Activation of peripheral CB_1 cannabinoid receptors in haemorrhagic shock

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Anandamide, an endogenous cannabinoid ligand[1], binds to CB_1 cannabinoid receptors[2] in the brain and mimics the neurobehavioural actions of marijuana[3,4]. Cannabinoids and anandamide also elicit hypotension mediated by peripheral CB_1 receptors[5–8]. Here we report that a selective CB_1 receptor antagonist, SR141716A[9], elicits an increase in blood pressure in rats subjected to haemorrhagic shock, whereas similar treatment of normotensive rats or intracerebroventricular administration of the antagonist during shock do not affect blood pressure. Blood from haemorrhaged rats causes hypotension in normal rats, which can be prevented by SR141716A but not by inhibition of nitric oxide synthase in the recipient. Macrophages and platelets from haemorrhaged rats elicit CB_1 receptor-mediated hypotension in normotensive recipients, and incorporate arachidonic acid or ethanolamine into a product that co-elutes with anandamide on reverse-phase high-performance liquid chromatography. Also, macrophages from control rats stimulated with ionomycin or bacterial phospholipase D produce anandamide, as identified by gas chromatography and mass spectrometry. These findings indicate that activation of peripheral CB_1 cannabinoid receptors contributes to haemorrhagic hypotension, and anandamide produced by macrophages may be a mediator of this effect.

Rats anaesthetized with urethane were subjected to standardized, stepwise bleeding until mean blood pressure stabilized at 40 mm Hg (refs 10, 11). Blood pressure remained stable at this level for 25–35 min, after which it started to decline and the animal died. At the beginning of the stable hypotensive phase, some rats received 3 mg kg^{-1} of SR141716A intravenously (i.v.), which caused a marked and prolonged pressor response that lasted 20–30 min (Fig. 1). The dose of SR141716A that caused a half-maximal pressor effect (0.37 ± 0.08 mg kg^{-1}, Fig. 1, inset) was similar to the dose that caused half-maximal inhibition of anandamide-induced hypotension (0.29 ± 0.14 mg kg^{-1}) (ref. 8) or Δ^9-tetrahydrocannabinol (THC)-induced neurobehavioural effects in rats (0.11–0.54 mg kg^{-1}) (ref. 9), which supports the involvement of CB_1 receptors in shock-related hypotension. Other rats received vehicle, which only caused a small and transient pressor effect (Fig. 1). In control animals, the same dose of SR141716A caused no significant change in blood pressure either when injected during normotensive conditions (-6 ± 3 mm Hg, $n = 6$), or after mean blood pressure had been reduced to 53 ± 3 mm Hg due to α-adrenergic blockade by phentolamine, 2 mg kg^{-1} i.v. ($n = 3$, not shown). Furthermore, in three rats subjected to haemorrhagic hypotension, injection of 300 μg kg^{-1} SR141716A into the cisterna magna or the IVth cerebral ventricle failed to influence blood pressure (not shown).

The specificity of the pressor effect of SR141716A during haemorrhagic hypotension is further indicated by the ability of a CB_1 receptor agonist to reverse it. WIN-55212-2 is a potent CB_1 receptor agonist[12], and it causes pronounced hypotension in urethane-anaesthetized control rats with a half-maximal effect at 0.1 mg kg^{-1} i.v. (ref. 8). In three rats in haemorrhagic shock, cumulative doses of WIN-55212-2 were given i.v. at the plateau of the pressor response elicited by 3 mg kg^{-1} SR141716A. WIN-55212-2 started to reverse the pressor response to SR141716A at a dose of 1 mg kg^{-1}, and rapid reversal to 40 mm Hg was achieved with a cumulative dose of 20 mg kg^{-1}. This dose range is similar to that needed to lower blood pressure in normal rats pretreated with the same dose of SR141716A (not shown), which suggests that the pressor effect of SR141716A in shock and its reversal by WIN-55212-2 are mediated by the same receptor. Taken together, these findings suggest that activation of peripheral CB_1 receptors by an endogenous ligand contributes to haemorrhagic hypotension.

