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Review article

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THE ANNEXIN-1 KNOCKOUT MOUSE: WHAT IT TELLS US ABOUT THE INFLAMMATORY RESPONSE.

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The 37kDa protein annexin 1 (Anx-1; lipocortin 1) is a glucocorticoid-regulated protein that has been implicated in the regulation of phagocytosis, cell signalling and proliferation, and postulated to be a mediator of glucocorticoids action in inflammation and in the control of anterior pituitary hormone release. Immuno-neutralisation or antisense strategies support this hypothesis as they can reverse the effect of glucocorticoids in several systems. We recently generated a line of mice lacking the Anx-1 gene noting that some tissues taken from such animals exhibited an increased expression of several proteins including COX-2 and $cPLA_2$. In models of experimental inflammation, Anx-$1^{-/-}$ mice exhibit an exaggerated response and a partial or complete resistance to the anti-inflammatory effects of glucocorticoids. Several other anomalies were noted including abnormal leukocyte adhesion molecule expression, an increased spontaneous migratory behaviour of PMN in Anx-$1^{-/-}$ mice and a resistance in Anx-$1^{-/-}$ macrophages to glucocorticoid inhibition of superoxide generation. This paper reviews these and other data in the light of the development of the 'second messenger' hypothesis of glucocorticoid action.

Key words. *Glucocorticoids, inflammation, annexins, transgenic animal models.*

INTRODUCTION

Although the potent anti-inflammatory actions of the glucocorticoid steroids have been known since the late 1940s, their actual mechanism of action eluded researchers for many years. Many early ideas were advanced to explain their action, one of the most influential of which was the hypothesis of Weismann and his colleagues who suggested that these drugs 'stabilised' lysosomal membranes (1) thereby reducing the severity of the inflammatory response.

Another, even more ancient, anti-inflammatory drug with an unexplained action at the beginning of the 1970s was aspirin. Whilst the pharmacology of the 'aspirin-like drugs' was well known, no one understood how the therapeutic and side effect profile of these drugs was linked. Vane's resolution of this problem depended upon the observation that prostaglandins could produce many of the symptoms of the inflammatory response and that their removal through inhibition of the prostaglandin forming cyclo-oxygenase could account for most aspects of their therapeutic action (2, 3) including their ability to inhibit hyperalgesia (4) and fever (5, 6). It also seemed likely that the mechanism-based side effects of aspirin, such as the gastric irritancy, could also be explained on the basis of prostaglandin involvement in this phenomenon (e. g. ref (7)). Indeed for a while in the 1970s, it seemed that prostaglandin synthesis was the key which unlocked most of the mysteries of the inflammatory response.

It was in keeping with the spirit of these discoveries therefore that the effects of several other anti-inflammatory drugs (the glucocorticoids and DMARDs) were tested on the cell-free cyclo-oxygenase enzyme system in the early 1970s with the finding that they were uniformly inactive (8). But, given the prominence of the 'prostaglandin' theory of inflammation, this posed a conceptual problem: if these drugs didn't inhibit prostaglandin generation, how did they bring about an anti-inflammatory effect? This mystery was compounded by another series of observations which suggested that the glucocorticoids could, under some circumstances, inhibit prostaglandin generation – but only in intact cells (9,10).

The most likely explanation for this was that these drugs inhibited some other facet of the prostaglandin generating pathway - other than the cyclo-oxygenase itself. Since arachidonic acid was thought to be the most abundant substrate for the generation of prostaglandin E_2 and other members of this series, a potential mechanism of action could be the inhibition of the release of substrate. Several studies demonstrated that this was indeed the case as glucocorticoids were seen to block the release of prostaglandins from intact cells or perfused organs whilst having no effect upon the conversion of arachidonic acid (10,11). It seemed that a likely mechanism was inhibition of substrate liberation probably through inhibition of the activity of the enzyme phospholipase A2, the most likely enzyme to be involved in cleavage of arachidonyl containing phosphatides in the cell. But how was this regulatory action of glucocorticoids achieved?

Despite the fact that specific glucocorticoid receptors had been observed in the nuclei of some cells (12) and the 'genomic' theory of sex steroid action was already beginning to be understood (13,14), few workers had applied this type of thinking to the problems of the anti-inflammatory action of the glucocorticoids. Building upon this type of approach we, and others (15-17) tested glucocorticoid receptor antagonists and inhibitors of RNA and protein synthesis, as inhibitors of the effect of glucocorticoids on prostaglandin release. We found that the ability of glucocorticoids to prevent arachidonic acid release (as well as other effects) was completely vitiated by such agents providing strong support for an alternative, genomic, action of these drugs.

