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# Endocannabinoid immune and vascular signaling[1]

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## HISTORY

Hemp has been cultivated for 7000 to 8000 years[1]. The Sumerian/Babylonian term for cannabis hemp is K(a)N(a)B(a), and it is one of the longest surviving root words in Indo-Semitic-European language. In 2700 BC, Shen Nung, a contributor to early Chinese medicine, mentions cannabis in the pharmacopoeia. Around 500 BC Zoroaster, a Persian prophet, in the Zend-Avesta, listing 10 000 medicinal plants, includes hemp. In the first century AD the Chinese begin making paper from hemp as an inexpensive means of preserving information. In 800 AD the Islamic prophet Mohammed allows cannabis use. In 1100, Moslems use cannabis to start Europe's first paper mill. In 1430 Saint Joan D'Arc is accused of using herbal drugs, ie, cannabis, to hear voices. Pope Innocent VIII (1484) labels cannabis as an unholy sacrament of the Satanic mass and issues a ban on cannabis. Queen Elizabeth I (1563) and King Phillip of Spain (1564) order land owners to grow cannabis. In America, the Jamestown Colony, Virginia (1619), enacts the New World's first marijuana legislation, ordering all farmers to grow Indian hemp seed. Mandatory hemp cultivation laws were passed in Massachusetts in 1631 and in Connecticut in 1632. Thus, hemp as a herbal medicine and commercial plant has long been apart of human history.

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## EVOLUTION: INVERTEBRATES

Many types of intercellular signal molecules first thought only to exist in vertebrates also exist in invertebrates[2]. These include catecholamines, indole amines, neuropeptides and opiate alkaloids, demonstrating that in all likelihood these had their origins in simpler organisms, i.e., invertebrates and/or single cell organisms. Thus, we can now conclude that they were maintained during evolution. In this regard, the operating and determining process stabilizing their presence during evolution is in all likelihood the redundancy of conformational matching in their synthesis, degradation and receptor interaction[2]. Given the large number of conformation matching events, serving as a safe guard, forces signaling to become conservative and stabilizes its presence. Thus, it is not all that surprising to find reports documenting the presence of endocannabinoids in simpler animals.

In invertebrates, the first glimmer of an endogenous cannabinoid presence was first noted by Acosta-Urquidi and Chase[3], working with the buccal and parieto-visceral ganglia of *Aplysia californica*, delta 9-tetrahydrocannabinol (THC) depressed nerve cell excitability. McClean and Zimmerman[4] showed that THC elicited actions on cellular growth in *Tetrahymena pyriformis*, involving cAMP[5]. In *Strongylocentrotus purpuratus* (sea urchin) THC reduced the fertilizing capacity of sperm[6]. Later, the presence of anandamide and two related acyl-ethanolamides (palmitoyl- and stearoyl-ethanolamides), as well as enzymatic activities potentially responsible for their biosynthesis and degradation, were found in *Paracentrotus lividus*[7]. Thus, anandamide or a related substance appears to be involved in sea urchin fertility[6,7]. Recently, Berdyshev[8] found that oleoyl- and linoleoyl-ethanolamide and THC, but not palmitoylethanolamide, inhibited sea urchin sperm fertilization by a non-cannabinoid receptor (CB1) manner, indicating either a novel receptor, CB2 or a non-specific effect.

In this regard, in 1996 membrane homogenates of

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*Mytilus edulis* (a marine bivalve mollusk) immunocytes revealed monophasic and specific anandamide binding sites[9]. Scatchard analysis showed a single, relatively high affinity binding site with a $K_d$ of 34.3 nmol/L, with $B_{max}$ of 441 fmol/mg membrane protein for the immunocytes. *Mytilus* microglia exhibited the same binding profile [a $K_d$ of 32.7 nmol/L, with $B_{max}$ of (458 ± 28) fmol/mg membrane protein], suggesting that they originate from this animal's immunocytes. Furthermore, a variety of diverse signal molecules were ineffective in displacing specifically bound $^3$H-anandamide. The cannabinoid agonist CP55940 and the antagonist SR-141716A were quite potent in displacing $^3$H-anandamide whereas Win 55212-2, another agonist, was less efficacious.

Given the fact that cannabinoid signal molecules have immune altering actions, it was of interest to determine if anandamide would induce the formation of nitric oxide (NO) as does morphine, which exhibits similar immunocyte suppressive actions[10-12]. Anandamide and CP55940 initiate the release of NO from *Mytilus* immunocytes, microglia, and human macrophages[9]. This process can be antagonized by coincubating the cells with the nitric oxide synthase inhibitor, $N^{\omega}$-nitro-*L*-arginine methyl ester (*L*-NAME), as well as exposing the cells to the cannabinoid antagonist SR 141716A[9]. Interestingly, CP55940 was more potent in this regard than anandamide[9]. Prior incubation with naloxone did not block the NO releasing action of CP55940 or anandamide.

