HDR-PJ580 camera (Sony, Tokyo, Japan) and analyzed using an automated IPLab script (Scanalytics, Milwaukee, WI). Drugs including ISO, NE, CG920712, and sodium nitroprusside (SNP) were suffused through ports on the surface of the cranial window. The concentrations of ISO, NE and CGP20712 were chosen based on earlier studies by our laboratory (Moore *et al.*, 2015). All drugs were prepared

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in PSS. Sodium nitroprusside (100 μmol/L) was suffused at the end of experiments to verify that arteries retained reactivity, and hence, viability.

Drugs

All drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise. The following drugs were prepared as stock solutions in water: CGP20712 10 mmol/L (Tocris, Bristol, UK); ISO 20 mmol/L; NE 20 mmol/L; DOB 20 mmol/L (Hospira, Lake Forest, IL); MET 10 mmol/L; SNP 100 mmol/L. For *ex vivo* diameter studies, drugs were diluted first in PSS when appropriate and added directly in 1:1000 dilutions to the perfusion chamber. Drug concentrations in the text refer to final bath concentrations. For cranial window experiments, drugs were diluted in PSS to their final concentration and directly suffused through ports of the cranial window.

Statistical Analysis

Statistics were calculated using GraphPad Prism 5 (San Diego, CA, USA). Data are expressed as mean ± standard deviation. Diameter results from vessel perfusion studies are reported as the change in outer vessel diameter as percent of calcium-free dilation from baseline. The results of *in vivo* cranial window experiments are reported as the percent change in outer vessel diameter from baseline. Data were analyzed using t-tests, or when appropriate, using one-way ANOVA or two-way ANOVA with *post hoc* Bonferroni t-tests. Sample sizes were chosen based on our previous experience to yield power of 0.8 - 0.9; $P<0.05$ was considered statistically significant. Sample size (*n*) represents the number of arteries used in *ex vivo* studies or the number of animals used in *in vivo* studies. In box and whisker plots, boxes represent the inner quartile range and whiskers represent minimum and maximum values.

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RESULTS

Metoprolol (MET) abolishes β1 adrenergic receptor (β1AR)-induced dilation *ex vivo*

Initially, we performed proof-of-principle studies *ex vivo* to determine whether clinically relevant plasma concentrations of MET antagonize β1AR-induced vasodilator responses in rat CA. The range of therapeutic plasma concentrations of MET in patients is between 20 - 600 ng/mL, which is comparable to a molar range of 0.07 – 2.24 µmol/L (Ritscher *et al.*, 2019). Although we have demonstrated that CGP20712, a highly potent and selective small molecule antagonist of β1AR, prevents β1AR-mediated vasodilation in rat CA, MET has a less favorable profile as a β1AR-selective antagonist. It is a reversible antagonist unlike CGP20712 and only 2.5- to 30-fold more selective for β1AR than β2AR (Smith and Teitler, 1999; Baker, 2005). Regardless of these shortcomings, we found that MET antagonized the progressive vasodilator responses of cannulated rat CA to β1AR agonist. For example, increasing log concentrations (10 nmol/L to 100 µmol/L) of the β1AR-selective agonist DOB (**Fig. 1A; left**) were attenuated in a concentration-dependent manner after pre-incubation of arteries with MET (1, 3 or 10 µmol/L) (**Fig. 1A; right**). The clinically achievable concentration of 1 µmol/L MET attenuated the maximal dilator response to 100 µmol/L DOB by 45.5±23.4%, whereas higher concentrations of 3 µmol/L and 10 µmol/L MET resulted in 72.1±23.4% and complete block of DOB-elicited vasodilation (**Fig. 1B**). The other statistical measures of half-maximal effective concentration (EC_{50}) and maximal effect (E_{max}) are tabulated in **Supplemental Table 1**.

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Subsequently, we used a similar protocol to demonstrate that MET also effectively antagonizes the dilator response of cannulated rat CA to the endogenous adrenergic agonist NE. In these studies, increasing log concentrations of NE (10 nmol/L to 100 μmol/L) resulted in progressively larger diameters that ultimately reached 40.7±19.0% of the maximal dilator response to Ca^{2+}-free PSS (**Fig. 2A, left**). Induction of β1AR blockade by 10 μmol/L MET resulted in a reversal of the progressive NE-induced dilation to a sustained constriction (**Fig. 2A, right**), which represented a 24.7±19.3% reduction in resting diameter (**Fig. 2B**).

A single oral dose of metoprolol (MET) acutely inhibits β1 adrenergic receptor (β1AR)-mediated dilation

The perioperative oral administration of MET to confer cardiac protection is correlated with an increased risk of stroke (Devereaux *et al.*, 2008). We sought to establish a rat model that mimicked the clinical scenario of perioperative MET administration in order to determine its impact on cerebrovascular reactivity. Based on earlier pharmacokinetic and pharmacodynamic analyses of MET in a variety of species, which we adapted to the body weight of our animal subjects, we estimated that oral MET doses of 10 and 30 mg/kg may exert acute β1AR antagonism in rats (Freireich *et al.*, 1966; El Beheiry *et al.*, 1985; Höcht *et al.*, 2005; Komura and Iwaki, 2005; Reagan-Shaw *et al.*, 2008; Yoon *et al.*, 2011). In order to verify these doses of MET acutely mitigated the heart rate response to β1AR agonism, rats were subjected to a DOB challenge, which consisted of progressive infusion of three doses (3.6, 11 and 18 μg/kg/min) of DOB (**Fig. 3A**). Heart rates at baseline and in response to an increasing infusion rate of DOB were compared between control rats and similar animals gavaged with a single dose of 10 or 30 mg/kg

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MET. In control animals, DOB maximally increased heart rate from 328±25 to 407±25 bpm (**Fig. 3B**). Values for resting heart rate and DOB-elicited heart rate in animals treated with 10 mg/kg MET were not significantly different from the control, averaging 318±23 and 396±21 bpm, respectively. However, rats gavaged with 30 mg/kg MET showed significantly lower resting heart rate and DOB-elicited heart rate, averaging 298±19 and 375±32 bpm, respectively (**Fig. 3B**). Mean arterial pressure in anesthetized rats recorded by femoral catheter was 94.7±6.5 mmHg at baseline and was reduced to 72.9±10.8 mmHg by 30 min after oral gavage of 30 mg/kg MET (**Supplemental Fig. 2A-B**).

