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## NOVEL INHIBITOR OF FATTY ACID AMIDE HYDROLASE NORMALIZES CARDIOVASCULAR FUNCTION IN HYPERTENSION WITHOUT ADVERSE METABOLIC EFFECTS

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### SUMMARY

The enzyme fatty-acid amide hydrolase (FAAH) catalyzes the *in vivo* degradation of the endocannabinoid anandamide, thus controlling its action at receptors. A novel FAAH inhibitor, AM3506, normalizes the elevated blood pressure and cardiac contractility of spontaneously hypertensive rats (SHR) without affecting these parameters in normotensive rats. These effects are due to blockade of FAAH and a corresponding rise in brain anandamide levels, resulting in CB<sub>1</sub> receptor-mediated decrease in sympathetic tone. The supersensitivity of SHR to CB<sub>1</sub> receptor-mediated cardiovascular depression is related to increased G-protein coupling of CB<sub>1</sub> receptors. Importantly, AM3506 does not elicit hyperglycemia and insulin resistance seen with other FAAH inhibitors or in FAAH<sup>-/-</sup> mice, which is related to its inability to inhibit FAAH in the liver due to rapid hepatic uptake and metabolism. This unique activity profile offers improved therapeutic value in hypertension.

### HIGHLIGHTS

Unique pharmacological profile of novel fatty acid amide hydrolase inhibitor  
Selectivity of AM3506 in FAAH inhibition in brain over liver *in vivo*

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AM3506 as centrally acting antihypertensive agent with minimal cardiovascular effects under normotensive conditions and without adverse metabolic effects

## INTRODUCTION

Endocannabinoids are polyunsaturated fatty acid amides such as arachidonoyl ethanolamide (anandamide) or esters such as 2-arachidonoyl glycerol (2-AG) that mobilize “on demand” and signal through two  $G_{i/o}$  protein-coupled cannabinoid receptors (Pacher, et al., 2006):  $CB_1$  receptors, highly expressed in the brain but also present at much lower yet functionally relevant concentrations in peripheral tissues, and  $CB_2$  receptors, expressed predominantly in immune and hematopoietic cells (Howlett, 2005).

The concentration of endocannabinoids at target receptors is tightly controlled by enzymes that generate and eliminate them. Anandamide is preferentially degraded *in vivo* by fatty acid amide hydrolase (FAAH)(Cravatt, et al., 1996), whereas 2-AG is metabolized primarily by monoacylglyceride lipase (MAGL)(Dinh, et al., 2002), with additional involvement of  $\alpha\beta$ -hydrolase domain-containing 6 (ABHD6)(Marrs, et al., 2010). Pharmacological blockade or genetic ablation of FAAH in mice result in elevated tissue levels of anandamide, as well as  $CB_1$  receptor-mediated reductions in anxiety-like behavior (Kathuria, et al., 2003; Moreira, et al., 2008), pain sensation (Cravatt, et al., 2001) and enhanced hypotensive responsiveness to exogenous anandamide (Pacher, et al., 2005). The FAAH inhibitor URB597 was also found to normalize blood pressure in rats with various forms of hypertension by decreasing cardiac contractility and total peripheral resistance through a  $CB_1$  receptor-mediated mechanism (Batkai, et al., 2004). In humans, higher anandamide plasma levels have been linked to lower blood pressure in young males with a functional FAAH gene variant (Sarvani, et al., 2008) that had been found to result in reduced enzymatic activity (Chiang, et al., 2004). FAAH deficiency also promotes excessive energy storage and insulin resistance through a food intake-independent mechanism (Tourino, et al., 2010). Thus, a dynamic relationship exists between endocannabinoid tone and cardiovascular and metabolic functions.

Here we report the structure and pharmacological profile of a novel, highly potent and *in vivo* effective FAAH inhibitor, AM3506. AM3506 is more potent than URB597 in reducing blood pressure, heart rate and cardiac contractility in hypertensive animals through activation of  $CB_1$  receptors, which correlates with the blockade of FAAH activity and the consequent increase in tissue anandamide levels. The cardiovascular effects of AM3506 can be attributed to central  $CB_1$  receptor-mediated reduction in sympathetic tone, and the much greater responsiveness of hypertensive compared to normotensive animals is related to increased coupling of  $CB_1$  receptors to G proteins in the CNS of the former. Finally, unlike URB597 which blocks FAAH in both brain and liver and causes hyperglycemia in rats and glucose intolerance in obese mice, AM3506 does not block FAAH activity in liver and has no obvious effect on these glycemic parameters.

## RESULTS

### Structure of AM3506

AM3506 (Fig. 1E) is a hydroxyl substituted phenylalkylsulfonyl fluoride (Deutsch, et al., 1997) structurally related to the broad spectrum serine protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF), but unrelated to the currently available *in vivo* effective FAAH inhibitors, such as the carbamate URB597 (Kathuria, et al., 2003) or the piperidine urea-based compounds (Ahn, et al., 2009).

## FAAH Inhibitory Activity and Target Specificity of AM3506

The effectiveness and target selectivity of AM3506 to inhibit FAAH activity was tested using several approaches. First, mice were treated with bolus i.p. doses of 0.1 or 1 mg/kg AM3506, and the activity of serine hydrolases in brain tissue obtained after 1 h was assayed using competitive activity-based protein profiling (Leung, et al., 2003). As illustrated in Fig. 1A, treatment of mice with 0.1 mg/kg AM3506 i.p. reduced brain FAAH activity with no change in the activity of any of a large number of other serine hydrolases whereas at 1 mg/kg, AM3506 completely blocked FAAH and partially inhibited MAGL and ABHD6. Using the same technique, AM3506 had similarly low 'off-target' effects in the liver, with only MAGL and an unidentified, ~58kD protein, showing partial inhibition at the higher, 1 mg/kg dose. The effect of AM3506 on hepatic FAAH could not be assessed using this technique, due to the presence of prominent interfering proteins of similar molecular weight.

In the assay measuring the release of [<sup>3</sup>H]ethanolamine from [<sup>3</sup>H]anandamide (Fig. 1B), AM3506 caused dose-dependent inhibition of brain FAAH activity (ID<sub>50</sub>=0.10 mg/kg). Surprisingly, AM3506 was devoid of substantial FAAH inhibitory activity in liver of the same mice in doses up to 1 mg/kg. In contrast, the reference compound URB597 had similar FAAH inhibitory activity in liver (ID<sub>50</sub>=0.45 mg/kg) as in brain (ID<sub>50</sub>=0.22 mg/kg).

Using this same assay, both AM3506 and URB597 were fully effective orally and produced the same pattern of FAAH inhibition as after parenteral administration. Thus, 1 mg/kg AM3506 suppressed the enzyme in brain (by 90±0.4%), but had little effect on hepatic FAAH activity (by 13±2.6%; n=5), whereas 10 mg/kg URB597 equally suppressed FAAH activity in brain (by 86±2%) and liver (83±0.5%; n=5). The duration of FAAH inhibition was also tested. Following treatment with 1 mg/kg AM3506 i.p., FAAH was inhibited by >99% for up to 2 hours, ~90% at 12 hours and ~80% 24 hours in agreement with the covalent nature of its interaction with its target, as reported earlier for fluorosulfonates, PMSF and some other fluorophosphonates (Seierstad and Breitenbucher, 2008).

The FAAH inhibitory activity of AM3506 and URB597 *in vitro* was also tested against human recombinant FAAH (Fig. 1C). In this assay, AM3506 was twice as potent as URB597 (IC<sub>50</sub>s of 48 nM and 118 nM, respectively), with PMSF being 3 orders of magnitude less potent. AM3506 weakly inhibited human recombinant MAGL with an IC<sub>50</sub> of 11 μM (Fig. 1D), for a FAAH:MAGL potency ratio of >220-fold.