In order to test this possibility further, we examined the effects of exchange transfusion of blood from heparinized donor rats on the blood pressure of recipient rats. Transfusion of 3 ml of blood from normal donor to normal recipient rats had no effect on blood pressure. However, in five rats, transfusion of the same volume of blood obtained following haemorrhage ('shock' blood) gradually lowered blood pressure to a plateau 33 ± 9 mm Hg below preinfusion levels, and the hypotension lasted more than 2 h. This effect was completely blocked by pretreatment of recipients with 3 mg kg^{-1} SR141716A (peak effect of 'shock' blood: $+5 \pm 7$ mm Hg, $n = 3$, $P < 0.005$).

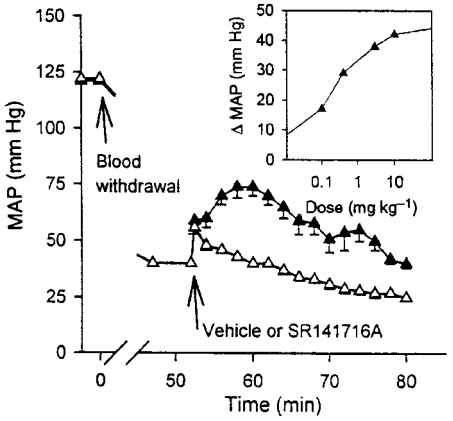

Figure 1 The effects of SR141716A (3 mg kg^{-1} i.v. in 0.2 ml, ▲, $n = 7$) or vehicle (0.2 ml, △, $n = 7$) on blood pressure in rats subjected to haemorrhagic shock. Inset: dose-dependence of the pressor effect of SR141716A in shock, each point representing the mean of 3–7 experiments. The ED_{50} value for the pressor effect of SR141716A was calculated by using the ALLFIT program. Vertical bars in Figs 1–3 represent s.e.m.

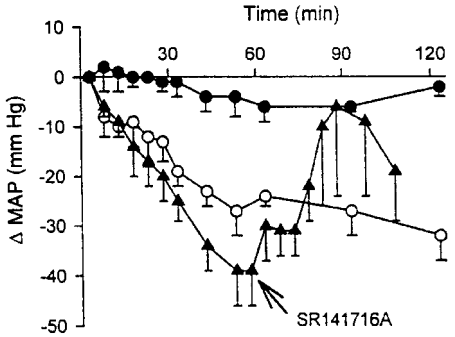

Figure 2 SR141716A inhibits the hypotensive response of control rats to i.v. administered mononuclear cells isolated from the blood of haemorrhaged rats. Recipient rats were either untreated (○, $n = 4$), pretreated with SR141716A, 3 mg kg^{-1} i.v. 10 min before receiving the injection of mononuclear cells (●, $n = 3$), or treated with the same dose of SR141716A after injection of mononuclear cells, as indicated by the arrow (▲, $n = 4$).

Plasma obtained by centrifugation of 3 ml of 'shock' blood caused only a minimal change in blood pressure (-6 ± 4 mm Hg, $n = 3$) when injected into recipient rats. In contrast, exchange transfusion of the cellular pellet resuspended to the original blood volume in saline caused marked and prolonged hypotension (>2 h, -30 ± 8 mm Hg, $n = 5$), which could be completely blocked by pretreatment of the recipients with 3 mg kg^{-1} SR141716A ($+8 \pm 2$ mm Hg, $n = 3$, $P < 0.005$). Injection of blood cells from normotensive donors caused minimal change in blood pressure (-8 ± 4 mm Hg, $n = 3$). Cells from 3 ml of blood were separated into mononuclear, granulocyte, and erythrocyte fractions, and each cell type resuspended in 1 ml of normal saline. Intravenous injection of the erythrocyte fraction did not affect blood pressure, whereas the granulocytes caused a transient (<1 min) hypotension and tachycardia, which was similar whether cells were obtained from normotensive or haemorrhaged donors, and was unaffected by SR141716A pretreatment. Injection of mononuclear cells from normotensive donors caused only minimal hypotension (-7 ± 1 mm Hg, $n = 7$), whereas the same cells from haemorrhaged rats caused prolonged hypotension that could be prevented by pretreatment of the recipients with 3 mg kg^{-1} of SR141716A (Fig. 2). Also, SR141716A was able to reverse the hypotension when administered following administration of the mononuclear fraction (Fig. 2).