Subsequently, we evaluated whether the oral doses of 10 or 30 mg/kg MET disrupted the *in vivo* vasodilator responses of rat CA to adrenergic agonists, ISO and NE. In these studies, rats were administered 10 or 30 mg/kg MET by oral gavage 30 minutes prior to surgery for installation of a cranial window (Moore *et al.*, 2014). Agonists were infused topically through a port in the window to avoid confounding systemic cardiovascular effects (Moore *et al.*, 2015). We reported earlier that increasing log concentrations of ISO (10 nmol/L to 10 μmol/L) progressively dilate CA by a maximum of 19.4±5.6% in untreated animals (Moore *et al.*, 2015). Animals given a single oral dose of 10 mg/kg MET showed a maximal dilation of 20.2±4.0%, which was similar to untreated animals (**Fig. 4A**). However, the maximal dilator response to ISO was significantly reduced to 10.2±3.1% in animals given a single dose of 30 mg/kg MET (**Fig. 4A**). Thus, the effective oral dose of 30 mg/kg MET that mitigates β1AR-driven tachycardia (**Fig. 3B**) also disrupts the *in vivo* cerebral vasodilator responses to the adrenergic agonist ISO. At this effective oral dose of 30 mg/kg MET, CA failed to exhibit the expected β1AR-

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mediated dilation in response to topical NE (Moore *et al.*, 2015), and instead exhibited a stepwise diameter loss reaching -9.3±11.2% (**Fig. 4B**). Thus, a single oral administration of MET that confers cardiac protection disrupts the β1AR-mediated dilator responses to the endogenous neurotransmitter NE *in vivo* and unmasks α1AR-mediated constriction (Moore *et al.*, 2015). Basal diameter as percent of the maximal diameter elicited by SNP were 63.4±1.2% and 62.1±1.9% in rats gavaged with 10 and 30 mg/kg MET, respectively. These values were not significantly different from the value of 61.3±2.1% obtained in control rats (**Supplemental Fig. 2C**).

Chronic administration of metoprolol (MET) also disrupts β1 adrenergic receptor (β1AR)-mediated dilation

A final set of studies employed two different 2-week dosing protocols to recapitulate the clinical use of MET as a long-term therapy for chronic cardiovascular diseases, a treatment scenario correlated with increased risk of stroke (Dahlöf *et al.*, 2002; Lindholm *et al.*, 2005; James *et al.*, 2014). As before, our goal was to define the impact of systemically administered MET on β1AR-mediated cerebral vasodilation. We chose our protocols for MET administration after normalizing MET doses used in humans (Reagan-Shaw *et al.*, 2008) to rat body weight, and also considering the more rapid metabolism of MET in rats (Yoon *et al.*, 2011). Accordingly, rats were administered MET for two weeks in drinking water (0.3 or 2.0 mg/ml) to mimic the oral route of MET administration in patients, or administered MET by subcutaneous mini-pump (5 or 15 mg/kg/day) to avoid potential fluctuations in plasma levels of MET caused by diurnal patterns of water consumption. Regardless of the route of administration, all *in vivo* experiments were conducted in animals within a 4-hour window after the dark period of

the light:dark cycle, which is associated with higher animal activity and water consumption.

For both routes of MET administration, DOB challenge was employed again at the end of the two-week drug treatment to identify MET doses that provided protection from β1AR-driven cardiac stimulation *in vivo*. Compared to control animals in which DOB maximally increased heart rate from 325±10 to 412±24 bpm, only the higher oral MET dose of 2.0 mg/mL significantly reduced resting heart rate to 299±23 bpm and DOB-induced heart rate to 383±26 bpm (**Fig. 5A**). The lower oral MET dose of 0.3 mg/mL did not significantly reduce resting (312±27 bpm) or DOB-induced heart rate (397±6 bpm). In animals administered MET by mini-pump infusion, resting heart rate in rats administered the lower subcutaneous dose of 5 mg/kg/day was significantly lower than that of control animals (296±12 and 325±10 bpm, respectively), but the tachycardia caused by DOB challenge was not significantly different from untreated animals (385±8 bpm and 412±23 bpm, respectively). Only the higher MET dose of 15 mg/kg/day significantly lowered both resting heart rate (273±9 bpm) and DOB-induced tachycardia (328±26 bpm) (**Fig. 5B**). Based on observed daily water consumption, the two oral doses of MET, 0.3 and 2.0 mg/mL in drinking water, were estimated to be 34±10 and 130±20 mg/kg/day of MET, respectively (**Supplemental Fig. 3A**). Neither the 0.3 nor 2.0 mg/mL oral dosing of MET significantly altered systolic blood pressure after one week (3.5±15.2 or -1.0±8.8 mmHg, respectively) or two weeks (0.0±14.1 or -7.1±14.4 mmHg, respectively) of treatment, relative to pre-treatment levels (**Supplemental Fig. 3B**).

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Subsequently, rats dosed for two weeks with MET in drinking water (2.0 mg/mL) or by osmotic mini-pump (15 mg/kg/day) were subjected to cranial windows to assess the impact of two-week MET administration on the cerebrovascular response to NE. Topical application of increasing concentrations of NE resulted in maximal constriction of cerebral arteries *in vivo* averaging 26.0±10.4% and 26.8±6.4% in rats treated with MET orally or by subcutaneous mini-pump, respectively, in contrast to a mild dilation of CA in untreated control rats reported previously (Moore *et al*., 2015) (**Fig. 5C**). Isolated CA from rats treated with 2.0 mg/mL MET for two weeks showed similar vasodilator responses to NE *ex vivo* as CA from untreated control rats (**Supplemental Fig. 4A**), presumably reflecting the return of β1AR-mediated vasodilation after washout of MET in the perfusion chamber. Resting and Ca^{2+}-free diameters at 80 mmHg also were not significantly different between CA isolated from control and MET-treated rats (**Supplemental Fig. 4B**). Finally, the resting diameter of CA in cranial windows *in vivo* expressed as percent of the maximal dilator response to SNP was not significantly different between untreated rats and rats treated with 2.0 mg/mL MET for two weeks (**Supplemental Fig. 4C**).

DISCUSSION

Reports of an increased risk of stroke associated with the perioperative or long-term use of beta-blockers resulted in revised guidelines by the AHA/ACC and JNC8 that advocated to limit the use of these medications (Fleischmann *et al.*, 2009; James *et al.*, 2014). However, the number of prescriptions for the β1AR-selective blocker MET continues to increase each year in the United States (Aitken *et al.*, 2014; IQVIA, 2018)

despite cautionary guidelines. This dichotomy may relate to the fact that we lack a mechanistic explanation for the positive correlation between beta-blockers and stroke. In this regard, the findings of the present study that acute and longer-term administration of MET disrupts β1AR-mediated cerebral vasodilation may provide clues to a mechanistic link. It's possible that the loss of β1AR-mediated cerebral vasodilation resulting from beta-blocker therapy predisposes to deficits in cerebral blood flow during times of stress when circulating levels of catecholamines and tissue oxygen demands are at their highest (Myers *et al.*, 1981; Cechetto *et al.*, 1989; Ragoonanan *et al.*, 2009; Akil *et al.*, 2015).