The assay based on the release of [<sup>3</sup>H]ethanolamine from [<sup>3</sup>H]anandamide was used to quantify FAAH activity in SHR and WKY brain (Fig. 2). Basal FAAH activity was similar in SHR (59.1±0.8) and WKY (62.6±1.1 fmol/mg/min, n=3–5), and was equally inhibited by AM3506, with ID<sub>50</sub> values close to 0.1 mg/kg. The dose-dependent inhibition of FAAH by AM3506 was accompanied by parallel increases in brain anandamide levels, whereas 2-AG levels remained unaffected by AM3506 (Fig. 3). In a similar, *ex vivo* MAGL assay measuring the release of [<sup>3</sup>H]glycerol from 2-oleoyl [<sup>3</sup>H]glycerol in brain homogenates of mice treated 1 hour prior to sacrifice with vehicle vs. different i.p. doses of AM3506, no inhibition was observed at 0.3 and 1.0 mg/kg (−5.2±4.8% and −1.9±4.8%, respectively, n=4 in each group), and moderate inhibition was observed only after 3 mg/kg (−38.8±7.1%, n=4). This indicates >30-fold *in vivo* selectivity for FAAH over MAGL and also that the maximally effective *in vivo* dose of 1 mg/kg caused complete inhibition of FAAH with no inhibition of MAGL.

AM3506 had low affinity for binding to CB<sub>1</sub> (5.77 μmol/L) and moderate affinity for CB<sub>2</sub> receptors (192 nmol/L), and did not affect anandamide transport at concentrations up to 20 μmol/L.

## Cardiovascular Effects of AM3506 are Mediated by Endogenous Anandamide Acting via CB<sub>1</sub> Receptors

In SHR, AM3506 (1 mg/kg i.v.) elicited a profound and sustained decrease in MAP as well as marked bradycardia (Fig. 3A), whereas only slight changes in MAP and HR were observed in WKY (Fig. 3B). The effects of AM3506 in SHR were dose-dependent, with hypotensive and bradycardic ED<sub>50</sub> values of 0.14 and 0.10 mg/kg, respectively, whereas URB597 was ~40 times less potent, with corresponding ED<sub>50</sub> values of 5.2 and 4.0 mg/kg. The effects of AM3506 were prevented by pretreatment with the CB<sub>1</sub> antagonist AM251, which also acutely reversed the fully developed effects (Fig. 3A). Similar acute reversal was elicited by the CB<sub>1</sub> antagonist rimonabant (3 mg/kg,  $\Delta$ MBP: +101±9 mmHg;  $\Delta$ HR: +129±14 bpm), but not by a novel, peripherally restricted CB<sub>1</sub> antagonist, AM6545 (Fig. 3A). In chronically cannulated, conscious SHR, AM3506 (0.5 mg/kg i.v.) reduced MAP and HR from 176 ±4 to 127±5 mmHg (P<0.01) and 355±6 to 286±22 bpm (P<0.05, n=4), and the effects lasted up to an hour.

AM3506 (0.5 mg/kg i.v.) also caused hypotension and bradycardia in awake, chronically cannulated SHR. Peak decreases in MAP and HR were from 172 ± 4 to 128 ± 5 mmHg (P < 0.001, n = 5) and from 345 ± 10 to 290 ± 15 bpm (P < 0.05), respectively, and the effects lasted up to 60 minutes.

As shown in Fig. 4A, 1 mg/kg AM3506 caused a marked decrease in cardiac contractility in SHR, as assessed by the end-systolic pressure-volume relation (ESPVR), a load-independent indicator of systolic contractility (Kass, et al., 1987), as well as by decreases in left ventricular end-systolic pressure (LVESP) (Fig 4B) and dP/dt<sub>max</sub> (Fig 4C), total peripheral resistance (TPR) (Fig 4D) and cardiac output (CO) (Fig 4E). No changes of these parameters were observed in normotensive WKY rats (Fig. 4B–E).

## Cardiovascular Effects of AM3506 Depend on Autonomic Nervous Tone

To test whether the effects of AM3506 are mediated via the autonomic nervous system, autonomic tone was disrupted by ganglionic blockade. Treatment of SHR with hexamethonium (C6, 20 mg/kg i.v) caused a profound drop in MAP and HR, which were then restored to the preexisting baseline by continuous infusion of vasopressin and isoproterenol, respectively. Blockade of autonomic tone was verified by the near-complete loss of reflex tachycardia or bradycardia in response to bolus injections of sodium nitroprusside or phenylephrine, respectively. In such animals, the hypotension and bradycardia elicited by 1 mg/kg AM3506 were reduced by 89.6±3.6% and 74.0±7.7%, respectively (Fig. 5A, n=5–13; P<0.001), indicating the dependence of these effects on intact autonomic tone. As a further indication that AM3506 decreases sympathetic tone, plasma norepinephrine levels in anesthetized SHR were reduced by 82±5%, from 311±41 to 53±12 pg/mL (n=5, P<0.01) 30 min after the i.v. administration of 1 mg/kg AM3506. In WKY, baseline plasma norepinephrine was lower than in SHR (198±11 pg/mL, P<0.05) and remained unaffected by AM3506 (167±35 pg/mL, P>0.2, n=3).

## Increased Cardiovascular Responsiveness of Hypertensive Rats to CB<sub>1</sub> Receptor Activation

The greater depressor responses to AM3506 in SHR vs WKY despite similar degree of FAAH inhibition and rise in brain anandamide suggest increased target-tissue responsiveness to CB<sub>1</sub> receptor activation in SHR. Indeed, the cannabinoid agonist HU210 also elicited much greater hypotension in SHR than in WKY (Fig. 5B). In both strains, the effects of HU210 were abolished by AM251 or by ganglionic blockade, indicating the involvement of neuronal CB<sub>1</sub> receptors.

The sympathoinhibitory effects of HU210 or AM3506 may be triggered in the CNS or at presynaptic CB<sub>1</sub> receptors on postganglionic sympathetic nerve terminals in the heart and vasculature. To distinguish between these possibilities, we tested the cardiovascular effects of a non-brain penetrant CB<sub>1</sub>/CB<sub>2</sub> mixed agonist, CB13 (Dziadulewicz, et al., 2007). The hypotensive effect of 0.3 mg/kg CB13 was similar in SHR and WKY ( $-40 \pm 6$  vs  $-36 \pm 10$  mmHg), suggesting that the increased hypotensive effectiveness of HU210 in SHR is due to a central mechanism.

### Increased Efficiency of CB<sub>1</sub> Receptor Coupling in SHR

The increased responsiveness of SHR to direct activation of CB<sub>1</sub> receptors suggested increased expression and/or increased coupling efficiency of CB<sub>1</sub> receptors. CB<sub>1</sub> receptor density, as quantified by [<sup>3</sup>H]-rimonabant binding, was similar in WKY and SHR brain ( $1.58 \pm 0.16$  vs  $1.60 \pm 0.06$  pmol/mg protein, respectively), with slightly higher affinity for the radioligand in SHR ( $0.71 \pm 0.09$  nmol/L) than in WKY ( $1.92 \pm 0.53$  nmol/L). However, CB<sub>1</sub> receptor coupling efficiency, as deduced from HU210-stimulated GTP $\gamma$ S labeling, was dramatically increased in brain membrane preparations from SHR vs WKY (Fig. 5C).

