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The elmiric acids: biologically active anandamide analogs

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Summary

As chemical entities, lipoamino acids have been known for some time. However, more recently their occurrence and importance in mammalian species has been discovered. They appear to have close relationships with the endocannabinoids not only structurally but also in terms of biological actions. The latter include analgesia, anti-inflammatory effects, inhibition of cell proliferation and calcium ion mobilization. To date about 40 naturally occurring members of this family have been identified and, additionally, several synthetic analogs have been prepared and studied. To facilitate their identity, a nomenclature system has been suggested based on the name elmiric acid (EMA). The prototypic example, N-arachidonoyl glycine does not bind to CB1, however it does inhibit the glycine transporter GLYT2a and also appears to be a ligand for the orphan G-protein-coupled receptor GPR18. It may also have a role in regulating tissue levels of anandamide by virtue of its inhibitory effect on FAAH the enzyme that mediates inactivation of anandamide. Its concentration in rat brain is several fold higher than anandamide supporting its possible role as a physiological mediator. Future studies should be aimed at elucidating the actions of all of the members of this interesting family of molecules.

Keywords

endocannabinoid; lipoamino acid; eicosanoid; elmiric acid; inflammation; anandamide

1. Lipoamino acids (fatty acid-amino acid conjugates): general considerations

The older literature on this topic is mainly concerned with lipoamino acids of bacterial origin (Batrakov et al., 2000; Kawai et al., 1990; Kawazoe et al., 1991; Lerouge et al., 1988; Miyazaki et al., 1993). These involve amino acid conjugation with complex and unusual fatty acids and little is known about their function in bacteria.

More recently, attention has been given to lipoamino acids present in mammalian species in part because of their possible relationships to the endocannabinoids. The closely related analog of anandamide, N-arachidonylglycine (NAGly), is in a sense, a comparable acid analog to anandamide as the THC metabolite, THC-11-oic acid is to THC. NAGly is an endogenous substance found in rat brain and other sites that occurs in amounts greater than the closely related endocannabinoid, anandamide (Huang et al., 2001). However, the origin of NAGly *in vivo* is not completely understood.

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Prior reports (Burstein et al. 1997; Huang et al., 2001), suggest that NAGly might have analgesic properties similar to those reported for anandamide (Pertwee, 2001; Walker et al., 1999) but would be inactive in assays for psychotropic action such as the "ring test (Pertwee, 1972). The latter was in agreement with a report showing a lack of affinity by NAGly for the cannabinoid receptor, CB1 (Sheskin et al., 1997).

Several possible targets for NAGly and perhaps the other elmiric acids* have been described. The action of the glycine transporter GLYT2a is inhibited by NAGly in a reversible and non-competitive manner (Wiles et al., 2006). Arachidonic acid, anandamide and R1-methanandamide showed no effect on glycine transport, however N-arachidonoyl-L-alanine was active. NAGly has been suggested to be the endogenous ligand for orphan G-protein-coupled receptor GPR18 based on preliminary data (Kohno et al., 2006;Samuelson et al., 1996). Like anandamide, NAGly is also a substrate for COX-2 giving rise to amino acid conjugates of the prostaglandins (Prusakiewicz et al., 2002).

Other amino acid conjugates have been found in diverse tissues (Bradshaw and Walker, 2005), for example, N-arachidonoyl-L-serine has recently been isolated from rat tissues (Bradshaw, 2005) and from bovine brain and reported to have vasodilatory effects in rat mesenteric arteries (Milman et al., 2006).

The origin of EMA-1 (20:4) has been a subject of interest for some time. Early observations raised the possibility that anandamide might be a precursor, which by an oxidative process could lead to EMA-1 (20:4) synthesis (Burstein et al., 2000). In a subsequent study, it was reported that deuterium labelled arachidonic acid and labelled glycine are converted to doubly labelled EMA-1 (20:4) by the P2 membrane fraction from rat brain (Huang et al., 2001). This suggests a direct condensation of glycine with arachidonic acid. Recent preliminary findings support a role for both FAAH and anandamide in EMA-1 (20:4) synthesis based on several lines of evidence (Bradshaw, 2006). The FAAH inhibitor URB-597 when used in vivo caused a decrease in EMA-1 (20:4) levels. Moreover, it was found that FAAH knockout mice had lower levels of EMA-1 (20:4) than wild type. Finally, incubation of RAW cells with deuterium labelled anandamide yielded labelled EMA-1 (20:4) by some unidentified process. If these inter relationships prove to be significant in vivo, they could be important factors in the physiological regulation of anandamide tissue levels.

