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Background and discovery of lipocortins

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Introduction

Unlike machines, living things are usually able to recover spontaneously from damage sustained during their lifetime, and they have evolved a sophisticated set of responses to infection and injury which have proven survival value. This repertoire of responses – collectively known as 'inflammation' – has as its main objectives the neutralisation and removal of pathogens, the rebuilding and restructuring of diseased or injured tissue, and ultimately the restoration of normal structure and function.

A biological response of the complexity of inflammation requires close control and integration. Phagocytic cells must be summoned to the inflammatory site, the immune system must be primed, there must be an increased access of cells and other components to the site of injury and many other concomitant changes must also occur.

The most plausible mechanistic explanation for the control of inflammation is the 'chemical theory', and during the last decade this has dominated the attention of most biomedical researchers. According to this idea, chemicals released from dead, dying, 'activated' or injured cells are responsible for initiating the familiar signs and symptoms of inflammation together with many of the underlying pathological changes which are also seen. Histamine, serotonin, and bradykinin, are amongst the local hormones which have been implicated in the development of inflammation but it is the lipid mediators such as the prostaglandins, leukotrienes and PAF acether which have currently captured the imagination of the research community.

It is not the author's intention to review the (very compelling) evidence that suggests a role for these mediators in the inflammatory response [cf. refs 1, 2 and 3 for a detailed guide to the bibliography covering this section] – but certain aspects of their biosynthesis have to be mentioned here so that the arguments made later in this paper will appear clear.

Both the prostaglandins and the leukotrienes are biosynthesised from polyunsaturated fatty acids, preeminent amongst which in land-dwelling mammals, is arachidonic acid. This fatty acid (as well as some other related compounds) can be transformed by the fatty acid cyclo-oxygenase enzyme into prostaglandins and by lipoxygenase and other enzymes into leukotrienes. The first of these transformations may occur in practically any cell in the body (with the exception of the erythrocyte) whilst the latter series of reactions occur mainly in leukocytes.

PAF-acether is synthesised in a two-stage process in which the ether phosphatide precursor is catalytically transformed to lyso-PAF by phospholipase A_2 and then acetylated by a specific acetyl transferase [4]. Recently it has been observed that a high proportion of the ether phosphatide precursor also contains arachidonic acid esterified to the 2′position such that cleavage by phospholipase liberates both the precursors for the eicosanoids, and that for PAF simultaneously [5].

Biologically active lipids are, of course, not the only chemicals involved in inflammation. For example, complement plays a role in cell migration and oedema formation, and

fever, a common sequel to inflammation, is regulated by the release from macrophages of yet another type of chemical, the polypeptide interleukine 1. Other, less well characterised factors regulating immune and other function, the lymphokines, are generated by lymphocytes and influence other cells such as monocytes. Finally, there must be some mechanism – as yet unidentified, but presumably also dependent on the release of chemicals – which terminates the inflammatory response and initiates the healing process. There is evidence for substantial interactions between inflammatory mediators [see for example ref. 6].

The action of drugs on the inflammatory response

Normally, the inflammatory, response is a spectacularly successful 'defence mechanism', resolving spontaneously with healing occurring and complete restoration of function. In many cases the process is so successful that an invading organism once eliminated can never again gain access to the host. Like all biological systems however the inflammatory response occasionally goes awry and under these circumstances the body's response to disease may become a 'disease' in itself. Chronic conditions such as rheumatoid arthritis probably fall into this category. Although the agent which triggers the original inflammation is seldom identified, and may well be completely innocuous, the resulting reaction causes severe damage to local tissue, thus exacerbating the inflammation which in turn causes more damage and so on. The inflammatory response never terminates under these circumstances. Little if any healing occurs, and a continuing cycle of inflammation–tissue destruction–more inflammation is established.

It is upon such occasions that the physician intervenes to quench the inflammation with drugs and other treatment. The most common anti-inflammatory drugs, the aspirin-like drugs, seem to work by preventing the generation of the pro-inflammatory prostaglandins [see ref. 7] thus relieving the symptoms of the illness, but the mechanism of action of the most potent drugs in our therapeutic armoury, the glucocorticoids, has always been something of a mystery.

The therapeutically active glucocorticoids are analogues of the endogenous steroid hydrocortisone. All healthy mammals have the hormone in their blood, there is a marked diurnal variation, and significantly, the secretion of these hormones into the blood stream rises sharply when the organism is 'stressed', injured or infected. Because of this, it was thought for many years that the function of the glucocorticoids in the body was to participate somehow in the *mounting* of the inflammatory response. The demonstration in the 1940s that the glucocorticoids were actually *anti-inflammatory* caused consternation because this action did not fit with the preconceived notions of their normal function. To get around this problem the scientific community adopted the somewhat schizoid idea that in 'physiological' amounts these hormones had one type of effect, whereas in 'pharmacological' amounts they had another, often opposing, action.

