MECHANISMS OF SIGNAL TRANSDUCTION:

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Anandamide Uptake by Human Endothelial Cells and Its Regulation by Nitric Oxide*

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Anandamide (AEA) has vasodilator activity, which can be terminated by cellular re-uptake and degradation. Here we investigated the presence and regulation of the AEA transporter in human umbelical vein endothelial cells (HUVECs). HUVECs take up AEA by facilitated transport (apparent K_m = 190 ± 10 nM and V_{max} = 45 ± 3 pmol·min^{-1}·mg^{-1} protein), which is inhibited by α-linolenoyl-vanillyl-amide and *N*-(4-hydroxyphenyl)-arachidonoylamide, and stimulated up to 2.2-fold by nitric oxide (NO) donors. The NO scavenger hydroxocobalamin abolishes the latter effect, which is instead enhanced by superoxide anions but inhibited by superoxide dismutase and *N*-acetylcysteine, a precursor of glutathione synthesis. Peroxynitrite ($ONOO^-$) causes a 4-fold activation of AEA transport into cells. The HUVEC AEA transporter contributes to the termination of a typical type 1 cannabinoid receptor (CB_1) -mediated action of AEA, *i.e.* the inhibition of forskolin-stimulated adenylyl cyclase, because NO/$ONOO^-$ donors and α-linolenoyl-vanillyl-amide/*N*-(4-hydroxyphenyl)-arachidonoylamide were found to attenuate and enhance, respectively, this effect of AEA. Consistently, activation of CB_1 cannabinoid receptors by either AEA or the cannabinoid HU-210 caused a stimulation of HUVEC inducible NO synthase activity and expression up to 2.9- and 2.6-fold, respectively. Also these effects are regulated by the AEA transporter. HU-210 enhanced AEA uptake by HUVECs in a fashion sensitive to the NO synthase inhibitor *N*ω-nitro-L-arginine methyl ester. These findings suggest a NO-mediated regulatory loop between CB_1 cannabinoid receptors and AEA transporter.

Anandamide (arachidonoylethanolamide, AEA)[1] belongs to an emerging class of endogenous lipids including amides and esters of long chain polyunsaturated fatty acids and is collectively termed "endocannabinoids" (1, 2). In fact, AEA has been isolated and characterized as an endogenous ligand for both CB_1 and, to a lesser extent, CB_2 cannabinoid receptor subtypes, and has been shown to mimic the psychotropic, antiemetic, and analgesic effects of cannabinoids (3). Recently, attention has been focused on the cardiovascular actions of AEA and their potential role in human shock conditions (4). In particular, a role for AEA has been proposed in both endothelium-dependent and -independent relaxations of vascular tissues, which involve several mechanisms including hyperpolarization of the smooth muscle cell membrane (5–7). A similar mechanism has been attributed in the past to a diffusible endothelium-derived hyperpolarizing factor (EDHF) different from nitric oxide (NO), whose chemical nature is still a matter of speculation (8). In fact, AEA has been proposed as an EDHF (5), though this hypothesis is still under debate (9, 10), and recent data strongly support the theory that EDHF is a cytochrome P450 metabolite (11). Whether or not an EDHF, AEA is likely to play an important role in the control of vascular tone (for reviews see Refs. 4 and 12), as suggested also by the observation that both endothelial cells and macrophages release this as well as the other endocannabinoid, 2-arachidonoyl-glycerol (2-AG) (1, 13–16).

The pharmacological effects of AEA on CB_1 and CB_2 receptors depend, as for any other extracellular transmitter, on its life span in the extracellular space, which is limited by a two-step process: (i) its rapid and selective uptake by cells through the action of a membrane transporter and (ii) intracellular degradation. In particular, AEA is hydrolyzed to ethanolamine and arachidonic acid by the enzyme fatty acid amide hydrolase (FAAH) (17, 18). Both components of this inactivation process of AEA are the objects of active investigations. Recent data seem to indicate that the uptake process is the rate-limiting step in AEA degradation (19–23). There is pharmacological evidence suggesting that also the hypotensive action of AEA *in vivo* is limited by its re-uptake (24). However, the existence of the AEA membrane transporter in endothelial cells has never been investigated.

Although cannabinoid receptor activation was recently shown to lead to AEA biosynthesis (25, 26), the possibility of a functional link between CB_1 and CB_2 receptors and the AEA transporter has not been tested. Such a functional coupling might trigger self-elimination of AEA following activation by this lipid of cannabinoid receptor-dependent signaling pathways and would represent a regulatory loop critical for the manifold actions of this compound. A possible mechanism for this coupling may be suggested by findings that AEA binding to

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[1] The abbreviations used are: AEA, anandamide (arachidonoylethanolamide); CB, cannabinoid; EDHF, endothelium-derived hyperpolarizing factor; 2-AG, arachidonoylglycerol; FAAH, fatty acid amide hydrolase; HUVEC, human umbelical vein endothelial cell; NO, nitric oxide; NOS, nitric-oxide synthase; NAC, *N*-acetylcysteine; BSO, DL-buthionine-[*S*,*R*]-sulfoximine; SOD, superoxide dismutase; L-NAME, *N*ω-nitro-L-arginine methyl ester; SNP, sodium nitroprusside; AM404, *N*-(4-hydroxyphenyl)-arachidonoylamide; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SIN-1, 3-morpholino-sydnonimine; linvanil, α-linolenoyl-vanillyl-amide; iNOS, inducible NOS; SPER-NO, spermine NONDate.

cannabinoid receptors leads to NO release (13, 27, 28), whereas AEA uptake is enhanced by NO donors (22).

The results reported here demonstrate a saturable and temperature-dependent transport of AEA into endothelial cells. AEA uptake by human umbelical vein endothelial cells (HUVECs) is enhanced by various NO donors and further potentiated by superoxide anions. Conversely, a major cellular antioxidant and NO scavenger, glutathione, reduces the NO effect on the AEA transport. The observation that exogenously added NO donors may link CB receptors and HUVEC AEA transporter, through a CB receptor-mediated up-regulation of inducible NO synthase (NOS) and intracellular release of nitric oxide, appears to be the main outcome of this investigation.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the purest analytical grade. AEA, phenylmethylsulfonyl fluoride, hydroxocobalamin, *N*-acetylcysteine (NAC), DL-buthionine-[*S*,*R*]-sulfoximine (BSO), superoxide dismutase (SOD, bovine liver), actinomycin D, cycloheximide, *N*ω-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP) and *N*-(4-hydroxyphenyl)-arachidonoylamide (AM404) were purchased from Sigma. 2-AG and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were from Research Biochemicals International, and spermine NONOate ((Z)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropyl-ammonio)-butyl]-amino}-diazen-1-ium-1,2-diolate) (SPER-NO) and 3-morpholinosydnonimine (SIN-1) were from Alexis Corp. (Läufelfingen, Switzerland). Peroxynitrite was from Calbiochem. *N*-piperidino-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR 141716) and *N*-[1(S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR 144528) were a kind gift from Sanofi Recherche (Montpellier, France). [^{3}H]AEA (223 Ci/mmol) was from NEN Life Science Products, L-[2,3,4,5-^{3}H]arginine (64 Ci/mmol) was from Amersham Pharmacia Biotech. [^{3}H]2-AG (5 Ci/mol) was synthesized as described previously (21). Linvanil (α-linolenoyl-vanillyl-amide) was synthesized as reported (29). HU-210 was kindly donated by Prof. R. Mechoulam (The Hebrew University of Jerusalem). Monoclonal antibodies against the inducible nitric oxide synthase (iNOS) were purchased from Transduction Laboratories. Rabbit polyclonal antibodies against the N-terminal region of the human CB_1 receptor were from Calbiochem, and rabbit polyclonal antibodies against the human apoptosis protease-activating factor 1 were purchased from Cayman Chemical. Goat anti-mouse antibodies conjugated with alkaline phosphatase were from Bio-Rad. Generation of superoxide anions (O_2^-) was achieved by adding to the culture medium 100 μM xanthine and 5 milliunit/ml xanthine oxidase (Sigma), which produces about 2 μM O_2^-/min (30).

