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Are formyl peptide receptors novel targets for therapeutic intervention in ischaemia–reperfusion injury?

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Ischaemia–reperfusion (I/R) injury is a common feature of several diseases associated with high morbidity and mortality, such as stroke and myocardial infarction. The damaged tissue displays cardinal signs of inflammation and microvascular injury that, unless resolved, lead to long-term tissue damage with associated dysfunction. Current therapies are limited and are often associated with many side effects. Increasing evidence suggests that members of the formyl peptide receptor (FPR) family, in particular human FPR2/ALX, might have an important role in the pathophysiology of I/R injury. It was recently demonstrated that several peptides and non-peptidyl small-molecule compounds have anti-inflammatory and pro-resolving properties via their action on members of the FPR family. Here I review this evidence and suggest that FPR ligands, particularly in the brain, could be novel and exciting anti-inflammatory therapeutics for the treatment of a variety of clinical conditions, including stroke.

Introduction

Ischaemia–reperfusion (I/R) contributes to the pathophysiology of many clinical problems such as myocardial infarction, stroke, resuscitation, coronary bypass surgery, frostbite, extension of burn injury and organ transplantation. According to the American Heart Association, more than 1 million people suffer a heart attack each year and approximately 795,000 suffer a stroke. These diseases significantly contribute to the mortality rate and full recovery is unlikely, with the single most important factor being the degree of ischaemic damage at the time of the event. Ischaemia refers to a reduction in blood flow and reperfusion injury is associated with an initial blood-borne neutrophil infiltration, giving rise to an inflammatory response and finally resulting in tissue injury [1–3].

Although restoration of blood flow to a previously ischaemic region is essential to prevent irreversible tissue damage, reperfusion itself is a double-edged sword and thus is not always beneficial. Although a great deal of damage occurs to the tissue because of reperfusion, a significant amount of injury occurs due to ischaemia itself. During myocardial injury, several events occur that mediate vascular injury, including oxygen free radical production by mitochondrial respiration, activated neutrophils and xanthine oxidase activity [3]. These are

all intimately involved in the inflammatory cascade, themselves activating leukocytes, inducing lipid peroxidation and increasing vascular permeability.

Leukocyte recruitment occurs in the microvasculature and involves a complex set of events that can occur both locally and systemically. Both *in vivo* and *in vitro* evidence (e.g. antibodies against adhesion molecules; chemotaxis and transmigration assays; flow chamber assays; and real-time studies to visualise cellular interactions in the circulation of anaesthetised animals using intravital microscopy) have demonstrated molecular and cellular pathways involved in this multi-step cascade. The leukocyte adhesion cascade involves: capture, rolling, slow rolling, leukocyte arrest, post-adhesion strengthening, intravascular crawling and paracellular or transcellular transmigration [4] (Figure 1).

Glossary

Lipoxin A$_4$ (LXA$_4$):
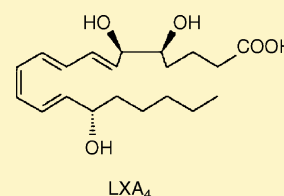
LXA$_4$

15-*Epi*-LXA$_4$: an aspirin-triggered lipoxin

15-*epi*-LXA$_4$ (ATL)
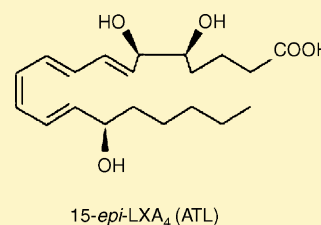
Annexin Ac2–26: N-terminal domain of annexin A1, representing the bioactive peptide spanning amino acids 2–26.
Complement 5a (C5a) receptor (C5AR): also known as complement component 5a receptor 1 and CD88 (cluster of differentiation 88); a G-protein-coupled receptor for C5a.
Dissociation constant (K_d): measure of how tightly a ligand binds to a particular receptor. Low ligand K_d values reflect a high affinity for the receptor.
G-protein-coupled receptors (GPCRs): proteins with an extracellular domain and an intracellular C-terminal tail, separated by three extracellular loops and three intracellular loops. They are the largest family of membrane receptors and couple to intracellular effector systems via a G protein.
Intravital microscopy (IVM): real-time qualitative and quantitative method for observing cell–cell interactions in the microcirculation of an anaesthetized animal.
Neutrophil: white blood cell.

