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Cannabinoid antagonist SR141716 inhibits endotoxic hypotension by a cardiac mechanism not involving CB_1 or CB_2 receptors

Sándor Bátkai*, Pál Pacher*, Zoltán Járai, Jens A. Wagner, George Kunos

Laboratory of Physiologic Studies, National Institute on Alcohol Abuse & Alcoholism, National Institutes of Health, Bethesda, MD, 20892

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Address for reprint requests and other correspondence: George Kunos, NIAAA, 12420 Parklawn Drive, MSC-8115, Bethesda, MD 20892-8115. E-mail gkunos@mail.nih.gov

*S. Bátkai and P. Pacher contributed equally to this work.

Abstract – Endocannabinoids and CB_1 receptors (CB_1) have been implicated in endotoxin (lipopolysaccharide [LPS])-induced hypotension: LPS stimulates the synthesis of anandamide in macrophages, and the CB_1 antagonist SR141716 inhibits the hypotension induced by treatment of rats with LPS or LPS-treated macrophages. Recent evidence indicates the existence of cannabinoid receptors distinct from CB_1 or CB_2 which are inhibited by SR141716 but not by other CB_1 antagonists such as AM251. In pentobarbital-anesthetized rats, i.v. injection of 10 mg/kg LPS elicits hypotension associated with profound decreases in cardiac contractility, moderate tachycardia and an increase in lower body vascular resistance. Pretreatment with 3 mg/kg SR141716 prevented the hypotension and decrease in cardiac contractility, slightly attenuated the increase in peripheral resistance and had no effect on the tachycardia caused by LPS, whereas pretreatment with 3 mg/kg AM251 did not affect any of these responses. SR141716 also elicited an acute reversal of the hypotension and decreased contractility when administered after the response to LPS had fully developed. The LPS-induced hypotension and its inhibition by SR141716 were similar in pentobarbital-anesthetized wild-type, $CB_1^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ mice. We conclude that SR141716 inhibits the acute hemodynamic effects of LPS by interacting with a cardiac receptor distinct from CB_1 or CB_2, which mediates negative inotropy and may be activated by anandamide or a related endocannabinoid released during endotoxemia.

Key Words: endotoxin; cannabinoids; hypotension; negative inotropy

ACTIVATION OF CANNABINOID RECEPTORS by plant-derived and endogenous cannabinoids elicits well documented cardiovascular effects (17, 25). In humans, acute ingestion of Δ^9-tetrahydrocannabinol (THC), the main psychoactive ingredient of marijuana, usually elicits tachycardia (24). However, prolonged use in humans and acute as well as chronic administration in most animal models causes long lasting hypotension and bradycardia (4, 26), and similar depressor effects have been also reported in response to the acute administration of the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) (22, 45). The introduction in 1994 of the first selective cannabinoid-1 receptor (CB_1) antagonist, SR141716 (40), has allowed these responses to be characterized as CB_1-mediated (45), a conclusion later borne out by the absence of cannabinoid-induced hypotension and bradycardia in mice with genetic ablation of CB_1 (23, 27). Although the overwhelming majority of CB_1 in mammals are located in the brain (16), the cardiovascular depressor effects of cannabinoids appear to involve CB_1 expressed in peripheral tissues, including blood vessels (13, 17, 29), the heart (5), and sympathetic nerve terminals (20, 30). The possible involvement in cardiovascular depressor responses of CB_2 (15), another cannabinoid receptor primarily expressed by immune cells (33), is less well documented.

Activation of CB_1 by certain synthetic cannabinoids can cause profound and prolonged hypotension (26), which has raised the possible involvement of the CB_1/endocannabinoid system in pathological states associated with hypotension, such as various forms of shock. Indeed, the CB_1 antagonist SR141716 has been reported to inhibit or reverse the hypotension associated with hemorrhagic (48), endotoxemic (46), and cardiogenic shock (47) and the hypotension that accompanies advanced liver cirrhosis (2, 41). There is also evidence that in these conditions, macrophage- and platelet-derived endocannabinoids, including anandamide and 2-arachidonoylglycerol (2-AG) are responsible for the activation of SR141716-sensitive receptors (2, 41, 46-48, 50).

