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COMMENTARY

BIOLOGICAL RELEVANCE OF LIPOCORTINS AND RELATED PROTEINS AS INHIBITORS OF PHOSPHOLIPASE A_2

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The therapeutic benefits of glucocorticoid treatment in the management of inflammation and other diseases are well established. Over the years, much time and effort have been expended to determine their mechanisms of action. "Lipocortins" and related proteins have recently received a great deal of attention as possible mediators of glucocorticoid action. A popular, but controversial hypothesis has been that lipocortins serve as specific inhibitors of phospholipase A_2, a proposed controlling enzyme in the production of prostaglandins, leukotrienes and other eicosanoids (see Ref. 1 for review), and thus control the inflammatory response. The origins and difficulties with this hypothesis will be the focus of this commentary.

Origin of the phospholipase A_2 inhibitor hypothesis

The idea that lipocortins are specific biological inhibitors of phospholipase A_2 stems from experiments in several laboratories offering circumstantial evidence for links between glucocorticoids, lipocortins, phospholipase A_2, and arachidonic acid release. The development of the idea began with the discovery that the anti-inflammatory effects of glucocorticoid drugs are dependent upon protein and mRNA synthesis [2]. Glucocorticoids were also found to inhibit synthesis of the inflammatory mediators, prostaglandins, in animal models of inflammation. However, they were inactive against the cyclooxygenase which produces prostaglandins if assayed directly. In addition, glucocorticoids were ineffectual in inhibiting prostaglandin production in the perfused-lung assay system if exogenous arachidonic acid precursor was supplied, and in cultured fibroblasts, which had been prelabeled with [^{3}H]arachidonic acid, glucocorticoids inhibited the release of [^{3}H]arachidonic acid in response to stimuli. For an extensive recent review and detailed references, see Ref. 3.

On the basis of the above and other experiments, it was deduced that glucucorticoids must affect some step prior to the cyclooxygenase and, having come to this conclusion, it was suggested that glucocorticoids act by inducing the synthesis of a protein which inhibits phospholipase A_2, thereby inhibiting the release of the cyclooxygenase substrate, arachidonic acid. In fact, many other possibilities existed, particularly since it was usually the inhibition of *stimulated* eicosanoid release that was measured (for examples, see Refs 4 and 5). As an example, the drugs might block the expression of cytokines like IL-1, or other molecules involved in the activation of phospholipase A_2, cyclooxygenase, or lipoxygenase. Any such effects on prostaglanding production would be expected to be overcome by excess exogenously added arachidonic acid, and could fit the experimental results. Alternatively, the release of arachidonic acid could be accomplished by any number of enzymes of lipid metabolism, and the glucocorticoids could affect any one of these enzymes, indirectly affecting the availability of arachidonic acid. These are only two out of many possibilities.

However, at the time of the early experiments, other possibilities were overlooked because anti-phospholipase A_2 activity was looked for and found in the lavage fluids or supernatant fractions of glucocorticoid-treated animals or cells [3, 4, 6]. The assays used employed either [^{3}H]oleate-labeled *Escherichia coli* cells, or [^{14}C]phosphatidylcholine (PC) as substrate, in very low amounts, in combination with porcine or bovine pancreatic phospholipase A_2. Anti-phospholipase A_2 activity was found and followed through several steps of protein purification using these *in vitro* assays, although, as pointed out by Flower and coworkers [5, 7], its correlation with anti-inflammatory activity was not always conclusive. From this point on, several proteins were further purified using only the anti-phospholipase A_2 assays, and the first to be cloned and sequenced was subsequently named lipocortin I [8]. Soon thereafter, the sequences of two protein tyrosine kinase substrates, called p36 and p35 (also named calpactin I and calpactin II), and another lipocortin (II) were published [9–12]. They were, surprisingly, all found to be related. The p35 protein, which is a substrate of the EGF receptor [13] and is also known as calpactin II, was found to be the same protein as lipocortin I. Furthermore, this protein was 50% identical to the p36 protein, which is a major substrate of the viral oncogene protein tyrosine kinase pp60$^{v\text{-}src}$ [14], and is itself the same as lipocortin II and the cytoskeletal element, calpactin I. In summary, lipocortin I = p35 = calpactin II and lipocortin II = p36 = calpactin I.