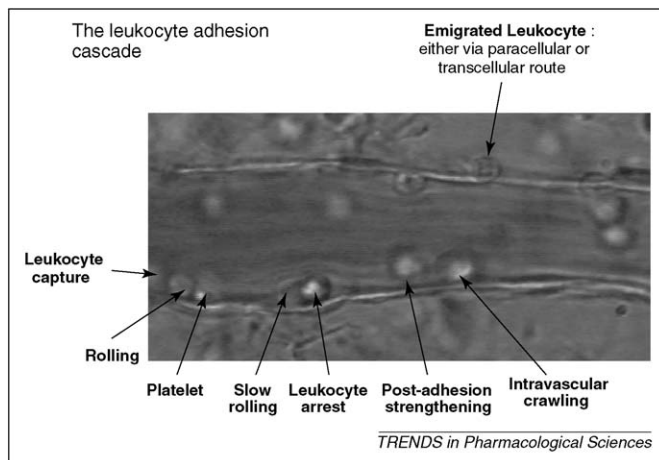*Corresponding author:* Gavins, F.N.E. (f.gavins@imperial.ac.uk).

Figure 1. Example of an inflamed murine mesenteric venule. This picture demonstrates the steps involved in the inflammatory cascade: leukocyte capture, rolling, slow rolling, arrest, post-adhesion strengthening, intravascular crawling and either paracellular or ranscellular migration of leukocytes into the surrounding tissue.

Living organisms are capable of recovering from different pathogens and noxious stimuli that enter the system. Injury to the body provokes a host acute inflammatory response of pain, fever, redness, swelling and, in the case of chronic inflammation, loss of function. The inflammatory response is characterised by leukocyte infiltration, which is achieved by integrins, adhesion, selectins and glycoprotein selectin ligands in response to cytokines and chemotactic factor gradients. These factors mediate the inflammatory cascade, involving leukocyte rolling, adhesion and trans-endothelial migration [3,4] (Figure 1). Numerous receptors modulate the host inflammatory response. Under abnormal situations, the body's response can assume the character of a disease itself [5], resulting in tissue damage, as observed in pathological conditions such as I/R injury.

Microvascular dysfunctions are observed on reperfusion of ischaemic tissue, including endothelial-dependent dilation of arterioles and increased fluid filtration and leukocyte plugging in capillaries, leading to a no-reflow phenomenon [3]. On the basis of current understanding of I/R, several potential treatments have been suggested based on mechanical (e.g. coronary angioplasty or stenting) or pharmacological (e.g. anti-platelet therapy, tissue plasminogen activator administration, neutralization of already secreted pro-inflammatory cytokines, administration of anti-inflammatory cytokines, factors favouring mesenchymal stem cell implantation and mobilization) restoration of blood flow [2,6,7]. Other therapeutic approaches have been explored such as targeting of receptors implicated in regulating and resolving the inflammation associated with I/R. One such family of receptors is the G-protein-coupled receptors (GPCRs) known as the formyl peptide receptors (FPRs), whose involvement in I/R injury has been demonstrated in a variety of different tissues, and thus ligands for FPRs might play a role in host defense.

The FPRs are promiscuous in their ability to bind different ligands, such as formyl-Met-Leu-Phe (fMLP) and lipoxin A_4 (LXA$_4$) (Box 1, Table 2). However, the receptors are expressed in different cells and tissues, raising the possibility that FPRs have far more diverse and complex roles in biology. Interestingly, resolution of inflammation involves the formation of endogenous anti-inflammatory mediators, which signals the termination of recruitment and removal of inflammatory cells from the inflammatory locus [8]. In this review, I summarise the evidence for proposing FPR ligands as novel anti-inflammatory and pro-resolving therapeutics and focus on their role in reducing the detrimental effects associated with I/R injury.