2. Cell proliferation effects

In view of the reported inhibition of cell proliferation by anandamide (*vide supra*), it was decided to look for such effects with the elmiric acids (Burstein et al., 2007). Like anandamide, many of the elmiric acids cause a profound reduction of cell proliferation when tested at concentrations of 1 and 10 uM (Fig, 1). The cell type studied, RAW264.7, is frequently used as a model for measuring the effects of potential anti-inflammatory agents. In this report, it was found that a complex relationship appears to exist between structure and activity. That is, in general unsaturated elmiric acids were better than saturated analogs, however, the amino acid portion could also influence anti proliferative activity. Since complete dose-response data were not obtained, it is not possible to arrive at any conclusions on this subject. However, it seems certain these compounds inhibit cell proliferation at moderate concentrations. Whether this relates to any in vivo action remains to be proven.

*These are defined as compounds that conform to the general structure shown in Table 1 for which a short hand nomenclature system is proposed. Using this system N-arachidonylglycine (NAGly) would be designated as: EMA-1 (20:4). EMA stands for elmiric acid; each amino acid constituent is assigned a number, e.g. 1 - glycine; 2 = alanine, etc. The identity of the acyl substituent is indicated in parentheses; e.g. (20:4) = arachidonoyl; (16:0) = palmitoyl, etc. We are proposing this nomenclature system in order to condense and simplify the naming of these compounds (Burstein et al., 2007); it has not been approved or adopted by any official body.

An interesting and unusual effect on the immune system was reported for EMA-1 (20:4) (Sipe et al., 2005). They observed that its inhibition of T-cell proliferation was controlled by a CB2 gene polymorphism in cells obtained from human subjects with this condition. This CB2 gene variation is thought to be a risk factor for autoimmune effects suggesting a possible therapeutic target for the elmiric acids in diseases such as rheumatoid arthritis and multiple sclerosis.

3. Inhibition of FAAH by EMA-1 (20:4) and several congeners

EMA-1 (20:4) showed substantial potency (EC-50 = 4-7 uM) as an *in vitro* inhibitor of FAAH (Huang et al., 2001) the enzyme primarily responsible for the degradation of anandamide to arachidonic acid and ethanolamine under physiological conditions. However, it had little effect on anandamide transport or on the vanilloid receptor. This suggests as one possibility that EMA-1 (20:4) may act as an endogenous regulator of tissue anandamide concentrations by virtue of its ability to inhibit FAAH and its presence in a number of tissue sites *in vivo.* FAAH -/- mice have 15 fold increased brain levels of anandamide and show decreased pain responses (Cravatt et al., 2001) underscoring the role of FAAH in anandamide activity.

A more recent publication (Cascio et al., 2004) extended the above-mentioned observations on EMA-1 (20:4). The inhibition of FAAH activity was studied in preparations from several sources with both EMA-1 (20:4) and several of its amino acid congeners. In the case of EMA-1 (20:4), it appears that there may be species differences in inhibitory action. The data indicate that there is an appreciable sensitivity to structure as well as species in the inhibition of FAAH. At this time, it is not certain that this activity of the elmiric acids is responsible for any of their vivo effects.

4. Pharmacological effect of EMA-1 (20:4) on anandamide levels

Based on the report by Huang et al. (Huang et al., 2001) showing that EMA-1 (20:4) is a potent inhibitor of FAAH, experiments were carried out in more complex models to see whether this property would result in a net increase in anandamide concentrations. An initial experiment using RAW cells indicated that this was, in fact, the case (Burstein et al., 2002). A second study in intact rats gave an even more robust increase in blood levels of anandamide. These findings using pharmacological models raise the possibility that EMA-1 (20:4) may serve as an endogenous regulator of FAAH activity and, therefore, of anandamide tissue levels. In turn, this further suggests that at least some of the actions of EMA-1 (20:4) may be due to the higher concentrations of anandamide produced in the model being studied. These effects could, in all probability, be observed with other substrates of FAAH such as 2-AG and the sleep mediator, oleamide. Thus, the elmiric acids may exhibit a much wider range of activities beyond their anti-inflammatory and analgesic effects. In addition, it appears from the report that oral administration of EMA-1 (20:4) is an effective route for dosing despite speculationsspeculations on its short half-life in vivo.