The elucidation of the relatedness of these proteins was potentially very exciting, as it appeared to link oncogenesis and growth regulation with a protein

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which was involved in the regulation of eicosanoid biosynthesis. However, since then, many more independently discovered members of this protein superfamily have been found. Six members have been given the name lipocortin (I–VI) [15]. Other names for the same and related proteins include annexins, chromobindins, calcimedins, calelectrins, calpactins, p35, p36, uteroglobin, placental anticoagulant proteins, endonexins, anchorin and calmotilin (for reviews, see Refs 16–19). A physiological role has not yet been rigorously established for any of these structurally related molecules, all of which bind Ca^{2+} and phospholipid. For this reason, the more neutral name, annexins, would be more appropriate in order to unite the nomenclature, but it has not yet been generally adopted.

Are the Ca^{2+} and phospholipid-binding proteins really lipocortins?

As originally defined [20], lipocortins are: (i) glycoproteins whose (ii) synthesis or secretion is stimulated by glucocorticoids and which (iii) specifically inhibit phospholipase A_2 *in vitro* and *in vivo*. Phosphorylated lipocortin is proposed to not inhibit phospholipase A_2 [21, 22]. Thus, phosphorylation has been said to be an additional mode of physiological regulation. Yet experiments in our laboratory [23, 24] show that lipocortins I and II are *de facto* inhibitors of phospholipase A_2 *in vitro* only under conditions where the substrate or cofactor of the enzyme is limiting. These results throw a new light on previous studies. Indeed, the *in vivo* studies purported to show that lipocortins are physiological inhibitors of phospholipase A_2 are also under fire from other directions. One problem is that, although lipocortins are defined as secreted proteins, there is no direct evidence that the "lipocortins" which have been characterized and sequenced are, in fact, secreted proteins. In fact, they are predominantly, if not solely, intracellular proteins [25] (see Ref. 16 for review). Then, there is the problem that purified "lipocortin" inhibits stimulated release of arachidonic acid from prelabeled cells, not basal release [26], leaving open the possibility of many other mechanisms besides direct inhibition of phospholipase A_2. Furthermore, an early and primary oversight in the experiments leading to the theory that glucocorticoids induce the synthesis of phospholipase A_2 inhibitors is that the effects of the steroids on the total phospholipid complement of the cells were never examined. Such complete and necessary studies are only now being carried out (see below).

Meanwhile, there is much controversy over the question of whether any of the recombinant or otherwise structurally characterized "lipocortins" are actually induced by glucocorticoids, especially in a way that correlates with anti-inflammatory activity. The mRNA for lipocortin I was shown by Wallner *et al.* [8] to be increased 6-fold in peritoneal cells of rats treated with dexamethasone, but no accompanying increase in protein was seen, and neither the mRNA nor the protein could be induced in cells in culture. By contrast, Northup *et al.* [27] could detect no induction of synthesis, or secretion of lipocortin I in rat or mouse peritoneal macrophages under conditions where dexamethasone caused the secretion of unidentified anti-inflammatory factors into the cell supernatant fractions. Similarly, Isacke *et al.* [28] found no increase in p36 or p35 in U937 cells upon dexamethasone treatment, nor was p36 increased by dexamethasone in AG1523 human fibroblasts, with or without serum in the culture medium.* Furthermore, recent work by Chap and coworkers [29] on human endothelial cells showed that, under conditions in which dexamethasone treatment led to a decrease in PGI_2, no change in "lipocortin" levels was observed. On the other hand, a preliminary report by Philipps *et al.* [30] indicated that lipocortin I expression could be stimulated by dexamethasone in Swiss 3T3 fibroblasts, so long as serum was added. These differing results await clarification. However, the latter results are interesting in light of recent reports by Bailey and coworkers [31, 32] in which it was found that some factors in serum, one of which is EGF, are needed in order to stimulate the recovery of cyclooxygenase in vascular smooth muscle cells following treatment with aspirin (a cyclooxygenase inhibitor). Dexamethasone and a bovine "lipocortin" both inhibited this EGF-dependent cyclooxygenase recovery, and the recovery was also dependent on protein synthesis. Furthermore, corticosteroids appeared to suppress cyclooxygenase mRNA levels. They therefore suggested a potentially different role for lipocortin, one in EGF-dependent translational control of cyclooxygenase activity. Also, see related new studies by Raz *et al.* [33].