Endothelial Cell Culture—HUVECs were purchased from BioWhittaker and were cultured in 75-cm^2 flasks at a density of 2500/cm^2 in EGM-2 Bulletkit medium (BioWhittaker). HUVECs were maintained at 37 °C in humidified 5% CO_2 atmosphere and were split every second day (at 70–80% confluency) with daily replacement of the culture medium.

Determination of Anandamide Uptake—The uptake of [^{3}H]AEA by intact HUVECs was studied essentially as described (22). Cells at the fourth to fifth passage were washed in phosphate-buffered saline, trypsinized with trypsin-EDTA (Life Technologies, Inc.), and resuspended in their serum-free culture media at a density of 1×10^6 cells/ml. Cell suspensions (1 ml/test) were incubated for different time intervals, at 37 °C, with 100 nM [^{3}H]AEA, and they were washed three times in 2 ml of culture medium containing 1% bovine serum albumin and were finally resuspended in 200 μl of phosphate-buffered saline. Membrane lipids were then extracted (31), resuspended in 0.5 ml of methanol, and mixed with 3.5 ml of Sigma-Fluor liquid scintillation mixture for nonaqueous samples (Sigma), and radioactivity was measured in a LKB1214 Rackbeta scintillation counter (Amersham Pharmacia Biotech). To discern noncarrier-mediated from carrier-mediated transport of AEA into cell membranes, control experiments were carried out at 4 °C (22). Incubations (15 min) were also carried out with different concentrations of [^{3}H]AEA, in the range 0–1000 nM, to determine apparent K_m and V_{max} of the uptake by Lineweaver-Burk analysis (in this case, the uptake at 4 °C was subtracted from that at 37 °C). Q_{10} value was calculated as the ratio of AEA uptake at 30 and 20 °C (19). AEA uptake was expressed as pmol of AEA taken up/min/mg of protein. The effect of different compounds on AEA uptake (15 min) was determined by adding each substance directly to the incubation medium at the indicated concentrations. In the case of BSO or NAC, cells were preincubated for 6 h before assaying AEA uptake. Cell viability after each treatment was checked with Trypan blue and was found to be higher than 90% in all cases. Uptake of [^{3}H]2-AG by HUVECs was determined as reported previously for other cell types (16, 32).

Enzymatic Assays— FAAH (E.C. 3.5.1.4) activity was assayed in HUVEC extracts by measuring the release of [^{3}H]arachidonic acid from [^{3}H]AEA, using reversed phase high performance liquid chromatography as reported (33). The activity of NOS (E.C. 1.14.13.39) was assayed by incubating cell extracts with the radiolabeled substrate [^{3}H]arginine and then measuring the reaction product [^{3}H]citrulline as described (34). FAAH and NOS activities were expressed as pmol arachidonate or pmol citrulline released/min/mg of protein, respectively. The effect of various compounds on FAAH or NOS activity was determined by adding each substance directly to the assay buffer, at the indicated concentrations, and incubating for 15 min at 37 °C. The expression of the iNOS at the protein level was determined by enzyme-linked immunosorbent assay, performed by coating the plate with cell homogenates (25 μg/well), prepared as described (34). Anti-iNOS monoclonal antibodies (diluted 1:400) were used as first antibody, and goat anti-mouse antibodies conjugated with alkaline phosphatase were used as second antibody, diluted 1:2000. Color development of the alkaline phosphatase reaction was followed at 405 nm, using *p*-nitrophenylphosphate as substrate (34). Controls included wells coated with different amounts of bovine serum albumin.

Determination of Glutathione Content in Endothelial Cells—The colorimetric assay based on 5,5′-dithiobis-(2-nitrobenzoic acid) was used to quantify glutathione, the only detectable thiol in endothelial cells (35). HUVECs (2.5×10^6 cells/test) were treated with different compounds (or vehicle alone in the controls) for 15 min, and they were washed in phosphate-buffered saline and centrifuged at 800 × *g*, and pellets were resuspended in 75 μl of trichloroacetic acid (5% in 0.1 M HCl, 10 mM EDTA). Supernatants from the 10,000 × *g* centrifugation were recovered and aliquots of 60 μl were mixed with 130 μl of stock buffer (125 mM Na_2PO_4, 6.3 mM EDTA, pH 7.4). Ten μl of stock buffer containing 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) were added to each sample, and after 30 min at room temperature in the dark the absorbance was read in a microtiter plate at 412 nm (extinction coefficient was 14.3 mM^{-1} cm^{-1}) (35). The glutathione content of cellular extracts was within the linearity range of the assay procedure, as assessed by calibration curves made with glutathione (Sigma). The sensitivity of the colorimetric assay was ascertained by incubating HUVECs for 6 h with 1 mM BSO or NAC, a selective inhibitor or a precursor of glutathione biosynthesis, respectively (35).

Nitrite Production Assay—Generation of NO was determined by measuring accumulation of the stable end product nitrite (NO_2^-) in culture supernatants (30, 36). HUVECs (5×10^6 cells/test) were treated with different compounds (or vehicle alone in the controls) for 15 min, and the nitrite levels were determined in the culture medium via spectrophotometric analysis, after using nitrate reductase (Alexis Corporation, LÄufelfingen, Switzerland) and the acid-catalyzed diazotation reaction with sulfanylamide and naphtylethylenediamine (Griess reaction) as described (35). Nitrite levels in culture supernatants were within the linearity range of calibration curves made from a solution of sodium nitrite.

Determination of cAMP Concentration—HUVECs (5×10^6 cells/test) were treated with different compounds (or vehicle alone in the controls) for 15 min, then medium was discarded, and the cells were trypsinized as described above. Cyclic AMP levels in acetylated HUVEC extracts were determined by the Cayman Chemical cAMP Enzyme Immunoassay kit (Alexis Corporation, Läufelfingen, Switzerland). Cyclic AMP in cellular extracts was within the linearity range of the method, calibrated with acetylated cAMP as suggested by the manufacturer.

Data Analysis—Data reported in this paper are the mean ± S.D. of at least three independent experiments, each performed in duplicate. Statistical analysis was performed by the Student's *t* test elaborating experimental data by means of the InStat program (GraphPAD Software for Science).

RESULTS

Characterization of AEA Uptake by Endothelial Cells and Its Modulation by NO—HUVECs were able to accumulate [^{3}H]AEA, a process which was temperature- ($Q_{10} = 1.6$), time- ($t_{1/2} = 4$ min) and concentration-dependent (Fig. 1*A* and data not shown). [^{3}H]AEA uptake at 37 °C was saturable (apparent $K_m = 190 \pm 10$ nM, apparent $V_{max} = 45 \pm 3$ pmol·min^{-1}·mg^{-1} protein) and was inhibited in a dose-dependent manner by the