Recent studies indicate that anandamide can elicit vasodilation through a number of mechanisms in addition to the possible activation of vascular CB_1 (17, 25), including the activation of vanilloid TRPV1 receptors on sensory nerve terminals (51). Of particular interest is a novel endothelial site of action which, similar to CB_1, is G_i/G_o coupled and inhibited by SR141716, but does not interact with other CB_1 or CB_2 agonists or antagonists (18, 23, 32, 34). A similar situation may exist in the heart, where the negative inotropic effects of cannabinoids including anandamide can be mediated both by CB_1 receptors (5) and by an SR141716-sensitive mechanism that does not involve CB_1 receptors (8). Shock-related hypotension, such as the hypotension induced by bacterial endotoxin (lipopolysaccharide [LPS]), may involve both vasodilation and decreased cardiac contractility, and the relative role of endocannabinoids and their receptors in these two mechanisms has not been determined. Furthermore, cannabinoid receptors have been implicated in endotoxin-induced hypotension based on the ability of SR141716 to prevent this effect, and the relative role of CB_1 versus SR141716-sensitive receptors distinct from CB_1 or CB_2 needs to be explored. Here we report that LPS elicits similar, SR141716-sensitive hypotension in anesthetized wild-type mice and in mice deficient in CB_1 or in both CB_1 and CB_2. In anesthetized rats, the acute (< 2h) hypotensive phase following LPS injection is primarily due to a decrease in cardiac contractility, and both the hypotension and the decreased contractility are prevented by SR141716 but are unaffected by another CB_1 antagonist, AM251 (12).

MATERIALS AND METHODS

Reagents. SR141716 [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 H-pyrazole-3-carboxamide] was provided by the National Institute on Drug Abuse drug supply service, Research Triangle Park, NC; AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 H-pyrazole-3-carboxamide was from Tocris Cookson, Baldwin, MO. SR141716 and AM251 were dissolved in a vehicle of corn oil:water (1:4) emulsified by the addition of Pluronic F-68 (40 mg/ml). The concentration of the antagonists in this vehicle was 10 mg/ml, and vehicle or drug were injected in a volume of 0.3 ml/kg body weight. LPS (*E. coli,* 0127:B8) and Pluronic F-68 were from Sigma Chemicals, St.Louis, MO.

Animals. Male Sprague-Dawley rats weighing 300-350 g were obtained from Harlan (Indianapolis, IN). $CB_1^{-/-}$ mice, their homozygous controls ($CB_1^{+/+}$) and $CB_1^{-/-}/CB_2^{-/-}$ double knockouts were developed and backcrossed to a C57Bl6/J background by Andreas Zimmer and Nancy E. Buckley, as described earlier (23). $CB_1^{-/-}$ mice were bred from heterozygote breeding pairs and genotyped using a PCR-based assay and DNA extracted from tail snips obtained at the time of weaning. $CB_1^{-/-}/CB_2^{-/-}$ double knockout mice were bred from breeding pairs that were homozygote knockouts for both receptor genes.

Hemodynamic measurements. Rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.) and tracheotomized to facilitate breathing. The animals were placed on heating pads, and core temperature measured via a rectal probe was maintained at 37°C. To measure left ventricular systolic pressure (LVSP) and the maximal slope of systolic pressure increment (+dP/dt), a microtip catheter (SPR-524; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle (LV) under pressure control as described (36-38). A P50 tube was also inserted into the right femoral artery and vein for measurement of arterial blood pressure and i.v. injections,