At this time, it cannot be concluded that the proteins which are currently known as lipocortins are either the primary agents of the anti-inflammatory actions of exudates from glucocorticoid-treated animals, or cells, or, even if induced, serve always to inhibit eicosanoid biosynthesis. Induction of intracellular lipocortin I in human amnionic cells has been reported by Mitchell *et al.* [34] to correlate with an *increase* in PGE_2 production. Perhaps significantly, these authors measured PGE_2 levels by radioimmunoassay, which is more convincing than prelabeling of cells for short times with arachidonic acid (for more on the subject of labeling, see below). In animal models of inflammation, there is further conflicting evidence concerning the possible functions of "lipocortins". Northup *et al.* [27] found that human placental lipocortin I did *not* inhibit arachidonate release from zymosan-stimulated macrophages, *or* decrease swelling in the carageenan rat-paw edema test. Rather, at higher concentrations, lipocortin I caused greater swelling. Cirino *et al.* [35] report that human recombinant lipocortin I does inhibit thromboxane A_2 (TXA_2) production in leukotriene C_4 (LTC_4) stimulated perfused lung, although only at lower doses of LTC_4. Also, this group reports that lipocortin I inhibits prostacyclin production in human umbilical arterial endothelial cells [36]. However, this is not synonymous with affecting arachidonic acid release by phospholipase A_2. Moreover, one wonders how specific these effects are, and whether other lipid-binding proteins added to these cells in culture would cause the same effects. Furthermore, experiments have not been

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reported showing that added lipocortin does not bind either the arachidonic acid or the eicosanoid product, although such binding could possibly explain some results. It should be obvious at this point that the complexity of the anti-inflammatory response, as well as the variability of results with the purified proteins do not yet allow any conclusions to be made as to which steps are directly affected by lipocortins (either the actual isolated proteins, or the glucocorticoid-inducible activities originally described).

Is phosphorylation involved in lipocortin action?

At least two members of the "lipocortin" family of proteins are phosphorylated in response to stimuli *in vivo*. However, the relationship of this phosphorylation to eicosanoid biosynthesis has not been proven, and is likely to be complex if it does exist. Lipocortin I (p35, calpactin II) is phosphorylated by the EGF receptor *in vivo* as well as *in vitro* [13, 37] and also has been shown to serve as an *in vitro* substrate for $pp50^{v\text{-}abl}$, the polyoma middle T antigen $pp60^{c\text{-}src}$ complex, protein kinase C, and cyclic-AMP-dependent protein kinase [37, 38]. Lipocortin II (p36, calpactin I) is an *in vivo* as well as *in vitro* substrate for $pp60^{src}$ and protein kinase C [14, 37, 39–41]. The phosphorylation of both of these proteins *in vitro* is dependent on the concentrations of Ca^{2+} and phospholipid present [41, 42], and the affinity of both proteins for phospholipid appears to be affected by the phosphorylation. Powell and Glenney [43] found that phosphorylation of calpactin I (lipocortin II) caused a decrease in its affinity for phosphatidylserine (PS) liposomes at all Ca^{2+} concentrations tested. On the other hand, Schlaepfer and Haigler [42] found that the Ca^{2+} concentration required for binding of lipocortin I to PS liposomes was lowered by phosphorylation. Whether phosphorylation results in any changes in membrane binding by lipocortins I or II *in vivo* is not known. However, the stoichiometry of phosphorylation *in vivo* is always low* and, therefore, only a small fraction of these very abundant intracellular proteins would be affected. The phosphorylation of p36 (lipocortin II) in cells is variable and depends on which cells are stimulated by which growth factors. Thorough studies by Isacke *et al.* [39] have shown that EGF-dependent phosphorylation of p36 occurs in A431, but not AG1523, cells. Furthermore, TPA or PDGF activation of AG1523 cells (both of which stimulate protein kinase C) results in phosphorylation of different sites on p36 [14, 39].