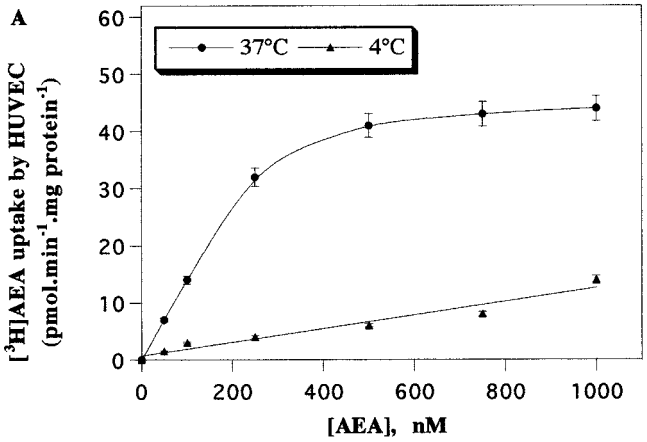


FIG. 1. **Uptake of [^{3}H]AEA by intact HUVECs.** *A,* dependence of [^{3}H]AEA uptake (15 min) on AEA concentration. *B,* effect of nitric oxide donors SNP, SNAP, and spermine NONOate on the uptake of 100 nM [^{3}H]AEA by HUVECs (15 min, 37 °C). Uptake was expressed as percentage over the control (100% =18.5 ± 1.2 pmol·min^{-1}·mg^{-1} protein). Values are reported as mean ± S.D. (*vertical bars*) of at least three independent experiments, each performed in duplicate. *, $p < 0.01$ compared with untreated control.

synthetic vanilloid linvanil (29), 10 μM of which reduced the transport to about 38% of the untreated control (Table I). A similar inhibition of AEA transport (42 ± 5% of the control) was observed also in the presence of 10 μM AM404 (not shown). It is noteworthy that 2-AG inhibited [^{3}H]AEA uptake by HUVECs in a dose-dependent manner, showing a IC_{50} of 400 ± 30 nM. Lineweaver-Burk analysis of double reciprocal plots showed that the inhibition was competitive and had an apparent inhibition constant (K_i) of 350 ± 30 nM. However, we could not find accumulation of [^{3}H]2-AG into HUVECs and observed instead that the compound was either immediately hydrolyzed to [^{3}H]arachidonic acid and glycerol (see below) or directly inserted into membrane phospholipids in a temperature-dependent fashion (data not shown), as previously reported for J774 macrophages and rat basophilic leukemia (RBL-2H3) cells (16, 32).

AEA uptake was dose dependently enhanced by the NO donors SNP, SNAP, or SPER-NO (Fig. 1*B*), which led to a 2.2-fold increase when used between 2.5 mM (SNAP) and 5 mM (SNP or SPER-NO). The NO scavenger hydroxocobalamin (1 mM) abolished the stimulation of AEA uptake by 5 mM SNP or 2.5 mM SNAP. NO can readily react with O_2^- generating peroxynitrite ($ONOO^-$), a potent oxidant and nitrosylating agent (30). Co-incubation of SNP or SNAP with a superoxide (O_2^-) generating system, such as xanthine-xanthine oxidase (see "Experimental Procedures"), led to a further enhancement of AEA transport compared with NO donors alone, *i.e.* up to 2.7- and 3.2-fold the control compared with 2.0- and 2.5-fold with SNP or SNAP, respectively (Fig. 2*A*). Superoxide ions alone hardly affected AEA uptake (not shown). To test the hypothesis that peroxynitrite was more efficient than NO as a stimulator of AEA transport, SIN-1, which generates $ONOO^-$ via simultaneous release of NO and O_2^- in stoichiometric amounts (37), was used. SIN-1 (1 mM) was twice as effective as SNP (5 mM) or SNAP (2.5 mM) in enhancing AEA uptake by HUVECs, leading to a 4-fold increase over the untreated control (Fig. 2*A*). When peroxynitrite was added directly to the medium, a dose-dependent increase in AEA uptake by HUVECs was also observed. The transport increased from 18.5 ± 1.2 to 32.3 ± 2.5 or 39.4 ± 3.4 pmol·min^{-1}·mg^{-1} protein, in the presence of 150 or 300 μM $ONOO^-$, respectively. The presence of SOD (100 units/ml) in the medium significantly reduced the effect of SNP or SNAP on AEA transport into the endothelial cells (Fig. 2*A*). The effect of SOD on AEA transport was more pronounced when the experiments were carried out with SIN-1 (from 4- to 1.5-fold of the control). Finally, the presence of O_2^- in excess over NO, as in the case of co-incubation of HUVECs with O_2^- and SIN-1, did not further potentiate AEA uptake by endothelial cells, which was instead slightly reduced (Fig. 2*A*).

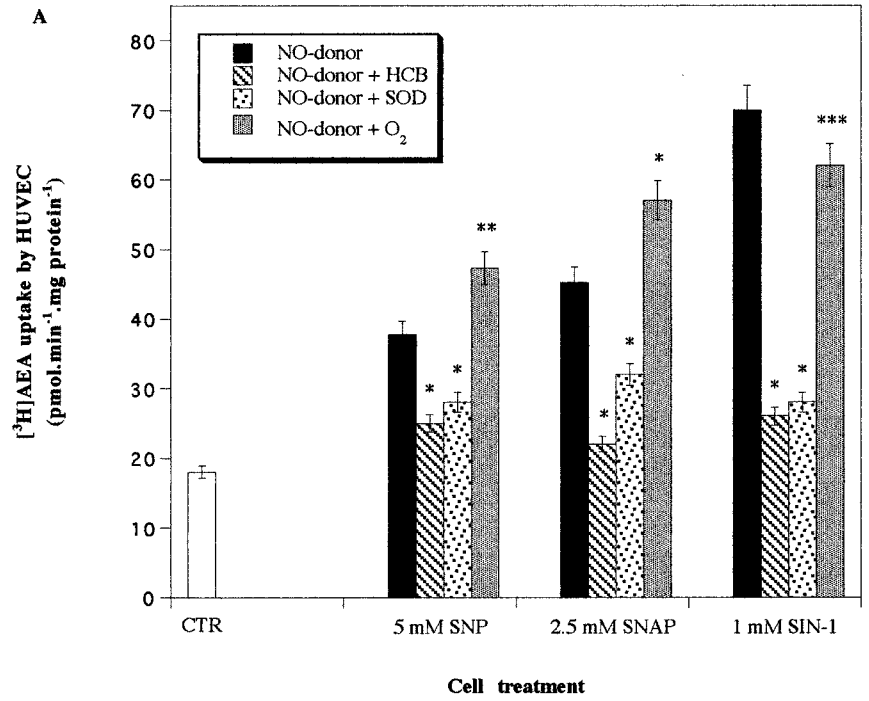
TABLE I
Effect of linvanil and HU-210 on AEA uptake by intact HUVECs
The uptake of 1 μM [^{3}H]AEA by intact HUVECs was measured after a 15-min incubation at 37 °C, in the absence or in the presence of each compound. Values in brackets represent percentage of the control.

Compound	[^{3}H]AEA uptake
	pmol · min^{-1} · mg $^{-1}$protein
None	44 ± 5 (100%)
+5 μM linvanil	23 ± 2 (52%)[a]
+10 μM linvanil	17 ± 2 (38%)[a]
+100 nM HU-210	60 ± 6 (137%)[b]
+1 μM HU-210	95 ± 10 (216%)[a]
+1 μM HU-210 + 400 μM L-NAME	51 ± 5 (116%)[c]
+1 μM HU-210 + 0.1 μM SR141716	52 ± 5 (118%)[c]
+1 μM HU-210 + 0.1 μM SR144528	90 ± 9 (204%)[a]

[a] $p < 0.01$ compared with control.
[b] $p < 0.05$ compared with control.
[c] $p < 0.05$ compared with control.

Modulation of AEA Uptake by Glutathione—To examine whether intracellular glutathione could affect the activation of AEA uptake by NO donors, HUVECs were treated with NAC or BSO, a precursor or a selective inhibitor of glutathione biosynthesis, respectively (35). NAC produced a 1.8-fold increase in intracellular glutathione (Fig. 3*A*). Under these conditions, the induction of AEA transport by SNP, SNAP, or SIN-1 was markedly attenuated (Fig. 2*B*). On the other hand, treatment with BSO reduced by 50% the glutathione content in HUVECs (Fig. 3*A*), further enhancing the AEA uptake by any of the NO donors used (Fig. 2*B*). It is worth noting that recently it has been shown that NO donors *per se* do not affect the intracellular glutathione concentration in endothelial cells (35).