### Hepatic FAAH is not Inhibited by AM3506 Due to its Rapid Hepatic Metabolism

Following *in vivo* administration, a maximally effective dose of AM3506 (1 mg/kg i.p.) blocked FAAH activity in brain but not in liver, whereas an equimolar dose of URB597 (1.38 mg/kg i.p.) blocked FAAH activity equally in both tissues (Fig. 6A). In contrast, when the compounds were added *in vitro* to tissue homogenates at a concentration of 100 nM, they were both equally effective in blocking FAAH activity in brain and liver (Fig. 6B). This suggests that the ability of AM3506 to block FAAH is similar in brain and liver, but the uptake and metabolism of AM3506 by intact hepatocytes is rapid enough to prevent its access to FAAH.

To further explore this possibility, we incubated hepatocyte suspensions of different cell density with a fixed concentration (100 nM) of AM3506 or URB597 versus vehicle for 30 min, and measured FAAH activity in the cell pellet (Fig. 6C). In a dilute suspension of hepatocytes (1 mg wet cell weight/mL medium), both AM3506 and URB597 nearly completely blocked FAAH, similar to what was observed in cell-free homogenates. When free drug was measured as the parent ion in the cell-free supernatant, 50–55% of the drug added at 0 min was still present for both AM3506 and URB597 at 10 min. In contrast, in dense suspensions of hepatocytes (40 mg/mL), URB597, but not AM3506 blocked FAAH activity, which replicates the findings in intact animals. Further analysis of such preparations indicated that both drugs were rapidly depleted from the medium (Fig. 6D, left panel). In the cell pellets, URB597 levels gradually declined over 10 min, whereas AM3506 levels were below threshold immediately after the addition of the drug and remained undetectable for the remaining incubation period (Fig. 6D, 2<sup>nd</sup> panel from left). In these experiments, the 0 min time point did include the time needed to separate the pellet (1 min of centrifugation at 0°C). FAAH activity was rapidly and nearly completely blocked in the URB597-exposed cells, whereas there was no measurable change in FAAH activity in AM3506-incubated cells (Fig. 6D, 3<sup>rd</sup> panel from left). Parallel to FAAH activity, anandamide levels in hepatocytes were markedly increased by URB597, but remained unchanged by AM3506 (Fig. 6D, right panel). The recovery of both drugs from cell-free medium remained constant from 0 to 30 min, so spontaneous degradation could not account for their time-dependent loss in the presence of hepatocytes.

### Blockade of Hepatic FAAH by URB597 Results in Glucose Intolerance in Obese Mice

High-fat diet (HFD)-induced obesity (DIO) is associated with increased activity of the hepatic endocannabinoid/CB<sub>1</sub> receptor system, which contributes to obesity-related glucose

intolerance/insulin resistance (Osei-Hyiaman, et al., 2008). We tested whether acute treatment with FAAH inhibitors can influence glycemic control in DIO mice. As illustrated in Fig. 7A, treatment of male DIO mice with 1 mg/kg AM3506 did not affect glucose tolerance, as tested using the i.p. glucose tolerance test, whereas an equimolar dose of URB597 (1.38 mg/kg) resulted in a marked increase in glucose intolerance, even though it was much less effective in producing hypotension. Similar treatment with URB597 failed to affect glucose tolerance in liver-specific  $CB_1^{-/-}$  mice on HFD (Fig. 7B). Increasing the dose of URB597 to 10 mg/kg, which is equieffective with 1 mg/kg AM3506 in causing hypotension, caused the same degree of glucose intolerance in wild-type mice on HFD as the lower dose, and it remained ineffective in liver-specific  $CB_1^{-/-}$  mice on HFD (not shown). These findings indicate that blockade of FAAH in liver results in hepatic  $CB_1$  receptor-mediated increase in glucose intolerance.

URB597 (10 mg/kg i.p.) also increased non-fasting blood glucose in both SHR (from  $104.0 \pm 7.4$  to  $144.0 \pm 9.6$  mg/dL,  $n=4$ ,  $P<0.05$ ) and WKY (from  $90.0 \pm 7.2$  to  $135.3 \pm 12.9$  mg/dL,  $n=4$ ,  $P<0.05$ ) whereas AM3506, 1 mg/kg i.p., had no such effect (SHR:  $97.8 \pm 5.6$  to  $96.8 \pm 2.9$ ,  $n=6$ ; WKY:  $114.5 \pm 5.3$  to  $108.2 \pm 4.5$ ,  $n=6$ ). In the same rats, 10 mg/kg URB597 blocked hepatic FAAH activity by  $93.1 \pm 0.3\%$  in SHR and  $92.6 \pm 0.2\%$  in WKY, whereas 1 mg/kg AM3506 only marginally reduced it, by  $11.1 \pm 0.4\%$  in SHR and  $6.7 \pm 0.2\%$  in WKY ( $n=3-5$ ).

## DISCUSSION

Here we report the unique activity profile of a novel, highly potent and *in vivo* effective FAAH inhibitor, AM3506. Similar to another FAAH inhibitor, URB597, AM3506 reverses the increased blood pressure and cardiac contractility of hypertensive rats without causing similar effects in normotensive rats. These effects could be attributed to sympathoinhibition mediated indirectly by endogenous anandamide acting at  $CB_1$  receptors in the central nervous system. Unlike the other FAAH inhibitor, AM3506 does not inhibit FAAH activity in the liver and does not elicit hyperglycemia and insulin resistance seen after global inhibition or genetic ablation of FAAH, thus offering an improved therapeutic profile as a potential antihypertensive agent.

AM3506 is structurally unrelated to currently available FAAH inhibitors, and is a close structural analog of the serine protease inhibitor PMSF. The original observation that PMSF prolonged the half-life of anandamide and increased its apparent affinity for  $CB_1$  receptors in membrane preparations was the first evidence supporting the existence of an endogenous amidase being involved in the *in vivo* biodegradation of anandamide (Deutsch and Chin, 1993). Lead optimization efforts starting from AM374, an early generation FAAH inhibitor based on the PMSF structure (Deutsch, et al., 1997; Janero, et al., 2009; Lu, et al., 2006), led to AM3506, a sulfonyl fluoride analog with enhanced affinity and favorable oral bioavailability and brain penetrability. AM3506 is selective for FAAH over MAGL, the maximally effective *in vivo* dose of 1 mg/kg causing 100% inhibition of FAAH with no inhibition of MAGL, as verified using *ex vivo* enzyme activity assays.

Earlier observations indicated that the endocannabinoid system remains quiescent under normotensive conditions, but becomes activated in various experimental models of hypertension as a compensatory mechanism (Batkai, et al., 2004). This allows for the use of SHR as a model to study the cardiovascular consequences of modulating the activity of the endocannabinoid system (Batkai, et al., 2004). AM3506 caused strong cardiovascular depression in SHR and only modest hypotension and bradycardia in normotensive WKY rats. While these effects are similar to the earlier reported effects of the reference FAAH antagonist URB597 (Batkai, et al., 2004), they expand those findings in several ways.

First, ganglionic blockade combined with restoration of baseline vascular tone and heart rate resulted in a near complete loss of the hypotensive and bradycardic responses to AM3506 in SHR. These findings make it unlikely that modest increases in CB<sub>1</sub> receptor expression in heart and aortic smooth muscle of SHR compared to WKY rats, as reported earlier (Batkai, et al., 2004), could account for the increased responsiveness of SHR to CB<sub>1</sub> receptor activation, and indicate the dominant role of neuronal CB<sub>1</sub> receptors. Reduction of sympathetic tone in SHR by AM3506 is further indicated by the marked reduction in plasma norepinephrine. This could result from a centrally triggered reduction in sympathetic outflow, or from activation of presynaptic CB<sub>1</sub> receptors located at sympathetic nerve terminals in the heart and vasculature (Varga, et al., 1996). The former mechanism is favored, however, by the observation that the cardiovascular depressor response to AM3506 could be reversed by both rimonabant and AM251, brain-penetrant CB<sub>1</sub> antagonists, but not by AM6545, a novel, peripherally restricted CB<sub>1</sub> antagonist (Tam, et al., 2010). Furthermore, the moderate hypotensive effect of a peripherally restricted cannabinoid agonist was similar in SHR and WKY.