The FPR family: tissue and cellular distribution

FPR1 is one of the best-studied GPCRs, dating back to the early 1970s [9], and its activation produces a range of inflammatory responses associated with I/R, such as stimulation of leukocyte migration [8]. FPR1 has been found in a variety of different tissues and cells (Table 2)

Box 1. FPR signalling and nomenclature

FPR signalling
Leukocyte responses to chemoattractants, such as fMLP, require binding and activation of pertussin-toxin-sensitive GPCR coupling to $G_i\alpha_2$ or $G_i\alpha_3$, which triggers multiple second messengers through phospholipase C (PLC), PLD and PLA$_2$ activation [8]. fMLP stimulation of leukocytes induces shape changes, chemotaxis, adhesion, phagocytosis and the release of superoxide anions and granule contents, leading to tissue damage, as observed in inflammation and infarction [86]. All major neutrophil functions stimulated by fMLF can be inhibited by treatment of the cells with pertussis toxin, indicating that the FPRs belong to the G$_i$ family of heterotrimeric G proteins [11].

All three human FPRs are clustered on chromosomal region 19q13.3 [11], whereas the murine equivalents are arranged in two clusters on chromosome 17 (A3.2) together with an additional pseudogene (ΨFpr2) [18] based not on receptor structure, but on receptor agonists (Table 2).

FPR nomenclature
Human: Table 1 demonstrates human FPR nomenclature [11,19]. FPR2/ALX shares 69% sequence homology with FPR1. Both FPR2/ALX and FPR3 possess high degrees of amino acid identity (69%) [15,20], but are activated by different ligands (Table 2). The *FPR3* gene encodes a putative protein with 56% amino acid similarity to FPR1 and 72% to FPR2/ALX.

Non-human: A high degree of specificity to human orthologue genes occurs in non-human primates (95–99%) [11]. The situation is complicated in mice, with gene clusters having undergone differential expansion. The mouse genome encodes for at least seven different receptors (Table 1). It is now agreed that murine Fpr1 is 77% identical to human FPR1 [11] and Fpr3 is 76% identical to human FPRL2. Fpr2 binds fMLF with low affinity [11,18]. Fpr-r3, 4, 6 and 7 seem to have no direct counterparts in the human genome [73]. *Fpr-rs4* encodes a receptor with a short COOH-terminal domain, whereas *Fpr-rs5* has a stop codon in the putative transmembrane domain 6 (more likely to be a pseudogene) [73]. Two additional genes were characterised from screening of a mouse BAC genomic library, termed *Fpr-rs6* and *Fpr-rs7* [105]. Other orthologues have been found at a molecular level in species such as rabbit and primates and at a functional level in rat [106], guinea pig and horse (as reviewed in [11]).

It is noteworthy that there is chemoattractant receptor-like molecule (CRTH2) from a human Th2 clone that has high amino-acid homology with members of the FPR family. However, the similarity is insufficient to classify CRTH2 as an FPR member.

Table 1. Tissue distribution and IUPHAR nomenclature for human and murine FPRs

Species	Previous nomenclature	IUPHAR nomenclature	Tissue and cellular distribution
Human	Formyl-peptide receptor; FPR; NFPR; FMLPR	FPR1	Adrenal glands, adrenal cortical cells, astrocytes, bone marrow, carcinoma cells, CNS, colon, endothelial cells, epithelial cells, eye, fibroblasts, heart, hepatocytes, immature DCs, kidney, Kupffer cells, liver, lung, macrophages, microglial cells, monocytes, neuroblastoma cells, neutrophils, ovary, placenta, platelets, spleen
	Formyl-peptide receptor like 1; FPRL1; lipoxin A4 receptor (LXA4R); ALXR; FPRH1; HM63; RFP; FMLPX; FPR2A	FPR2/ALX	Astrocytes, bone marrow, brain, endothelial cells, epithelial cells, fibroblasts, hepatocytes, immature DCs, lung, macrophages, microglial cells, monocytes, neuroblastoma cells, neutrophils, placenta, spleen, T and B lymphocytes, testis
	Formyl-peptide receptor like 2; FPRL2; FPRH2; FMLPY; RMLP-related receptor 1	FPR3	Adrenal gland, DCs, HL-60 cells, liver, lung, lymph nodes, macrophages, monocytes, placenta, small intestine, spleen, trachea
Mouse	Fpr1	Fpr1	Adrenal gland, anterior pituitary, DCs, hippocampus, hypothalamus, liver, lung, microglia, mononuclear cells, neutrophils, spleen
	Fpr-rs2	Fpr2	Anterior pituitary, adrenal gland, DCs, hippocampus, hypothalamus, lungs, microglia, neutrophils, spleen
	Fpr-rs1; mALXR; fprL1	Fpr3	Adrenal gland, anterior pituitary, heart, hippocampus, hypothalamus, liver, lung, microglia, neutrophils, spleen
	Fpr-rs3	Fpr-rs3	Skeletal muscle
	Fpr-rs4	Fpr-rs4	?
	Fpr-rs5	Fpr-rs5	?
	Fpr-rs6	Fpr-rs6	Brain, skeletal muscle, spleen, testis
	Fpr-rs7	Fpr-rs7	Heart, liver, lung, pancreas, smooth muscle, spleen
	Fpr-rs8	Fpr-rs8	?