The 'second messenger' hypothesis

If the glucocorticoids were acting in such a fashion, how did they do it? By modifying gene expression they clearly had the potential to bring about a profound change in the biology of the cell by altering the synthesis of many cellular proteins. Our group took one facet of glucocorticoid action – the ability to interfere with prostaglandin generation in intact cells – and further analysed the mechanism. Our studies revealed that the glucocorticoids caused the release and synthesis from cells and tissues of a 'second messenger protein' which had the ability to inhibit arachidonic acid release in other cells and tissues (18). Initially this protein was dubbed 'macrocortin' (19), but later the name was changed to 'lipocortin' (20) to accommodate the fact that two other groups had also discovered second messengers of glucocorticoids which seemed to be identical or at least closely related proteins (21, 22).

From farther studies it seemed that there were several lipocortins that could be released from cells following glucocorticoid treatment (23). The protein responsible for the majority of the biological activity was characterised as a monomeric phosphorylated species of approximately 40kDa. It could be prepared from glucocorticoid-stimulated cells or from the peritoneal lavages of glucocorticoid-treated rats by ion-exchange and affinity chromatography (23-25).

The protein was eventually sequenced and cloned (26) and the 37kDa recombinant human protein shown to share the anti-inflammatory and eicosanoid suppressive properties of the naturally occurring protein (27). The other 'lipocortins' were likewise sequenced and found to be closely related species (28).

It was recognised that lipocortins belonged to a much larger family of proteins characterised by the presence of several (usually 4) repeating domains that conferred calcium and phospholipid binding properties on the protein. This super family is now termed the 'annexins' and eventually, the name lipocortin 1 was changed to 'Annexin-1' (Anx-1) to accommodate this finding. In addition to the characteristic repeating conserved 70aa calcium binding domain each member of

the family has a unique N-terminal tail. The presence of annexins in many different plant and animal species suggests a function that is fundamental to the biology of many different cell types (29).

Since its discovery, our work, together with that from other laboratories, has implicated Anx-1 in control of cell growth (30) and differentiation (31), signal transduction and arachidonic acid release (32, 33), as well as intracellular vesicle trafficking (34-36). In mammals, glucocorticoids regulate the synthesis, phosphorylation and cellular disposition of Anx-1 and work from our laboratory, as well as from others, has provided further evidence for the involvement of Anx-1 in the regulation by these drugs of leukocyte migration (37), acute (38) and chronic (39) inflammation, ischaemic damage (40-42), pain (43) and fever (44).

It is not just the host defence system that is a target for Anx-1. The glucocorticoids themselves have crucial actions in the neuroendocrine system where they act to inhibit the release of pituitary hormones. In the rodent anterior pituitary gland for example, the inhibition of ACTH (45) and other (46) hormone release by glucocorticoids is mediated through an Anx-1 dependent mechanism.

The protein is elevated or released in man after systemic (47, 48), or local (49) administration of glucocorticoids and endogenous levels in circulating monocytes correlate with the activity of the HPA axis in normal, cushingoid and addisonian patients (50) and there have been many reports that defects in Anx-1 function are implicated in human diseases; familial Mediterranean fever (51, 52), fragile X syndrome (53) and Weber-Christian disease (54), for example. In other disorders, such as in cystic fibrosis (55) and other lung pathologies (56-58) Anx-1 appears to have an abnormal metabolism and it has also been implicated, directly or indirectly, in the control of human cell division (30) or differentiation (31) and tumour development, (59) skin disorders (60, 61) and CNS pathology (62, 63). Auto antibodies to Anx-1 may be responsible for some forms of glucocorticoid resistance in rheumatoid patients, (64) associated with the pathology of SLE (65) and Crohn's disease (66) and could be a diagnostic marker for certain type of tumours (67).

Our previous attempts to delineate the role of Anx-1 in physiopathology tested the human recombinant protein (or peptidomimetics) as a putative anti-inflammatory agent or employed acute passive immunisation strategies to probe the involvement of Anx-1 in glucocorticoid action. Anx-1 showed great selectivity of action in inflammation being mainly active in models of inflammation where the involvement of PMN was crucial to the development of the response (e. g. ref (68)). When neutralising antibodies were used in models of rodent inflammation such as the zymosan-inflamed air pouch, our chief findings were that they exacerbated inflammation as assessed by PMN influx, cytokine and eicosanoid synthesis and resolution of the response (69). The results of many of these studies are shown in *Table 1* and it is clear that whilst duplicating some of the effects of glucocorticoids, Anx-1 does not account for all their effects. This is what we anticipated as the whole thrust of our work was to replace the rather

Table 1. Some models of Inflammation where Anx-1 is Active or Implicated in the Mechanism of Glucocorticoid Action.