AEA Enzymatic Hydrolysis in Endothelial Cells—Once taken up by HUVECs, AEA (and possibly 2-AG (32, 38)) can be degraded by a FAAH. This FAAH, found and characterized here for the first time, shows an apparent K_m and a V_{max} of 7 ±



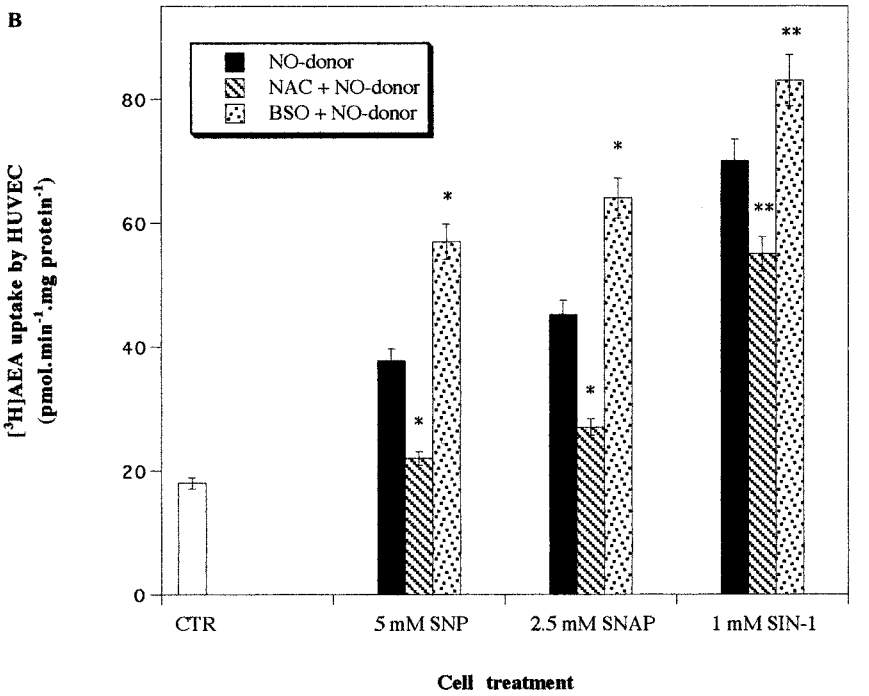
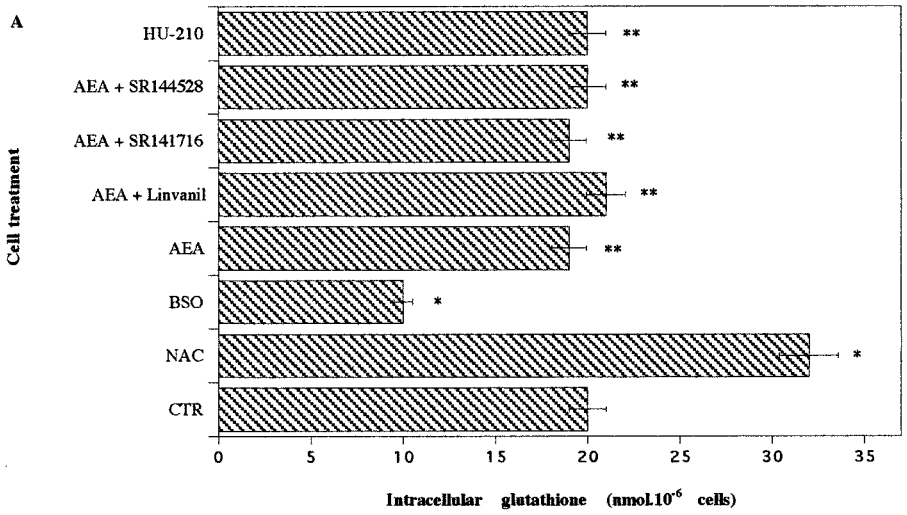
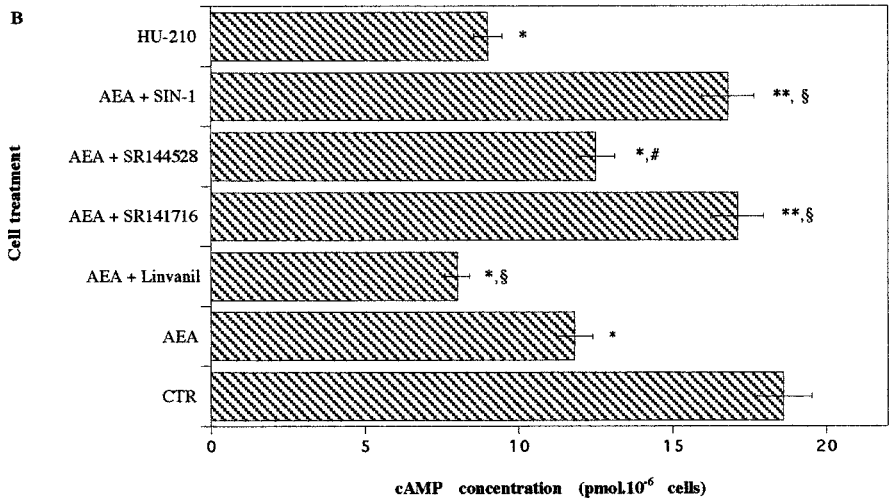


FIG. 2. **Effect of various compounds on the activation of [^{3}H]AEA uptake by nitric oxide donors.** *A,* uptake of 100 nM [^{3}H]AEA by HUVECs was measured after a 15-min incubation at 37 °C with NO donors SNP (5 mM), SNAP (2.5 mM), or SIN-1 (1 mM), in the presence or absence of hydroxocobalamin (1 mM), superoxide dismutase (100 units/ml), or xanthine-xanthine oxidase (see "Experimental Procedures"). *B,* the effect of pretreatment of HUVECs for 6 h with *N*-acetylcysteine (1 mM) or buthionine-[*S*,*R*]-sulfoximine (1 mM) on the activation of [^{3}H]AEA uptake by nitric oxide donors was determined in the same conditions as in *A*. Control experiments (*CTR*) were performed by exposing HUVECs to medium alone. *HCB*, hydroxocobalamin. Values are reported as mean ± S.D. (*vertical bars*) of at least three independent experiments, each performed in duplicate. *, $p < 0.01$ compared with NO donor alone; **, $p < 0.05$ compared with NO donor alone; ***, $p > 0.05$ compared with NO donor alone.

0.7 μM and 25 ± 3 pmol·min^{-1}·mg^{-1} protein toward AEA, respectively. It was unaffected by NO donors or by glutathione concentration (data not shown). The properties of FAAH in HUVECs resembled those previously reported for human neuroblastoma CHP100 cells (22).