involved in inflammation, such as endothelial cells, platelets and dendritic cells (DCs) [10] (possibly important for modulation of T-cell activation) [11]. FPR2/ALX is equally widely distributed, with an interesting presence, in addition to peripheral cells and tissues, in brain and spinal cord, and is functionally expressed in glial cells, fibroblasts and astrocytes [12–15].

Functional FPRs are widely expressed in non-lymphoid tissues, including normal human lung and skin fibroblasts and the human fibrosarcoma cell line HT-1080. Van Compernolle demonstrated that fibroblasts express FPRs that respond in a chemoattractant manner on treatment with fMLP [15], thus opening up a new area for a role of non-leukocyte cell types in innate immune responses (recently reviewed in [11]). FPR3 seems to be present not in neutrophils, but in monocytes [11]. Fascinatingly, this tissue distribution varies with the monocyte differentiation stage [10,11], with mature DCs mainly expressing FPR3 [11].

Cui *et al*. [14] used primary microglia cells to demonstrate expression of FPR1 and FPR2/ALX genes; on stimulation with bacterial lipopolysaccharide (LPS), these cells undergo FPR2/ALX-mediated activation, leading to the suggestion that the blood brain barrier (BBB), under normal conditions, protects microglial cells. When endotoxaemia occurs, however, microvessels form an incomplete BBB and LPS is able to stimulate microglial cells, which assume macrophage characteristics and thus play a role in the inflammatory process [16]. Increased inflammatory events, such as leukocyte rolling and adhesion, occur in the murine cerebral microcirculation after endotoxaemia (something not observed under normal conditions) and seem to be mediated by FPR2/ALX (Gavins *et al.*, unpublished observations). The increase in inflammatory events

such as leukocyte rolling and adhesion observed following endotoxaemia also occurs during stroke. The role of FPR2/ALX in stroke is of great interest to my research team and the importance of this receptor as a potential therapeutic target is currently being investigated. The role of the FPRs in cerebral I/R is discussed in more detail later in this review.

A variety of different agonists and antagonists are currently availbale for the FPR family (Table 2). It is not possible to cover all of them within this review, so I have focussed on the more widely used ones.

FPR pharmacology
Agonists
The emergence of more sensitive techniques, such as computer-assisted 3D modelling, has helped us to understand how ligands bind to different receptors. Several natural formylated peptides have been purified from bacterial supernatants and exhibit the ability to activate human leukocytes [17]. fMLP is a widely used agonist of the FPR family and binds to FPR1 with approximately 1000-fold higher affinity than FPR2/ALX (K_d values in the picomolar to low nanomolar range), resulting in calcium mobilization and neutrophil activation [18]. Evidence suggests that FPR1 and FPR2/ALX activate the same signal response downstream of the receptor, resulting from activation produced by different ligands [11,19]. FPR2/ALX is a low-affinity receptor for fMLP (K_d 430 nM) [11,20] that produces a chemotactic response at micromolar concentrations. (FPR3 has a K_d value of $\sim$10 μM [21]). LXA$_4$ also interacts with FPR2/ALX (K_d $\sim$0.5 nM) [22,23], inhibiting the pro-inflammatory responses induced by both FPR2/ALX ligands in neutrophils and non-FPR2/ALX ligands in