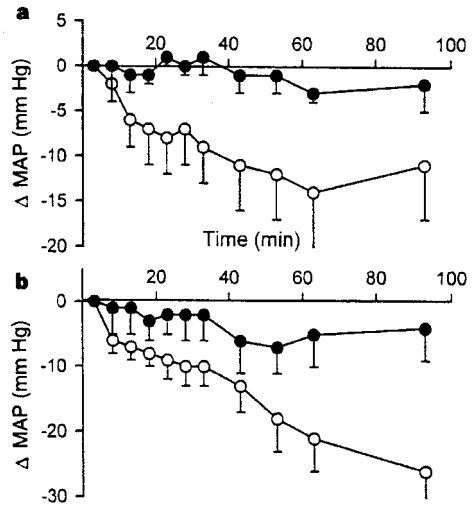

Figure 3 SR141716A inhibits the hypotensive response of control rats to i.v. injection of mononuclear cells from platelet-depleted blood (**a**) or of platelet-rich plasma (**b**) obtained from animals in shock. Symbols as in Fig. 1, $n = 3$–5 (**a**) or 7–12 (**b**).

Several lines of evidence suggest that changes in NO synthase activity and/or in circulating levels of nitrosothiols[13] cannot explain the CB_1 receptor-mediated hypotension observed in shock, although some contribution of the NO system cannot be definitively excluded. First, inhibition of endothelial NO synthase in recipient rats by pretreatment with 20 mg kg^{-1} nitro-L-arginine methyl ester (L-NAME)[14] caused a rapid and sustained (>2 h) increase in basal blood pressure by 20 ± 4 mm Hg, but did not prevent the hypotensive response to 'shock' blood (peak change: -34 ± 12 mm Hg, $n = 4$). Second, we found no significant difference between control and 'shock' cells in the rate of nitrite (1.32 ± 0.16 versus 1.36 ± 0.15 nmol h^{-1} per 10^6 macrophages) and nitrate production (3.56 ± 0.61 versus 4.47 ± 1.04 nmol h^{-1} per 10^6 macrophages, $n = 4$) during the first 3 h following their isolation, which agrees with a previous report[15]. Third, pretreatment of donor animals with 20 mg kg^{-1} L-NAME before haemorrhage did not block the transfer of hypotensive activity by macrophages (not