Exposure of invertebrate immunocytes to 2-arachidonyl-glyceral (2-AG), another endocannabinoid, obtained by aspiration from the marine mussel *Mytilus edulis*, resulted in releasing NO[13]. Again, SRl41716A could block this process and not the CB2 antagonist, indicating its coupling to a CB1 mediated phenomenon. In these cells 2-AG-stimulated NO release was blocked by prior exposure to *L*-NAME. Additionally, 2-AG down regulated the spontaneously active immunocytes (7.2 % ± 1.3 % SEM); exhibiting form factor (FF: $4 \times \pi$ area/perimeter$^2$; FF of 0.42 ± 0.04 and mobile) to become round and immobile (FF 0.87 ± 0.06). The level of spontaneous activation was reduced to 1.4 % ± 0.5 % and 0.8 %, respectively. We also determined if arachidonic acid liberated NO[13]. In an examination of this phenomenon we found it to stimulate NO release but only at high concentrations, suggesting that it may not be involved in a prime signaling event[13].

In a recent report published from our laboratory we noted that NO release/presence also induces these same immunocytes to become round[11,12]. Following anandamide addition, amoeboid immunocytes and microglia changed their FF from 0.51 ± 0.08 to 0.82 ± 0.05 (SEM) and 0.46 ± 0.05 to 0.85 ± 0.7, respectively. Human amoeboid macrophages exhibit the same conformational shift to a more rounded shape upon anandamide exposure (0.51 ± 0.04 to 0.87 ± 0.06 SEM). Prior exposure of these cells to SR141716A or *L*-NAME prevented the cellular rounding[9]. Thus, as with opiates, cannabinoids may exert their biological actions via coupling to NO production. This study[9] is complemented by another recent study that demonstrated the presence of long chain acylethanolamides, eg, anandamide and palmitoylethanolamide in bivalve mollusks[14]. Furthermore, anandamide cell rounding results in a lack of adherence (Tab 1). In *Mytilus* immunocytes and human granulocytes adhering to heart and internal thoracic artery endothelium, anandamide inhibited their adherence in an NO and CB1 specific manner, respectively (Tab 1).

**Tab 1. Anandamide acutely diminishes immunocyte adherence via nitric oxide.**

| Drug | Cell adherence % of total | |
|---|---|---|
| | I Immunocytes | Human granulocytes |
| Control | 36 ± 6.3 | 56 ± 8.4 |
| Anan | 13.4 ± 3.6 | 19.2 ± 4.5 |
| Anan + *L*-NAME | 32.4 ± 7.9 | 48.4 ± 8.8 |
| Anan + SR 141716A | 37.3 ± 6.9 | 50.3 ± 7.3 |

These experiments were performed and described in the following references[44,65,82]. I Immunocytes = invertebrate immunocytes were examined as they adhered to *Mytilus* heart tissue in a application concentration of (100 ± 10) cells per 100 μL whereas human granulocytes were adherent to saphenous vein endothelium in a concentration of (147 ± 12) cells per 100 μL. Anan = anandamide (1 μmol/L); *L*-NAME (10 mmol/L); SR 141716A (1 μmol/L).

In yet another study, stereoselective binding sites for anandamide were found in leech (*Theromyzon tessulatum and Hirudo medicinalis*) central nervous system[15]. The anandamide binding site was monophasic and of high affinity exhibiting a $K_d$ of approximately 32 nmol/L with a $B_{max}$ of 550 fmol/mg protein in both animals. These sites are highly selective as demonstrated by the inability of other types of signaling molecules to displace $^3$H-anandamide. Furthermore, this binding site is also coupled to NO release. A deduced amino acid sequence (153 residues) analysis from a 480 pb amplified RT-PCR

fragment cDNA exhibited a 49.3 % and 47.2 % sequence identity with human and rat cannabinoid receptors (CB1R), respectively, in these animals. Thus, the leech cannabinoid receptor may be a G-protein coupled receptor with 7 transmembrane domains as in CB1R. Moreover, this sequence exhibits highly conserved regions, particularly in the putative transmembrane domains 1 and 2[15]. More strikingly, within the sequence, there are two highly conserved motifs-between amino acids 1 – 97 and 128 – 153-which show 80 % and 58 % homology to human CB1 recognition[15].

Anandamide has also been shown to influence ganglionic monoamine release[16]. We have previously reported that preloaded tritiated monamines can be released from invertebrate tissues by 50 mmol/L KCl[17]. This release process is sensitive to the presence of calcium[17]. In an earlier study[16] we demonstrated the KCl-induced release of preloade $^3$H-serotonin (5-HT) and $^3$H-dopamine (DA) in the leech *Hirudo medicinalis* and in the pedal ganglia of *Mytilus edulis*[16,17]. Anandamide, in a concentration-dependent manner, suppressed the potassium-stimulated release of $^3$H-DA, but not 5-HT[16]. The effect of anandamide was blocked by pre-exposing the neural tissues of both animals to the cannabinoid receptor antagonist SR141716A. SR 141716A by itself had no effect[16]. Prior treatment of both sets of ganglia with the nitric oxide synthase inhibitor *L*-NAME, significantly diminished the effect of anandamide. These data suggest that cannabinoids and their endogenous effectors play a prominent role in the regulation of catecholamine release in invertebrates as well as in mammals (Fig 1).