The AEA Transporter Quenches the Activity of AEA toward Endothelial Cells and Is Linked to the Activation of CB_1 Cannabinoid Receptors—HUVECs have been recently shown to express the CB_1 messenger RNA (15). The physiological importance of the AEA transporter in limiting AEA activity in HUVECs was investigated by its effect on the intracellular concentration of cyclic AMP, a second messenger in cannabinoid





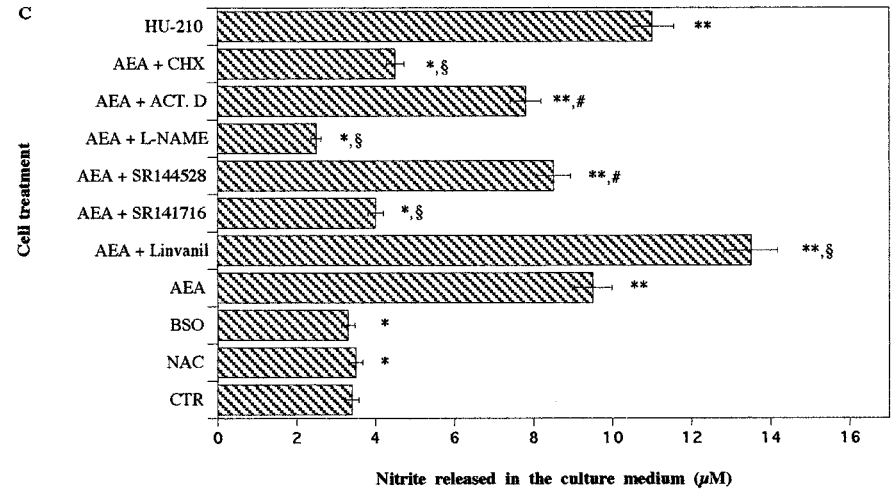


FIG. 3. **Effect of AEA and related compounds on intracellular glutathione, cAMP concentration, and nitrite release by HUVECs.** *A,* glutathione content in HUVECs was determined after a 15-min treatment at 37 °C with AEA (1 μM), alone or in the presence of linvanil (10 μM), SR141716 (0.1 μM), or SR144528 (0.1 μM). The effect of HU-210 (1 μM) was also determined under the same experimental conditions. NAC (1 mM) or BSO (1 mM) represented the positive and negative control, respectively. *, $p < 0.01$ compared with control; **, $p > 0.05$ compared with control. *B,* cyclic AMP concentration in HUVECs treated as in *A*, or in cells exposed for 15 min at 37 °C to AEA (1 μM) in the presence of SIN-1 (1 mM). *, $p < 0.01$ compared with control; §, $p < 0.01$ compared with AEA; **, $p > 0.05$ compared with control; #, $p > 0.05$ compared with AEA. Control experiments (*CTR*) were performed by exposing HUVECs to medium alone. *C,* release of nitrite was determined in the same samples as in *A* and also in HUVECs exposed to AEA (1 μM) for 15 min at 37 °C in the presence of L-NAME (400 μM) or pretreated for 4 h with actinomycin D (*ACT. D*) or cycloheximide (*CHX*) (10 μg/ml each) and then exposed to AEA under the same conditions. *, $p > 0.05$ compared with control; **, $p < 0.01$ compared with control; §, $p < 0.01$ compared with AEA; #, $p > 0.05$ compared with AEA. Values are reported as mean ± S.D. (*vertical bars*) of at least three independent experiments, each performed in duplicate.

TABLE II

Effect of AEA and related compounds on the activity and expression of nitric-oxide synthase in HUVECs

NOS activity was determined using [^{3}H]arginine as substrate and quantitating the reaction product [^{3}H]citrulline. NOS expression was determined by enzyme-linked immunosorbent assay, and by reacting cell homogenates (25 μg of proteins/well) with anti-inducible NOS monoclonal antibodies.

Sample	Nitric-oxide synthase	
	Activity	Protein level
	%	
Control	100[a]	100[b]
+1 μM AEA	250 ± 25[c]	220 ± 22[c]
+1 μM AEA + 10 μM linvanil	325 ± 32[c,d]	280 ± 28[c,d]
+10 μM linvanil	105 ± 10[e]	110 ± 11[e]
+1 μM AEA + 0.1 μM SR141716	112 ± 11[e,f]	120 ± 12[e,f]
+1 μM AEA + 0.1 μM SR144528	237 ± 24[c,g]	210 ± 21[c,g]
+1 μM AEA + 1 mM SIN-1	125 ± 13[h,f]	145 ± 15[h,f]
+1 mM SIN-1	110 ± 11[e]	115 ± 12[e]
+1 μM HU-210	290 ± 30[c]	260 ± 26[c]

[a] 100% = 40 ± 4 pmol citrulline · min^{-1} · mg $^{-1}$protein.
[b] 100% = 0.250 ± 0.030 absorbance units at 405 nm.
[c] $p < 0.01$ compared to control.
[d] $p < 0.05$ compared to AEA.
[e] $p > 0.05$ compared to control.
[f] $p < 0.01$ compared to AEA.
[g] $p > 0.05$ compared to AEA.
[h] $p < 0.05$ compared to control.

signaling pathways (3, 39). AEA (1 μM) significantly decreased forskolin-induced cAMP in endothelial cells, and this effect was reversed by 0.1 μM SR141716 but not SR144528. The potent CB_1 agonist HU-210 (1 μM) (1) also reduced cAMP content in endothelial cells (Fig. 3*B*). More importantly, this effect of AEA was potentiated by 10 μM linvanil and canceled by 1 mM SIN-1 (Fig. 3*B*), which inhibits or stimulates AEA transport, respectively. The other AEA transport inhibitor, AM404 (10 μM), also enhanced AEA inhibition of cAMP levels (45 ± 5% of control).

We found that AEA increased nitrite release from HUVECs in a dose-dependent manner (not shown), to ~2.6-fold above the controls at 1 μM AEA. The CB_1 antagonist SR141716 (0.1 μM), but not the CB_2 antagonist SR144528 (0.1 μM), fully reversed the effect of AEA, whereas the agonist HU-210 (1 μM) also led to a remarkable increase in nitrite release (Fig. 3*C*). The NOS inhibitor L-NAME (13, 34) fully reverted the AEA-induced nitrite release when used at 400 μM. A similar inhibitory effect was observed adding the protein synthesis inhibitor cycloheximide, but not the transcription inhibitor actinomycin D, both used at 10 μg/ml. Interestingly, inhibition of the AEA transporter by either 10 μM linvanil or 10 μM AM404 enhanced AEA-induced NO release up to 3.9- and 3.7-fold over the untreated control, respectively (Fig. 3*C* and data not shown). In keeping with these observations, exposure of HUVECs to 1 μM AEA or HU-210 significantly increased NOS activity (up to 250% over the untreated control), an effect which was blocked by 0.1 μM SR141716, but not 0.1 μM SR144528, and, in the case of AEA, again enhanced by 10 μM linvanil (325% of the control) (Table II). Changes in NOS activity were always paralleled by changes of (inducible) NOS protein content (Table II). Thus, 10 μM AM404 potentiated the effect of AEA on both NOS activity (310 ± 31% of the control) and content (270 ± 27% of the control) in a way superimposable to that observed with 10 μM linvanil (Fig. 3, *B* and *C,* and Table II). Conversely, activation of the AEA transporter by 1 mM SIN-1 inhibited AEA-induced NOS activity and content (Table II). Finally, AEA alone or in the presence of SR141716, SR144528, linvanil, or AM404 was always unable to modulate the intracellular glutathione concentration, as was HU-210 (Fig. 3*A*).

To check a possible NO-mediated functional link between the CB_1 receptor and the AEA transporter, the effect of HU-210 on AEA uptake was measured. HU-210 dose dependently enhanced AEA accumulation into cells, up to about 2.2-fold over the untreated control in the presence of 1 μM of the agonist (Table I). The CB_1 antagonist SR141716 (0.1 μM) counteracted the effect of 1 μM HU-210, whereas the CB_2 antagonist SR144528 was ineffective at the same concentration (Table I). Moreover, rabbit anti-human CB1 receptor antibodies (in the range 0–15 μg/ml or 0–0.1 μM) counteracted the effect of 1 μM HU-210 on AEA uptake in a dose-dependent manner; at 0.1 μM these antibodies significantly ($p < 0.05$) reduced AEA uptake by HUVECs from 216 to 150% of the untreated control. Rabbit anti-human apoptosis protease-activating factor 1 antibodies were ineffective under the same experimental conditions. Finally, the NOS inhibitor L-NAME also blocked HU-210-induced enhancement of AEA uptake (Table I).