5. Analgesic effects of EMA-1 (20:4)

The possibility that some of the elmiric acids might have pain relieving properties was first disclosed in a preliminary report using the mouse hot plate assay at done 50°C (Burstein, 1997; Burstein et al., 2000). In this study, it was shown that oral administration of EMA-1 (20:4) at 40 mg/kg produced 85% of the maximal possible effect (MPE). Subsequent studies using other assays have confirmed the analgesic effects of EMA-1 (20:4) (Bradshaw et al., 2006; Burstein et al., 2000; Huang et al., 2001). A 40% decrease in tonic inflammatory pain was seen in Phase 2 of the formalin model in the rat following treatment with 275nm of the drug. Control treatment with either arachidonic acid or glycine had no analgesic effect.

In a related study (Bradshaw et al., 2006), it was reported that there were gender and hormonal cycle differences in rat brain levels of pain-related lipid mediators including EMA-1 (20:4) and EMA-21 (20:4). In an elegant series of experiments, using LC/MS/MS analysis, the levels of these molecules as well as other endocannabinoids were measured in various brain regions in both male and female rats. The processes involved in the production of elmiric acids in the midbrain may have implications for understanding the neurophysiology of pain modulation.

Efficacy for EMA-1 (20:4) in a rat model for neuropathic pain independently extends and confirms the above reports on elmiric acid induced analgesia (Vuong et al., 2007). Allodynia induced by partial sciatic nerve ligation was reduced by the intrathecal administration of EMA-1 (20:4) in a doe-dependent manner. The response was not affected by pretreatment with either CB1 or CB2 antagonists suggesting a novel mechanism different from HU-210, the comparison cannabinoid. Other controls such as arachidonic acid and glycine had no analgesic effects showing that the metabolites of EMA-1 (20:4) were not responsible. Finally, EMA-1 (20:4) had no effect on rotarod latency in contrast to HU-210 further supporting a novel mechanism for this prototypic elmiric acid.

6. Potential anti-inflammatory activity of the elmiric acids

In a recently reported study (Burstein et al., 2007), data were presented supporting the possibility that members of the elmiric acid family are candidates for drugs to treat various inflammatory conditions. The authors have developed a mechanism-based in vitro assay for screening libraries of elmiric acids for potential anti-inflammatory activity based on their stimulatory action on prostaglandin J_2 levels (Fig. 2). The details of this mechanism are discussed below in section 8, however, it is noteworthy that, in contrast to the nonsteroidal anti-inflammatory drugs (NSAIDS), the elmiric acids are not COX-2 inhibitors. Thus, it could be expected that their side effect profile, if any, would be different.

In this same study (Burstein et al., 2007), several of the EMAs showed in vivo efficacy in mouse models such as paw edema, leucocyte migration (Fig. 3) and ear edema. The agents were administered orally in safflower oil in the first two assays suggesting good bioavailability by this route. Some indication of a structure-activity profile where inhibition of ear edema was compared with prostaglandin ratios was also obtained from these experiments (Fig. 4). An important factor seems to be the presence of double bonds in the fatty acid portion of the molecule; however, the structure of the amino acid group can also influence its anti-inflammatory action.

7. Calcium mobilization in dorsal root ganglion cells and primary beta cells

A somewhat different structure-activity profile was seen when a group of elmiric acids was studied for their effect on calcium mobilization in F11-DRGxNeuroblastoma cells (Rimmerman, 2006). Those with saturated acyl groups were more active in mobilizing intracellular calcium than were those with unsaturated groups. EMA-1 (16:0) was the most active; interestingly, L-EMA-2 (16:0), its alanine analog, was inactive. CB1 and CB2 antagonists failed to reduce the effect, however, ruthenium red, a transient potential (TRP) channel blocker was able to reduce the EMA-induced mobilization of calcium. The authors concluded that this action of EMA-1 (16:0) was receptor mediated and that a TRP channel was the likely target. EMA-1 (16:0) is most abundant in skin suggesting that it may have a role in the firing of nociceptive neurons in this tissue.

In primary beta cells, EMA-1 (20:4) has been reported to increase [Ca2+]i through stimulation of voltage-dependent Ca^{++} channels and the effect was dependent on extracellular glucose levels (Ikeda et al., 2005). The authors suggested that EMA-1 (20:4) could be considered a novel insulin secretagogue and may offer possibilities for treatment of Type 2 diabetes.