As noted above, anandamide initiates the release of NO from leech and mussel ganglia. SR141716A, a cannabinoid CB1 antagonist, blocks the anandamide-stimulated release of NO from these tissues[15]. Methyl arachidonyl fluorophosphonate (MAFP), a specific anandamide amidase inhibitor, when administered to either invertebrate ganglia with anandamide did not increase the

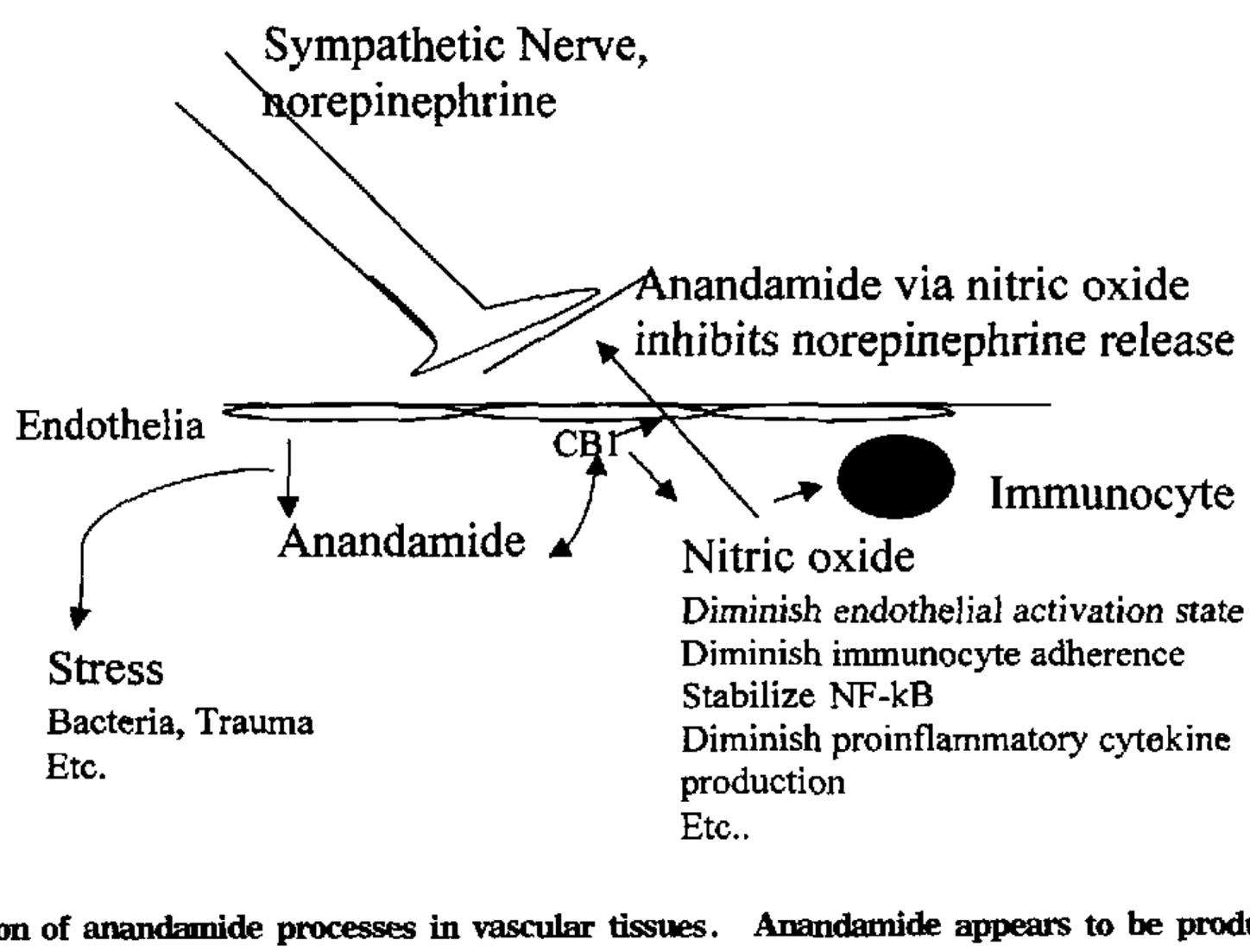


Fig 1. Illustration of anandamide processes in vascular tissues. Anandamide appears to be produced in vascular endothelial cells where it can exert an autoregulatory pathway by stimulating eNOS via the CB1 receptor. The nitric oxide so generated can then alter immunocyte activity, vascular activity and the interaction of the two tissues. eNOS-derived NO may then alter sympathetic nerve release of norepinephrine (NE). Furthermore, within the immune and vascular cells the NO so generated may stabilize the transcription factor NF-kB, thus diminishing the likelihood of proinflammatory cytokine generation. Under these circumstances the key question is what type of stimulus initiates anandamide synthesis/release. Here, it is probably some form of stress, ie, bacteria, faster heart beat, etc since pulsations can release NO[70,83]. It can even be surmised, since immunocyte respond to anandamide by releasing NO, that the immune origin of NO may enhance that derived from endothelial cells to enhance the NO driven processes already noted.