Second, there are both quantitative and qualitative differences between the FAAH inhibitory actions of AM3506 and URB597. AM3506 is more potent than URB597 in reducing blood pressure and heart rate as well as in inhibiting FAAH activity in brain, and its effects are much longer lasting. Unexpectedly, AM3506 failed to inhibit FAAH in the liver, whereas URB597 inhibited FAAH with similar potency and efficacy in brain and liver. A plausible reason for the lack of effect of AM3506 in the liver is its rapid uptake and metabolism by hepatocytes. Due to the covalent binding of AM3506 to its target, it is not possible to quantitatively analyze its metabolic fate in intact cells. Instead, we used the approach of varying the ratio of liver tissue to drug in suspensions of isolated hepatocytes as a way to explore the effect of cellular uptake and metabolism on the pharmacological action of a compound (Kunos, 1984). In dilute suspensions of hepatocytes, the capacity of the liver to eliminate AM3506 was not sufficient to prevent its access to and inhibition of FAAH. However, in a much more dense suspension of cells, which approximates the conditions in the intact liver, AM3506 was taken up and metabolized very rapidly, which prevented its ability to reach and block FAAH (Fig. 6D). URB597 was eliminated more slowly, and as a result it was able to fully block FAAH with the resulting increase in hepatic anandamide (Fig. 6D).

Formation of adducts of AM3506 with small molecules, as reported for PMSF (Fontana, et al., 1995), is unlikely to account for its lack of inhibition of FAAH in the liver, as AM3506 was able to fully block FAAH in the brain or in cell-free extracts of liver. Furthermore, interaction with 'off-target' serine proteases in liver is also unlikely to explain the lack of FAAH inhibition, because AM3506 had fewer such targets (Fig. 1A) than did URB597 (see Fig. 8 in (Ahn, et al., 2007), yet URB597 was able to block hepatic FAAH.

The lack of *in vivo* effect of AM3506 on hepatic FAAH turns out to be an advantageous feature, as the development of diet-induced fatty liver and insulin resistance have been linked to inhibition of FAAH activity in the liver and the resulting increase in hepatic anandamide levels (Osei-Hyiaman, et al., 2005; Osei-Hyiaman, et al., 2008; Tam, et al., 2010). The predisposition of FAAH-deficient mice to diet-induced hyperglycemia and insulin resistance (Tourino, et al., 2010) also supports this possibility. Indeed, URB597, but not AM3506, caused hyperglycemia in both SHR and WKY. Furthermore, URB597 even at a sub-hypotensive dose caused glucose intolerance in mice with diet-induced obesity, whereas an equimolar dose of AM3506, which elicited pronounced hypotension, caused no change in glucose tolerance. The absence of a similar effect of URB597 in hepatocyte-specific CB<sub>1</sub> knockout mice indicates that this effect is likely mediated by anandamide acting via hepatic CB<sub>1</sub> receptors (Fig. 7B).

Unlike in heart and aorta, CB<sub>1</sub> receptor expression in the SHR brain is not increased. Instead, there was a marked increase in CB<sub>1</sub> stimulated GTPγS binding in SHR compared to WKY brain tissue, indicating enhanced G protein activation. CB<sub>1</sub> receptors are coupled to G<sub>i</sub>/G<sub>o</sub> proteins, and upregulation of G<sub>iα</sub> protein isoforms has been documented in the heart and vasculature of rats with different forms of hypertension, including SHR (Ge, et al., 2006; Kost, et al., 1999; Marcil, et al., 1998; Marcil, et al., 1997). A similar mechanism in the brain may account for the enhanced cardiovascular responsiveness of hypertensive animals to CB<sub>1</sub> receptor activation. Nevertheless, a small contribution of vascular and cardiac CB<sub>1</sub> receptors is likely in view of the significant hypotensive response to the peripherally restricted CB receptor agonist, CB13. The fact that CB13 produced a moderate hypotensive effect in SHR and WKY also suggests that peripheral CB<sub>1</sub> receptors are functional and may be subject for modulation in certain cardiovascular pathologies.

The location in the brain of CB<sub>1</sub> receptors mediating cardiovascular depressor responses is unclear. Microinjection of anandamide or an anandamide uptake inhibitor into the brainstem nucleus of the solitary tract (NTS) of anesthetized normotensive rats was found to enhance the baroreflex inhibition of renal sympathetic nerve activity, an effect prevented by rimonabant, and increases in blood pressure resulted in enhanced anandamide content in the NTS (Seagard, et al., 2004). However, cannabinoids microinjected into the NTS caused less sympathoinhibition in SHR than in normotensive rats (Brozoski, et al., 2009), which is evidence against the NTS being the site of the antihypertensive effect of CB<sub>1</sub> receptor activation. Another potential site is the rostral ventrolateral medulla, the location of premotor sympathetic neurons. It has been reported that microinjection of the synthetic cannabinoid agonist WIN55,212-2 into this site elicits hypotension in normotensive rats, whereas similar microinjection into the NTS had no effect on blood pressure (Niederhoffer, et al., 2003). The core central sympathetic network can also be regulated at hypothalamic nuclei, circumventricular organs and area postrema (Guyenet, 2006). Further studies are needed to identify the CNS location of CB<sub>1</sub> receptors that mediate the enhanced cardiodepressor response to endogenous anandamide in SHR.

## SIGNIFICANCE

The findings in this study provide an impetus to the use of FAAH inhibitors to treat hypertension. First, the absence of cardiovascular effects under normotensive conditions would minimize unwanted side effects, such as orthostatic hypotension. Second, FAAH inhibitors produce significant antianxiety effects in rodents (Kathuria, et al., 2003), which may provide added benefit in neurogenic hypertension. At the same time, behavioral effects that predict addictive potential, such as catalepsy, are not amplified by FAAH inhibition (Justinova, et al., 2008; Kathuria, et al., 2003), an important safety consideration. Third, a major component of the antihypertensive action of AM3506, similar to the previously reported effect of URB597 (Batkai, et al., 2004), is the marked reduction of the inappropriately increased cardiac contractility. This suggests that chronic inhibition of FAAH may not only normalize blood pressure but also mitigate the development of the associated cardiac hypertrophy, an independent risk factor for cardiovascular events and mortality. Finally, the distinctive pharmacological profile of AM3506 suggests a broader safety spectrum, especially with regard to absence of mechanism-based metabolic effects, which should make it attractive for potential therapeutic use as an antihypertensive in obese subjects.



## EXPERIMENTAL PROCEDURES

### Animals

Male 12–16 week-old spontaneously hypertensive rats (SHR) and age-matched Wistar Kyoto (WKY) rats were obtained from Charles River Laboratories; male C57Bl/6J mice were from Jackson Laboratories. Liver specific  $CB_1^{-/-}$  mice were generated as described (Osei-Hyiaman, et al., 2008). Experimental protocols were in accordance with the ethical guidelines for animal research and approved by the Institutional Animal Care and Use Committee of NIAAA.