8. Putative mechanism for anti-inflammatory actions

Inflammation: a biphasic process

Following some type of initiation event, inflammation is characterized by an induction phase that is eventually succeeded by a resolution phase. In preclinical models of both acute and chronic inflammation, COX-2 inhibition suppresses endogenous 15d-PGJ_2 production and prolongs the induction phase whereas administration of 15d-PGJ_2 has been shown to promote resolution of inflammation (Cuzzocrea et al., 2002; Gilroy et al., 2003). Prostaglandins synthesized during an inflammatory response result from the action of COX-2 on free arachidonic acid released from pools of cellular phospholipids and the actions of specific terminal synthases on the transient, intermediary endoperoxide, PGH_2. The unregulated conversion product of PGD_2, 15d-PGJ_2, down regulates pro-inflammatory pathways mediated by the transcription factors NF-kappa-B, AP-1, and STAT, and suppresses production of matrix metalloproteinases (MMPs) and other mediators of inflammation, thus preventing joint damage in rats with adjuvant arthritis, and generally acts to facilitate resolution of inflammation serving as a negative regulator of inflammation (Gilroy et al., 2004a). PPAR-g, as a binding site for 15d-PGJ_2, has been implicated in the resolution of some, but not all inflammatory conditions.

Ajulemic acid (AJA)

The mechanisms responsible for the anti-inflammatory and joint protective properties of the synthetic cannabinoid AJA are have been studied (Zurier, 2003). Although the elmiric acids are structurally different than AJA, there are certain similarities in their actions so that a comparison of AJA with the elmiric acids may be helpful in understanding both. The addition of AJA to human fibroblast-like synovial cells (FLS) *in vitro* increased arachidonic acid release and COX-2 expression with a subsequent marked increase in 15d-PGJ_2 production without an appreciable change in PGE_2 synthesis (Stebulis et al. unpublished findings). The stimulatory effect of AJA could be reduced by pretreatment with the CB2 antagonist SR144728 but not with the CB1 antagonist SR141716a. These remarkable results are potentially important in light of accumulating evidence that the timing of COX-2 expression may determine its potential to promote either induction or resolution of inflammation by regulating the production of prostaglandins, depending on the stage and progression of the inflammatory response (Willoughby et al., 2000). Although an earlier report suggests a mechanism for AJA involving PPAR-g (Liu et al., 2003), a more recent study shows that this is not always the case (Johnson et al., 2007) so that AJA may act by either PPAR-g dependent or independent mechanisms.

The elmiric acids

It was observed that EMA-1 (20:4) causes the mobilization of free arachidonic acid in RAW cells from sources other than the elmiric acid added to the cells (Burstein and Karim, unpublished data; Fig 5). Cannabinoids generally have a stimulatory action on the phospholipases responsible for the release of esterified arachidonic acid and its subsequent conversion to one or more eicosanoids (Burstein and Hunter, 1978,1981;Burstein et al., 1983;Burstein et al., 1994;Pestonjamasp and Burstein, 1998;Shivachar et al., 1996). The data in Figure 5 show that EMA-1 (20:4) is no exception. The increase in free, radiolabelled arachidonic acid from pre labelled cells could not have been due to hydrolysis of EMA-1 (20:4) since the EMA-1 (20:4) used in this experiment was not radiolabelled whereas the released arachidonic acid was radiolabelled. Thus, the elmiric acids may act by very similar mechanisms to those mediating the anti-inflammatory actions of AJA.

Based on these considerations, a putative mechanism is proposed and is shown in Figure 6. It is believed that there are two phases of arachidonic acid release: the first occurs during the induction of inflammation leading to an increase in pro inflammatory eicosanoid levels, while the second happens during resolution and results in the production of anti-inflammatory

eicosanoids (Gilroy et al., 2004b). The initiation of this second phase may be accelerated by the EMA-induced activation of a presently unknown receptor that, in turn mobilizes Ca^{++}. Precedent for this type of EMA action has been reported in which the authors suggested that a TRP channel is involved in their model (Rimmerman, 2006). Phospholipase A_2 is then activated as shown in Figure 5, in a RAW cell model, promoting the release of free arachidonic acid (Burstein and Karim, unpublished findings). The action of COX-2 followed by PGD_2 terminal synthase results in a transient elevation of this prostaglandin, which in turn is converted in an unregulated manner to 15-deoxy-delta-12, 14-PGJ_2 (Bell-Parikh et al., 2003). This latter prostaglandin has been suggested to be an important promotor of the resolution phase of inflammation possibly through activation of PPAR-gamma, however, this last point is being actively debated (Nosjean and Boutin, 2002). Related to this debate are the reports that mediators other than PPAR-gamma have been implicated in some of the actions of 15-deoxy-delta-12, 14-PGJ_2 (Emi and Maeyama, 2004) (Trivedi et al., 2006). Thus, this putative mechanism for EMA action will no doubt undergo many modifications as new data become available. Nevertheless, it provides a credible hypothesis on which to base future studies.