Recently a new proposal has been put forward by ALANN MUNCK [8] and his colleagues which suggests that the physiological function of the glucocorticoids secreted during stress or injury is *not to protect against the source of the stress itself* (as was originally believed) *but to protect that organism against the normal defence mechanisms which are activated by stress.* In other words, glucocorticoid secretion is one arm of a homeostatic mechanism which checks and controls the activity of the inflammatory response.

When the inhibitory action of aspirin on the prostaglandin forming cyclo-oxygenase was discovered, many other types of drugs were tested as putative inhibitors. Amongst inactive compounds screened were the narcotic analgesics such as morphine and the anti-inflammatory glucocorticoids [9] (although some inhibition was seen at high concentrations by some other workers: ref. 10). It is important to point out at this stage that these experiments were all performed on broken cell preparations. The negative effects observed with the glucocorticoids were puzzling, because although the steroidal drugs were many times more active in experimental models of inflammation, they were all inactive against the cyclo-oxygenase enzyme.

Studies on the mechanism of action of glucocorticoids

Whilst these findings suggested that the glucocorticoids had no effect on the prostaglandin system, a number of observations

appeared in the literature which apparently contradicted this conclusion. In 1974, K. HERBACZYNSKA-CEDRO and J. STASZEWSKA-BARZCAK [11] demonstrated that a release of a prostaglandin-like substance into the venous blood of exercising dogs was blocked by hydrocortisone. Shortly after this came a report by G.P. LEWIS and P.J. PIPER in which it was observed that steroids could antagonise the release from isolated fat pads of prostaglandin E_2 which accompanies ACTH induced vasodilation [12]. The suggestion made at that time to explain these findings was that the glucocorticoids were preventing the release of prostaglandins from the adipocytes, rather than their biosynthesis, but some other experiments reported by different groups suggested another interpretation. In 1975, R. GRYGLEWSKI and his colleagues [13] found that two glucocorticoids, hydrocortisone and dexamethasone, prevented the noradrenaline-induced release of prostaglandins from rabbit perfused mesenteric vascular bed as well as the immunologically-induced release of prostaglandins from guinea-pig lungs. The direct conversion of arachidonic acid into prostaglandins in these preparations was not blocked by the glucocorticoids indicating that they had no inhibitory activity on the cyclo-oxygenase itself.

Because the rate-limiting step in prostaglandin synthesis seems to be the release of arachidonic acid from some intra-cellular lipid pool – probably the phosphatide pool – it was suggested that the glucocorticoids were interfering with substrate release from membrane phospholipids. This conclusion was supported by some elegant experiments from L. LEVINE's group published in 1976 [14]. Using cultured fibroblasts labelled with tritiated arachidonic acid, this group was able to demonstrate that the anti-inflammatory glucocorticoids prevented phospholipid deacylation and arachidonic acid release which accompanied stimulation by various agents. Levine's group proposed that the mechanism of action of these drugs was to prevent substrate release and suggested that this might underline their inflammatory action. Several other groups also reported that steroids could prevent synthesis of prostaglandins by intact cells and in each case the mechanism was consistent with an action on the supply of substrate.

Further studies on the mechanism of steroid action

In addition to the studies on steroid action by Gryglewski and Levine's group the author and his colleagues, at the Wellcome Foundation, were also studying the effect of steroids upon prostaglandin synthesis and release [15]. The system used was the guinea-pig perfused lung, a simple technique which has been extremely useful to workers in the prostaglandin field.

The perfused lungs can be induced to release thromboxane and other prostaglandin endoperoxide derivatives in two ways: firstly, by the injection or infusion into the lungs of the substrate arachidonic acid, and secondly by the injection of 'releasing factors' such as histamine, bradykinin, SRS-A (as it used to be known) or antigen. All these substances apparently release prostaglandins (as detected by superfusion bioassay) by liberating arachidonic acid by some ill-defined mechanism. Another releasing factor under investigation at this time was RCS-RF (an acronym of Rabbit Aorta Contracting Substance–Releasing Factor) a low molecular weight peptide found in immunologically-shocked lung effluent. RCS-RF had no direct effect upon the assay tissues but when injected into the lung it caused a release of biologically active substances identified as a mixture containing chiefly thromboxane and prostaglandin endoperoxide. Arachidonic acid injections induced a similar effect. When a glucocorticoid such as dexamethasone was infused, it was observed that the generation of biologically active substances elicited by the releasing factor was blocked whereas the conversion of arachidonic acid was unimpaired. There was always a time delay with the first effects of the steroid being observed after about 30 minutes of infusion. To produce greater than 50% inhibition it was often necessary to continue the infusion for 45–60 minutes or even longer. Of course, when indomethacin was given to the lung preparation, the generation of products in response to both arachidonic acid and RCS-RF was blocked.