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peak level of NO release but did significantly extend NO release from 12 to 18 min ($P<0.05$)[18]. Lower levels of anandamide ($10^{-8}$ and $10^{-7}$ mol/L) do not stimulate the release of significant amounts of NO from these tissues. However, in the presence of MAFP (2.5 nmol/L), the lower anandamide concentrations were able to release significant peak amounts of NO. In mussel neural tissues the peak NO release increased from ($2.2 \pm 1.3$) nmol/L to ($8.6 \pm 2.1$) nmol/L. Taken together, the results indirectly demonstrate the presence of anandamide amidase in these tissues, suggesting that the enzyme may serve as an endogenous regulator of anandamide action. This result in *Mytilus* was substantiated by Sepe *et al*[14].

Cannabinoid signaling has even been found in the coelenterate *Hydra vulgaris*[19]. Hydra contain anandamide, 2-arachydonel glycerol (2-AG), the theoretical anandamide precursor *N*-arachidonoyl-phosphatidylethanolamine, and they also have anandamide amidohydrolase activity[19]. Hydra cell membranes exhibit specific binding sites for CB1 ligands and the CB1 antagonist SR141716A. Furthermore, this Hydra cannabinoid system appears to be involved with the organisms feeding behavior[19].

Taken together, it appears that cannabinoid signaling originated earlier than previously thought. Furthermore, this signaling system has been maintained during evolution, suggesting that specific determinants exist that support the preservation of this common signaling. Certainly, within the context of this signal molecule conservation, functional properties/actions are also important and will be considered at the end of the next section.

## VERTEBRATE VASCULAR AND IMMUNE CANNABINOID SIGNALING

**Vascular** In regard to cardiovascular actions, prolonged use of THC elicits a decrease in blood pressure and heart rate[20,21]. At that time more than 20 years ago, these effects were first believed to occur via the central nervous system. THC, in this regard, was even considered for use as an antihypertensive medication[22]. Needless to say, given its substance abuse association, this potential has never materialized to any great extent. Anandamide, later on was found to stimulate blood pressure responses and bradycardia[23], as did THC[24]. Interestingly, the hypotension stimulated by anandamide is absent in normotensive rats[25,26], but present in spontaneously hypertensive rats[27], suggesting a sympatho-inhibitory mechanism. Indeed, the modulation of peripheral norepinephrine release via anandamide stimulated NO release may help explain this mechanism in vascular tissues[28,29] (Fig 1).

In recent times we have demonstrated that endocannabinoid signaling occurs in mammalian vascular tissues[28]. The rat kidney contains both *N*-acylphosphatidylethanolamine (NAPE) and long-chain *N*-acylethanolamines (NAE) in a ratio of approximately 10:1. Anandamide amounts to 4.4 % of total NAE [($0.29 \pm 0.13$) pmol/$\mu$mol lipid P; ($2.79 \pm 1.11$) ng/g wet weight] and the corresponding 20:4 *N*-acyl groups in NAPE are 11.2 % of the amide-linked fatty acids. In contrast, cultured mesangial (MC) and endothelial cells (EC) contain approximately equal amounts of NAE and NAPE containing much smaller percentages (< 1 %) of *N*-arachidonoyl groups. Furthermore, both the MC and EC contain anandamide amidase activity, ($56 \pm 2$) and ($19 \pm 2$) nmoles anandamide hydrolyzed per hour per mg protein, respectively[28], demonstrating an important component of intercellular signaling. The MC and EC also exhibited synthase activity ($290 \pm 59$) pmoles/h per mg protein and ($298 \pm 72$) pmoles/h per mg protein, respectively)[28]. Because the amidase inhibitor MAFP also inhibited synthesis, it is likely that both hydrolytic and biosynthetic activities were catalyzed by the same enzyme[28].

In this study[28], Southern analysis of the RT/PCR amplified products indicates that CB1 mRNA was present in rat MC and EC. RT/PCR products of CB2 were only found in the MC and spleen, but not in EC. Accordingly, MC have messages for both the brain type receptor (CB1) and the spleen type receptor (CB2) while endothelial cells only have CB1 receptor mRNA. Supporting this data is the finding that membrane homogenates of rat renal microvascular EC contain anandamide binding sites[28]. Scatchard analysis showed a single, relatively high-affinity binding site with $K_d$ of 27.4 nmol/L, with $B_{max}$ of 623.3 fmol/mg membrane protein. Furthermore, a variety of diverse signal molecules were found to be ineffective in displacing specifically bound $^3$H-anandamide. However, this radioligand can be displaced by the agonists CP55940 and WIN 55,212-2, and the antagonist SR141716A[28].