Our recent preclinical studies describe a powerful β1AR-dependent vasodilator pathway in rat CA (Moore *et al.*, 2015). Importantly, earlier studies also provide evidence that β1AR-dependent vasodilation is a feature of the human cerebral circulation. Pharmacological studies (Edvinsson *et al*., 1976) reported prominent β1AR-mediated relaxations in strips of human pial artery. Radio-ligand binding studies (Nakai *et al.*, 1986; Tsukahara *et al.*, 1986) suggested co-expression of β1AR with β2AR in human basilar and middle cerebral arteries. Collectively, these studies indicate the presence of a β1AR-mediated vasodilator pathway in the human cerebral circulation, which potentially could be compromised by systemic administration of beta-blockers.

Our initial experiments verified that clinically relevant plasma concentrations of MET (Ritscher *et al.*, 2019) can antagonize β1AR-mediated vasodilation in isolated CA, which were removed from native influences that may confound interpretation of changes in cerebrovascular reactivity *in vivo* (Gorshkova *et al.*, 2011). Notably, these studies also revealed that antagonism of β1AR by MET reverses NE-elicited vasodilation to a

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vasoconstrictor response. A similar “NE reversal” in isolated rat CA has been attributed to the experimental β1AR blocker CGP20712 (Moore *et al.*, 2015) and to the non-selective beta-blocker, propranolol, in bovine caudal CA *ex vivo* (Ayajiki and Toda, 1990). This anomalous vasoconstriction to adrenergic stimulation in the presence of beta-blockers may create an environment of risk for individuals predisposed to cerebral ischemia. Indeed, hemodilution studies in rats suggest that MET is associated with reduced cerebral tissue oxygen tension (Myers *et al.*, 1981; Cechetto *et al.*, 1989; Ragoonanan *et al.*, 2009; Akil *et al.*, 2015).

Next, we designed *in vivo* experiments to mimic clinical MET uses as either acute or chronic therapies. Interestingly, in perioperative use of beta-blockers, the risk of stroke is greatest in healthy, otherwise low-risk patients undergoing non-cardiac surgery where high fixed doses of beta-blockers are used on the day of surgery (Fleischmann *et al.*, 2009; Poldermans *et al.*, 2009). Starting from standardized conversion of doses from humans to rats (Reagan-Shaw *et al.*, 2008; Yoon *et al.*, 2011), we evaluated two acute oral doses of MET to blunt baseline heart rate and DOB-induced tachycardia. The effective dose of MET (30 mg/kg) that antagonized cardiac β1ARs and lowered heart rate also effectively prevented ISO-induced dilation of CA *in vivo*. The inhibition of CA dilation by 30 mg/kg MET was similar to that produced by a topical application of the highly-selective β1AR blocker CGP20712 in our previous study (Moore *et al.*, 2015), suggesting MET mainly acts on β1AR to limit adrenergic-mediated dilation of CA. Under these conditions, a concomitant NE-induced contraction mediated by α-AR becomes apparent, similar to our findings in isolated CA *ex vivo*. Although mean arterial pressure in anesthetized rats was significantly less after an acute oral dose of 30 mg/kg MET, the

resting diameters of CA *in vivo* were not significantly different from untreated control rats.

In accordance with the new guidelines from ACCF/AHA that limit acute perioperative use of beta-blockers, these medications are now initiated at least two weeks before surgery rather than acutely in the pre-operative period (Devereaux *et al.*, 2018). Furthermore, MET is taken chronically for several cardiovascular conditions (Lindholm *et al.*, 2005; Frishman, 2008; Fleischmann *et al.*, 2009). Thus, our second set of MET dosing involved two-week administration using two different routes: oral *ad libitum* in drinking water and by subcutaneous osmotic mini-pump. Although rats do most of their drinking during their nocturnal waking hours (Johnson and Johnson, 1991), the timing in individual rats in our study between the last drink of MET and the start of cranial window experiments undoubtedly was variable, presumably mimicking the situation of outpatients on oral MET, who present to clinics at different timepoints after their last dose of MET. On the other hand, delivery by subcutaneous osmotic mini-pumps allows for a consistent exposure to a known dose of MET, thereby eliminating the time-of-day variations in drug plasma concentration analogous to intravenous infusion of MET in inpatient settings. Unlike a single acute oral dose, prolonged use of MET is associated with upregulation of β1AR in the cardiac tissue of heart failure patients (Heilbrunn *et al.*, 1989). Despite this confounding factor, we again relied on the DOB challenge to find effective doses of MET that would blunt resting heart rate and DOB-induced tachycardia. At the doses of 2.0 mg/mL in drinking water or 15 mg/kg/day subcutaneously at which MET lowered resting heart rate and blunted the DOB-induced tachycardia, it also converted the mild vasodilator response to NE of CA *in vivo* to a

marked vasoconstriction. These results suggest that even chronic MET doses that blunt cardiac β1AR stimulation as their intended therapeutic effect concomitantly block β1AR in CA as an off-target effect, thereby unmasking a vasoconstrictive response to adrenergic stimulation.

Roughly 80% of primary stroke events are associated with thrombosis and embolism (Albers, 1995). In this regard, MET-facilitated CA constriction by adrenergic agonists may increase the risk and/or severity of stroke in multiple ways. By reversing a physiological vasodilation response to vasoconstriction, it may significantly elevate arterial resistance in situations where more blood flow to the brain is needed. Notably, a 10% change in diameter leads to >40% change in arterial resistance (Cassot *et al.*, 1995). In the presence of an existing partial obstruction of CA, vasoconstriction can result in a complete or near-complete blockage of blood flow causing a direct hypoperfusion or ischemia and also may significantly damage the arterial wall from turbulent blood flow. If larger CA such as the middle cerebral artery is constricted, small emboli that would otherwise cause small infarcts in higher order branches with no significant symptoms, may get lodged in lower-order arteries depriving a larger area of adequate cerebral blood flow (Traverse *et al.*, 1995). Notably, in SD rats, it has been reported that β-AR density is at its highest in lower order branches of middle cerebral arteries and show significant loss of dilation to intravenous NE by propranolol only in the large distributive branches of pial arteries (Gorshkova *et al.*, 2011).

There are a number of limitations in the present study. First, due to high variability in the diameters of CA observed in cerebral windows, we could not compare resting diameters between untreated and MET-treated rats. This raised the question of whether

differences in adrenergic responsiveness to NE were influenced MET treatment. Indeed, we attempted to answer this question by measuring the diameter of CA in a single animal before and after acute oral doses of MET. However, the delicacy of the brain exposed in cranial windows did not allow repositioning of the animal in the stereotaxic apparatus for oral dosing post-craniectomy. Instead, we compared resting diameters as percent of the maximal vessel diameter attained by SNP-induced vasodilation. Using this strategy, we found no statistical difference between untreated and MET-treated groups. This finding concurs with our previous observation that application of topical beta-blockers to the cranial window chamber does not alter the resting diameter of rat CA under anesthesia (Moore *et al.*, 2015). Second, the adrenergic stimulation was pharmacologically simulated with a topical application of exogenous NE rather than direct neuronal stimulations. However, it is interesting to note that constriction of CA occurred at 1 – 10 μmol/L of NE comparable to NE levels measured in the brain during stroke in rat models (Cechetto *et al.*, 1989) or during exercise or acute illness in humans (Silverberg *et al.*, 1978). Third, our study only examined the effect of MET treatment on cerebral adrenergic responsiveness in male rats. Potential interactions between estrogen and β1AR signaling pathways (Machuki et al., 2018) may add an extra layer of complexity that is beyond the scope of the current studies and requires additional research. Fourth, we only examined the effect of MET administration on NE-induced diameter responses of CA. Under normal physiologic conditions, epinephrine plays a minimal role in determining cerebral arterial diameter due to its exclusion by the blood-brain barrier (Weil-Malherbe et al., 1959; Myburgh et al., 2002). However, after the onset of a pathologic state such as after ischemic stroke,