Dexamethasone was not the only steroid able to produce this effect: all the common glucocorticoids shared this activity. Mineralocorticoids and most sex steroids were inactive in this test. Further experiments revealed that the releasing activity of some other agents such

as SRS-A and histamine was also blocked by the steroids.

Studies in which the arachidonic acid content of the perfusate was measured indicated that when releasing factors were injected into the perfused lung there was a transient release of arachidonic acid from the lung, and that during steroid infusion this release was inhibited or reduced. This inhibition was easily reversed when the steroid infusion was discontinued.

The most widely accepted idea at this time was that the major store of polyunsaturated fatty acids in the cell was the phospholipids and that it was the liberation of arachidonic acid from this pool under the influence of the enzyme phospholipase A_2 which was the first step in the generation of prostaglandins. Virtually all mammalian cells contain phospholipases A_2. There are several different types which differ in their pH optima, calcium requirement and subcellular location, but it seemed highly likely that the enzyme most relevant to the generation of arachidonic acid was the plasma membrane phospholipase A_2; an enzyme which is a constituent of all plasma membranes, and has a requirement for calcium and a neutral or alkaline pH optimum [16]. If such a potent hydrolytic enzyme were simply embedded in the membrane it would digest the membrane phospholipids very rapidly. Since this obviously does not happen, the enzyme must be present as some functionally inactive complex which can be 'switched on' in some way by stimuli known to release arachidonic acid.

To test if this concept obtained in the lung a specifically labelled phospholipid substrate of phospholipase A_2 was synthesized with a radioactive fatty acid in the 2'-position. If a cell membrane phospholipase was attached to the surface of some cell-type in the lung (as it is with several other cell types) then it could be revealed by examining the hydrolysis of the radioactive fatty acid. When aliquots of the labelled isotope (suspended as micelles in buffer) were injected at regular intervals into the pulmonary circulation of the lung and the amount of isotope hydrolysed determined, we found that there was a background hydrolysis of the labelled phospholipid and this was strongly inhibited by infusions of glucocorticoids such as dexamethasone. Again, the onset of the steroid effect occurred after a time delay and was reversible upon termination of the infusion. Experiments of a similar nature also determined that phospholipase A_2-like activity was increased by the injection of releasing factors such as RCS-RF, histamine and SRS-A and that this elevated hydrolytic activity was also blocked glucocorticoids [17].

The obvious question was whether or not the glucocorticoids caused the inhibition of phospholipase activity by the 'classical pathway' of steroid action [cf. 18, 19, 20]. We found that there was indeed a high affinity glucocorticoid binding protein in the cell-free supernatants of the guinea-pig lung [21]. The receptor was detected using tritiated dexamethasone as a ligand, and this was displaced from the receptor with unlabelled dexamethasone, hydrocortisone or the glucocorticoid receptor antagonist cortexolone. Interestingly, this latter compound also partially inhibited the effect of the steroid in the perfused lung preparation. Further experiments established that the anti-phospholipase effect of steroids in the lungs could be reversed by inhibition of RNA synthesis (by agents such as actinomycin D) and of protein synthesis (by agents such as cycloheximide and puromycin). At about this time A. DANON and G. ASSOULINE also demonstrated that the glucocorticoid effects on prostaglandin production by renal interstitial cells depended upon unimpaired RNA and protein synthesis [22], and going even further F. RUSSO-MARIE and her colleagues demonstrated unequivocably that the inhibitory action of corticosteroids on prostaglandin synthesis by rat reno-medullary cells is mediated through receptor occupancy and requires RNA and protein synthesis [23].

All these experiments strongly suggested that steroids inhibited phospholipase A_2 by a mechanism depending upon glucocorticoid receptor occupancy, followed by *de novo* RNA and protein synthesis and begged the question of whether the glucocorticoids were inducing the synthesis or release of an inhibitor of phospholipase A_2.