respectively. In some experiments, a saline-filled cannula connected to a pressure transducer was inserted into the right jugular vein for monitoring central venous pressure. Aortic blood flow was measured using the transit time technology, by placing a flow probe (2.5SB733, Transonic Systems, Ithaca, NY) on the abdominal aorta below the renal arteries following midline laparotomy. Blood flow in the abdominal aorta was used to calculate hindquarter vascular resistance, representing predominantly skeletal muscle, skin and bone. After stabilization for 20 minutes, the signals were continuously recorded with a Powerlab/4SP A/D converter at 1 kHz (AD Instruments, Mountain View, California), stored, and displayed on a personal computer (36-38). The heart rate, maximal LVSP, mean arterial pressure (MAP), and +dP/dt were calculated as previously described (36-38). Peripheral resistance index (PRI) was calculated as MAP/mean aortic blood flow/100 g body weight and changes expressed as % of control.

Mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Polyethylene cannulae (P10) were inserted into the carotid artery and jugular vein for the measurement of mean arterial pressure and for drug injections, respectively.

All animal procedures were in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee.

Data analyses. Values are expressed as mean ± SEM. Time-dependent variables were analyzed by ANOVA followed by Bonferroni's *post-hoc* test. Differences were deemed statistically significant when $p<0.05$.

RESULTS

Hemodynamic effects of LPS in anesthetized rats are inhibited by SR141716. In pentobarbital-anesthetized rats, i.v. injection of 10 mg/kg LPS caused an acute hypotensive response that lasted up to 1 hour. As shown in Fig. 1, the hypotension was associated with moderate tachycardia and a major decrease in left ventricular contractility, as indicated by dramatic decreases in +dP/dt and LVSP. Aortic blood flow, an indicator of cardiac output, also decreased, and the peripheral resistance index (PRI) increased. Pretreatment of the rats with either vehicle or 3 mg/kg SR141716 10 min prior to the injection of LPS had no significant hemodynamic effect by itself, as also illustrated by the 0 min values in Fig. 1 which were similar in the vehicle- and in the SR141716-treated groups. However, SR141716 pretreatment nearly completely prevented the hypotension without influencing the tachycardic response to LPS, and also completely prevented the LPS-induced decrease in cardiac contractility, but only slightly delayed the decrease in aortic blood flow and the increase in PRI.

SR141716 was also able to reverse the effects of LPS when administered after the acute hemodynamic effects of LPS had developed. As illustrated in Fig. 2, in 5 rats the i.v. injection of 3 mg/kg SR141716 15 min following LPS treatment induced an immediate and rapid increase in arterial pressure that significantly exceeded the gradual increase in pressure in 5 other LPS-treated animals injected with vehicle only. SR141716 also caused a rapid increase in LVSP and +dP/dt that not only reached but then exceeded pre-LPS baseline levels.

Differential effects of SR141716 and AM251 on the hemodynamic response to LPS. To explore the role of CB_1 in the acute hypotensive response to LPS, rats were pretreated with a 3 mg/kg i.v. dose of the CB_1 antagonists SR141716 or AM251 ten minutes prior to the similar administration of 10 mg/kg LPS. This dose of SR141716 was shown to completely block the hypotensive response of anesthetized rats to a near-

maximal dose (10 μg/kg i.v.) of the potent synthetic CB_1 agonist HU-210 (26), and in separate experiments 3 mg/kg AM251 caused a similar, complete blockade of the hypotensive effect of 10 μg/kg HU-210 (not shown). As illustrated in Fig. 3, there was a dramatic difference between the ability of the two antagonists to antagonize the effect of LPS. Unlike SR141716, which nearly completely prevented the hypotensive response to LPS (see also Figs. 1 and 2), AM251 had no effect at all: LPS caused as great a hypotensive response in the absence as in the presence of AM251.

LPS-Induced hypotension in wild-type and CB receptor-deficient mice. The inability of AM251 to inhibit the hypotensive response to LPS suggested that the SR141716-sensitive receptors involved are not CB_1. To further test this, the acute hypotensive response to LPS was tested in pentobarbital-anesthetized mice deficient in CB_1 ($CB_1^{-/-}$) or both CB_1 and CB_2 ($CB_1^{-/-}/CB_2^{-/-}$), and in their wild-type littermates. As illustrated in Fig. 4, i.v. injection of 100 μg/kg LPS caused similar hypotension in the three groups and, in all three, the hypotensive response to LPS could be prevented by pretreatment with 3 mg/kg SR141716.