DISCUSSION

We have shown that HUVECs have the ability to rapidly take up AEA in a temperature-dependent and saturable way. The AEA transporter in HUVECs exhibited a maximum velocity (apparent V_{max} = 45 ± 3 pmol·min^{-1}·mg^{-1} protein) closer to that reported for human neuroblastoma CHP100 cells (30 ± 3 pmol·min^{-1}·mg^{-1} protein) than that reported for human lymphoma U937 cells (140 ± 15 pmol·min^{-1}·mg^{-1} protein) (22). As previously shown in murine central neurons (17) and leukocytes (14), as well as in human neuroblastoma and lymphoma cells (22), the facilitated uptake process in HUVECs is likely to be followed by AEA hydrolysis catalyzed by FAAH (18). In fact, FAAH in HUVECs had kinetic properties similar to the hydrolase in CHP100 cells (22). The somewhat lower V_{max} value for FAAH toward AEA (25 ± 3 pmol·min^{-1}·mg^{-1} protein) should not forbid an efficient hydrolysis of AEA by endothelial cells, because the uptake rate into cells was similar. Moreover, we cannot rule out the possibility that other AEA-hydrolyzing activities with a different optimal pH, such as the enzyme recently described by Ueda's group (40), are present in endothelial cells besides FAAH. Interestingly, the HUVEC AEA transporter could be inhibited not only by the previously reported inhibitor of AEA-facilitated transport, AM404 (20), but also by a long chain fatty acid capsaicin analogue, linvanil, previously shown to inhibit the RBL-2H3 cell AEA transporter (29). This compound was selected for this study instead of other capsaicin analogues, such as olvanil and arvanil (29, 41, 42), because it exhibits very low affinity for cannabinoid receptors (29).

2-AG is another putative endogenous ligand for cannabinoid receptors (1, 15). This compound is produced and released by HUVECs on stimulation with the calcium ionophore A23187 or thrombin (15) and biosynthesized by aortic endothelial cells after treatment with carbachol (43). 2-AG was also shown to be produced and inactivated by rat platelets and macrophages (16). Because this compound also exerts a vasodilatory action (43, 44), we investigated 2-AG uptake by endothelial cells. Although we observed that 2-AG competitively inhibited AEA uptake, we did not get evidence for its temperature-dependent accumulation into intact HUVECs, possibly because this process may have been obscured or made unnecessary by the rapid esterification into membrane phospholipids and/or hydrolysis of 2-AG that were observed here as well as in other cell systems (16, 32).

NO donors SNP, SNAP, and SPER-NO are chemically unrelated compounds, which at millimolar concentrations release nanomolar concentrations of NO in solution (45, 46). We have previously shown that NO donors can enhance the activity of the AEA transporter in human neuroblastoma cells and platelets (22, 47). Accordingly, in HUVECs we found that NO donors activate the AEA transporter in a way proportional to their

ability to release NO (48), 2.5 mM SNAP being approximately as effective as 5 mM SNP or SPER-NO (Fig. 1*B*). Therefore, 2.5 mM SNAP or 5 mM SPER-NO were chosen to further characterize the sensitivity of AEA uptake to NO. Interestingly, superoxide anions and intracellular glutathione modulate the stimulation of AEA transporter by NO donors in cultured endothelial cells (Fig. 2). Superoxide anions (O_2^-) enhanced the effect of SNP or SNAP on AEA uptake, whereas SOD significantly reduced the effect of both NO donors (Fig. 2*A*). Because NO rapidly reacts with O_2^- to give peroxynitrite ($ONOO^-$), we investigated the possibility that $ONOO^-$ might activate AEA transporter better than NO. To this end, SIN-1, which generates $ONOO^-$ via simultaneous release of NO and O_2^- in stoichiometric amounts (37), was used and was found to be more effective than SNP or SNAP (Fig. 2*A*). Also, peroxynitrite directly added to the incubation medium led to a concentration-dependent increase in AEA uptake. It should be stressed that $ONOO^-$ may contribute to *S*-nitrosylation of target proteins *in vivo* better than NO does (30). Moreover, it has been proposed that NO synthase activity favors the formation of $ONOO^-$ rather than that of NO (49). In this context, it seems noteworthy that generation of O_2^- in excess over NO, as in the case of co-incubation of HUVECs with O_2^- and SIN-1, failed to potentiate the effect of SIN-1 on AEA uptake, which was instead slightly reduced (Fig. 2*A*). Indeed, excess superoxide anions have been shown to inhibit the nitrosylation reaction *in vitro* (30). Taken together, our results suggest a possible involvement of $ONOO^-$ in enhancing AEA uptake *in vivo* and indicate that this effect can be attenuated by preventing the direct interaction between NO and O_2^- through scavengers of these two radical molecules. Consistent with this hypothesis, depletion or enhancement of intracellular glutathione concentration potentiated or attenuated, respectively, the effect of NO donors on AEA transport into HUVECs (Fig. 2*B*). Indeed, although it is commonly accepted that NO diffuses freely in tissues, a recent report considers that, to reach its targets, NO needs to diffuse through the intracellular environment where glutathione levels are in the millimolar range (50). Glutathione is the most important cellular nonprotein thiol and constitutes the major cellular antioxidant (35). Moreover, glutathione binds to NO and forms *S*-nitrosoglutathione, a long lived NO derivative found in a variety of organ systems and biological fluids (51). In blood, nitrosoglutathione participates with *S*-nitroso serum albumin and *S*-nitroso hemoglobin in controlling transport, delivery, and disposal of nitric oxide (52, 53). Our data strongly suggest that, under certain conditions, glutathione may prevent NO from activating the AEA transporter.

A previous pharmacological study (24) had suggested that termination of the hypotensive effect of AEA *in vivo* could be effected through a re-uptake process. In this study we have provided biochemical evidence to this observation by showing that the AEA transporter regulates the activity of AEA in living endothelial cells. In fact, inhibition of AEA uptake by the selective AEA transport inhibitors, linvanil and AM404, or its activation by the peroxynitrite donor, SIN-1, enhance or inhibit, respectively, AEA effects on both forskolin-induced adenylyl cyclase and NO synthesis (Table II and Fig. 3). However, when another AEA effect, *i.e.* endothelium-dependent vasodilation, is monitored instead, inhibition of the transporter may also result in the reduction of AEA activity (54), possibly because this action requires the interaction of the endocannabinoid with an intracellular target.