Table 2. Non-exhaustive list of agonists and antagonists of human and murine FPRs and examples of specific effects[a]

Receptor	Agonist or antagonist	Ligand	Example of a specific effect	Refs
FPR1	Agonists	Ac2–12	Cardio-protective in murine model of myocardial infarction	[53]
		Ac9–25	Activates neutrophil release of O_2^-	[76]
		Ac2–26	Decreases neutrophil–endothelium interactions in flow chamber	[77]
		Annexin 1	Decreases neutrophil–endothelium interactions in flow chamber	[77]
		Cathepsin G	Chemoattractant for phagocytic leukocytes	[78]
		fMLP and analogues	Defective PMN chemotaxis in juvenile peridontitis *in vivo* Chemotaxis, lysozyme release, O_2^- production, including endothelial interaction, all in neutrophils	[79]
		HIV-1 T20 (DP178)	Chemoattractant and activator of peripheral phagocytes	[47,48]
		HIV-1 T21 (DP107)	Chemoattractant and activator of peripheral phagocytes (low affinity)	[47]
		HIV gp41	Induces directional migration and calcium mobilization in human monocytes and neutrophils	[49]
		HSV gG-2p20	Chemoattractant for monocytes and neutrophils	[50]
		LL-37	Chemoattractant for human peripheral blood neutrophils, monocytes and T cells	[23]
		SRSRY	Directional cell migration on vitronectin-coated filters	[80]
		WKYMVM	Activates neutrophils (low affinity)	[28]
		WKYMVm peptide	Activates neutrophils (low affinity)	[28]
FPR1	Antagonists	CDCA	Inhibits neutrophil chemoattraction and migration (high affinity)	[58]
		CHIPS	Inhibits chemotaxis in *S. aureus* infection (low affinity)	[55]
		Coronavirus 229E peptides	Ligand binding studies using transfected CHO cells demonstrated antagonism of FPR2	[81]
		Coronavirus peptides	Inhibits fMLP interaction in CHO cells	[81]
		Cyclosporine A	Inhibits fMLF-stimulated degranulation, chemotaxis, calcium mobilization of neutrophils	[82]
		Cyclosporine H	Decreased neutrophil activation (high affinity)	[83]
		DCA	Inhibits fMLP-induced monocyte and neutrophil chemotaxis and calcium mobilization	[57]
		Ebola peptides	Inhibits fMLP interaction in CHO cells	[81]
		FLIPr	FPRL1 inhibitory protein (FLIPr) inhibits leukocyte responses to FPR2 agonists	[56]
		HIV-2 peptides	Inhibits fMLP interaction in CHO cells	[81]
		Isopropylureido-FLFLF	Inhibits chemotaxis	[84]
		Spinorphin	Inhibits calcium mobilization in mouse-FPR transfected human embryonic kidney cells	[85]
		tBOC	Decreased neutrophil activation (low affinity)	[83]
FPR2/ALX	Agonists	$A\beta_{42}$	Chemotaxis of mononuclear cells	[86]
		Annexin 1	Decreased neutrophil–endothelium interactions (firm adhesion) in flow chamber	[77]
		Antiflammin 2	Decreased neutrophil–endothelium interactions	[87]
		CRAMP	Chemoattractant for leukocytes	[88]
		D2D3$_{88-274}$	Inhibits monocyte chemotaxis and integrin-dependent cell adhesion	[89]
		F peptide	Downregulates expression and function of CCR5 and CXCR4 in monocytes	[50]
		fMLP and analogues	Induces chemotaxis, lysozyme release, O_2^- production in neutrophils	[11]
		Formylated humanin	Chemotaxis of human FPR2-transfected CHO cells	[90]
		HIV-1 T21 (DP107)	Chemoattractant and activator of peripheral phagocytes (low affinity)	[86]
		Humanin	Chemotaxis of human FPR2-transfected CHO cells	[90]
		Lipoxin A4	Attenuated hind limb I/R-induced lung injury	[69]
		LL37	Chemoattractant for monocytes, neutrophils and T cells	[23]
		MMK-1	Chemotaxis and calcium mobilization in monocytes and neutrophils	[31]
		N36 peptide	Chemotaxis and calcium mobilization in monocytes and neutrophils	[49]
		NADH dehydrogenase	Chemotaxis and calcium mobilization in human FPR2-expressing HL-60 cells	[91]
		PACAP27	Neutrophil chemotaxis and upregulation of CD11b	[92]
		PRP106-126	Endocytosis in glial cells	[93]
		Quin-C1	Chemotaxis and secretion of β-glucuronidase in peripheral neutrophils	[94]
		Rana-6	Chemoattractant of phagocytes	[57]
		Serum amyloid A (SAA)	Chemoattractant for neutrophils	[41]
		T20 (DP178)	Chemoattractant and activator of peripheral phagocytes	[47,48]
		T21 (DP107)	Chemoattractant and activator of peripheral phagocytes (high affinity)	[47]
		Temporin A	Chemoattractant of phagocytes	[95]
		uPAR$_{84-95}$	Basophil chemotaxis	[96]
		V3 peptide	Inhibits monocytic response to chemokines	[97]
		WKYMVM	Activates neutrophils (high affinity); O_2^- production	[32]
		WKYMVm peptide	Activates neutrophils (high affinity)	[98]
		sCKb8-1	Alters protein pattern of PMN cells	[99]
		Hp2–20	Migration and proliferation of gastric epithelial cell lines MKN-28 and AGS	[96]