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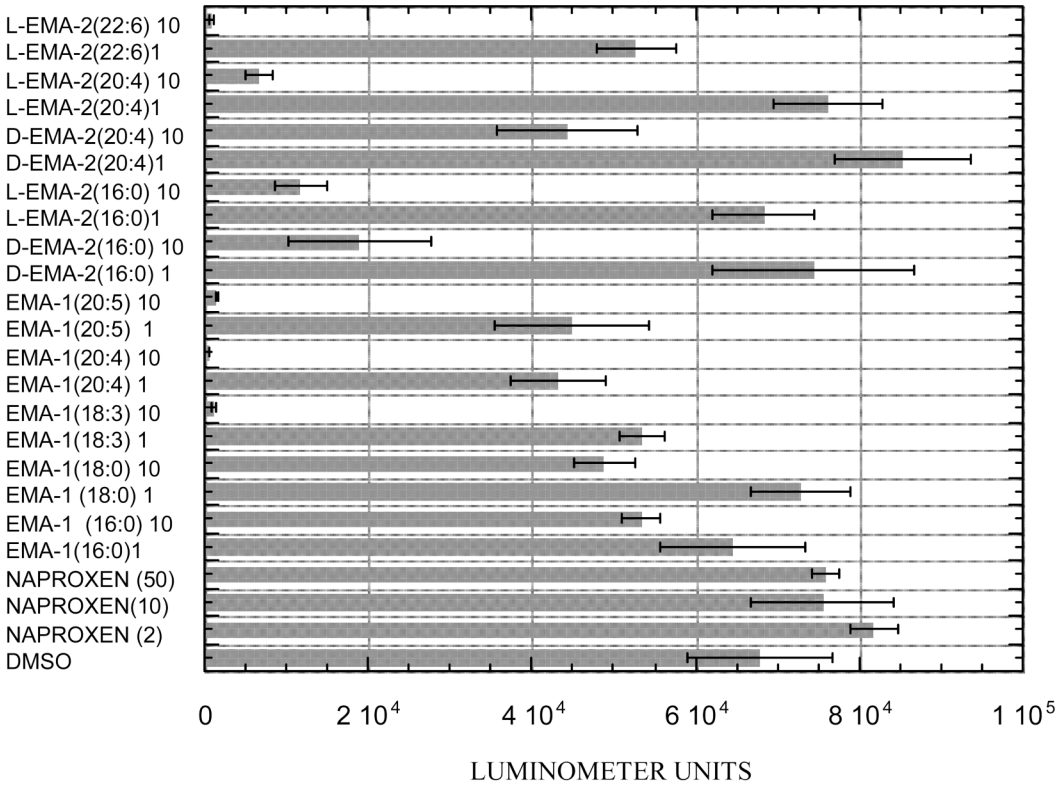


Figure 1. Effects of elmiric acids on the proliferation of mouse macrophage RAW cells

The data shown were previously reported in Burstein et al. (2007). The cells were treated with 1 and 10 uM elmiric acid whose identity is shown on the Y axis (designated as 1 or 10 on the Y axis). Luminometer units are directly proportional to the number of metabolically active cells and are a measure of ATP levels.