The identification of a 'second messenger' of steroid action in the lung and macrophage

To search for an inhibitor of phospholipase we devised a bioassay experiment in which two guinea-pig isolated lungs were perfused in series [21]. The design of the experiment was such that the effluent from one lung (generator lung) was connected, reoxygenated

and pumped into a second (test) lung in which the phospholipase activity was assessed by the radiochemical assay or bioassay (i.e. release of prostaglandins). The second lung was rendered insensitive to steroids by the continuous infusion of cycloheximide. This meant that when the steroid was infused directly into the second lung, there was little or no effect on phospholipase activity, but if it was infused into the first lung, then the phospholipase activity of the second test lung began to decline after a short lag period. The most reasonable interpretation of these experiments was that a soluble inhibitor of phospholipase was being secreted from the first lung under steroid stimulation and was being transported in the Krebs' perfusate into the second lung.

In another type of experiment which also reinforced the idea that steroid stimulated perfused lungs released a soluble phospholipase inhibitor, two lungs were independently perfused. One of these received steroid, the other, vehicle. The perfusates were collected and later tested on a third cycloheximide-treated lung. The perfusate from the steroid-treated lung blocked phospholipase activity in the cycloheximide-treated lung but the perfusate from the control lungs was inactive. If the perfusates containing the anti-phospholipase factor were boiled or treated with proteolytic enzymes, then the biological activity was destroyed strongly suggesting that the inhibitory factor was a protein.

In other systems too, the action of glucocorticoids was found to be more complex than was originally envisaged: BRAY and GORDON [24] had demonstrated in 1976 that corticosteroids blocked prostaglandin synthesis by guinea-pig macrophages. M. DI ROSA and his colleagues at the University of Naples [25] demonstrated in 1979 that rat peritoneal lavage cells (about 80% macrophages) were also highly sensitive to the prostaglandin inhibitory effect of steroids and that this effect too was dependent on *de novo* RNA and protein synthesis. In addition, there was evidence that this effect was also caused by the release from the cells of a phospholipase inhibitory protein [26].

The Wellcome/Naples group established some collaborative studies with the object of comparing the biological profile of the lung and macrophage-derived inhibitors, and quickly determined that they were both proteins, and had similar molecular weights (about 15 k) as judged by gel chromatography. Both inhibitors were interchangeable in their actions, that is to say the macrophage-derived material was active in the guinea-pig lung system and vice versa. Finally, both proteins shared a similar resistance to heating being stable for 10 minutes at 70°C but being destroyed at higher temperatures [27].

These and other results encouraged us to believe that we were dealing with a single protein which we christened 'macrocortin': 'cortin' to indicate that the protein was induced (or released) specifically by the glucocorticoids and mimicked their action in the two systems, while the prefix 'macro' indicated the most likely cellular origin (macrophages) also that their molecular weight (15 k) was considerably in excess of that of the steroids themselves.

Two other protein inhibitors of phospholipase discovered

Two other groups had independently arrived at a similar conclusion concerning the mechanism of action of steroids on phospholipase. F. HIRATA and J. AXELROD at the National Institute of Health in Bethesda had been investigating the mechanism of neutrophil chemotaxis [28]. When neutrophils are stimulated with chemoattractants such as f-met-leu-phe there are rapid changes in membrane biochemistry which include phospholipid methylation and arachidonic acid release. These authors demonstrated that both events were preceded by an activation of phospholipase A_2 in the cell membrane, and that drugs which inhibited this enzyme such as the anti-malarial mepacrine blocked both the phospholipase A_2 activation and chemotaxis. When steroids were tested as inhibitors in the cell chemotaxis assay, both cell chemotaxis and phospholipase A_2 were blocked. Only glucocorticoids (dexamethasone, fluocinolone, hydrocortisone, and cortisone) were effective, oestradiol 17-β, testosterone and progesterone were without activity. As in the lung experiments, steroid receptors were demonstrated in the neutrophils and displacement of labelled dexamethasone from the receptor was observed in the presence of inducing steroids. The inhibitory activity of the steroids was also abrogated by inhibitors of protein and RNA synthesis.

When the particulate fractions of the steroid-treated neutrophils were solubilised with the non-ionic detergent NP40, it was found to con-

tain a phospholipase inhibitory protein which chromatographed on Sephadex with an apparent molecular weight of 40 k [29]. If neutrophils were first incubated with labelled lysine the Sephadex fractions containing the 40 k protein were found to have a greater incorporation of the isotope when prepared from steroid treated cells than did similar fractions derived from untreated cells. The conclusion from these experiments was that glucocorticoids blocked chemotaxis by inhibiting phospholipase A_2 and this was the result of an induction (or at least an increase) of a protein inhibitor of this enzyme. This protein was subsequently christened 'lipomodulin' by the NIH group.