### General Surgical Procedures

Rats were anesthetized with isoflurane via a nose cone (induced with 3–4% and followed by 1.5% in pure oxygen). Adequacy of anesthesia was verified by the absence of tail-flick reflex. Animals were placed on a controlled heating pad and core body temperature, measured by a rectal probe, was maintained at 37°C. For experiments in conscious animals, rats were implanted with indwelling catheters in the femoral artery and femoral vein, as described earlier (Lake, et al., 1997), and allowed to recover. For other experiments, rats remained anesthetized and instrumented as described (Batkai, et al., 2004). Briefly, a left femoral artery was cannulated and the catheter connected to a pressure transducer (Abbott Park, IL) for monitoring mean arterial pressure (MAP). Heart rate (HR) was derived from the pressure wave. Signals were amplified through a 13–4615-71 Differentiator Plug-in Module (Brush-Gould) and recorded on PowerLab 4/30 data acquisition system (AD Instruments). Left femoral vein was cannulated for i.v. bolus injection of drugs in a volume of 0.25 mL/kg. In some experiments, the right femoral vein was prepared for drug infusion by means of a multichannel syringe pump (KD Scientific) and right carotid artery was catheterized for blood withdrawal. Mean arterial pressure (MAP) and heart rate (HR) were recorded. After 20 min stabilization, animals received a bolus intravenous (i.v.) dose of FAAH antagonist, HU210 or vehicle and mean arterial pressure (MAP) and heart rate (HR) were recorded. In some experiments,  $CB_1$  antagonist was injected i.p. 60 min before experiment or i.v. at the peak of the hypotensive response to AM3506.

### Autonomic Blockade

Ganglionic blockade was achieved with hexamethonium, as described (Schreihofner, et al., 2007). Briefly, a bolus of 20 mg/kg hexamethonium was injected i.v., followed by a continuous maintenance infusion of 4 mg/kg/h. Vasopressin (0.04–0.4 IU/kg/h) and/or isoproterenol (0.3–0.5 µg/kg/h) were also infused to offset the hexamethonium-induced fall in basal MAP and HR, respectively. Blockade of the autonomic nervous system was verified using the phenylephrine/sodium nitroprusside sensitivity test (Schreihofner, et al., 2007). The baroreflex was quantified as a change HR/MAP ratio in response to phenylephrine or sodium nitroprusside.

### Hemodynamic Analyses

Cardiac contractility was assessed in anaesthetized rats by a pressure-volume microcatheter system (Millar Instruments) as described (Batkai, et al., 2004; Pacher, et al., 2008). Briefly, a microtip pressure-volume catheter (SPR-838; Millar Instruments) was inserted into the right carotid artery and advanced into the left ventricle (LV) under pressure control. Signals were continuously recorded with an ARIA pressure-volume (PV) conductance system (Millar Instruments) coupled to a Powerlab/4SP A/D converter (AD Instruments), stored, and displayed on a computer. After stabilization for 20 minutes, AM3506 1 mg/kg or vehicle was injected i.v. and cardiac hemodynamic parameters were recorded 20 min later and computed using a cardiac PV analysis program (PVAN2.9; Millar). Maximal pressure

developed by the ventricle at any given left ventricular volume was defined as the end-systolic pressure-volume relationship (ESPVR) and represented the inotropic state of the ventricle. Cardiac output (CO) was calculated and corrected according to *in vitro* and *in vivo* volume calibrations with PVAN2.9 and normalized to body weight (cardiac index). The maximal slope of systolic pressure increment ( $+dP/dt_{max}$ ) was a time-derivative of left ventricular pressure used as a cardiac contractility parameter. Total peripheral resistance (TPR) was calculated as MAP/CO.

### Catecholamine Assay

Blood was collected from left carotid artery before and after i.v. injection of 1 mg/kg AM506 and plasma norepinephrine levels were determined by liquid chromatography with electrochemical detection, as described (Eisenhofer, et al., 1986).

### FAAH and MAGL activity

Antagonists were tested using inhibitor screening kits (Cayman Chemical Co.), based on the release of a fluorophore from a synthetic substrate by recombinant human FAAH and MAGL. AM3506 was also tested *ex vivo* using competitive Activity-Based Protein Profiling (Leung, et al., 2003) or the release of [ $^3$ H]ethanolamine from [ $^3$ H]anandamide for FAAH inhibition (Fowler, et al., 2000), or the release of [ $^3$ H]glycerol from 2-oleoyl-[ $^3$ H]glycerol in the presence of 1  $\mu$ M URB597 for MAGL inhibitory activity (Dinh, et al., 2002). Drug or vehicle was injected i.p. or given by gavage and enzyme activity was determined in harvested tissues. In some experiments, FAAH activity was measured in brain and liver homogenates, adjusting the protein levels to yield equal amounts of FAAH in the assay (10  $\mu$ g protein for brain and 3  $\mu$ g for liver to compensate for the higher FAAH expression in liver).

### Endocannabinoid Tissue Levels

Endocannabinoids were extracted and quantified using liquid chromatography/in-line mass spectrometry, as described (Batkai, et al., 2004). [ $^2$ H $_4$ ]anandamide was used as internal standard.

### Isolation and treatment of hepatocytes

Mouse hepatocytes were isolated by *in situ* liver perfusion with Collagenase type I (Sigma-Aldrich) and Percoll (GE Healthcare) density gradient centrifugation as described (Osei-Hyiaman, et al., 2008). Hepatocytes were then re-suspended in DMEM medium, containing 10% fetal bovine serum and L-glutamine (Invitrogen). Concentrated (40 mg of wet cell weight per mL of medium) and dilute (1 mg/mL) hepatocyte suspensions were prepared and incubated with AM3506, URB597 (each 100 nM) or vehicle (DMSO; 1:1000v/v) at 37°C. Aliquots were removed at the indicated time points, quickly chilled on ice and centrifuged at 400 x g, 4°C for 5 min. Drugs were extracted from supernatant and hepatocyte pellets using the same protocol as for endocannabinoids.

### Measurement of drug levels by LC/MS/MS

Drugs were quantified by LC-MS/MS using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, USA) coupled with an Agilent 1200 LC system (Agilent Technologies, Germany). Liquid chromatographic separation was obtained using 2.5  $\mu$ l injections of samples onto a Zorbax SB-C18 rapid resolution HT column (2.1mm $\times$ 50 mm, 1.8  $\mu$ m) from Agilent Technologies. The autosampler was set at 4°C and the column was maintained at 34°C during the analysis. Gradient elution mobile phases consisted of A (0.1% formic acid in water) and B (0.1% formic acid in methanol). Gradient elution (250  $\mu$ L/min) began and was held at 10% B for the first 0.5 min, followed by a linear increase

towards 85% B at 1 min; this was maintained until 12.5 min and then increased linearly to 100% B at 13 min; this was maintained until 14.5 min. At minute 16, the gradient changed linear to the initial setting which was maintained for 6 min. The mass spectrometer was set for ESI in positive ion mode. The source parameters were as follows; capillary voltage, 4000V; gas temperature, 350°C; drying gas, 10 L/min; nitrogen was used as the nebulizing gas with a pressure of 40 psig. Collision-induced dissociation (CID) was induced using nitrogen. Levels of each compound were analyzed by multiple reactions monitoring (MRM). Mass spectrometric conditions were optimized for AM3506, URB597, [<sup>2</sup>H<sub>4</sub>]AEA and AEA with injection of synthetic standards of each compound by using MassHunter Workstation Optimizer software (Agilent Technologies, USA). The molecular ion and fragments for each compound measured were as follows: m/z 247.1 → 107.0 and 247.1 → 77.1 for AM3506 (CID-energy: 12V and 52V, respectively), m/z 339.2 → 214.1 and 339.2 → 197.1 for URB597 (CID-energy: 8V and 20V, respectively), m/z 352.3 → 66.1 and 352.3 → 91 for [<sup>2</sup>H<sub>4</sub>]AEA (CID-energy: 12V and 56V, respectively) and m/z 348.3 → 62.1 and 348.3 → 91 for AEA (CID-energy: 12V and 48V, respectively). The acquisition and quantitation of analytes were achieved using MassHunter Workstation LC/QQQ Acquisition and MassHunter Workstation Quantitative Analysis softwares, respectively (Agilent Technologies, USA). Calibration curves were produced by using synthetic AEA, URB597 and AM3506. The amounts of AEA and AM3506 in the samples were determined by using linear regression of standard curves, whereas URB597 was determined by using quadratic regression of standard curve. Each time point represents hepatocytes pulled from 3–4 animals measured in duplicates. The limit of detection for AM3506 and URB597 was 1 pmol per injection.