In contrast to the above-cited studies, the reports claiming a link between phosphorylation of lipocortins and phospholipase A_2 regulation have failed to provide complete characterization of the phosphorylated products. Moreover, they have relied on *in vitro* assays of pancreatic phospholipase A_2 in trying to make a connection to cellular phospholipases A_2. The cellular or inflammation-related phospholipases A_2 are, however, likely to be significantly different from the pancreatic enzyme.† In two often-quoted studies claiming that phosphorylation affected "lipocortin" inhibition of phospholipase A_2, no Ca^{2+} was added to the phospholipase A_2 assays according to the original reports [21, 22]. Ca^{2+} is a required cofactor of this enzyme, but is bound with high affinity by "lipocortins" in a way that is interrelated, at least *in vitro*, with phospholipid binding and phosphorylation. Thus, the "lipocortins" and enzyme were probably in varying states of competition for trace Ca^{2+} (as well as phospholipid) and very little can be concluded from the experiments. In the only other study propounding a phosphorylation effect on inhibition [44], a 40 kD protein fraction from platelets was extracted before and after platelet stimulation by thrombin and assayed for anti-phospholipase A_2 activity. The thrombin-stimulated 40 kD pool was more phosphorylated and had less phospholipase A_2 inhibitory activity than the unstimulated pool, an effect which could be reversed by alkaline phosphatase. However, the identity of the phosphorylated protein was not directly ascertained. Furthermore, Crouch and Lapetina [45] have shown that, although α- and γ-thrombin both cause similar protein phosphorylation, only α-thrombin could induce significant release of arachidonic acid from prelabeled cells. Therefore, they reasoned that phosphorylation of this protein is not a control mechanism for phospholipase A_2 activity. In another study of phosphorylation and its effects on eicosanoid mobilization, Wijkander and Sundler [46] found that stimulation of mouse peritoneal macrophages by zymosan ionophore A23187 and other agents did indeed cause phosphorylation of a 45 kD protein (among others), but *not* lipocortin I or lipocortin II. So, even though phosphorylation of lipocortins may seem an attractive model for a control mechanism, evidence of a correlation between levels of phosphorylation of a "lipocortin" and either eicosanoid biosynthesis *in vivo*, or phospholipase A_2 inhibition in a relevant *in vitro* assay system is simply lacking.

Basis of phospholipase A_2 inhibition: substrate depletion model

Despite the paucity of evidence from any biological studies, the belief that lipocortins function as inhibitors of phospholipases A_2 persists. The idea has taken root largely because of the earlier biochemical studies, which in the face of the results found in our laboratory [23, 24] are now seen to have been seriously flawed. For example, early reports on *in vitro* phospholipase A_2 studies did not take into account all possible models for the results, experiments were not carried out over a wide enough concentration range of substrates, and sometimes inappropriate assay systems were employed. In fact, some of the very earliest *in vitro* studies indicated that lipocortins were not specific for phospholipase A_2, for they reportedly could also inhibit phospholipase C and phospholipase D under similar assay conditions [22]. For some reason, this information appears to have been downplayed. Inhibition of Ca^{2+}-independent mammalian phospholipase D has now also been reported [47] under the same conditions in which a snake venom phospholipase A_2 is

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inhibited. Such inhibition is consistent with the non-specific mechanism we [23] suggested, which entails binding of the "lipocortin" to the minute amounts of phospholipid used in these assays, thus making it unavailable, or else unsuitable as substrate.

The "substrate depletion model" [23] is illustrated by Eqn 1:

$$\begin{array}{l} E + \overset{K_s}{S \rightleftarrows} ES \overset{k_p}{\rightarrow} E + P \\ \quad + \\ \quad I \\ K_D \updownarrow \\ \quad SI \end{array} \qquad (1)$$

Here E is the phospholipase A_2 enzyme, S the substrate phospholipid, ES the Michaelis complex, P the hydrolysis products, and I the inhibitor lipocortin. The lipocortin binds to the phospholipid substrate forming the SI complex; this is especially tight for anionic phospholipids and enhanced by Ca^{2+}. Only when the substrate concentration is sufficiently high relative to I is the equilibrium driven to the right to form the ES complex. It should be made clear that the S and I symbols do not necessarily imply a stoichiometry of 1:1; rather each lipocortin molecule may bind many phospholipid molecules or interact with a membrane or micelle, perhaps causing a phase change, thereby affecting many phospholipid molecules or changing K_s.