DISCUSSION

We have previously reported that the CB1 antagonist SR141716 inhibits the acute hypotension and decreases mortality in response to LPS treatment in rats (46). The present findings confirm the ability of SR141716 to inhibit LPS-induced hypotension, yet indicate that this effect and the underlying hemodynamic changes are not mediated by CB_1. AM251 is a selective CB_1 antagonist at least equipotent with SR141716 (12), and at the *in vivo* dose used here it was reported to block the hemodynamic effects of potent CB_1 agonists in rats (11). Thus, its complete inability to influence the LPS response (Fig. 3) is not compatible with CB_1 involvement. Furthermore, LPS-induced hypotension in mice and its sensitivity to inhibition by SR141716 were not influenced by the genetic ablation of CB_1 or both CB_1 and CB_2 (Fig. 4).

Several recent studies point to the existence of additional cannabinoid receptors distinct from CB_1 or CB_2. At least two of these, a putative G_i/G_o-coupled endothelial receptor mediating vasodilation in certain vascular beds (18, 23, 32, 34), and a receptor postulated to be present on glutamatergic terminals in the hippocampus (14), have been shown to be uniquely sensitive to inhibition by SR141716 but not by other CB_1 antagonists such as AM251, although their differential sensitivity to the agonist WIN55,212-2 (14, 32, 34) suggests that they are distinct molecular entities. LPS is a potent stimulant of anandamide synthesis in macrophages (28, 46), and LPS-treated macrophages were found to elicit SR141716-sensitive hypotension when injected into normal control rats (28, 46). Since anandamide can interact with SR141716-sensitive non-CB_1/non-CB_2 receptors described above (23, 32, 34), one might postulate that such a receptor rather than CB_1 may mediate the acute hemodynamic effects of LPS.

Macrophages isolated from mice deficient in fatty acid amidohydrolase, the enzyme responsible for anandamide metabolism, and stimulated *in vitro* with LPS have higher anandamide levels and elicit a greater decrease in blood pressure in recipient rats

than similarly treated cells isolated from wild-type littermates (28). Although these findings suggest that macrophage-derived anandamide or a related fatty acid amide may mediate the acute hemodynamic effect of LPS, other mechanisms, such as a reported LPS-induced increase in target organ sensitivity to endocannabinoids (35) may also play a role.

The present findings also indicate, however, that the hypotensive effect of LPS is due to decreased cardiac contractility, which leads to a decrease in stroke volume and cardiac output, rather than vasodilation, and the decrease in contractility is so profound that arterial pressure decreases despite a parallel increase in peripheral resistance. This hemodynamic pattern of a primary decrease in cardiac contractility is similar to that reported in several recent studies in both anesthetized (19, 31, 36, 39) and conscious rats (42), although an LPS-induced vasodilation in certain vascular beds, such as the renal vasculature (11), the heart and the brain (44) would not be detected in the present experiments due to the positioning of the aortic flow probe. The primary cardiodepressor effect of LPS suggests that SR141716 must have a myocardial site of action as well. Indeed, SR141716 not only prevents the marked decrease in dP/dt and LVSP of subsequently administered LPS, but elicits an immediate reversal of the decline in these parameters to levels above control values when it is administered once the hemodynamic response to LPS has fully developed. Similarly, cardiac contractility increased above control values when LPS was administered after pretreatment with SR141716 (Fig. 1). This is an interesting phenomenon in which increased sympathetic nervous system drive may play a role: LPS has been reported to increase sympathetic tone in rats, as indicated by a rise in plasma catecholamines (21). Thus, the net effect of LPS on cardiac contractility may be determined by the balance between endocannabinoid-mediated negative and sympathetically mediated positive inotropy, although the involvement of additional mechanisms cannot be excluded.