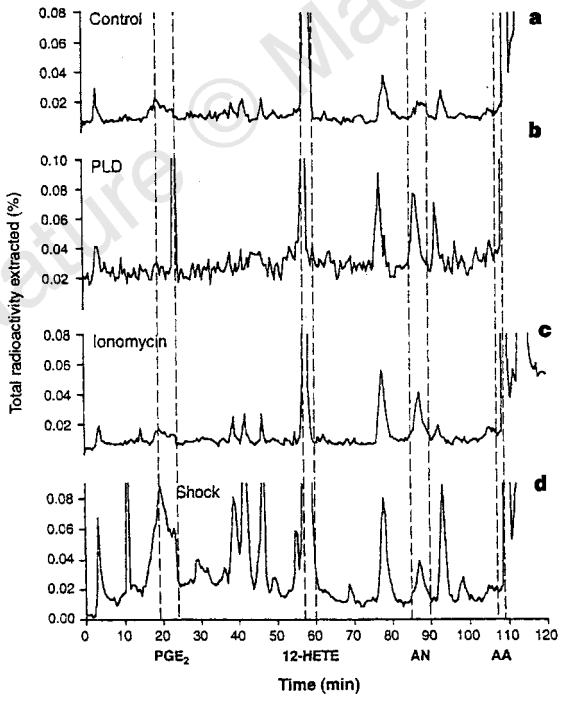

Figure 4 HPLC analysis of ^{3}H-arachidonic acid metabolites generated *in vitro* by macrophages isolated from rat blood. Cells isolated from a normotensive (**a** to **c**) and a haemorrhaged rat (**d**) were plated, preincubated with ^{3}H-arachidonic acid for 16 h (**a** to **c**) or 2 h (**d**), and then treated with vehicle (**a** and **d**), phospholipase D (**b**) or ionomycin (**c**) as described under Methods. Elution times of radiolabelled standards are indicated by double dashed lines. The radioactivity (d.p.m.) in the peaks coeluting with anandamide was 370 (**a**), 1,491 (**b**) and 899 (**c**). In another similar preparation the corresponding values were 295 (**a**), 785 (**b**) and 1,029 (**c**).

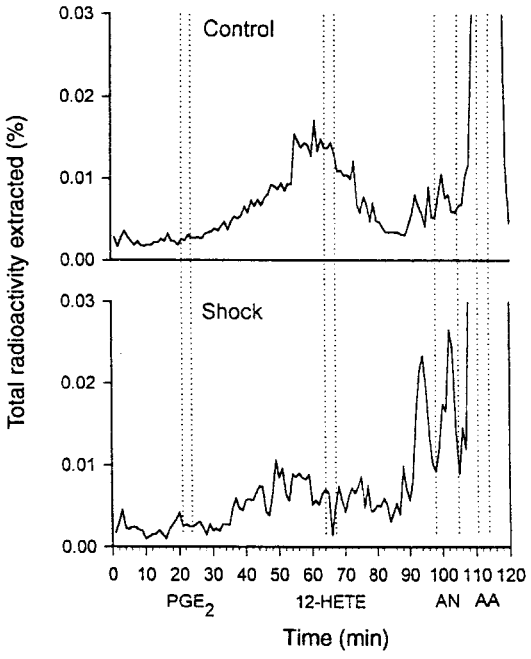

Figure 5 HPLC analysis of ^{3}H-ethanolamine metabolites generated *in vitro* by macrophages isolated from 3 ml blood from a control (top) or a haemorrhaged rat (bottom). Cells were preincubated for 16 h with ^{3}H-ethanolamine, followed by 30 min in serum-free medium containing PMSF and unlabelled anandamide (see Methods). Processing and analysis of the samples was as in Fig. 4, except for using a different HPLC machine and column. This is one of three experiments with similar results.

shown). Fourth, the hypotensive response to the i.v. injection of 5 $\mu g\,kg^{-1}$ sodium nitroprusside, a nitric oxide donor, was the same before and after the administration of 3 $mg\,kg^{-1}$ SR141716A (-57 ± 5 versus -63 ± 5 mm Hg, $n = 3$).

The mononuclear fraction was further separated by allowing the macrophages and most of the contaminating platelets (10–20 platelets per macrophage) to adhere to a plastic culture dish. Intravenous injection of the non-adherent cells, mainly lymphocytes, did not significantly alter the blood pressure of recipient rats. To test the effect of macrophages and platelets separately, platelet-rich plasma was prepared from 3 ml of whole blood, yielding $8–10 \times 10^8$ pure platelets. The platelets were removed and replaced with saline to prepare the mononuclear fraction as before, which now contained $5–6 \times 10^6$ macrophages plus 2–5 platelets per macrophage. Injection of either the platelet-rich plasma (Fig. 3a) or the platelet-depleted monocyte fraction (Fig. 3b) from haemorrhaged rats into normal recipients caused prolonged hypotension that could be prevented by pretreatment of the recipient with SR141716A. Injection of either fraction obtained from normal donors had no effect on blood pressure (not shown). These findings indicate that both macrophages and platelets contribute to the hypotensive activity of the mononuclear cell fraction.