Since the substrate depletion model was first suggested following an examination of the *E. coli* and pancreatic phospholipase A_2 assay system [23], the findings have been confirmed by others [48, 49]. In all of these studies, it was found that inhibition was overcome by raising the substrate concentration in amounts consistent with overcoming depletion of the substrate by the "lipocortins" used in the assays. In addition, no binding of inhibitor protein to the phospholipase A_2 was detected, but extensive binding to substrate was seen. Also, under similar conditions to those in which "lipocortins" were shown to bind *E. coli* membranes [23], Peers *et al.* [50] found that membrane-bound ^{125}I-labeled pancreatic phospholipase A_2 was displaced by lipocortin I. A paired study is needed and is currently under way in our laboratory. However, the results are in accord with the accumulated evidence, that inhibition of the pancreatic phospholipase A_2 by "lipocortin" in the *E. coli* assay system is due to binding of substrate by the inhibitor. Further evidence for the substrate depletion model in a different system comes from the study of phospholipid binding by placental anticoagulant proteins, which include lipocortin II. Tait *et al.* [51] have shown that the potencies of these proteins as inhibitors of pancreatic phospholipase A_2 on phosphatidylcholine/phosphatidylserine liposomes correlated with their relative affinities for the liposomes. Indeed, the anticoagulant properties of lipocortins *in vitro* are now widely attributed to their phospholipid binding capacities [51–53].

Even when non-anionic phospholipid bilayers have been chosen as substrates, for intracellular as well as extracellular phospholipases A_2 [24, 49], a dependence of the inhibition on substrate concentration has been seen. Inhibition has only been observed at low substrate concentrations and, in terms of the *maximum* possible surface concentrations, the IC_{50} values occurred at "lipocortin": phospholipid molar ratios of greater than 1:8. Lipocortins I and II do bind the zwitterionic PC, although more weakly than they do anionic lipids [24]. Thus, inhibition is consistent with substrate depletion, although it could also be due to generalized phospholipid phase changes associated with the partitioning of the "lipocortin" into the bilayers. In the studies just cited, competitive inhibition could not always be ruled out by the kinetics. However, if it occurs in these assay systems, it entails rather high protein binding constants [24]. Indeed, Ahn *et al.* [54] have shown that pancreatic phospholipase A_2 and members of the lipocortin family do exhibit very weak affinities for each other in the absence of lipid, and the high K_d values determined (about 10^{-5} M) would explain why binding of "lipocortins" to affinity columns of phospholipase A_2 is sometimes seen, yet would not account for inhibition of the negatively charged substrate assay systems where so little protein is used. In the PC assay, however, relatively more lipocortin must be used in order to see inhibition. This would be expected of substrate depletion inhibition anyway, given the lower affinity of lipocortins I and II for the substrate, but it cannot be ruled out that, when such high amounts of protein are thus used, inhibition by a classical competitive mode may occur.

Ca^{2+} *depletion and deoxycholate effects*

Depletion of cofactor Ca^{2+} appears to also have been a probable cause of inhibition in some of the early studies of "lipocortin" inhibition of pancreatic phospholipase A_2. It was apparently not realized that Ca^{2+} is absolutely required by the enzyme and, according to the original publications, it was not added [6, 22]. Thus, the "lipocortins" may have sequestered the essential cofactor (Ca^{2+} is almost always present in solutions in trace amounts). Ca^{2+} depletion continues to be a potential source of artifacts in deoxycholate/PC systems because of the ability of the bile salt itself to bind tightly to Ca^{2+}. This can leave only trace amounts of the free cation in solution for which to compete. This factor, and also the extraordinarily complex physicochemical properties of bile salt systems and the kinetic behaviour of phospholipase A in them, make deoxycholate/PC mixtures probably the least interpretable of all possible phospholipase A_2 assay systems; this makes the analysis of kinetic studies of lipocortin action in them unreliable [24].