### CB<sub>1</sub> and CB<sub>2</sub> Receptor Binding and Anandamide Transport Assays

The affinity of AM3506 for CB<sub>1</sub> and CB<sub>2</sub> receptors was tested in radioligand displacement assays, using [<sup>3</sup>H]CP,55940 as the radioligand and membrane preparations from rat forebrain and spleen, respectively, as describe (Janero, et al., 2009; Lu, et al., 2006). The effect of AM3506 on anandamide transport was assessed by measuring its ability to inhibit [<sup>3</sup>H]anandamide uptake by cultured CCF-STTG1 astrocytoma cells, as described (Fegley, et al., 2004).

### [<sup>35</sup>S]GTP S Binding

Basal and agonist-stimulated [<sup>35</sup>S]GTPγS binding was measured as described (Tam, et al., 2010).

### Glucose Tolerance Test

Male C57B16/J mice, wild-type or LCB<sub>1</sub><sup>-/-</sup>, were kept on a diet containing 60% of calories as fat (Research Diets Inc) for 14 weeks, by which time both strains have become fat due to increased adipose mass. Mice were then fasted overnight, followed by an i.p. injection of vehicle, 10 mg/kg URB597 or 1 mg/kg AM3506. Sixty minutes later the mice received 1.5 g/kg glucose i.p. and blood glucose levels were monitored for 2 hours, as described (Osei-Hyiaman, et al., 2008).

### Drugs

URB597 was from Cayman; AM251, HU210 and CB13 were from Tocris Bioscience; rimonabant was provided by the NIDA Drug Supply program. [<sup>3</sup>H]anandamide and 2-oleoyl [<sup>3</sup>H]glycerol were from American Radiolabeled Chemicals. All other chemicals were from Sigma-Aldrich. AM3506 (5-(4-hydroxyphenyl)pentanesulfonyl fluoride) was synthesized in nine steps starting from commercially available 4-phenoxybutyl bromide and 4-

anisaldehyde. Details of the synthesis along with a full structure-activity relationship study involving closely related analogs will be published elsewhere.

AM3506, URB597, CB13, rimonabant, AM251 and AM6545 were dissolved in DMSO, Tween 80 and saline (1:1:18). For gavage, AM3506 and URB597 were dissolved in DMSO and corn oil (1:19). HU210 was dissolved in DMSO and saline (1:19). All other drugs were dissolved in saline.

## Data Analysis

Statistical analyses were performed using Prism 4 (GraphPad). T test for unpaired or paired data was used for comparison of values between two groups and one-way ANOVA followed by Dunnett's test was used for comparison of multiple independent groups. When several variables were considered, two-way ANOVA with Bonferroni's post-hoc test was applied. Differences were considered significant when  $P < 0.05$ . Values were presented as means  $\pm$  s.e.m.).

## Acknowledgments

We thank Prof. B. Lutz and Dr. G. Marsicano for providing CB<sub>1</sub> floxed mice used to generate the liver-specific CB<sub>1</sub><sup>-/-</sup> mice.

### Source of Funding

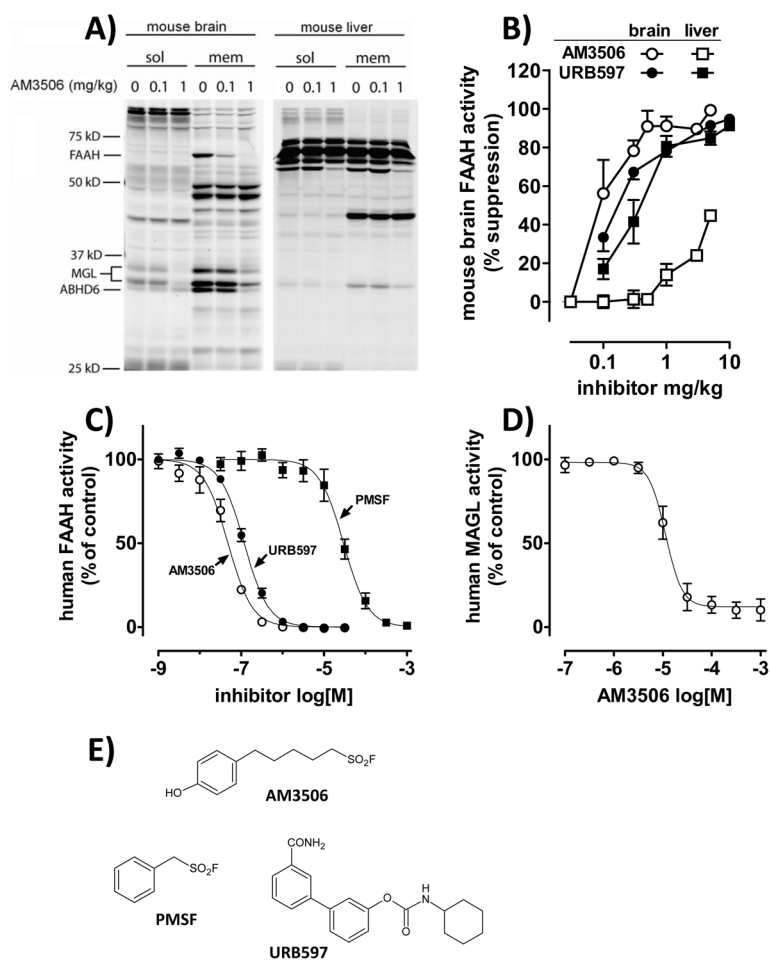
This work was supported by funds from the intramural program of NIAAA.