Endorphins are known to contribute to hypotension in various forms of shock through an action at central opiate receptors, and naloxone was reported to inhibit the hypotension as well as to improve survival[11]. Unexpectedly, pretreatment of rats with 3 $mg\,kg^{-1}$ SR141716 before bleeding reduced the survival time (measured from the last blood removal before blood pressure stabilized at 40 mm Hg for at least 5 min) from 30 ± 5 min ($n = 9$) to 8 ± 1 min ($n = 5$, $P < 0.005$). In contrast, pretreatment with 1 $mg\,kg^{-1}$ of THC, or 2 $\mu g\,kg^{-1}$ of HU-210, a potent hypotensive cannabinoid devoid of an initial pressor effect[8], significantly increased survival to 51 ± 8 ($n = 5$, $P < 0.05$) or 58 ± 8 min ($n = 5$, $P < 0.05$), respectively. This suggests that, in contrast to the effect of endogenous opioids, activation of CB_1 receptors is beneficial for survival in shock. Although the underlying mechanism is unclear, a favourable redistribution of cardiac output or improved microcirculation by localized vasodilation[16,17] are possibilities.

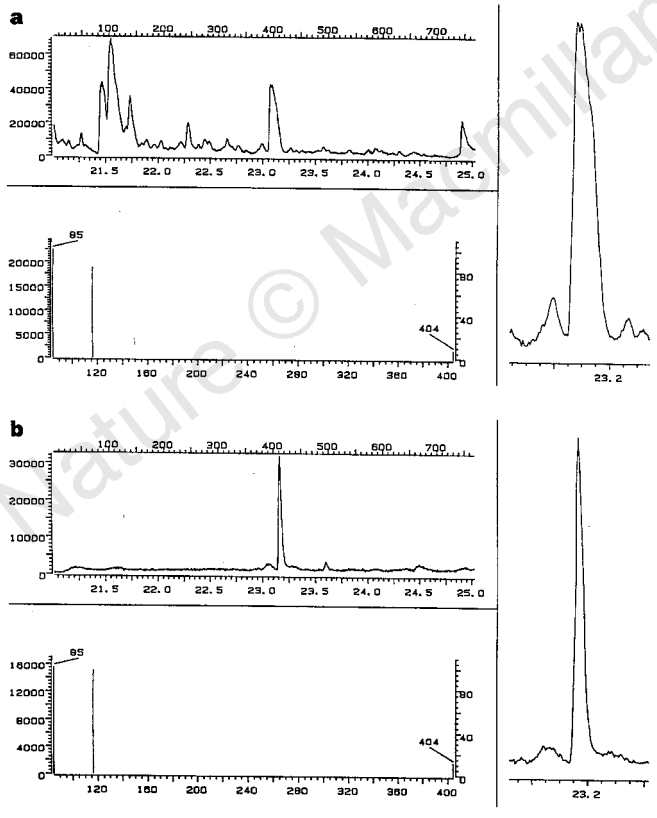

Figure 6 Identification by GC/MS of anandamide generated by macrophages and platelets. **a**, 5×10^7 macrophages plus $5–10 \times 10^8$ platelets isolated from 260 ml control rat blood were incubated for 20 min in serum-free medium containing 200 μM PMSF, 10 U ml^{-1} PLD and 5 μM ionomycin. Cells plus medium were extracted with ethylacetate, dried and resuspended in ethanol for separation by reverse phase HPLC. Fractions corresponding to the elution profile of anandamide were pooled and subjected to GC/MS (see Methods). **b**, GC/MS analysis of 100 ng anandamide. Upper left panels, combined currents obtained by selected ion monitoring on *m/z* 85, *m/z* 116 and *m/z* 404; lower left panels: ratio of the monitored ions at the apex of the anandamide peak; right panels: expanded view of the anandamide region of the ion chromatogram.