Experiments utilizing the *in vitro* juxtamedullary (JM) nephron preparation segments 5 – 10 min after ex-

posure to anandamide at 60, 100, and 140 mmHg perfusion pressure revealed the following: A) The dose-response to anandamide was significantly influenced by the level of perfusion pressure; B) Anandamide was involved in baseline vascular tone through normal autoregulatory adjustments in lumen diameter; C) Significant vasodilation was observed at 1 μmol/L anandamide at 140 mmHg, and at 10 μmol/L at 60 and 100 mmHg; D) Anandamide stimulated vasodilation was reversible within 10 minutes[28]; E) The vasodilatory response to anandamide was inhibited by pretreatment for 10 minutes with the NO synthase inhibitor *L*-NAME, demonstrating that the vasorelaxation was mediated by NO; and lastly, F) The anandamide action was also inhibited by pretreatment with SR141716A, a specific antagonist for the CB1 receptor. Importantly, SR141716A had no effect on the *in vitro* juxtamedullary nephron preparation whereas marked vasoconstriction resulted from pretreatment with *L*-NAME, suggesting that anandamide does not exert a *significant influence over basal vascular tone in renal afferent arterioles perfused in vitro*[28]. In the above experiments anandamide-stimulated vascular endothelial NO production was verified with NO-sensitive amperometric electrodes in segments of rat arteries[28]. This anandamide stimulated constitutive nitric oxide synthase (cNOS) NO release was inhibited by the CB1 receptor blocker SR141716A. The absence of anandamide-stimulated hypotension in CB1 receptor knockout animals supports the present hypothesis[30,31] as does the recent finding of CB1 receptors by others in vascular endothelial cells[32,33].

As previously demonstrated (see invertebrate section), exposing tissues *in vitro* to 50 mmol/L KCl induces a calcium-dependent release of preloaded tritiated monoamines. In this regard, we demonstrate that anandamide suppresses KCl-stimulated release of $^{3}$H-norepinephrine from rat renal arterial segment[28] (Fig 1). This action of anandamide can be antagonized by preexposing the tissue to SR141716A[28]. SR141716A, when applied alone, does not alter the KCl-stimulated release of $^{3}$H-NE. Furthermore, the NOS inhibitor *L*-NAME, also antagonizes the anandamide inhibition of $^{3}$H-NE release, demonstrating that anandamide exerts this neurosuppressive effect via nitric oxide. Thus, vascular neural elements respond to NO, and in part, this circuit may represent a mechanism whereby vascular endothelial cells control peripheral sympathetic activity. Supporting this data is a study that demonstrates that anandamide modulates neurotransmitter release[34] and twitch responses in vas deferens[35]. The present results indicate a linkage between cannabinoid-induced suppression of the renal sympathetic nerves and NO release, a finding consistent with previous findings that NO suppresses NE release in heart and kidney[36]. The neuromodulatory role of NO seems to be especially important in the renal circulation, as the hypertension induced by NOS inhibition is ameliorated by renal denervation[37]. In addition, our results suggest that the vasodilatory action of anandamide may be amplified in states where renal sympathetic activity is high; behavior consistent with this concept has been observed in the systemic circulation of rats by Lake, *et al*[26,27].

In 1998[38] these observations were extended by the presence of both anti-anandamide and anti-CB1-R immunopositive material on the human saphenous vascular endothelium. This finding complemented the earlier study demonstrating that anandamide stimulates NO release in human saphenous vein[39]. In human internal *thoracic artery fragments and right atrium endothelia*, anandamide also stimulated NO release that was antagonized by the NOS inhibitor, *L*-NAME, as well as by the cannabinoid receptor 1 antagonist SR141716A[38]. Additionally, varying concentrations of MAFP plus anandamide stimulated a higher peak level of NO that remained elevated for a longer period of time[38].

Recently, Randall, *et al*[40], reported that anandamide may be an endothelial-derived hyperpolarizing factor acting on potassium channels, ie, activation in vascular smooth muscles. They showed that bradykinin stimulated NO-independent vasodilation in mesenteric arteries and renal vasculature could be inhibited by SR141716A. Interestingly, in this report[41] *L*-NAME did not antagonize anandamide's ability to stimulate vasodilation. The report appears, at first glance, to contradict the findings of the present study. However, the differences may be resolved by considering the experimental methodologies. Our experiments measured NO directly, demonstrating that it is of short duration. Further, in applying anandamide simultaneously with *L*-NAME, it did not completely block the cannabinoid or opiate stimulated release of NO. Indeed, this action only took place when *L*-NAME administration preceded that of anandamide. Furthermore, regarding the ability of anandamide to influence potassium channels, we have demonstrated in invertebrates that NO donors influence potassium channels negatively[42]. Clearly, the phenomena reported here are complex and at the present time

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many explanations are possible. In this regard, a recent report[43] demonstrates that SR141716A can increase blood pressure in rats subjected to hemorrhagic shock, indicating the involvement of endogenous cannabinoids. It is important to also note that a new abnormal cannabidiol (abn-cbd[22], molecule has been described that does not bind to CB1 receptors, yet it is SR141716A-sensitive and induces endothelium-dependent hypotension[31]). This introduces a new degree of complexity.