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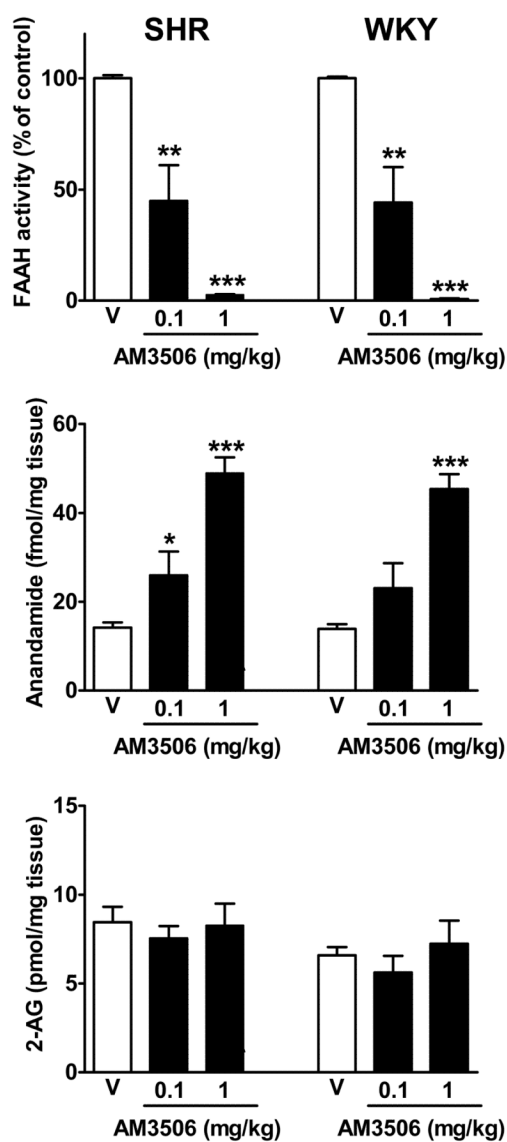
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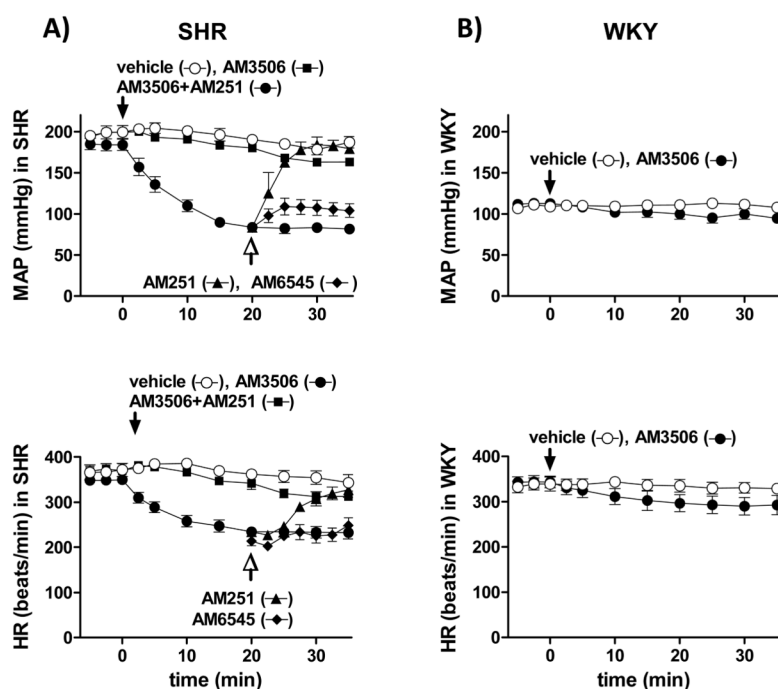
**Figure 1.** Inhibition of FAAH activity by AM3506. A) Activity-Based Protein Profiling in mouse brain (left) and liver (right), soluble (sol) and membrane fractions (mem); B) Comparison of FAAH inhibition in mouse brain (○, ●) and liver (□, ■) by AM3506 (open symbols) and URB597 (filled symbols), as assessed by [<sup>3</sup>H]ethanolamine release from [<sup>3</sup>H]anandamide; C) Effect of inhibitors on human recombinant FAAH activity; D) Effect of AM3506 on human recombinant MAGL activity. Values in panels B–D are means±s.e.m. from 3–6 experiments in triplicates. E) Chemical structure of FAAH inhibitors.



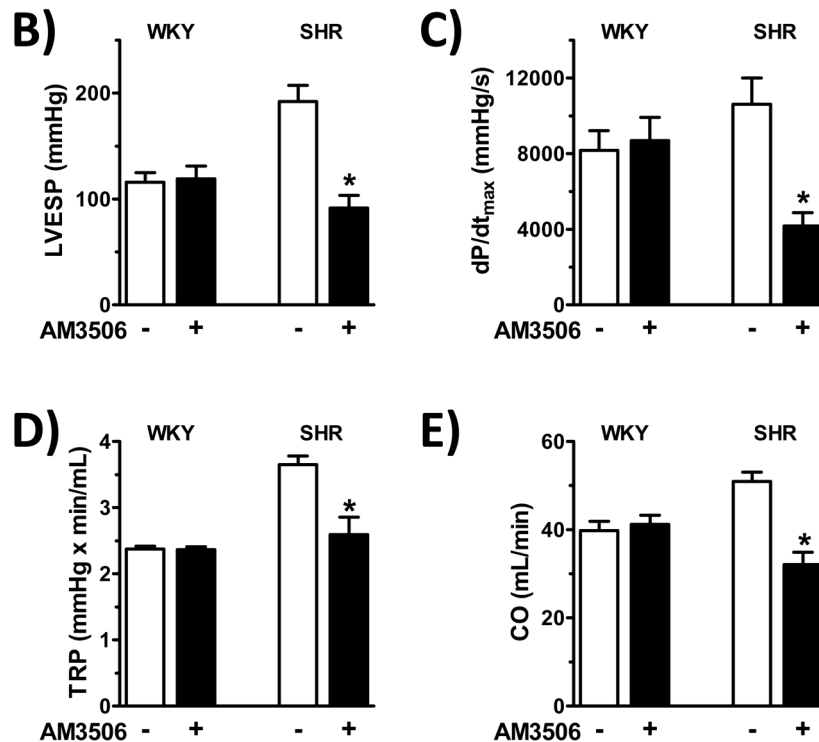
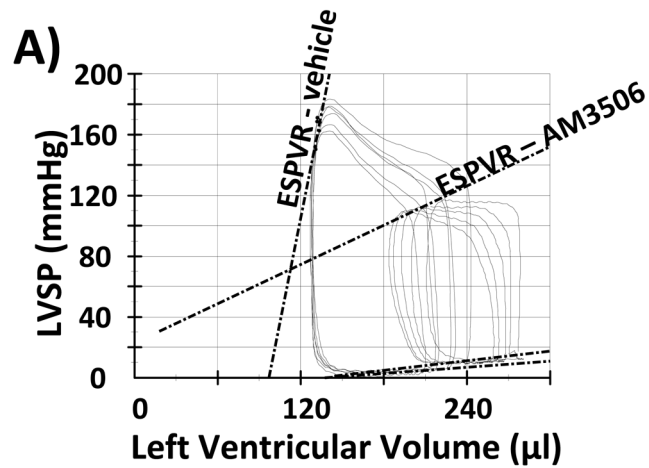
**Figure 2.**

Inverse relationship between FAAH activity and anandamide, but not 2-AG, levels in brain tissue is similar in SHR and WKY rats. Rats were treated with the indicated bolus i.p. doses of AM3506 and were sacrificed 1 h later to measure FAAH activity and anandamide and 2-AG levels in brain tissue. Values are means $\pm$ s.e.m. from 5–6 experiments. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\*  $P$ <0.001, compared to vehicle (V).



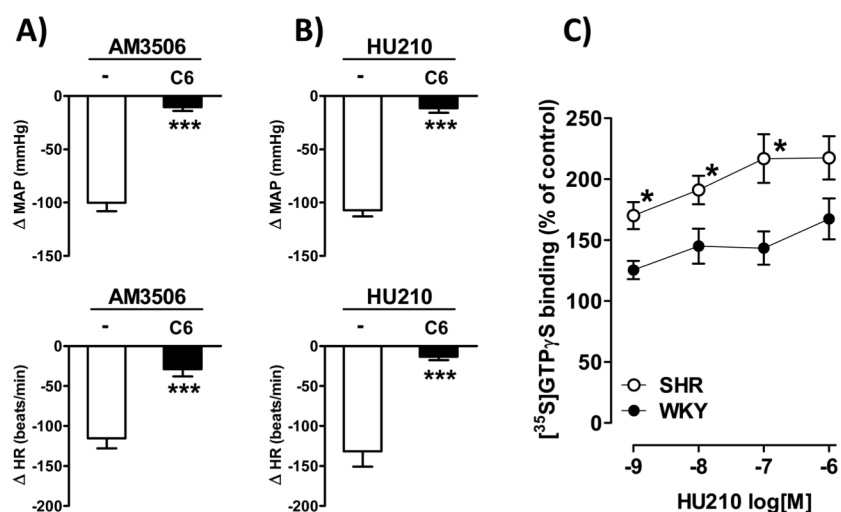


**Figure 3.** Cardiovascular effects of AM3506 in anaesthetized SHR are mediated by CB<sub>1</sub> receptors. Solid arrows indicate the i.v. injection to SHR (A) and WKY (B) of vehicle (○), 1 mg/kg AM3506 (●) and 1 mg/kg AM3506 following i.p. injection of 3 mg/kg AM251 60 min earlier (■). Open arrows indicate the i.v. injection of 3 mg/kg AM251 (▲) or 3 mg/kg AM6545 (◆) to a separate group of SHR, at the peak hypotensive response to AM3506. Values of MAP and HR are means±s.e.m. from 4–8 experiments.