Table 2 (*Continued*)

Receptor	Agonist or antagonist	Ligand	Example of a specific effect	Refs
		Ac2–26	Decreased neutrophil–endothelium interactions (firm adhesion) in flow chamber	[77]
		Ac2–12	Cardioprotective in a murine model of myocardial infarction	[53]
FPR2/ALX	Antagonists	CDCA	Inhibits neutrophil chemoattraction and migration (low affinity)	[95]
		Coronavirus 229E peptides	Inhibits neutrophil chemoattraction and migration	[58]
		FLIPr	Inhibits calcium mobilization	[56]
		Isopropylureido-FLFLF	Inhibits chemotaxis	[84]
		PBP10	Inhibits granule mobilization and oxygen radical secretion	[100]
		tBOC	Decreased neutrophil activation (high affinity)	[83]
		WRWWWW	Inhibits oxidative burst from neutrophils, measured as a release of superoxide anions	[100]
FPR3	Agonists	Formylated peptides	Triggers dose-dependent migration of fibroblasts *in vitro*	[15]
		WKYMVM	Activates neutrophils	[32]
		WKYMVm peptide	Activates neutrophils	[98]
		Annexin 1	Initiates chemotactic responses in human monocytes	[39]
		Hp(2–20)	Chemoattractant for basophils	[101]
		Humanin	Induces chemotaxis of mononuclear phagocytes *in vitro*	[102]
		F2L	Promotes calcium mobilization and chemotaxis of monocytes and monocyte-derived DCs	[103]
FPR3	Antagonists	ETYIKPWWWVWL (from coronavirus 229E)	Inhibits fMLP interaction in CHO cells	[81]
		WRWWWW	Inhibits calcium flux	[32]
Fpr1	Agonists	Ac2–26	Protect against experimental myocardial I/R	[53]
		Annexin 1	Protect against experimental myocardial I/R	[53]
		fMLP and analogues	Chemoattractant for leukocytes	[51]
		T20 (DP178)	Induced chemotaxis in primary PMNs and transfected cells *in vitro*	[48]
		WKYMVm	Induced chemotaxis of transfected cells *in vitro*	[99]
Fpr1	Antagonists	Isopropylureido-FLFLF	Inhibits chemotaxis	[84]
		Spinorphin	Inhibits chemotaxis	[84]
		tBOC	Prevents the protective effect of AnxA1 peptides in murine mesenteric I/R preparations *in vivo*	[36]
Fpr2	Agonists	Aβ$_{42}$	Induced chemotaxis of transfected cells *in vitro*	[102]
		Ac2–26	Decreases adhesion and emigration in inflamed mesentery *in vivo*	[36]
		Annexin 1	Cerebroprotective in a murine stroke model *in vivo*	[54]
		CRAMP	Chemoattractant for leukocytes	[88]
		F2L	Induced chemotaxis in transfected cells and primary PMNs *in vitro*	[74]
		fMLP and analogues	Chemoattractant for neutrophils	[105]
		Humanin	Induces chemotaxis of mononuclear phagocytes *in vitro*	[102]
		MMK-1	Induced chemotaxis of PMNs into air pouch *in vivo*	[20]
		SAA	Induced chemotaxis of primary PMNs *in vitro*	[85]
		T20 (DP178)	Induced chemotaxis in transfected cells but not primary PMNs *in vitro*	[48]
		V3 peptide	Chemoattractant	[14]
		WKYMVm	Potent stimulant for murine neutrophils	[51]
Fpr2	Antagonists	Isopropylureido-FLFLF	Inhibits chemotaxis	[84]
		tBOC	Decreased neutrophil activation (high affinity)	[83]
		Aspirin-triggered lipoxins	Inhibited migration of PMNs into inflamed air pouch *in vivo*	[20]
Fpr3				