JPET Fast Forward. Published on October 25, 2020 as DOI: 10.1124/jpet.120.000176
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plasma epinephrine concentrations acutely increase (Meloux et al., 2018) and epinephrine may gain access to cerebral arteries due to increased blood-brain barrier permeability (Liebner et al., 2018). Thus, the effects of MET on the adrenergic responsiveness of CA may potentially be different in disease states associated with a disrupted blood brain barrier. This possibility draws attention to the fact that our study explored the effect of MET treatment only in young, healthy SD rats rather than rats with high blood pressure or in a rat model of stroke.

It is important to acknowledge that the findings of our study are specific to second-generation, β1AR-selective blockers such as MET and should not be generalized to all forms of beta-blocker therapies. The newer third-generation beta-blockers include drugs that exhibit pharmacological properties additional to βAR-blockade, such as nitric oxide production, β2AR-agonism, α1AR-antagonism, Ca^{2+} channel blockade and antioxidant activity (Brunton *et al.*, 2011). Importantly, these third-generation “vasodilating” beta-blockers have shown a better stroke risk profile compared to second-generation “cardio-selective” β1AR blockers (Poole-Wilson *et al.*, 2003; Remme, Cleland, *et al.*, 2007; Remme, Torp-Pedersen, *et al.*, 2007). However, MET continues to rank the highest of all beta-blockers in prescription volume in the U.S., whereas no third-generation beta-blockers were included in the top 20 (IQVIA, 2018). Investigation of the effects of vasodilating beta-blockers on the cerebral arteries represents an important future direction.

In conclusion, we have provided evidence that systemic administration of MET at clinically relevant doses directly blunts β1AR-mediated dilation of rat CA resulting in abnormal vasoconstriction to adrenergic stimulation by NE. These results suggest

JPET Fast Forward. Published on October 25, 2020 as DOI: 10.1124/jpet.120.000176
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β1AR-mediated vasodilation in CA represents a significant off-target site of action for "cardio-selective" β1AR blockers. This effect offers a possible mechanism to account for how conventional beta-blocker therapy may increase the risk of stroke as observed in clinical settings.

ACKNOWLEDGMENTS

None

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Moore, Henry, Rusch, Rhee

Conducted experiments: Moore, Henry, McClenahan, Ball

Performed data analysis: Moore, Henry, McClenahan, Ball, Rhee

Wrote or contributed to the writing of the manuscript: Moore, Henry, Rusch, Rhee

JPET Fast Forward. Published on October 25, 2020 as DOI: 10.1124/jpet.120.000176
This article has not been copyedited and formatted. The final version may differ from this version.

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FOOTNOTES

This work was supported in part by NIH [R01HL97107], American Heart Association grants [17GRNT33670970], [19TPA34880019] (SWR), and [13PRE17070035] (CLM).

Portions of this work was previously presented in Moore, C.L. (2015). The role of postsynaptic density-95 scaffolding in cerebral vasodilation: Implications for stroke in beta-blocker therapy (Doctoral dissertation).

Figures 4A, 4B and 5C modified from Moore CL, McClenahan SJ, Hanvey HM, Jang DS, Nelson PL, Joseph BK, and Rhee SW. J Cereb Blood Flow Metab 35 (9):1537–1546 (2015). Reprinted by Permission of SAGE Publications, Ltd.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

JPET Fast Forward. Published on October 25, 2020 as DOI: 10.1124/jpet.120.000176
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FIGURE LEGENDS

Figure 1. Metoprolol (MET) inhibits dobutamine (DOB)-induced dilation of rat cerebral arteries (CA) *ex vivo*. (A) Diameter response of an isolated, pressurized CA to cumulative log concentrations of DOB before (*left trace*) and 10 minutes after (*right trace*) superfusion with 10 μmol/L MET. **(B)** Average diameter responses to DOB in the absence of MET (control) and after 1, 3 or 10 μmol /L MET; *n*=6 each. One-way ANOVA at the highest concentration of MET with *post hoc* Bonferroni t-tests; significant difference from control: ***P*<0.05, ****P*<0.001.

Figure 2. Norepinephrine (NE)-induced dilation of isolated cerebral arteries (CA) is reversed to constriction by metoprolol (MET). (A) Diameter response of an isolated, pressurized rat CA to cumulative log concentrations of NE before (*left trace*) and 10 minutes after (*right trace*) superfusion with 10 μmol/L MET. **(B)** Average diameter responses to NE in the absence of MET (control) and after 10 μmol/L MET; *n*=6 (control), *n*=5 (MET). Two-way ANOVA with *post hoc* Bonferroni t-tests; significant difference from control: ****P*<0.001.

Figure 3. A single oral dose of metoprolol (MET) attenuates the heart rate response to dobutamine (DOB) challenge. **(A)** Sample plot of the control heart rate response to infusion of three cumulative doses (3.6, 11 and 18 μg/kg/min) of the β1AR agonist, DOB, which was administered by a syringe pump into the tail vein. Initiation of a new dose of DOB is indicated by ▼. **(B)** Average heart rate at baseline and at the