Which phospholipase A_2 should be studied?

Even if phospholipase A_2 is found to be involved in glucocorticoid action, a key question is "Which one?" There are a large number of different phospholipases A_2 [55] and the extracellular phospholipases A_2 likely to be involved in inflammation [1] have been found to belong to the Group II phospholipases A_2, not the Group I enzymes to which the pancreatic phospholipase A_2 belongs. Since Group I and II phospholipases A_2 have been found to be confined to different physiological compartments so far, there is as yet no reason to think that their physiological regulators will be the same.

Furthermore, some intracellular phospholipases A_2 may belong to a whole new structural group. Therefore, before we can understand phospholipase A_2 regulation, more information is needed about the identities of different phospholipases A_2 in different tissues, fluids, cells and organelles (see, for example, Ref. 56). Information is also lacking, and much needed, on their basal expression rates, and their expression in growth, mitogenesis, chemotaxis and inflammation, all processes in which the potential roles of "lipocortins" are being investigated.

Wider implications

None of the evidence from the biochemical studies of "lipocortin" inhibition of phospholipase A_2 so far is compelling enough to *conclude* that lipocortin I or II is likely to be a specific inhibitor of a phospholipase A_2. If *in vitro* studies are pursued, then more relevant phospholipase A_2/lipocortin combinations from appropriate sources must first be identified from biological studies, and then examined with a thoroughness matching or exceeding that of the studies described herein. At present, however, it seems that the "lipocortins" do not inhibit phospholipases A_2, so long as substrate or cofactors are not limiting. The broader implication of this is that, if lipocortins do function by affecting the availability of either of these (and we are not implying that they do), then they would affect far more than just the phospholipase A_2. However, the primary conclusion of the biochemical studies discussed is that the research on "lipocortins", phospholipase A_2, and glucocorticoid effects must be approached from a broader perspective. Specifically, those basic questions must be addressed which were essentially skipped over in the simplified model of glucocorticoid action that was originally proposed.

As one example, there are suggestions that glucocorticoids may exert some of their effects by depressing intracellular Ca^{2+} concentrations [57]. Activation of phospholipase A_2 in neutrophils by formyl-Met-Leu-Phe has been shown to be dependent on Ca^{2+} [58], as are also arachidonic acid mobilization in peritoneal macrophages [46] and 5'-lipoxygenase [59]. Thus, if glucocorticoids do mobilize any of the "lipocortins", it may relate only indirectly to phospholipases A_2. Given the submembranous location of lipocortin II, its Ca^{2+} affinity, and its affinity for those phospholipids which are implicated in fusion, lipocortin II would not be an unreasonable candidate for being a controlling protein in Ca^{2+} transport, or secretion.

Studies by Medow *et al.* [60] could also serve as a model for further investigation of glucocorticoid effects. These authors examined the effects of dexamethasone on total phospholipid composition in rabbit coronary microvascular endothelial (RCME) cells. They found, as have others, that prelabeling of cells for short times with [^{3}H]arachidonic acid led to artifacts due to non-equilibrium labeling when the cells were examined for [^{3}H]eicosanoid release. On the other hand, if the cells were labeled for very long times, so that equilibrium of labeling was achieved, or mass effects were measured directly rather than radioactive tracer, then there was no effect of dexamethasone on the arachidonic acid actually released by Ca^{2+} ionophore stimulation. Clearly, the overall metabolism of arachidonic acid and all of the enzymatic steps potentially involved in regulating arachidonic acid incorporation, release, and distribution must be evaluated.

Future directions and concluding remarks

In conclusion, members of the "lipocortin" family may be connected in some way to growth regulation and oncogenesis, or may play a role in inflammation, but there is not sufficient evidence to say that they are direct regulators of phospholipases A_2. They may have totally different functions in membrane structure, or secretion, or any of a number of possibilities and the name "lipocortin" as originally defined no longer seems appropriate for this group of lipid-binding proteins.

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