^{a}*CHIPS,* chemotaxis inhibitory protein of *S. aureus*; CHO, Chinese hamster ovary; Hp(2–20), *H. pylori* peptide Hp(2–20); N36, synthetic peptide derived from HIV-1; PMN, polymorphonucleocyte; spinorphin, LVVYPWT; V3, synthetic peptide derived from HIV-1.

epithelial cell lines [23–25]. These low K_d values demonstrate high affinity for the receptor.

To characterise further FPR interactions in phagocytes and subsequent cellular activation, several fMLP-OMe analogues have been synthesized [26] including for-Met-Leu-Cys(OMe)-Cys-Leu-Met-fpr, which binds to FPR1 [27]. Recently, fMLP-OMe analogues with receptor affinity greater than that of the parent fMLP-OMe have been synthesized, creating the potential for use as carriers for drugs [11].

Construction and screening of random peptide libraries have become useful tools in developing biologically active agents such as WKYMVm, which stimulates human B lymphocyte and monocytic cell lines and neutrophils [28,29] through both FPR1 and FPR2/ALX. In 1998, Klein isolated many small peptide sequences that react with FPR1 and FPR2/ALX [30], such as MMK-1 (LESIFRSLLFRVM), a potent FPR2/ALX-specific agonist [31]. Other synthetic peptides such as His-Phe-Tyr-Leu-Pro-Met-NH2 (HFYLPM) stimulate both monocytes and neutrophils via FPR2/ALX binding [32]. The peptide fragment of NADH dehydrogenase subunit 1 (MYFINILTL) is an agonist specific for FPR2/ALX. Other recent screening studies include a 152 GPCR screen that identified a novel 21-amino-acid peptide agonist of FPR2/ALX and FPR3 termed CGEN-855A (TIPMFVPESTSKLQKFTSWFM-amide) [33] and a screen for unique non-peptide agonists of FPR1/FPR2/ALX [33]. These screens demonstrate that

agonists of FPR1/FPR-ALX have wide chemical diversity and analysis of these and future agonists will enhance our knowledge of ligand–FPR interactions [34].

Both exogenous and endogenous ligands interact with the FPR family. One particular endogenous ligand that has been widely studied is the glucocorticoid-regulated protein annexin A1 (AnxA1), which counteracts leukocyte extravasation (Figure 1) [35–37]. Using calcium binding assays and L-selectin shedding protocols, Walther *et al.* demonstrated that AnxA1 N-terminal-derived peptides act on human neutrophils through FPR1 [38]. It was then shown that AnxA1 peptides initiate chemotactic responses in human monocytes, desensitizing cells to subsequent stimulations [39].

Other endogenous ligands with physiological relevance because of their involvement in pathological conditions are the acute-phase protein serum amyloid A (SAA) [40,41], and $A\beta_{42}$ [14,42] and PrP106–126 (a prion protein fragment) [43], found in prion disease, all of which are required in high micromolar concentrations to activate FPR2/ALX. Serum SAA concentrations increase during acute-phase responses, causing tissue and organ amyloidosis in chronic inflammation [44,45] by inducing chemotaxis of phagocytic leukocytes through FPR2/ALX [40,41]. It is thought that pathophysiological concentrations of SAA activate FPR2/ALX and could therefore be used as biomarker for inflammatory disease [46,47].