Species	Inflammatory model	Parameter	Effect of Anx-1	Effects of GCs	Effect of anti-annexin abs.	Ref.
Rat	Carrageenin paw oedema	Swelling	Inhibits	Inhibits	Reverses GCs	(68,81)
	Dextran paw oedema	Swelling	None or slight.	NT	NT	(68)
	48/80 paw oedema	Swelling	Inhibits moderately	Inhibits	NT	(68)
	PLA2 paw oedema	Swelling	Inhibits	NT	NT	(68)
	PAF paw oedema	Swelling	None	Inhibits	NT	(68)
	Bradykinin	Swelling	None	Inhibits	NT	(68)
	5HT	Swelling	None	Inhibits	NT	(68)
	Brain ischemia	Oedema	Inhibits	NT	Exacerbates	(40)
	Myocardial reperfusion	PMN damage	Inhibits	Inhibits	Reverses GCs	(82)
	Splancnic reperfusion injury	PMN damage	Inhibits	Inhibits	Reverses GCs	(41)
	ILIb hyperalgesia	Pain	Inhibits	Inhibits	Reverses GCs	(43)
Mouse	Air pouch (IL 1 (3)	PMN migration	Inhibits	Inhibits	Reverses GCs	(83)
	Air pouch (zymosan)	PMN migration	NT	NT	Exacerbates	(69)
	Air pouch (ovalb)	Eosinophil migration	None	Inhibits	None	(84)
	Zymosan blister	Oedema	NT	NT	Exacerbates	(69)
Hampster	Intravital microscopy	PMN migration	Inhibits	Inhibits	Reverses	(85)
Rabbit	Fever (poly 1C)	Body temperature	Inhibits	Inhibits	NT	(44)

simplistic physico-chemical account of glucocorticoid action with a model that depends upon the action of many genes.

However, inflammation was not the only system where Anx-1 was implicated as a mediator of glucocorticoid action. In models designed to mimic activation of the HPA axis during infection or inflammation, passive immunisation blocked the inhibitory effects of exogenous corticosterone or dexamethasone on IL-l induced increases in ACTH (70, 71). Similarly, passive immunisation studies have suggested that the capacity of glucocorticoids to counter the regulatory action of cytokines on the secretion of growth hormone and prolactin is dependent on Anx-1 (71).

A transgenic approach.

Recently, we decided to employ a transgenic strategy to study farther Anx-1 function in rodents (72). We were encouraged in this approach by the observation that in the case of some other members of the annexin family, such as Anx-6 (73) and (possibly) Anx-7 (74) (75), gene deletion was not a lethal mutation and that animals grow to sexual maturity without any obvious developmental problem. Therefore, using conventional gene targeting techniques, we raised a line of mice that lacked the Anx-1 gene.

There was no obvious physical difference between the Anx-l$^{-/-}$ Anx-1$^{+/-}$ and Anx-1$^{+/+}$ littermate control mice in terms of gross physical appearance or behaviour and we saw no significant differences in weight between any of the (sex matched) groups at any time point examined. All animals appeared healthy, bred normally and produced healthy Anx-1$^{-/-}$ offspring.

There were, however, striking changes in this study in the altered expression in some tissues of other members of the annexin family together with the over expression of COX-2 and cPLA$_2$ in the lung and thymus. The idea that there may be some form of reciprocal regulation between annexin family members and that inducible COX-2 and cPLA$_2$ enzymes is congruent with some previously published observations (76, 77).

In inflammation, many of our early findings were echoed, and confirmed by this gene ‘knockout’ study. In mouse paw carrageenin-induced oedema, for example, the inflammatory response exhibits a characteristic biphasic pattern (78). The first phase peaks at 4-6h and resolves at 24h whilst the second phase peaks at 72h and resolves at >96h. Dexamethasone (l0μg/kg x3, i.p.) strongly inhibited the first phase of oedema (mainly PMN-dependent: ref 78) in Anx-1$^{+/+}$ mice (*Fig. 1*) but was completely without effect in Anx-l$^{-/-}$ mice. In contrast, the glucocorticoid generally maintained its efficacy in the second phase (mainly macrophage - lymphocyte - and eosinophil - dependent (78)) of the oedema. An exaggerated response to zymosan was also seen in another zymosan peritonitis model with greatly increased PMN migration and an increase in IL1β observed during the course of the inflammatory response. Once again dexamethasone was