In another study, we demonstrated that activation of human EC, obtained from the saphenous vein, with morphine or anandamide stimulated NO that was of cNOS origin[39]. Furthermore, significant release of NO, from EC stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN) occurred after a 2.5-h post-exposure and remained significantly elevated over basal levels for 24 – 48 h, consistent with inducible NOS (iNOS) activation. Preincubation of EC with morphine or anandamide prior to, but not after, the addition of LPS + IFN, blocked iNOS activity. Exposure of EC to the NO donor, SNAP, prior to the addition of LPS + IFN, blocked iNOS induction, whereas preincubation of EC with inhibitors of NOS, prior to morphine or anandamide exposure, restored LPS + IFN induction of iNOS, suggesting a direct impact of NO on the regulation of iNOS activity. Morphine and anandamide stimulation of EC did not stimulate cAMP accumulation, whereas a marked increase in cAMP was observed in EC treated with LPS + IFN. Treatment of EC with LPS + IFN did not induce cAMP accumulation in EC treated with morphine, anandamide or SNAP prior to LPS + IFN exposure. These data suggest that cAMP is required for the induction of iNOS in EC and that NO may directly impair adenylate cyclase activity, preventing iNOS activation[39]. Taken together it would appear that anandamide actions are important in the regulation of vasodilation and immunocyte-endothelial interactions[44,45]. In 2000, we extended these observations by noting that iNOS expression was diminished by anandamide[46]. The anandamide vasodilation is consistent with the known systemic depressor actions of $\Delta^9$-THC and anandamide in humans and animals[26,47].

**Immune** With regard to immunocytes, THC inhibits macrophage cell line contact-dependent cytolysis of tumor cells[48]. THC also appears to alter antigen processing[49] and the expression of select proteins whose induction is associated with macrophage activation as well as the expression of tumor necrosis factor[50,51]. THC was found to increase supernatant interleukin-1 (IL-1) bioactivity in cultures of mouse resident peritoneal macrophages stimulated with lipopolysaccharide[52]. Incubating P388D1 macrophage cell cultures with THC results in a dose-dependent inhibition of cell propagation, DNA synthesis and phagocytosis[53]. In earlier reports, THC was found to inhibit human peripheral blood macrophage spreading and phagocytosis of yeast[54-56].

The inhibition of cell spreading[55-57] is in agreement with observations made by Stefano and colleagues[9], namely that anandamide receptor coupling to NO may be the mechanism initiating this cell rounding[10,12,58]. Given the fact that naloxone does not antagonize or bind to the anandamide receptor, these signal systems appear to be distinct. Thus, naturally occurring cannabinoids may share the NO-producing effector system with opiate alkaloids[10-12]. In this regard, the cannabinoid signaling system exhibits many biological similarities with that of opiate molecules. As with morphine, this psychoactive agent has been used by man for thousands of years. Biomedical properties it shares with morphine include analgesia, anti-inflammation and immunosuppression[9,59]. Another similarity this compound shares with morphine is that its receptors are found on neurons and immunocytes, suggesting autoimmunoregulating and neuroimmune actions. In this regard the cloning of a receptor for cannabinoids includes one found in macrophages[59].

Other common effects of cannabimimetic agents and opiates are in the inhibition of N18TG2 neuroblastoma cell adenylate cyclase[60]. The delta opioid receptor subtype on the N18TG2 membranes is unaltered by cannabimimetic drugs. Furthermore, opioid and opiate agents also inhibit this enzyme in the N18TG2 cells. Therefore it was concluded that both molecules were using diverse receptors but the same effector process, since naloxone only blocked the opioid action[60]. This observation is supported by the work of Stefano and colleagues[9]: naloxone, an opiate receptor antagonist, does not block the invertebrate cannabinoid receptor nor does it antagonize cannabinoid release of NO. This link between opioids and canabinboids is further strengthened by the observation that both bind to G-protein-coupled receptors to inhibit adenylyl cyclase in neurons[61]. In cerebellar granule cells, both cannabinoid and opioid receptors appear to exist on the same cells and their respective activation produces similar biological responses[61]. A recent report[62] provides evidence for a possible link with kappa receptors, however the exact nature of the interaction of the cannabinoids and the kappa receptors needs to be elucidated.