In contrast, SR141716 only slightly delays but does not significantly inhibit the LPS-induced decrease in aortic blood flow and increase in peripheral resistance. Although these findings do not exclude the possibility that SR141716 may antagonize LPS-induced vasodilation in vascular beds where they do occur, it appears that the primary site of action of SR141716 in this experimental model is cardiac, not vascular. An SR141716-sensitive, AM251-insensitive negative inotropic response to anandamide has been described in the rat Langendorff heart preparation (8), and a similar receptor site may be involved in the *in vivo* effects described here. The molecular identity of this site and its possible relation to the G_i/G_o-coupled endothelial receptor for anandamide remain to be determined.

Interestingly, our recent observations indicate that in various forms of hypertension a compensatory hypotensive endocannabinergic tone is activated, which can be reversed equally effectively by SR141716 and AM251, and is associated with upregulation of vascular and cardiac CB_1 (3). Thus, endocannabinoids are hypotensive regulators whose tonic effects may be mediated by different types of receptors in different pathological conditions.

Previous studies have implicated tumor necrosis factor-α (TNFα)(10, 43) and platelet-activating factor (PAF)(1) in the cardiodepressor effects of endotoxin. SR141716 does not inhibit PAF or TNFα receptors (unpublished observations). However, TNFα and/or PAF may act via the release of an endocannabinoid such as anandamide, which would then interact with an SR141716-sensitive receptor. Indeed, we have recently shown that a component of the hypotensive response of rats to PAF can be inhibited by SR141716 (28). Further studies are needed to identify the SR141716-sensitive myocardial site apparently involved in the acute hemodynamic effects of LPS.

CB_1 receptors in the central nervous system have been implicated in the control of appetite (7) and the rewarding effects of nicotine (6) and alcohol (49). Based on such findings, the antagonist SR141716, recently named rimonabant, is being developed for

the treatment of obesity, nicotine dependence and alcoholism. The present findings indicate that SR141716 has additional peripheral sites of action which are not shared by other CB_1 antagonists, but which may also be of therapeutic importance.

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Legend for figures

Figure 1. Hemodynamic effects of LPS in anesthetized rats in the absence and presence of the CB_1 antagonist SR141716. LPS (10 mg/kg i.v.) was injected at 0 min, 10 min following the injection of vehicle (●) or SR141716 (3 mg/kg i.v., O). Mean arterial pressure (MAP), heart rate (HR), left ventricular systolic pressure (LVSP), maximal slope of systolic pressure increment (+dP/dt), mean aortic blood flow (MAF) and peripheral resistance index (PRI) were monitored or computed as described in Methods. Points and bars represent means ± SEM from experiments in 4-5 separate animals. Significance ($p<0.05$) is indicated for difference from corresponding baseline value in the vehicle + LPS group (*) or between corresponding values in the two treatment groups (#).

Figure 2. SR141716 reverses the hemodynamic effects of LPS. SR141716 (3 mg/kg) or vehicle were injected i.v. 15 min following the i.v. injection of 10 mg/kg LPS, as indicated by arrows. Mean ± SEM from 5 rats treated with SR141716 (O) or 5 other animals treated with vehicle (●). Mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), and maximal slope of systolic pressure increment (+dP/dt) were continuously monitored and calculated at the indicated time points.

Figure 3. LPS-induced hypotension is inhibited by SR141716 but not by AM251. Representative tracings of the effects of LPS on MAP in a rat pretreated with vehicle (left), SR141716 (3 mg/kg i.v., center), and AM251 (3 mg/kg i.v., right) are shown on top, mean ± SEM from similar experiments from 4-5 animals are indicated by the columns and bars in the bottom. * indicates significant difference from values in vehicle + LPS-treated rats, $p<0.05$.