Another group interested in the question of steroid inhibition prostaglandin synthesis, was that of F. RUSSO-MARIE and her colleagues at the Necker Hospital in Paris [23, 30]. Rat renomedullary interstitial cells in culture produce large amounts of prostaglandins, and both nonsteroidal and steroidal drugs have inhibitory effects on the generation of these lipids. In a series of beautiful experiments they were able to demonstrate that the glucocorticoids, dexamethasone and corticosterone, inhibited prostaglandin synthesis and the concentrations required to do this generally correlated well with the concentrations required to occupy glucocorticoid binding sites. Once again, only steroids with the glucocorticoid properties prevented prostaglandin secretion, this was not caused by an effect on cell growth or cyclooxygenase activity and was blocked by inhibitors of macromolecule synthesis.

Later experiments by this group indicated that the inhibition of prostaglandin synthesis was secondary to phospholipase inhibition and this in turn was due to the generation of a heat-labile non-dialysable factor subsequently dubbed 'renocortin'. Analysis of renocortin demonstrated that it was a mixture of two proteins, one having a molecular weight of 15 k and the other a molecular weight of 30 k [31].

At the Children's Hospital of Philadelphia more recently, GUPTA and his colleagues have demonstrated that dexamethasone induces the formation of phospholipase inhibitory proteins ('PLIP') in cultures of embryonic palate cells and thymocytes. The molecular weight species observed (55 k, 40 k, 28 k and 15 k) are very similar to those observed by the other groups [32, 33].

A change in nomenclature

Initially, it seemed that macrocortin, lipomodulin and renocortin were three different proteins induced by the glucocorticoids, but a subsequent comparison of the conditions of their generation and a more rigorous examination of the distribution of molecular weights, their anti-enzyme and immunological properties led the Wellcome group, the NIH group and the Necker Hospital group to the conclusion that all these proteins are functionally identical and all active fragments of the same precursor. A unified nomenclature was agreed and the name 'lipocortin' proposed [34]. It is suggested that the disparity in molecular weights is a consequence of proteolysis.

Final reflections

The glucocorticoids inhibit our 'defence reactions' at many levels, and they do this by inhibiting the synthesis of chemicals involved in the promotion of the inflammatory response. The production of many mediators involved in the response to infection, injury, haemorrhage or metabolic disturbances are under glucocorticoid control such that elevated levels of hormone in the blood suppresses the formation of these mediators. In many cases the action of these mediators is blocked as well.

It might be thought that the glucocorticoids act simply by decreasing the synthesis rate of these protein mediators of inflammation such as the lymphokines, or of the enzymes which make prostaglandins, but whilst this undoubtedly does occur, another mechanism is also employed: that is, the glucocorticoid-induced synthesis of inhibitory proteins.

Lipocortin then is a sort of 'second messenger' of the glucocorticoids. It is only one of the many such regulatory proteins but it is a particularly important one, controlling as it does the development of the symptoms of the inflammatory response. It is undoubtedly an important component of this inbuilt mechanism for terminating the inflammatory response which the physician exploits, for when he gives his patients relatively large doses of steroids to control an inflammatory response he is in reality increasing the synthesis of these 'second messenger' proteins such as lipocortin to a near maximum.

All the early studies on lipocortin were performed *in vitro*, that is under conditions

in which steroids were not normally present. Under these circumstances the generation and appearance of lipocortin was absolutely dependent upon the presence of glucocortids in the perfusing medium. These findings led perhaps to the erroneous notion that lipocortin was only present following treatment with exogenous steroids.

Of course, all healthy mammals have circulating glucocorticoids and thus it is more rational to expect that lipocortin is a normal constituent of plasma and tissues as, indeed, it appears to be, although the amount present in the cells can be increased by raising the concentration of endogenous or exogenous steroids.

There has been a corresponding change in our appreciation of the function of lipocortin. Originally, it was regarded mainly as an 'anti-inflammatory protein' but today it is recognised that it is probably a key protein occurring in all cells and that its function is to inhibit phospholipase A_2 and allow lipid hydrolysis only under strictly controlled circumstances. This reversible inhibitory function of lipocortin is controlled by the phosphorylation and dephosphorylation cycle described by HIRATA [35]. Naturally, during inflammation phospholipase is substantially activated and thus there is a requirement for a greater than normal supply of the inhibitory protein, hence the relationship between the rate of synthesis and the release of steroid hormones.

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