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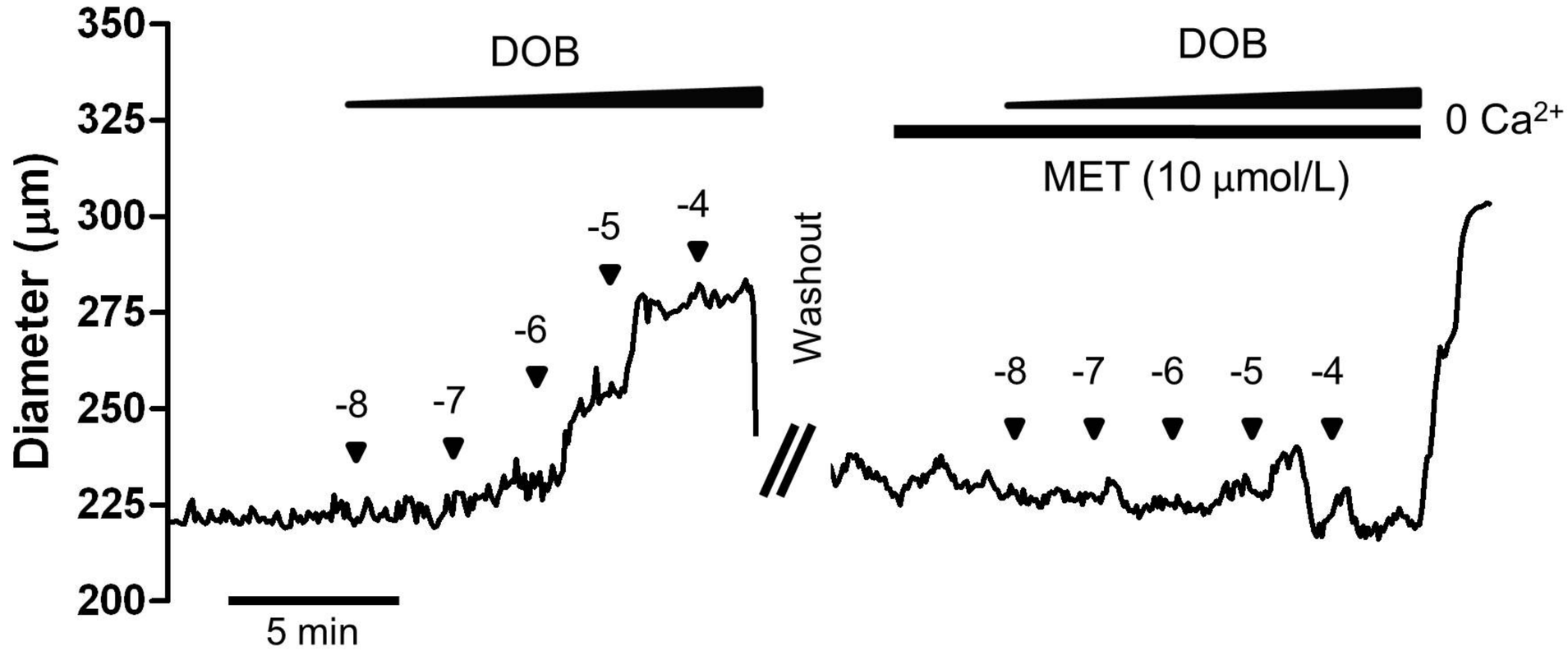
highest dose of 18.4 µg/kg/min DOB in control rats (Control) and in rats gavaged 30 minutes before the DOB challenge with a single dose of 10 or 30 mg/kg MET. (- - -) indicates 85% of estimated maximal heart rate; *n*=9 (control), *n*=10 (MET). One-way ANOVA with *post hoc* Bonferroni t-tests; significant difference from control: ***P*<0.05.

Figure 4. A single oral dose of metoprolol (MET) inhibits the dilator response to isoproterenol (ISO) of rat cerebral arteries (CA) and reverses norepinephrine (NE)-induced dilation to constriction *in vivo*. (A) Average diameter response to topical suffusion of cumulative concentrations of ISO. Suffusion was initiated 30-minutes after rats were gavaged with either 10 or 30 mg/kg MET. Vessels were imaged through a cranial window; *n*= 6 (control), 5 (10 mg/kg MET), 6 (30 mg/kg MET) **(B)** Average diameter response to topical suffusion of cumulative concentrations of NE initiated 30-minutes after rats were gavaged with 30 mg/kg MET; *n*= 7 (control), 5 (10 mg/kg MET), 6 (30 mg/kg MET). Two-way ANOVA with *post hoc* Bonferroni t-tests; significant difference from control: ***P*<0.01, ****P*<0.001. Control data figures modified from Moore *et al*., 2015 with permission.

Figure 5. Systemic administration of metoprolol (MET) for two weeks attenuates the heart rate response to dobutamine (DOB) and results in a cerebral vasoconstrictor response to topical norepinephrine (NE) *in vivo*. Heart rate at baseline and in response to a DOB challenge after 2 weeks of either **(A)** MET administration in drinking water (0.3 or 2.0 mg/ml) or **(B)** MET administration by subcutaneous mini-pump (5 or 15 mg/kg/day). Data are shown only for the highest

dose of DOB (18.4 µg/kg/min) administered during the challenge; *n*=11 (control); *n*=11 (2.0 mg/mL MET); *n*=6 (0.3 mg/mL MET); *n*=6 (15 mg/kg/day MET), *n*=4 (5 mg/kg/day MET). (- - -) indicates 85% of estimated maximal heart rate. One-way ANOVA with *post hoc* Bonferroni t-tests; significant difference from control: ******P*<0.05**, ******P*<0.001. **(C)** Constriction response of middle cerebral artery (MCA) branches in cranial window to suffused NE after 2-week MET treatment; *n=7* (control), *n*=8 each MET. Two-way ANOVA: no differences between routes of administration (*P*>0.05). Bonferroni t-tests; significant difference from control: *******P*<0.01, ********P*<0.001. Control data in (C) is modified from Moore *et al*., 2015 with permission.