Figure 4. The effect of LPS on mean arterial pressure (MAP) in wild-type (A), $CB_1^{-/-}$ (B) and $CB_1^{-/-}/CB_2^{-/-}$ mice (C) pretreated with vehicle (●) or SR141716 (3 mg/kg i.v. O)**.** The number of animals in the three groups were 5 (A), 5 (B), and 4 (C). * indicates significant difference from corresponding value in the vehicle + LPS group.

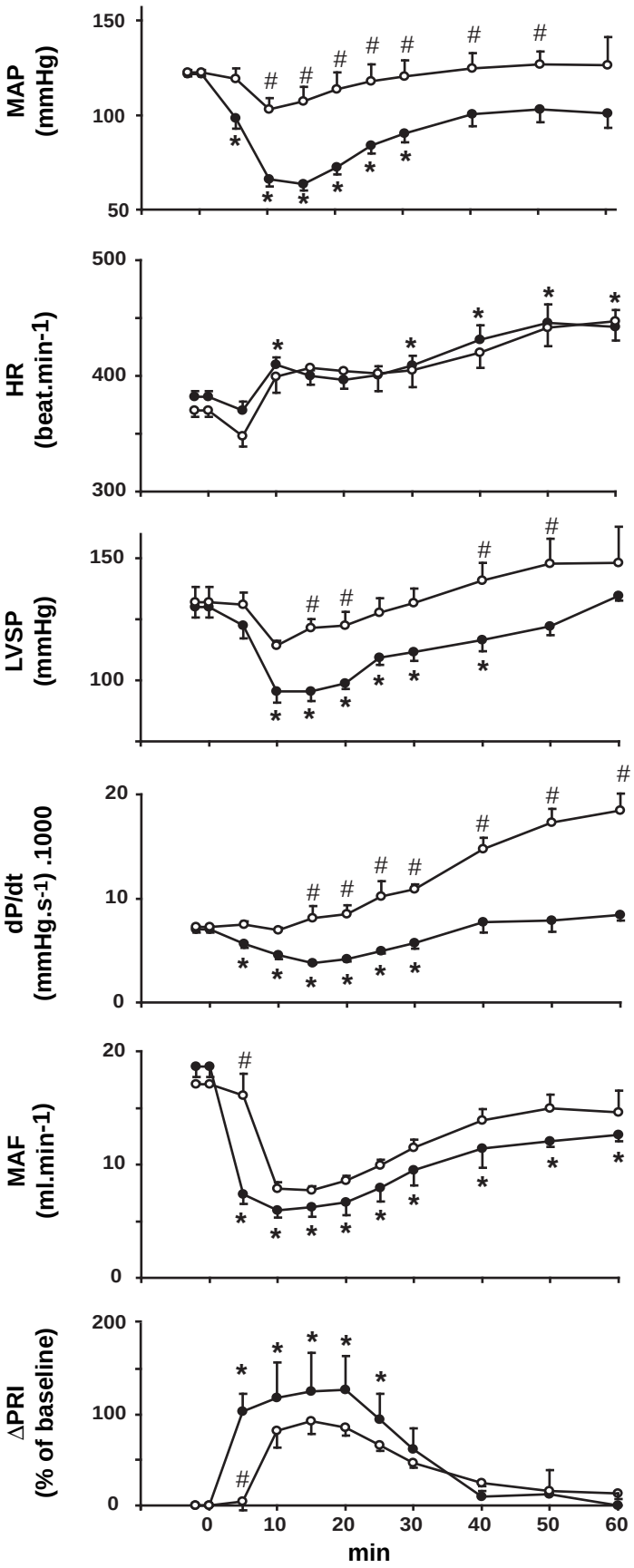
MAP (mmHg)
150
100
50
HR (beat.min-1)
500
400
300
LVSP (mmHg)
150
100
dP/dt (mmHg.s-1) .1000
20
10
0
MAF (ml.min-1)
20
10
0
ΔPRI (% of baseline)
200
100
0
0
10
20
30
40
50
60
min

Figure 1.