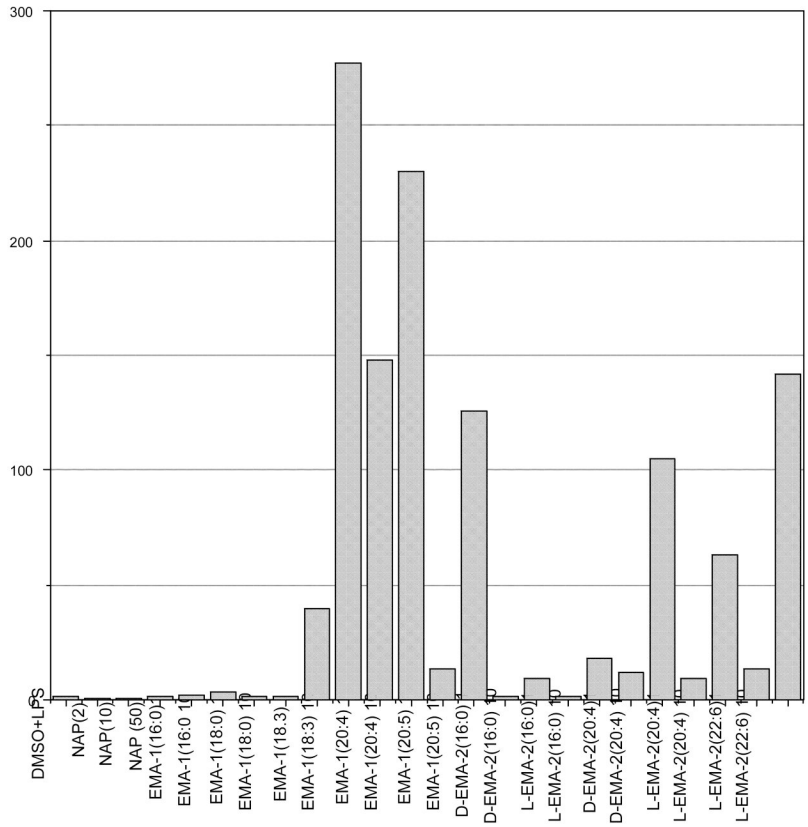


Figure 2. Effects of elmiric acids on the ratio of iPGJ/iPGE* in RAW cell media

The data shown were previously reported in Burstein et al. (2007). All of the ratio values greater than 20 are significant at the 95% confidence level when compared to the DMSO-LPS control by ANOVA.

Note: see Table 1 for structures.

*i denotes immunoreactive.

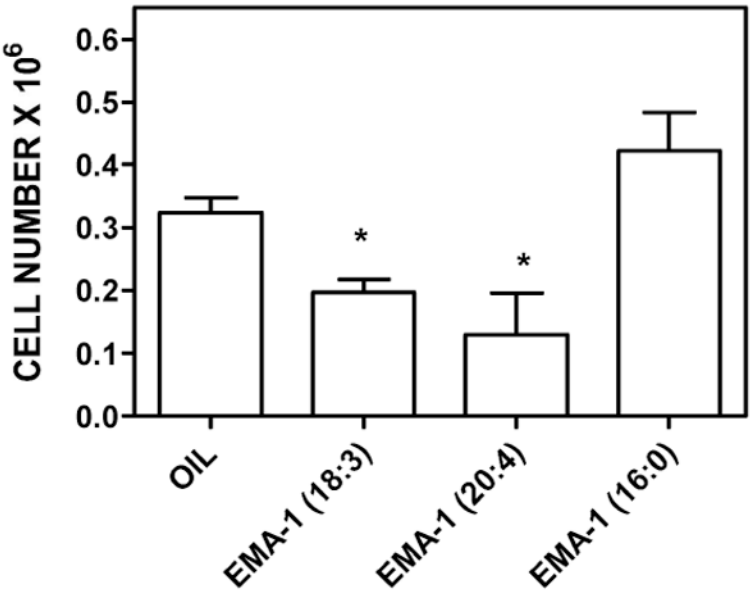


Figure 3. Effects of elmiric acids in the mouse subcutaneous pouch assay

The data shown were previously reported in Burstein et al. (2007). $P<0.05$ by ANOVA vs. the oil control.

Note: see Table 1 for structures.

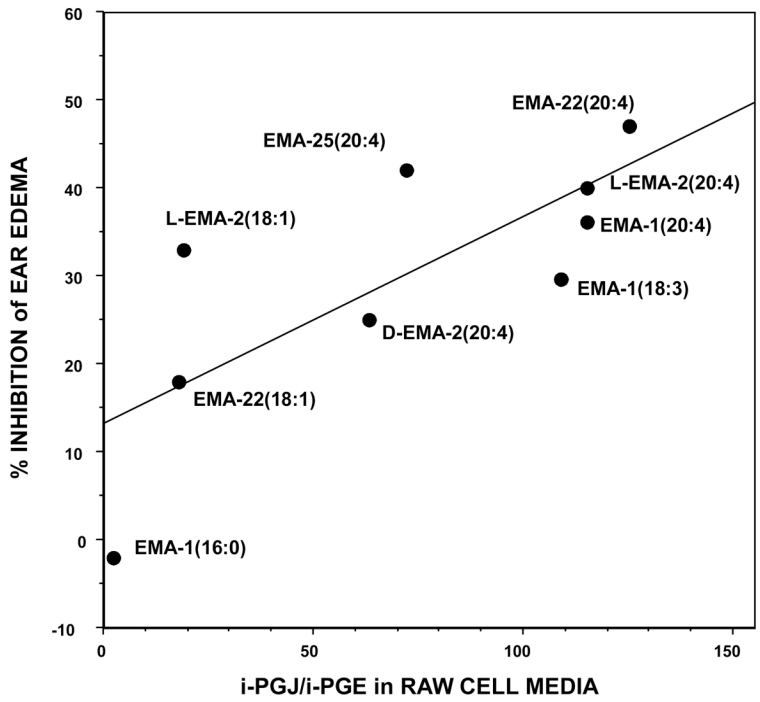


Figure 4. Effects of elmiric acids on ear edema compared with increases in prostaglandin ratio

The data presented here were previously reported in Burstein et al. (2007). The values shown in this comparison represent the maximum response in each case.