Anandamide has been shown to be formed in and released from neurons in a calcium-dependent manner by phospholipase D-mediated cleavage of *N*-arachidonoyl phosphatidylethanolamine[18]. A similar process has been documented in a macrophage cell line[19,20]. We tested whether anandamide was formed in macrophages obtained from circulating blood. The cells were maintained in primary culture and preincubated with ^{3}H-arachidonic acid. Reverse phase high-performance liquid chromatography (HPLC) separation of radioactive products generated by unstimulated cells from control rats yielded a small, composite peak coeluting with authentic anandamide (Fig. 4a). However, a much larger and more distinct peak emerged in the same position in aliquots of the same cells briefly exposed to bacterial phospholipase D (Fig. 4b) or to the calcium ionophore, ionomycin (Fig. 4c), agents that have been shown to release anandamide from a macrophage cell line[19,20]. A similar peak also increases in size in cells isolated from a rat in haemorrhagic shock (Fig. 4d). Because other arachidonate products may coelute with anandamide in this HPLC system[21], in additional experiments we incubated the macrophage/platelet preparation with ^{3}H-ethanolamine and then separated the products by reverse phase HPLC. A radiolabelled product coeluting with authentic anandamide appeared in cells from haemorrhaged rats, and the size of this peak was much smaller in the same number of cells form control rats (Fig. 5). This double labelling strongly suggests that the product generated is anandamide. We next examined the ability of the same cells to generate anandamide in the absence of exogenous precursors. A macrophage/platelet preparation stimulated *in vitro* by ionomycin plus PLD generated a product that could be identified as anandamide by gas chromatography/mass spectrometry, based on its retention time and *m/z* ratios (compare Fig. 6a and b). When a 10 times larger number of pure platelets was used in a similar experiment, the peak at the retention time of anandamide was much smaller and could not be clearly identified as anandamide, based on *m/z* ratios (not shown). This suggests that the major source of anandamide in Fig. 6 was macrophages.

Most of the anandamide formed and released by neurons or a macrophage cell line remains cell-associated because of a high-affinity uptake system[18,19]. A similar behaviour of circulating macrophages may explain the lack of hypotensive activity found in the plasma of animals in shock. Shock is known to be associated with sludging of blood cells and increased direct contact between macrophages, platelets and the vascular wall[10]. Endothelial or vascular smooth muscle cells may therefore be the site of the hypotensive action of macrophage-derived anandamide. However, platelets from rats in shock also elicited CB_1 receptor-mediated hypotension, yet produced much less, if any, anandamide. Autologous platelets have been reported to greatly enhance cytokine production by mononuclear cells[22], and platelets or platelet-derived factors may similarly induce anandamide production in activated macrophages. The lack of 'tonic' activity of this CB_1 receptor-mediated mechanism and its persistence after blockade of endothelial NO synthase distinguishes it from NO-mediated vasodilation. How this new vasodilator mechanism is activated in haemorrhagic shock, remains to be determined. □

Methods

Haemorrhagic shock. Male Sprague–Dawley rats (300–350 g) were anaesthetized with urethane, $0.7\,g\,kg^{-1}$ i.v. + $0.3\,g\,kg^{-1}$ i.p., heparinized ($500\,IU\,kg^{-1}$), and kept on a 37 °C heating pad. Polyethylene cannulae were placed into both femoral arteries for monitoring arterial blood pressure and for withdrawal of blood, respectively, and into the femoral vein for drug injections. Rats were then subjected to gradual bleeding, in steps of 0.5 ml gradually tapered to 0.1 ml, until mean arterial pressure (MAP) stabilized at 40 mm Hg within 40–45 min of withdrawal of a total of 3.1 ± 0.1 ml blood per 100 g body weight (52% of the total blood volume[23]).

Isolation of blood cells. Cells and plasma were separated by centrifugation. The combined cells were washed three times and resuspended to the original volume in saline. Erythrocyte, granulocyte and mononuclear cell fractions were prepared from whole blood by ficoll/isopaque density gradient centrifugation, as described by Böyum[24]. Erythrocytes were separated from the granulocytes by sedimentation through Dextran 500 (ref. 24). Platelet-rich plasma was prepared by centrifugation of whole blood at 64 g for 15 min.

Measurement of nitrite and nitrate production. Macrophages isolated from control and haemorrhaged rats (10^6 cells plus contaminating platelets) were plated and maintained in 1 ml Krebs buffer containing 10 mM glucose. The buffer was removed at 1, 2 and 3 h and replaced with fresh buffer. Nitrite concentrations were determined by the Griess reaction[25] in duplicate aliquots of the buffer, in one of which nitrates were first reduced using the cadmium method[25]. Nitrate concentrations were calculated as the difference between the two values.