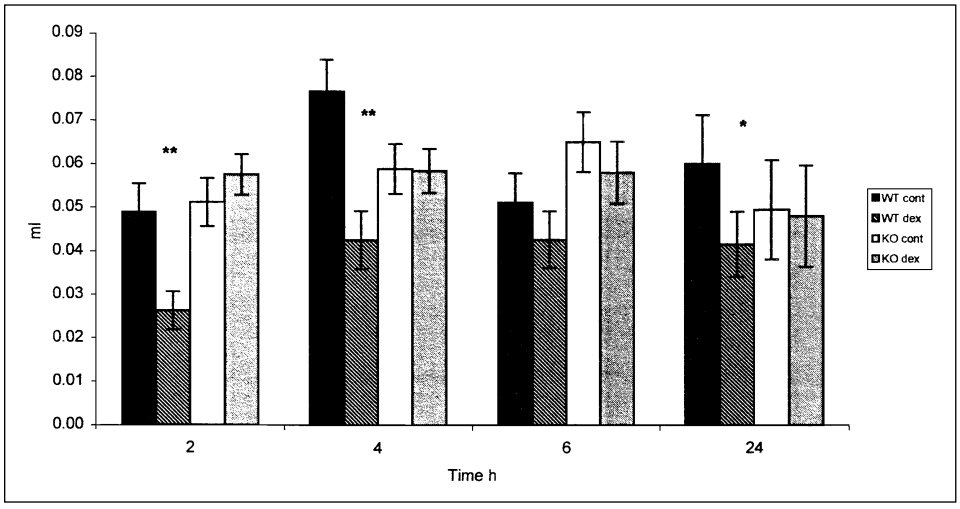

Figure 1. Failure of dexamethasone to inhibit the PMN dependent phase of carrageenin paw oedema in Anx-1$^{-/-}$ mouse.

Black columns, wild type mice. White columns Anx-1-/- mice. Cross-hatched columns, 10μg dexamethasone i.p. at 2, 6 and 24 h. Data analysed relative to time-matched, vehicle-treated control using student's t-test, n= 17-18 per group; ** P<0.01.

noticeably less efficacious in the Anx-1$^{+/-}$ and Anx-1$^{-/-}$ mice than in the wild type controls.

We also saw changes in the behaviour of leukocytes including alterations in adhesion molecule expression and defects in zymosan phagocytosis. Interestingly, hydrocortisone, which inhibits the activation of PMN superoxide generation by aggregated IgG in Anx-1$^{+/+}$ mice was completely ineffective in the Anx-1$^{-/-}$ animals. At the level of the microcirculation an increased basal adherence and migration of PMN was observed using intravital microscopy techniques but the velocity of rolling was not altered. A summary of many of these changes is given in *Table 2*.

Modulation of the synthesis and release of Anx-1 is, of course, not the only way in which glucocorticoids operate; they can act through a variety of mechanisms including inhibition of NF-κB activation, direct genomic actions mediated through GREs as well as other signalling effects (32, 79) to bring about their effects. There are also several ways in which the activated glucocorticoid receptor can function to effect cellular changes. We recently described a rapid receptor-dependent, genome-independent, signalling effect of glucocorticoids in the A549 human carcinoma cell line mediated by *src* specifically involving Anx-1 and demonstrated that this pathway is utilised preferentially by some, but not all glucocorticoids (80). Because of the apparent redundancy of glucocorticoid mechanisms we were not surprised that not all effects of these drugs were suppressed in the Anx-1$^{-/-}$ animals. Such results suggest that glucocorticoids employ separate but parallel pathways to regulate inflammation

Table 2. Summary of differences between Anx-1$^{+/+}$ and Anx-1$^{-/-}$ mice in response to inflammatory stimuli.

Parameters measured	Behaviour in Anx-1$^{-/-}$ Mouse	Effect ofGCs
Tissue protein expression*		
Cox-2	Increased	NT
$CPLA_2$	Increased	NT
Adhesion molecules		
Basal PMN/MonoCD11b	Reduced	NT
Basal PMN/Mono L selectin	Elevated	NT
Stimulated PMN/Mono CD11b	Exaggerated	NT
Macrophage phagocytosis		
Uptake ofunopsonised zymosan	Greatly diminished	NT
Uptake ofopsonised zymosan	Diminished	NT
PMN superoxide generation		
IgG stimulated	Unchanged	Abolished
Red Oxyburst assay	Unchanged	Abolished
Intravital microscopy		
Basal PMN rolling	Unchanged	NT
Basal PMN adherence	Increased	NT
Basal PMN migration	Increased	NT
Inflammatory models		
Carrageenin induced paw oedema		
PMN dependent phase	Slightly increased	Abolished
PMN independent phase	Slightly increased	Unchanged
Zymosan peritonitis		
PMN migration	Greatly exaggerated	Diminished
IL 1β production	Increased	NT
TNFα production	Slightly increased	NT

and is congruent with the observation that the recombinant protein exerts anti-inflammatory effects in some, but not all, inflammatory models (68).

CONCLUSION

In summary the overall pathophysiological picture of Anx-1 gene deletion that emerges from our studies suggests a heightened sensitivity to inflammatory and other environmental stimuli. This is no doubt exacerbated *in vivo* by the ineffectiveness of endogenous glucocorticoids adequately to control the inflammatory response because of the absence of this protein.

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