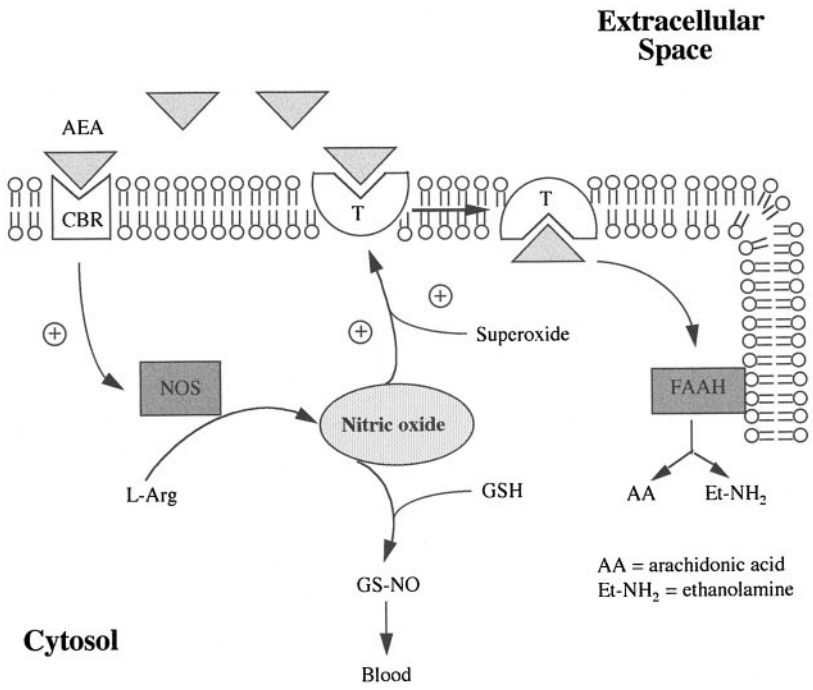
Given their sensitivity to the selective CB_1 antagonist SR141716, the effects of AEA and HU-210 on NO release and forskolin-induced cAMP formation are likely to be mediated by activation of CB_1-like receptors, whose presence in HUVECs had been suggested by Sugiura's group (15) by using reverse transcriptase-polymerase chain reaction. Additionally, a recent study suggested the presence, in endothelial cells, of an SR141716-sensitive, non-CB_1-non-CB_2 site of action for AEA and the nonpsychotropic cannabinoid, abnormal cannabidiol (55). Although more potent than AEA on CB_1, HU-210 does not activate this new site of action. This may explain why, in HUVECs, the synthetic cannabinoid appeared to be as efficacious as AEA (Fig. 3). However, only a full dose-response evaluation of the effects of both AEA and HU-210 on cAMP and NO levels in HUVECs would establish which of the two compounds is more potent in these cells.

Whereas the inhibition of forskolin-induced cAMP formation in endothelial cells by AEA was never reported before, previous studies (13, 27, 28) have shown that the endocannabinoid can induce NO release in these cells by acting at CB_1-like receptors. This effect was because of the activation of endothelial (constitutive) NOS and possibly resulted in the inhibition of cAMP formation (27). However, the present report is the first showing that AEA can cause NO release also by stimulating the activity and expression of the endothelial-inducible NOS isoform. In fact, AEA-induced NO release was not only reduced by the NOS inhibitor L-NAME but even required protein, but not messenger RNA, synthesis, as demonstrated by the experiments with cycloheximide and actinomycin D (Fig. 3*C*). NOS activity was paralleled by iNOS expression in the same cells (Table II) showing that the inducible form of NOS was part of the signaling pathway initiated by the CB_1 receptor. This finding extends previous observations showing that iNOS in HUVECs is rapidly modulated by growth factors, vasoactive hormones and estrogens (56–58). However, we could not establish to what extent the NO release was because of activation of either of the two NOS isoforms.

We suggest that NO donors might play a physiological regulation of AEA uptake, possibly linked to the activation of CB1 receptors by AEA. In fact, we found that the selective cannabinoid receptor agonist HU-210, while inducing NO release from HUVECs, significantly enhances [^{3}H]AEA uptake in a process sensitive to the NOS inhibitor L-NAME (Table I). Apart from a low concentration of the CB_1 receptor antagonist SR141716, this effect was also reduced by co-incubation with a polyclonal antibody against the extracellular domain of CB_1, thus pointing to the possible functional link between activation of cannabinoid receptors and withdrawal of AEA by the selective transporter identified in this study. According to this model depicted in Scheme I, AEA binding to cannabinoid receptors enhances the expression and the activity of NOS, which generates NO. The latter mediator then activates the AEA transporter even more in the presence of superoxide anions, whereas glutathione reduces the effect of NO by entrapping it into *S*-nitrosoglutathione. Once taken up by endothelial cells, AEA can be degraded by FAAH to arachidonic acid and ethanolamine. This scheme may represent a new, interesting mechanism through which AEA can limit its own CB_1-mediated actions. Indeed, preliminary experiments carried out in our laboratory[2] show that the endocannabinoid is toxic to some non-CB_1-containing tumor cells, probably also because they may be less efficient in the disposal of AEA through enhanced uptake.

The dependence of AEA transport into cells on NO/O_2^- formation might also represent an oxidative stress-induced mechanism for the reduction of extracellular AEA levels, while an increase of the anti-oxidative defense, through *N*-acetylcys-

[2] M. Maccarrone, T. Lorenzon, M. Bari, G. Melino, and A. Finazzi-Agrò, manuscript in preparation.



SCHEME I. **Regulatory loop between cannabinoid receptor and AEA transporter**. Binding of extracellular AEA to cannabinoid receptors (*CBR*) leads to activation of NOS and intracellular nitric oxide production from L-arginine (*L-Arg*). NO, or better peroxynitrite derived from its reaction with superoxide, activate transporter (*T*)-mediated uptake of AEA. Once uptaken, AEA can be rapidly cleaved to arachidonic acid and ethanolamine by membrane-bound FAAH. GSH can bind nitric oxide leading to *S*-nitrosoglutathione (*GS-NO*), thus inhibiting its effect.

teine and glutathione, would prevent the stress response by inhibiting $ONOO^-$-induced AEA uptake thus leading to an enhancement of AEA concentration. This hypothesis is in agreement with a cell-protecting role of AEA, for example during ischemic conditions (for reviews see Refs. 4 and 59).

Finally, it seems noteworthy that scheme I (with NO release promoting the termination of AEA signaling) establishes an inverse relationship between nitric oxide and anandamide, two relaxing factors derived both, at least in part, from endothelial cells. This type of relationship between endothelial-derived relaxing substances is not unprecedented in the literature as NO was shown to inhibit the release of the as yet uncharacterized EDHF (60), whereas activation of endothelial CB_1 receptors was recently reported to be negatively coupled to the production of EDHF (10). Although the physiological and pathological significance of these compensatory mechanisms remains to be established, our findings demonstrate that the potency and duration of AEA action in living cells are modulated by physiopathological stimuli coupled to NO release.