Note: EMA-1 = glycine; EMA-2 = alanine; EMA-22 = 1,1-dimethylglycine; EMA-25 = 1-amino-cyclohexane-carboxylic acid.

*i = immunoreactive.

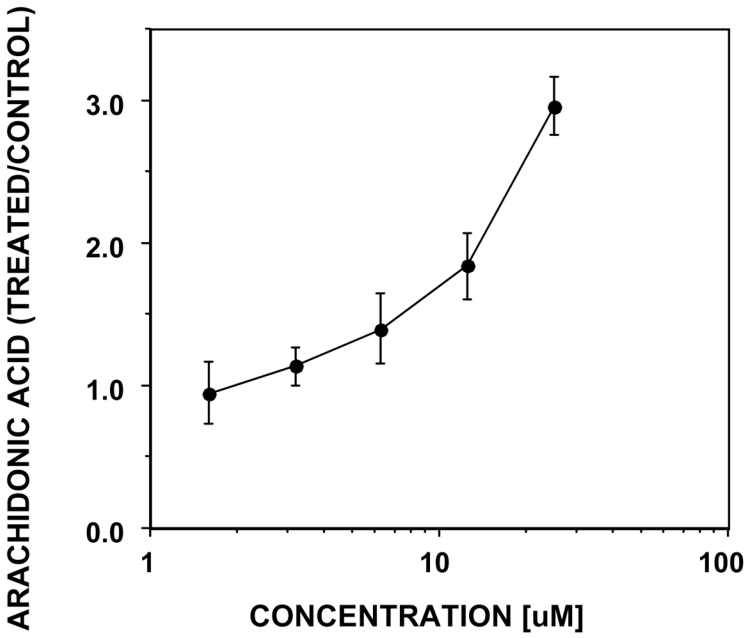


Figure 5. EMA-1 (20:4) – induced stimulation of arachidonic acid release from RAW cells (Burstein, unpublished findings)

Cells were grown and maintained as described previously (Pestonjamasp and Burstein, 1998). Following a 2 hr labeling period with ^{14}C-arachidonic acid, the media (RPMI+0.1% BSA) were changed and the cells treated for 1 hr with EMA-1 (20:4) in 10 ul of DMSO. The control was 10 ul of DMSO and there were 4 replicates of each treatment concentration. Release was measured by liquid scintillation counting on a 0.1 ml aliquot of medium. Values shown are the means (∓) SD.

Note: The cells were treated with non-radioactive EMA-1 (20:4).