Given the above remarkable parallelism between opiate and cannabinoid signaling cascades and the overall array of physiological systems they affect, including NO release, we must ask the question why are two such "redundant" systems required? Again, both types of compounds have analgesic, anti-inflammation and immunosuppressive properties[59]. Based on the above discussion, namely, that both compounds can release NO by separate processes, we speculate that the answer may be found in the degree of this action. Morphine in diverse tissues and animals appears to be a more potent stimulator of cNOS-derived NO release than cannabinoids on a same dose basis[10-12]. We surmise that the cannabinoid system is "activated" at times requiring a milder analgesic, anti-inflammatory or immunosuppressive action. This hypothesis is supported by the findings concerning cannabinoid tolerance and addiction[63]. O'Brien summarizes that tolerance to cannabinoids disappears rapidly, and without withdrawal symptoms, and "few patients seek treatment for marijuana addiction". Indeed, this hypothesis can also be used to explain the presence of the cannabinoid receptor in invertebrates.

## FUNCTIONS: SIGNIFICANCE OF ENDOCANNABINOIDS

In other studies, we have demonstrated that anandamide via NO release can down regulate both macrophage and endothelial excitation, consequently their interaction as well[45,64,65]. Anandamide, when presented acutely, releases endothelial cNOS[9], thereby inhibiting macrophage adherence[45]. Importantly, we demonstrate that the initial exposure to anandamide uncouples the ability of these agents to stimulate constitutive endothelial NO release further, thus enhancing macrophage adherence[39,64,65]. Thus, as with morphine, anandamide's actions are biphasic[64-66]. At first, via NO, they are inhibitory, however, following this inhibitory phase the tissues become hyper-excitable, ie, rebound from inhibition. In this regard, we have demonstrated that this biphasic phenomenon is a function of cNOS-derived NO with the use of NO-donors as well[64,65,67]. The physiological significance of this rebound may lie in the fact that following activity suppression the various cells are hyperactivated, that is, they are exhibiting enhanced surveillance compensating for their "downtime"[66]. Clearly, then this biphasic response to anandamide has evolutionary value, hence its presence in both invertebrates and vertebrates.

In other reports we have demonstrated that estrogen also has the ability to stimulate cNOS derived NO[68,69] and this is significant since NO is also considered as an important inhibitory agent that diminishes immunocyte adhesion and the vascular endothelium's capability to adhere immunocytes as well as down regulating various immunocytes both before and after proinflammatory events[45,70] (Tab 2). In this regard, estrogen is acting in parallel with endogenous morphine and the endocannabinoid anandamide[45,71].

**Tab 2.**

| | |
|---|---|
| **Basal NO** | |
| | Maintain cells in a mildly inhibitory state |
| | Stabilizes cell shapes |
| | Inhibits the induction of proinflammatory signal molecules |
| | Limits intercellular action and communication |
| **cNOS Enhancement** | |
| | Enhances basal NO functions |
| | May emerge to prevent overriding basal NO function |
| | Emerges to restore basal NO homeostasis |
| **iNOS Enhancement** | |
| | Extreme challenge, bacteria, trauma, acute and chronic inflammation, surgery, etc. |

Again, it may appear that we have a redundant immunovascular down regulating process. However, we believe that each of these signaling systems performs this common function, ie, cNOS derived NO release (Tab 2), under different circumstances. Morphine, given its long latency before increases in its levels are detected, arises after trauma/inflammation to down regulate these processes in neural, vascular and immune tissues[45,72,73]. Anandamide constitutively expressed, by being part of the always present arachidonate and eicosanoid signaling processes, serves to maintain an immediate burst in basal NO in vascular tissues since morphine levels only rise after a latency period[74,75] (Fig 2). We surmise that estrogen, since testosterone or progesterone do not exert this NO generating action, provides an extra-degree of immunocyte and vascular down regulation in females. This is most probably due to both the immune and vascular trauma associated with cyclic reproduction activities, ie, endometrial buildup, when a high degree of vascular and immune activities are occurring. Given the high degree of proliferative growth capacity during estrogen peak levels in this cycle, NO may func-

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tion to enhance down regulation of the immune system to allow for these changes. In this regard, it is not difficult to understand the reports documenting various cancers with blocking estrogen actions and conversely reports documenting its anti-cancer protective actions[76].