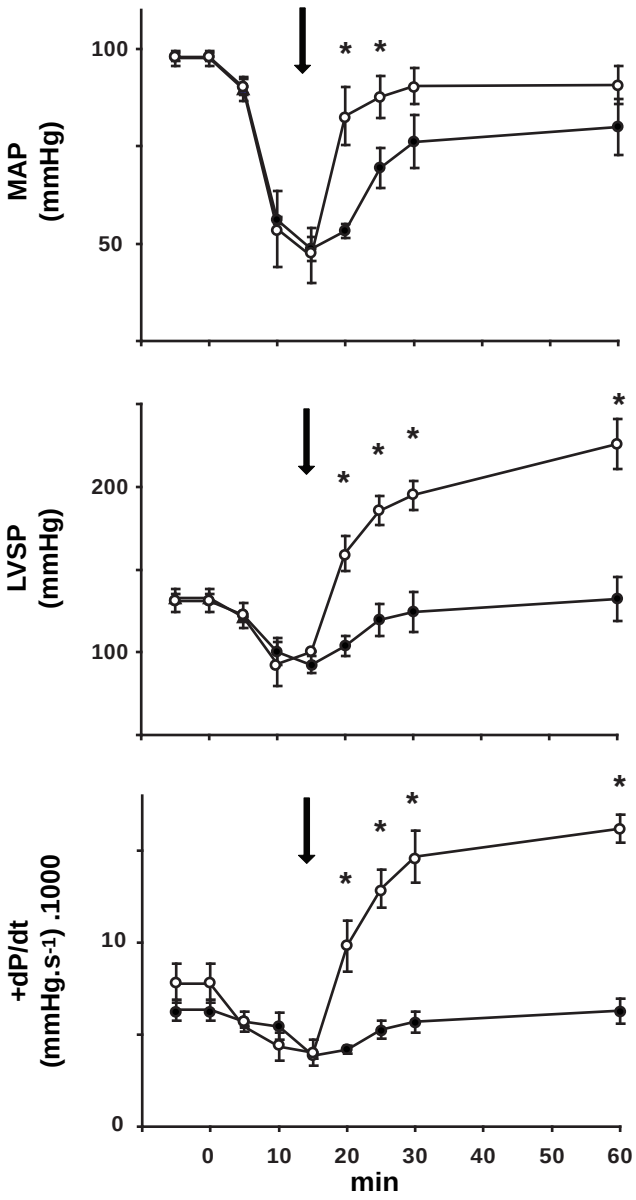
MAP
(mmHg)
100
50
LVSP
(mmHg)
200
100
+dP/dt
(mmHg.s-1) .1000
10
0
0
10
20
30
40
50
60
min

Figure 2.

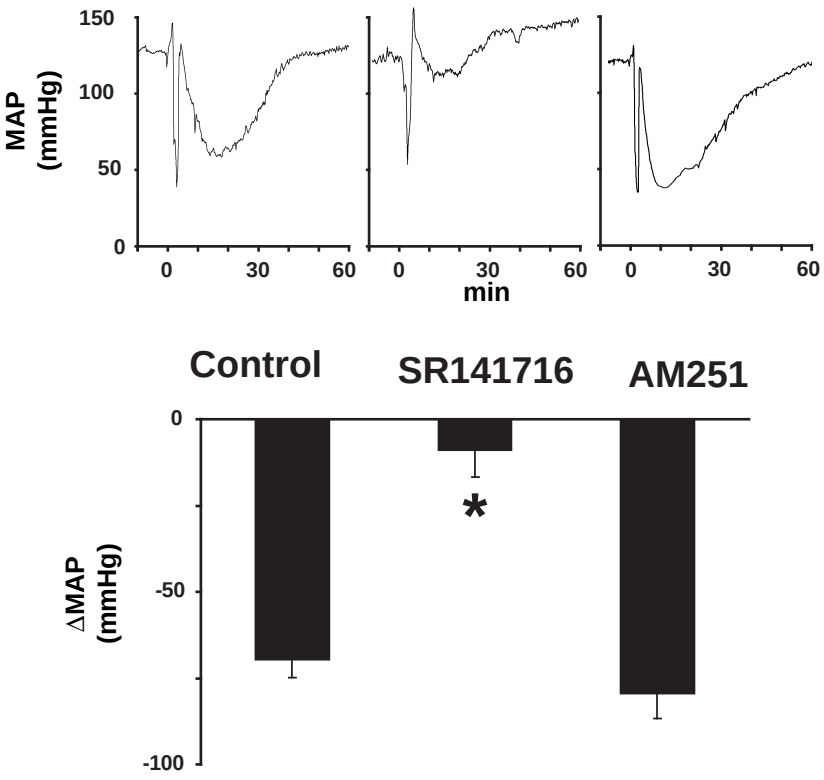
MAP
(mmHg)
150
100
50
0
0
30
60
0
30
60
0
30
60
min
Control
SR141716
AM251
0
-50
-100
ΔMAP
(mmHg)
*