A



B

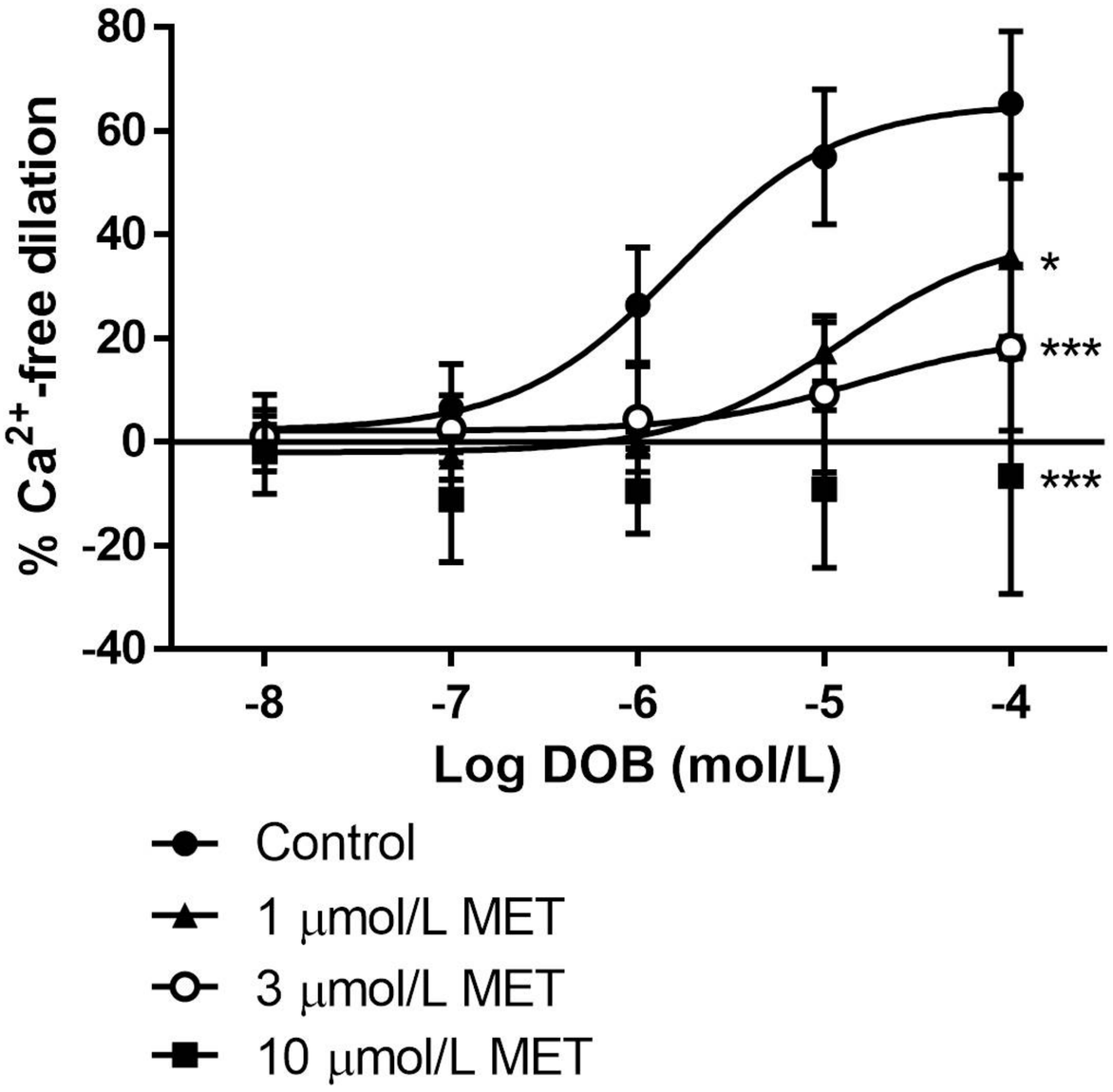


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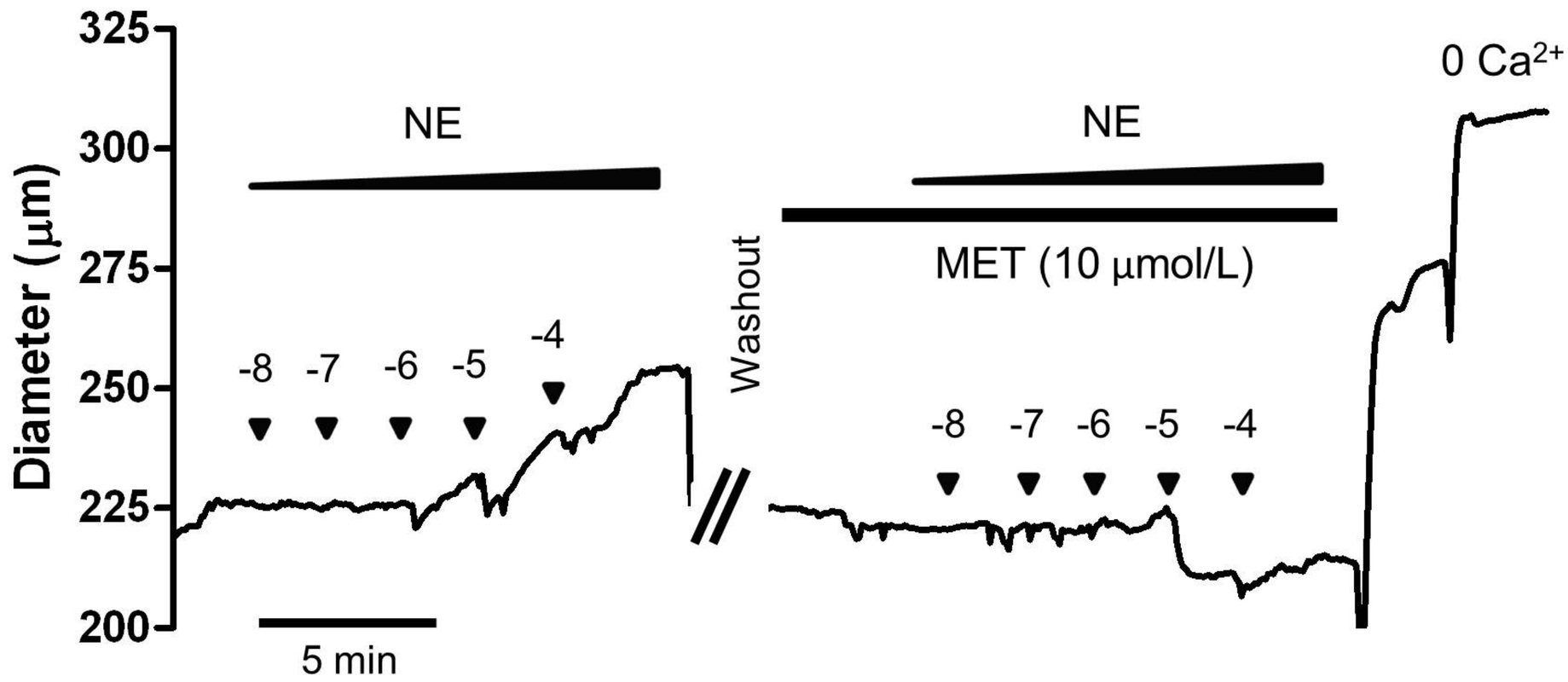
Figure 1