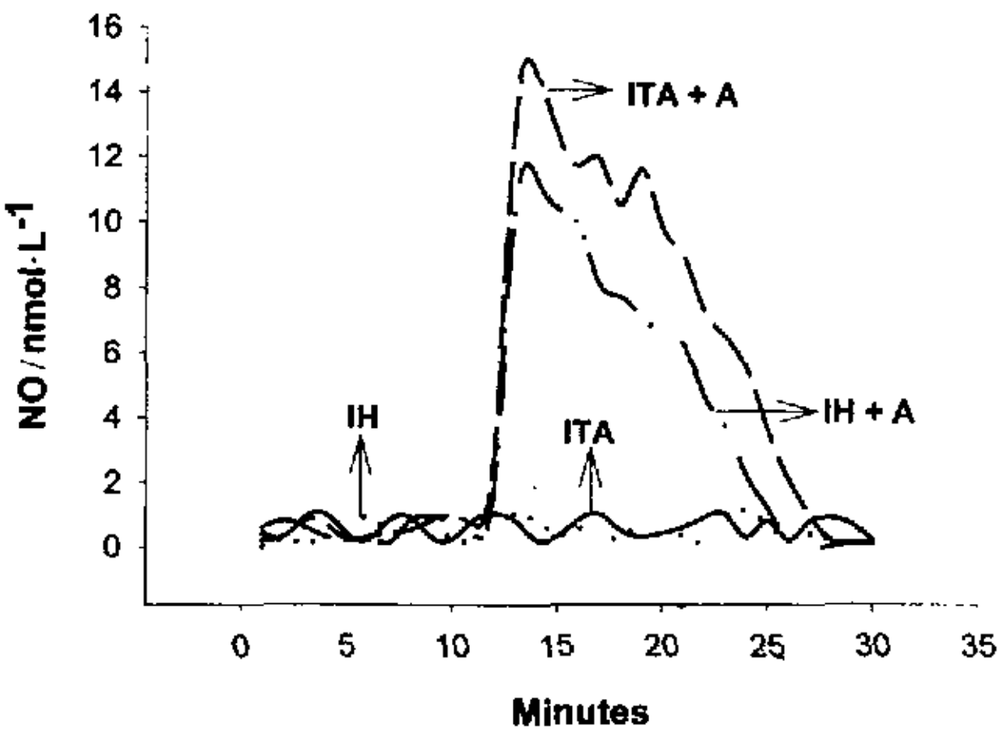


**Fig 2. Basal nitric oxide levels, measured amperometrically, from *Mytilus* heart (IH) and human internal thoracic artery (ITA) and following anandamide (A) exposure at 11 min. Note that basal levels (values plotted as a spline curve) *in vitro* exhibit a rhythmic pattern, faster for the human tissue. In this regard, this basal rhythmic pattern of NO release may help stabilize tissue for over-excitation, limit background noise, thus controlling the tissues ability to respond[70]. Clearly, anandamide exposure (μmol/L) immediately enhances basal NO release, suggesting that this mechanism enhances the stabilizing/homeostasis of this microenvironment (Tab 2).**

Thus, anandamide working via the CB1 receptor enhances a tissues capacity to down regulate itself as well as its interaction with other tissues, ie, immune-vascular. Again, invertebrates and vertebrates can benefit from this activity. Even though invertebrates have an open circulatory system, many have pulsatile hearts, immune-like cells and cell-lined organ systems that normally benefit from open tissue spaces that allow for the flow of hemolymph and immune cells that move, enhancing their surveillance capabilities (Tab 1, 2). Furthermore, NO is both antibacterial and antiviral[77], thus cNOS-derived NO bursts, as caused by anandamide, have an immediate protective value for survival.

In this regard, anandamide derived NO may be involved with an autoregulatory pathway that would further diminish cellular excitability. Constitutive NOS derived NO can depress iNOS-derived NO expression[39,45,46]. Furthermore, cNOS-derived NO can stabilize NF-kB levels, thereby preventing proinflammatory cytokine production, again diminishing the capacity for excitation[70,78,79]. Taken together, endocannabinoids may serve to boost basal cellular, i.e., vascular endothelial, immunocyte, etc, NO levels thereby inhibiting or preventing and limiting excitation[70], ie, setting a higher threshold. The need for this action in all organisms centres on the concept that while excitatory cascades are required to protect cells, tissues, organs and organisms, so are down regulating cascades. These down regulating cascades may prevent over excitation in an immune response as well as prevent a cell from responding to back ground noise, that may prove lethal to an organism[70]. Again, the presence of endocannabinoids in invertebrates and vertebrates may serve to demonstrate their significance in this regard. The fact that plants make THC may serve to indicate that they too require this activity. It may also indicate that this system evolved and remained in evolution because it arose in a common ancestor to both plants and animals.

As also noted with morphine, various stressors may trigger anandamide synthesis. Thus, it is not hard to predict, that as with morphine, endocannabinoids may participate in coping processes[80,81]. In this regard, since coping via cognition evolved only recently in evolution, it is not surprising to surmise anandamide involvement in this new neural activity since it represents its' ability to diminish excitation. Furthermore, there is a potential for immune cells, accumulating on vascular tissues, to also influence the activation state of vascular endothelial cells and other tissues. Thus, endocannabinoid signaling is old in regard to signal system evolvement and new in terms of our study of its involvement in intercellular signaling and physiological regulation. In this regard, we cannot let the stigma of substance abuse limit the biomedical exploration of these compounds for medical benefit.

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