Figure 3

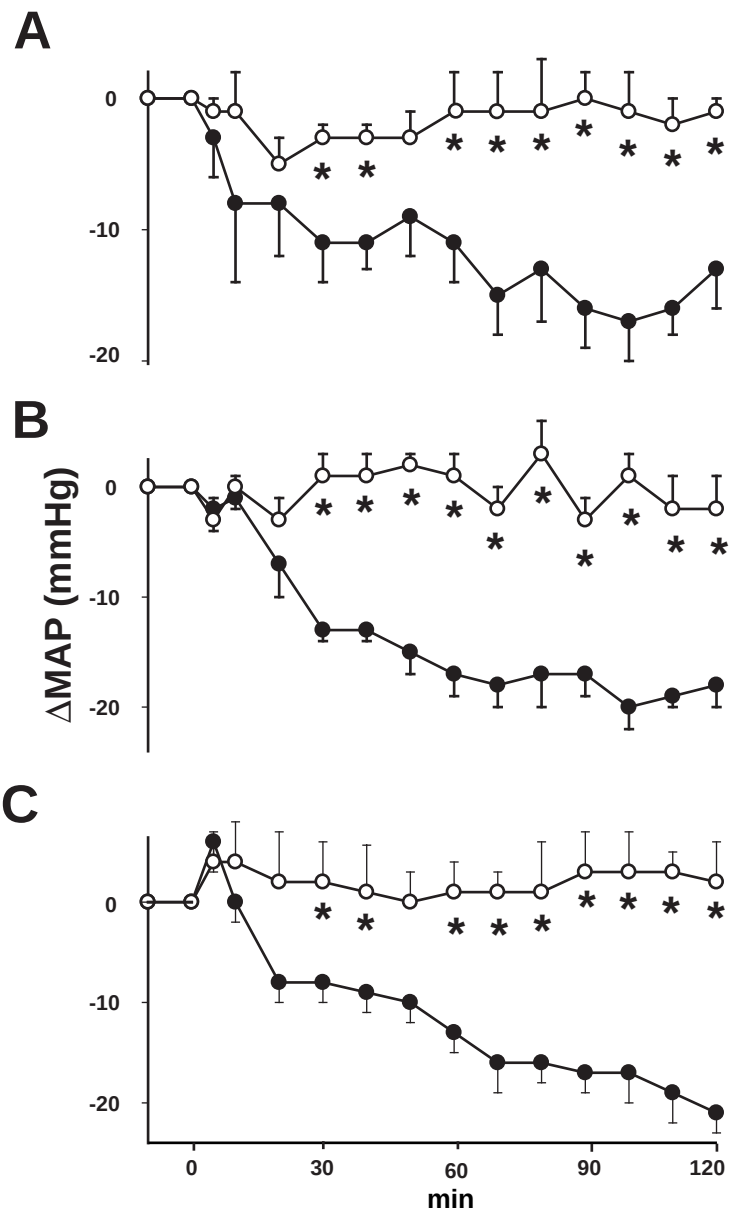

Figure 4