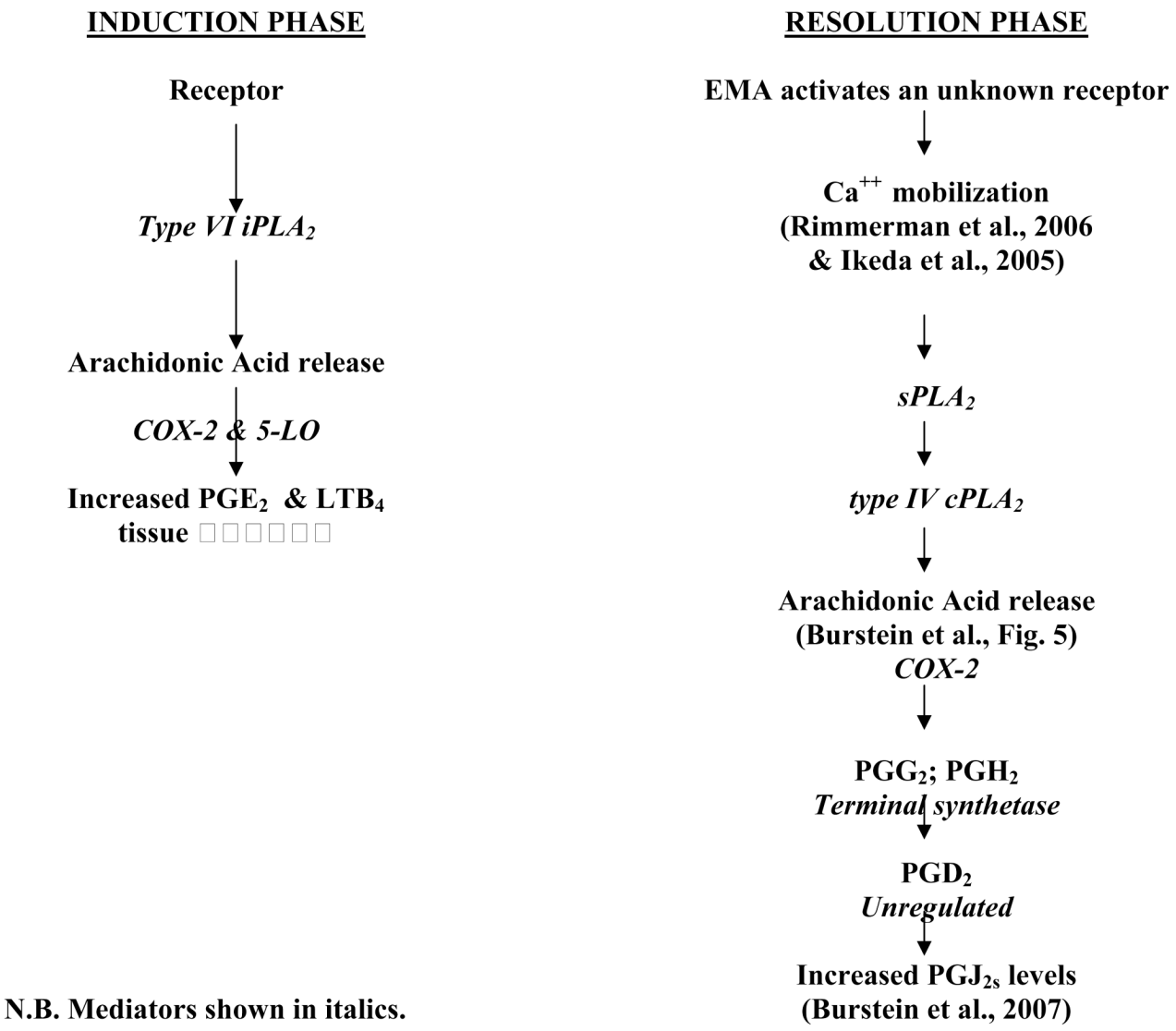


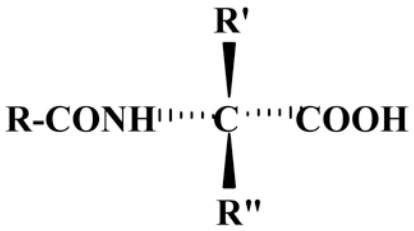
Figure 6. Putative mechanism for the anti-inflammatory actions of the elmiric acids

Induction of inflammation. A wide range of pro inflammatory agonists can activate various forms of phospholipase A_2 causing the release of free arachidonic acid from pools of phospholipids. Several routes of regulated biotransformation can then lead to elevated levels of various mediators of inflammation.

Resolution by elmiric acids. An elmiric acid, activating a receptor, promotes the mobilization of Ca^{++} that in turn causes the release of free arachidonic acid. COX-2 action then results in the production of PGG_2 and PGH_2 that is converted by a terminal synthetase to PGD_2. In a non-enzymic process, PGD_2 is transformed into 15-deoxy-PGJ_2, a potent anti inflammatory mediator.

Table 1

Structures and names of the elmiric acids



R = a long-chain saturated or unsaturated alkyl substituent.
R′ or R″ = H, or any alkyl group, carbocyclic or heterocyclic ring and combinations therof.

Examples made up of naturally occurring components

Fatty acid	Abbreviation	Amino acid	EMA name
Myristic	14:0	Glycine	EMA-1
Palmitic	16:0	Alanine	EMA-2
Stearic	18:0	Valine	EMA-3
Oleic	18:1	Leucine	EMA-4
Linoleic	18:2	Isoleucine	EMA-5
Linolenic	18:3	Serine	EMA-6
Eicosatrienoic	20:3	Threonine	EMA-7
Arachidonic	20:4	Methionine	EMA-8
Eicosapentenoic	20:5	Phenylalanine	EMA-9
Docosatetraenoic	22:4	Tyrosine	EMA-10
		Proline	EMA-11
		Lysine	EMA-12
		Aspartic acid	EMA-13
		Glutamic acid	EMA-14
		Asparginine	EMA-15
		Glutamine	EMA-16
		Cysteine	EMA-17
		Tryptophan	EMA-18
		Histidine	EMA-19
		Arginine	EMA-20
		Gama-aminobutyric acid	EMA-21