HPLC analyses. Mononuclear cells were isolated and plated on plastic Petri dishes in 4 ml RPMI 1640 medium containing 10% fetal calf serum, $10\,\mu g\,ml^{-1}$ gentamycin and $10\,\mu g\,ml^{-1}$ fungizone. After 60 min, lymphocytes were removed by aspiration, and the adherent macrophages ($5–6 \times 10^6$ cells per dish) plus contaminating platelets ($\sim 10^8$ cells per dish) were preincubated with ^{3}H-arachidonic acid ($220\,Ci\,mmol^{-1}$, $5\,\mu Ci\,ml^{-1}$) or ^{3}H-ethanolamine ($18\,Ci\,mmol^{-1}$, $10\,\mu Ci\,ml^{-1}$), for 2 or 16 h, as indicated. Following preincubation, the cells were washed 4 times with serum-free RPMI 1640 medium containing 200 μM phenylmethylsulphonylfluoride (PMSF) and 100 μM unlabelled anandamide to prevent the breakdown and cellular reuptake of anandamide formed by the cells, respectively[18]. The cells were then treated for 20 min with vehicle or phospholipase D (PLD) from *Streptomyces chromofuscus* ($10\,U\,ml^{-1}$), or for 5 min with ionomycin (5 μM), as indicated. At the end of the treatment the cells plus medium were extracted 3 times with 2 vol ethyl acetate, the organic phase was dried under nitrogen, resuspended in 400 μl methanol/water (3:1) and analysed by reverse phase HPLC on a Radial-Pak C_{18} column. Samples were eluted at a flow rate of $1\,ml\,min^{-1}$, using a continuous gradient of water/acetic acid (100:0.05, vol/vol) and methanol/acetic acid (100:0.05, vol/vol), as described[21]. Fractions (0.5 ml) were collected and radioactivity determined by liquid scintillation spectrometry.

Gas chromatography/mass spectrometry. Ethylacetate extracts of macrophages plus contaminating platelets were prepurified by reverse phase HPLC. The fractions corresponding to the elution profile of anandamide were pooled, evaporated to dryness under nitrogen, and derivatized with bis[trimethylsilyl]trifluoroacetamide (BSTFA) for 1 h at 70 °C. Samples were then dried again and resuspended in *n*-hexane. The trimethylsilylether (TMS) derivatives were injected in 'spitless' mode into a Hewlett-Packard 5890 gas chromatograph (GC) equipped with an HP-1 capillary column ($25\,m \times 0.2\,mm \times 0.33\,\mu m$) and interfaced with a Hewlett-Packard 5988A mass spectrometer. Four minutes after injection the oven temperature was ramped from 90 °C to 300 °C at $10\,°C\,min^{-1}$. Helium was used as the carrier gas. The injector and detector temperatures were maintained at 260 °C, and the source temperature was 200 °C. The TMS derivative of anandamide eluted from the GC at ~23.1 min. Based on the electron impact spectrum, selected ion monitoring analyses were performed on *m/z* 85, *m/z* 116 and *m/z* 404 (M-15).

Drugs. The vehicle for SR141716A [*N*-(piperidil-l-yl)-5-(4-chlorophenyl)–(2,4-dichloro-phenyl)-4-methyl-1H-pyrazol-3-carboxamide HCl][9] or anandamide (arachidonyl ethanolamide) was emulphor:ethanol:saline 1:1:18. Emulphor is a polyoxyethylated vegetable oil.

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Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels

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Leptin, the protein encoded by the obese (*ob*) gene, is secreted from adipose tissue and is thought to act in the central nervous system to regulate food intake and body weight[1,2]. It has been proposed that leptin acts in the hypothalamus[3–5], the main control centre for satiety and energy expenditure[6]. Mutations in leptin or the receptor isoform ($Ob\text{-}R_L$) present in hypothalamic neurons result in profound obesity and symptoms of non-insulin-dependent diabetes[7–10]. Here we show that leptin hyperpolarizes glucose-receptive hypothalamic neurons of lean Sprague–Dawley