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REFERENCES

1. Mechoulam, R., Fride, E., and Di Marzo, V. (1998) *Eur. J. Pharmacol.* **359,** 1–18
2. Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998) *Trends Neurosci.* **21,** 521–528
3. Di Marzo, V. (1998) *Biochim. Biophys. Acta* **1392,** 153–175
4. Wagner, J. A., Varga, K., and Kunos, G. (1998) *J. Mol. Med.* **76,** 824–836
5. Randall, M. D., Alexander, S. P. H., Bennett, T., Boyd, E. A., Fry, J. R., Gardiner, S. M., Kemp, P. A., McCulloch, A. I., and Kendall, D. A. (1996) *Biochem. Biophys. Res. Commun.* **229,** 114–120
6. Wagner, J. A., Varga, K., Jarai, Z., and Kunos, G. (1999) *Hypertension* **33,** 429–434
7. Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H.-h., Sorgård, M., Di Marzo, V., Julius, D., and Högestätt, E. D. (1999) *Nature* **400,** 452–457
8. Mombouli, J.-V., and Vanhoutte, P. M. (1997) *Trends Pharmacol. Sci.* **18,** 252–256
9. Plane, F., Holland, M., Waldron, G. J., Garland, C. J., and Boyle, J. P. (1998) *Br. J. Pharmacol.* **121,** 1509–1511
10. Fleming, I., Schermer, B., Popp, R., and Busse, R. (1999) *Br. J. Pharmacol.* **126,** 949–960
11. Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., and Busse, R. (1999) *Nature* **401,** 493–497
12. Randall, M. D., and Kendall, D. A. (1998) *Trends Pharmacol. Sci.* **19,** 55–58
13. Deutsch, D. G., Goligorsky, M. S., Schmid, P. C., Krebsbach, R. J., Schmid, H. H. O., Das, S. K., Dey, S. K., Arreaza, G., Thorup, C., Stefano, G., and Moore, L. C. (1997) *J. Clin. Invest.* **100,** 1538–1546
14. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L., and Di Marzo, V. (1997) *J. Biol. Chem.* **272,** 3315–3323
15. Sugiura, T., Kodaka, T., Nakane, S., Kishimoto, S., Kondo, S., and Waku, K. (1998) *Biochem. Biophys. Res. Commun.* **243,** 838–843
16. Di Marzo, V., Bisogno, T., De Petrocellis, L., Melck, D., Orlando, P., Wagner, J. A., and Kunos, G. (1999) *Eur. J. Biochem.* **264,** 258–267
17. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., and Piomelli, D. (1994) *Nature* **372,** 686–691
18. Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996) *Nature* **384,** 83–87
19. Hillard, C. J., Edgemond, W. S., Jarrahian, A., and Campbell, W. B. (1997) *J. Neurochem.* **69,** 631–638
20. Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A., and Piomelli, D. (1997) *Science* **277,** 1094–1097
21. Bisogno, T., Sepe, N., Melck, D., Maurelli, S., De Petrocellis, L., and Di Marzo, V. (1997) *Biochem. J.* **322,** 671–677
22. Maccarrone, M., van der Stelt, M., Rossi, A., Veldink, G. A., Vliegenthart, J. F. G., and Finazzi-Agrò, A. (1998) *J. Biol. Chem.* **273,** 32332–32339
23. Piomelli, D., Beltramo, M., Glasnapp, S., Lin, S. Y., Goutopoulos, A., Xie, X. Q., and Makriyannis, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5802–5807
24. Calignano, A., La Rana, G., Beltramo, M., Makriyannis, A., and Piomelli, D. (1997) *Eur. J. Pharmacol.* **337,** 1–2
25. Shivachar, A. C., Martin, B. R., and Ellis, E. F. (1996) *Biochem. Pharmacol.* **51,** 669–676
26. Pestonjamasp, V. K., and Burstein, S. H. (1998) *Biochim. Biophys. Acta* **1394,** 249–260
27. Stefano, G. B., Liu, Y., and Goligorsky, M. S. (1996) *J. Biol. Chem.* **271,** 19238–19242
28. Fimiani, C., Liberty, T., Aquirre, A. J., Amin, I., Ali, N., and Stefano, G. B. (1999) *Prostaglandins Other Lipid Mediat.* **57,** 23–34
29. Melck, D., Bisogno, T., De Petrocellis, L., Chuang, H.-h., Julius, D., Bifulco, M., and Di Marzo, V. (1999) *Biochem. Biophys. Res. Commun.* **262,** 275–284
30. Van der Vliet, A., 't Hoen, P. A., Wong, P. S.-Y., Bast, A., and Cross, C. E. (1998) *J. Biol. Chem.* **273,** 30255–30262
31. Maccarrone, M., Nieuwenhuizen, W. F., Dullens, H. F. J., Catani, M. V., Melino, G., Veldink, G. A., Vliegenthart, J. F. G., and Finazzi-Agrò, A. (1996) *Eur. J. Biochem.* **241,** 297–302
32. Di Marzo, V., Bisogno, T., Sugiura, T., Melck, D., and De Petrocellis, L. (1998) *Biochem. J.* **331,** 15–19
33. Maccarrone, M., Bari, M., and Finazzi-Agrò, A. (1999) *Anal. Biochem.* **267,** 314–318
34. Maccarrone, M., Fantini, C., Ranalli, M., Melino, G., and Finazzi-Agrò, A. (1998) *FEBS Lett.***434,** 421–424
35. Foresti, R., Clark, J. E., Green, C. J., and Motterlini, R. (1997) *J. Biol. Chem.* **272,** 18411–18417
36. Waksman, Y., Olson, J. M., Carlisle, S. J., and Cabral, G. A. (1999) *J. Pharmacol. Exp. Ther.* **288,** 1357–1366
37. Kelm, M., Dahmann, R., Wink, D., and Feelisch, M. (1997) *J. Biol. Chem.* **272,** 9922–9932
38. Goparaju, S. K., Ueda, N., Yamaguchi, H., and Yamamoto, S. (1998) *FEBS Lett.***422,** 69–73
39. Bayewitch, M., Avidor-Reiss, T., Levy, R., Barg, J., Mechoulam, R., and Vogel, Z. (1995) *FEBS Lett.* **375,** 143–147
40. Ueda, N., Yamanaka, K., Terasawa, Y., and Yamamoto, S. (1999) *FEBS Lett.* **454,** 267–270
41. Di Marzo, V., Bisogno, T., Melck, D., Ross, R., Brockie, H., Stevenson, L., Pertwee, R., and De Petrocellis, L. (1998) *FEBS Lett.* **436,** 449–454

42. Beltramo, M., and Piomelli, D. (1999) *Eur. J. Pharmacol.* **364,** 75–78
43. Mechoulam, R., Fride, E., Ben-Shabat, S., Meiri, U., and Horowitz, M. (1998) *Eur. J. Pharmacol.* **362,** 1–3
44. Jarai, Z., Wagner, J., Goparaju, S. K., Wang,, L., Razdan, R. K., Sugiura, T., Zimmer, A. M., Bonner, T. I., Zimmer, A., and Kunos, G. (2000) *Hypertension* **35,** 679–684
45. Bauer, J. A., Booth, B. P., and Fung, H.-L. (1995) *Adv. Pharmacol.* **34,** 361–381
46. Matthews, J. R., Botting, C. H., Panico, M., Morris, H. R., and Hay, R. T. (1996) *Nucleic Acids Res.* **24,** 2236–2242
47. Maccarrone, M., Bari, M., Menichelli, A., Del Principe, D., and Finazzi-Agrò, A.(1999) *FEBS Lett.***447,** 277–282
48. Singh, R. J., Hogg, N., Joseph, J., and Kalyanaraman, B. (1996) *J. Biol. Chem.* **271,** 18596–18603
49. Schmidt, H. H. H. W., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D., and Feelisch, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14492–14497
50. Hogg, N., Singh, R. J., and Kalyanaraman, B. (1996) *FEBS Lett.* **382,** 223–228
51. Upchurch, G. R., Jr., Welch, G. N., and Loscalzo, J. (1995) *Adv. Pharmacol.* **34,** 343–349
52. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996) *Nature* **380,** 221–226
53. Halliwell, B., and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, pp. 73–104, Oxford University Press, Oxford
54. Chaytor, A. T., Martin, P. E., Evans, W. H., Randall, M. D., and Griffith, T. M. (1999) *J. Physiol.* **520,** 539–550
55. Jarai, Z., Wagner, J. A., Varga, K., Lake, K. D., Compton, D. R., Martin, B. R., Zimmer, A. M., Bonner, T. I., Buckley, N. E., Mezey, E., Razdan, R. K., Zimmer, A., and Kunos, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14136–14141
56. Kroll, J., and Waltenberger, J. (1998) *Biochem. Biophys. Res. Commun.* **252,** 743–746
57. Schena, M., Mulatero, P., Schiavone, D., Mengozzi, G., Tesio, L., Chiandussi, L., and Veglio, F. (1999) *Am. J. Hypertens.* **12,** 388–397
58. Cho, M. M., Ziats, N. P., Pal, D., Utian, W. H., and Gorodeski, G. I. (1999) *Am. J. Physiol.* **276,** C337–C349
59. Schmid, H. H., Schmid, P. C., and Natarajan, V. (1996) *Chem. Phys. Lipids* **80,** 133–142
60. McCulloch, A. I., and Randall, M. D. (1998) *Br. J. Pharmacol.* **123,** 1700–1706