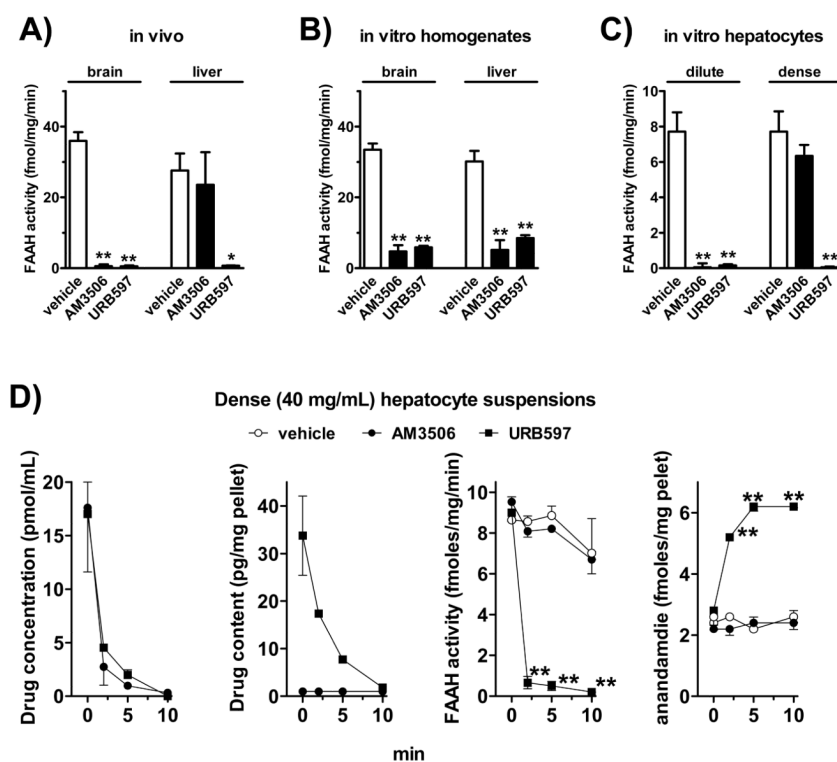


**Figure 4.**

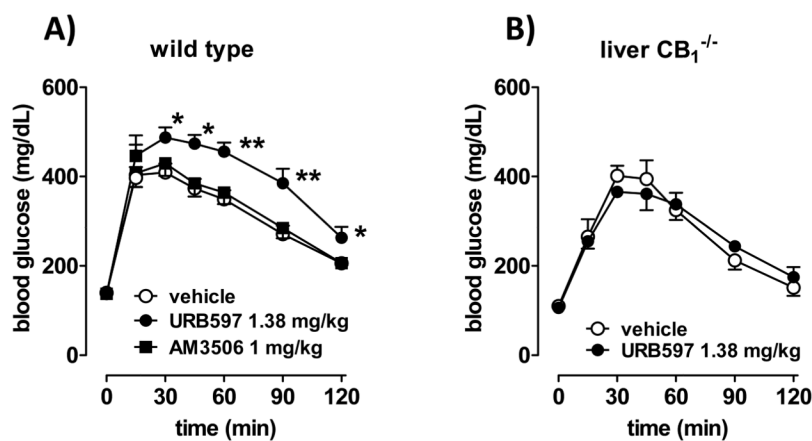
Hemodynamic effects of AM3506 in anesthetized SHR and WKY rats. Anesthetized rats were instrumented with an intraventricular pressure/volume microcatheter system for measurement of A) end-systolic pressure-volume relations (ESPVR); B) left ventricular end-systolic pressure (LVESP); C)  $dP/dt_{max}$ ; D) total peripheral resistance (TPR); and E) cardiac output (CO). Effects of vehicle (-, open columns) and 1 mg/kg AM3506 (+, filled columns) are shown. Panel A illustrates real-time pressure-volume loops recorded in a single SHR, whereas values in B to E are means  $\pm$  s.e.m. from 6 to 8 experiments. \* $P < 0.05$  compared to vehicle.



**Figure 5.** Neuronal CB<sub>1</sub> receptor coupling to G proteins is increased in hypertension. A bolus i.v. dose of 1 mg/kg AM3506 (A) or 1  $\mu$ g/kg HU210 (B) was injected to anaesthetized control SHR (open columns) or SHR receiving hexamethonium+vasopressin+isoproterenol (C6; filled columns). Values are means $\pm$ s.e.m. from 5–10 experiments, \*\*\*P<0.001 compared to control. C) Coupling efficiency of CB<sub>1</sub> receptors in membrane preparations from whole brain of SHR and WKY rats was assessed by measuring HU210-induced increases in GTP $\gamma$ S labeling, as described in Methods. Values are means $\pm$ s.e.m. from 4 experiments in triplicates. \*P<0.05 compared to WKY rats.

**Figure 6.**

Selective resistance of hepatic FAAH to inhibition by AM3506 *in vivo* is related to rapid uptake and degradation of AM3506 by the liver. A) FAAH activity in brain and liver 1 h following *in vivo* treatment of mice with 1 mg/kg AM3506 or 1.38 mg/kg URB597. B) FAAH activity following *in vitro* incubation of brain or liver homogenates with 100 nM AM3506 or URB597. C) FAAH activity in cell pellets following incubation of dilute (1 mg cells/mL) or dense hepatocyte suspensions (40 mg cells/mL) with 100 nM AM3506 or URB597. D) Analysis of the fate and activity of AM3506 and URB597 in dense suspension of hepatocytes. Drugs or vehicle were added to hepatocyte suspensions at 0 min. At the indicated times, cells were rapidly centrifuged, and drug levels were determined in the cell-free supernatant (left) or pellet (2<sup>nd</sup> from left). The pellet was also used to determine FAAH activity (3<sup>d</sup> from left) and anandamide levels (right). Each point represents the means from 4–5 separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$  relative to corresponding 0 min value. For explanations, see text.



**Figure 7.**

URB597, but not AM3506, increases glucose intolerance in mice with diet-induced obesity.

A) Male mice kept on a high-fat diet for 14 weeks were subjected to an intraperitoneal glucose tolerance test (ipGTT) 60 min following the i.p. injection of vehicle, 1.38 mg/kg URB597 or 1 mg/kg AM3506, as described in Methods. Area under the curve (AUC, mg h/dL): 352±28 (vehicle), 570±45\* (URB597), 411±35 (AM3506). \*P<0.01 relative to value in vehicle treated mice; n=12–14 mice in each group. B) Liver-specific CB<sub>1</sub> knockout (LCB<sub>1</sub><sup>-/-</sup>) mice subjected to ipGTT following vehicle or URB597 treatment. AUC: 322±37 (vehicle) vs 337±14 (URB597), P>0.5, n=4.