A



B

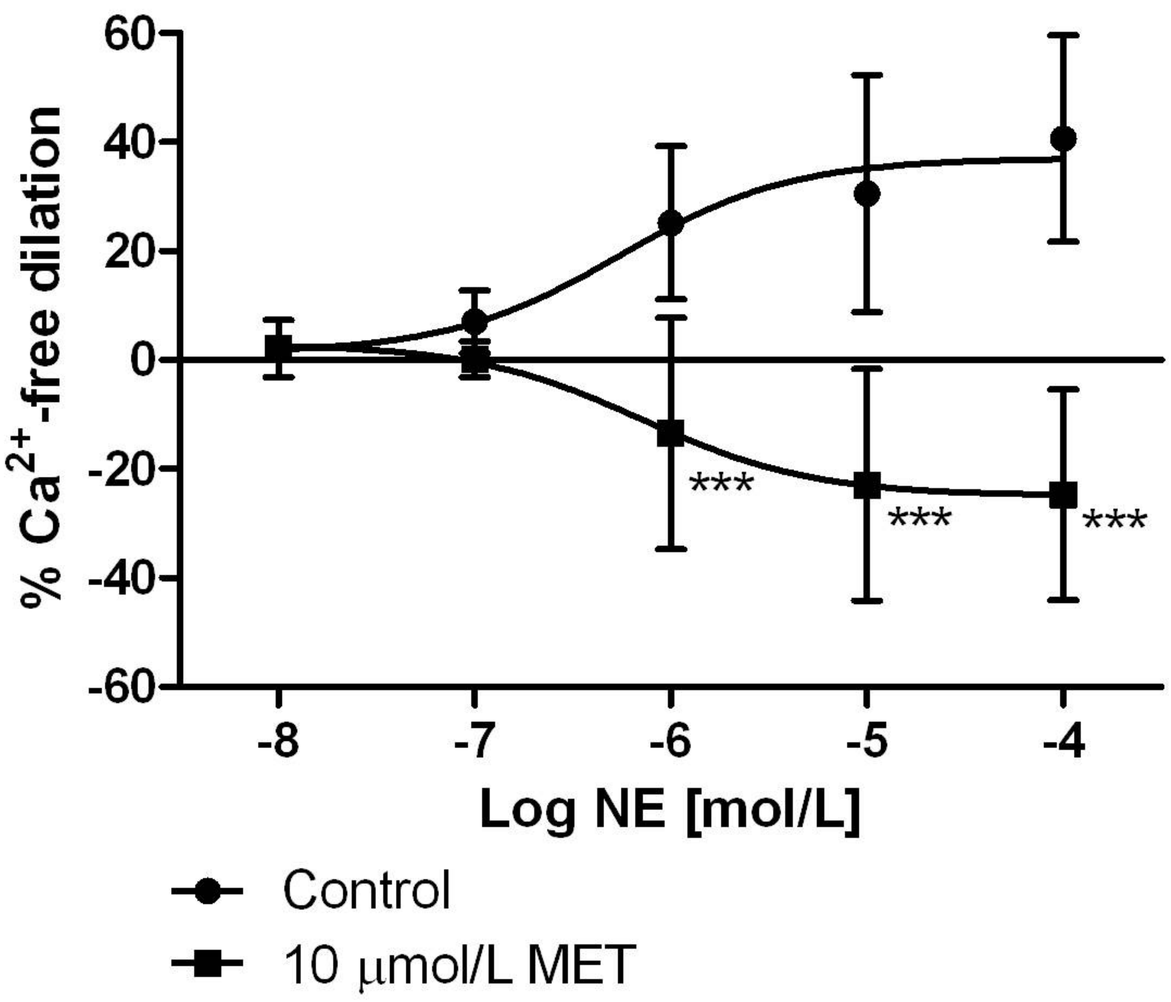


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Figure 2

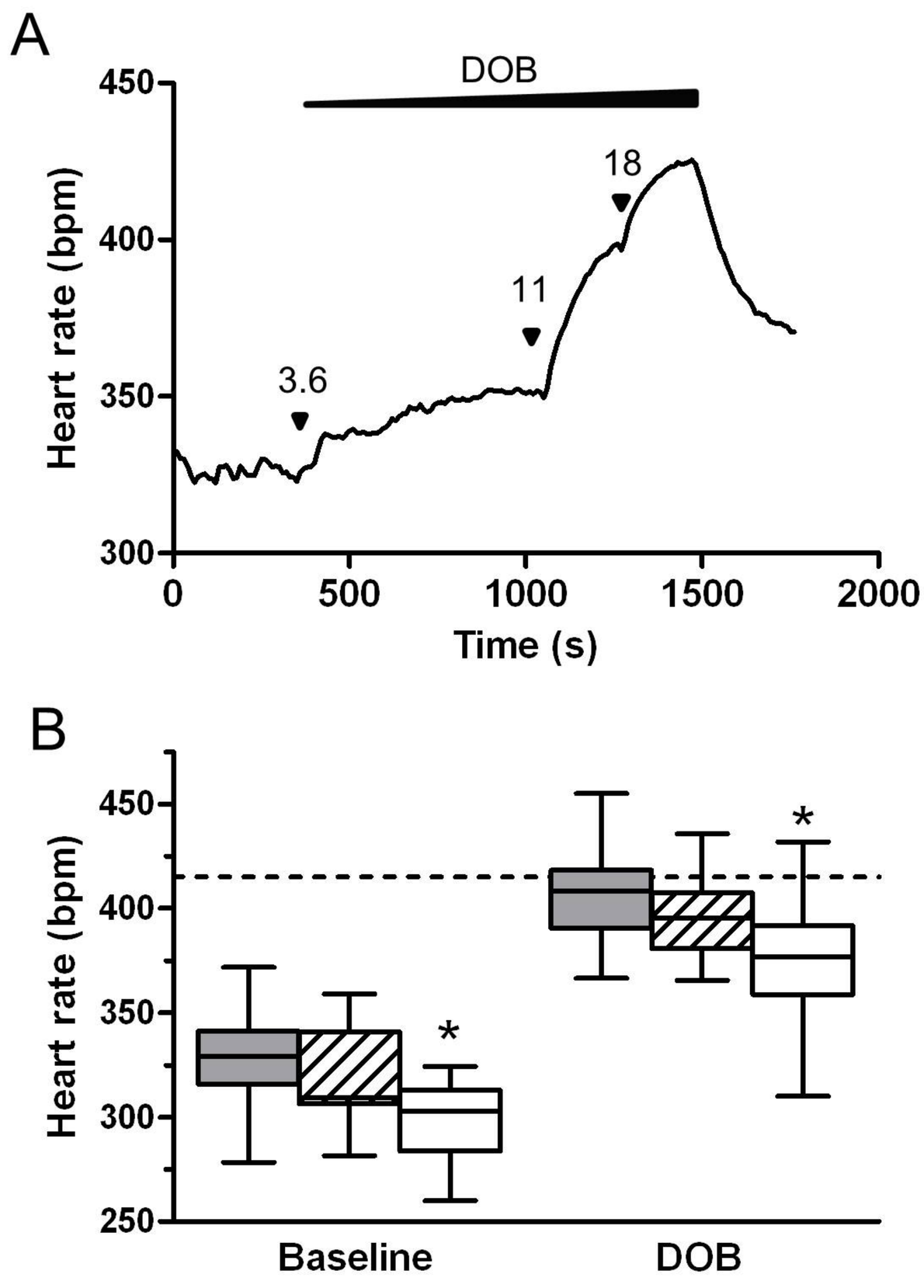

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Figure 3

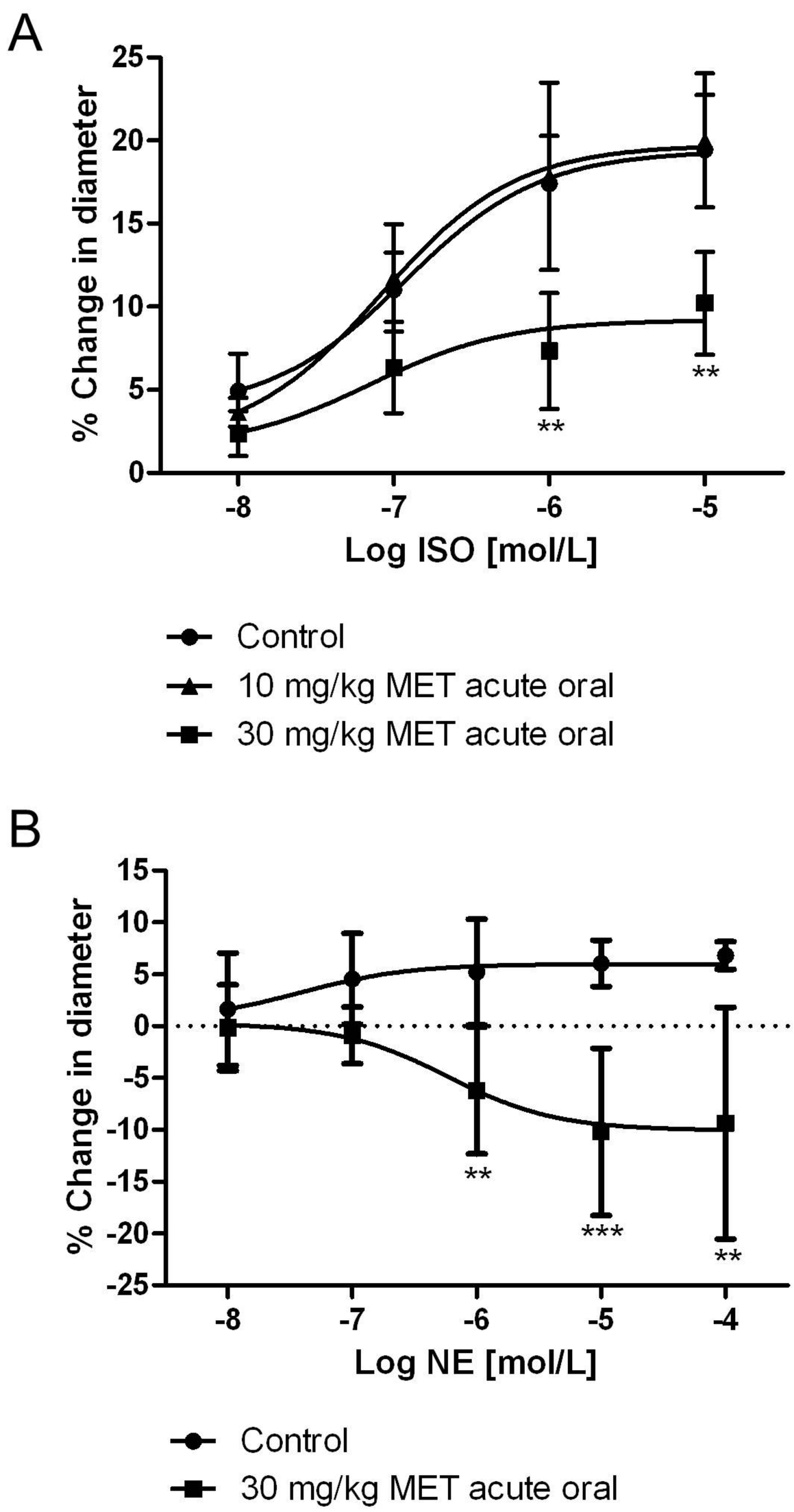

Figure 4

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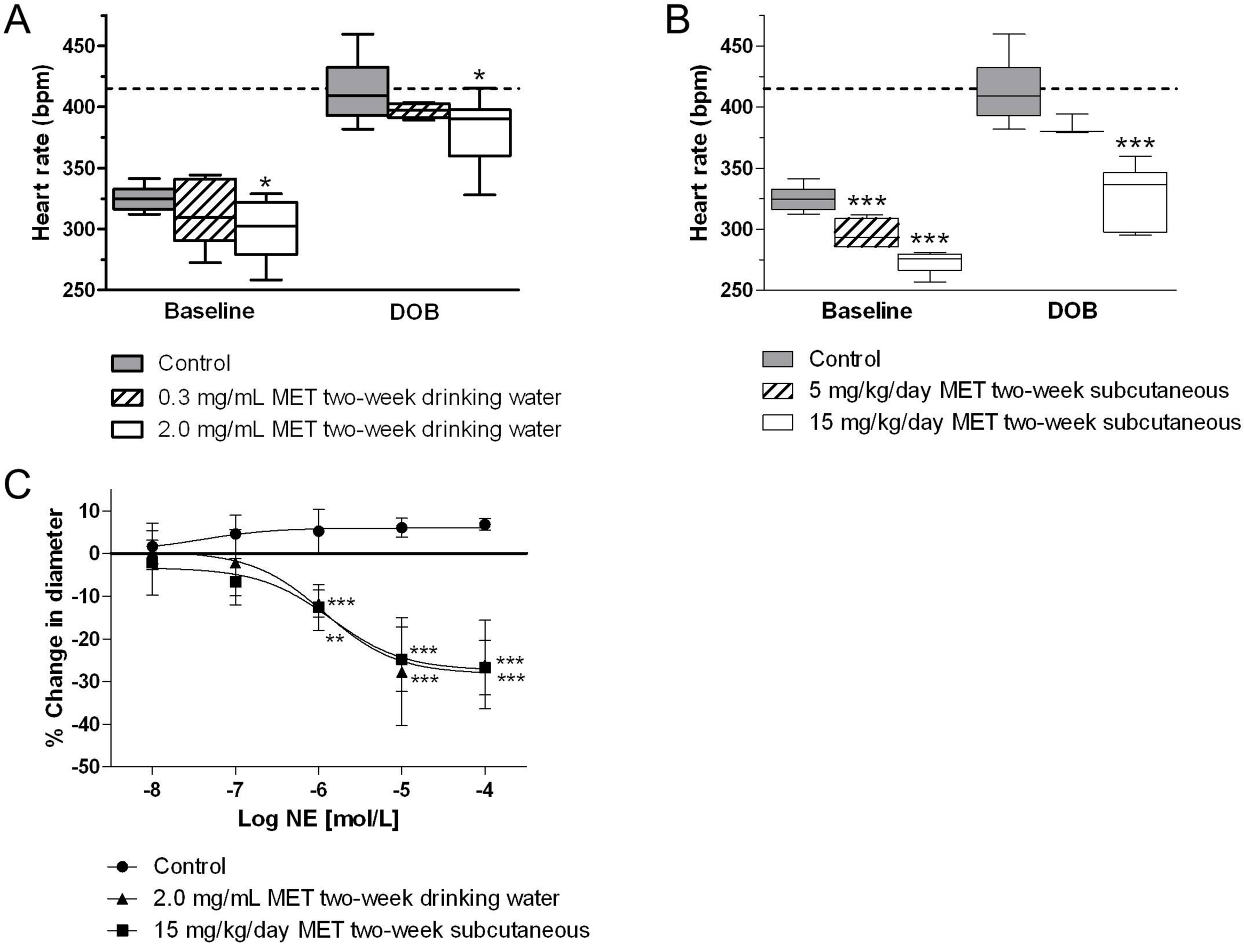

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Figure 5