$A\beta_{42}$ is an enzymatic cleavage fragment of the amyloid precursor protein (APP). Its aggregated form is the major component of senile plaques, found in brain tissue of patients with Alzheimer's disease (AD). $A\beta_{42}$ has been implicated in neurodegeneration and pro-inflammatory responses observed in AD; it activates FPR2/ALX, suggesting that FPR2/ALX might represent a potential therapeutic target for AD treatment [14,42]. PrP106–126 stimulation of FPR2ALX on human monocytes caused internalization of the receptor and a release of pro-inflammatory mediators [43].

Other potential ligands, including HIV-envelope proteins gp41 (T20/DP178, T21/DP107 and N36) and gp120 (F and V3 peptides), interact with FPR1 and/or FPR2/ALX. T20/DP178 interacts with FPR1 *in vitro* [47] and mouse Fpr1 *in vivo* [48]; T21/DP107 uses both FPR1 and FPR2/ALX (higher efficacy FPR2/ALX) [47] and N36 (overlaps with T21/DP107) signals through FPR2/ALX [49]. In terms of the gp120-derived peptides, both F peptide (20 amino acids) and V3 peptide (33 amino acids) interact with FPR2/ALX [50]. The *Helicobacter pylori* peptide Hp(2–20) also acts via FPR2/ALX, inducing NADPH oxidase activation in human neutrophils [51].

It is clear from the above that a large amount of research (still ongoing) has been performed to design a successful ligand for the FPR family. A key aspect for this process relies on target specificity, namely a ligand with high affinity for FPR2/ALX and almost no affinity for FPR1. Ligand target specificity can also reduce the risk of harmful side effects produced by, for example, non-specific binding.

Antagonists

There are various antagonists, such as those formed by replacing the *N*-formyl group of fMLP with *t*-butyloxycar-bonyl (tBOC) or isopropyl ureido, yielding peptides that block fMLP interaction with its receptor [14,52]. Several studies have used these Boc derivatives (that are non-selective antagonists to all FPR family members) to demonstrate FPR family involvement in diseases such as myocardial infarction and stroke [53,54].

More selective antagonists are available, such as the chemotaxis inhibitory protein (C5aR) of *Staphylococcus aureus* (CHIPS), which is present in over 50% of clinical strains of *S. aureus* and antagonises FPR2/ALX and the complement component 5a receptor 1. More specifically, a peptide fragment of CHIPS (FTFEPFPTNEEIESN) is selective for FPR2 and not C5aR [55]. Another protein of *S. aureus* that exhibits selective binding of and activation by MMK-1 is FPRL1 inhibitory protein (FLIPr), which binds directly to FPR1 and FPR2, but not FPR3 [56].

The bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) antagonise both FPR1 and FPR2/ALX [57], possibly suppressing anti-bacterial responses in individuals with cholestasis [58]. DCA inhibits fMLP-induced monocyte and neutrophil migration and calcium mobilization though fMLP binding to its receptors, whereas CDCA selectively inhibits monocyte chemotaxis and calcium flux induced by fMLP and W peptide, suggesting a mechanism for inhibition of inflammation and suppression of innate immune response [57,58]. Quin-c7, a synthetic non-peptide antagonist of FPR2/ALX, has been developed through chemical modification of Quin-C7 (an FPR2/ALX agonist) [59].

Although different ligands continue to be identified for the FPRs, there is a clear need to develop more potent and specific agonists and antagonists to offer a greater therapeutic potential devoid of major side effects.

Involvement of FPRs in I/R

Our understanding of the complexity and need for a balance between pro- and anti-inflammatory pathways to retain a homeostatic environment [60–63] has given rise to a great number of possible drug targets of the FPR family. In particular, targets activated by endogenous anti-inflammatory mediators could be used for drug discovery for both I/R and other disease states because they are likely to have fewer side effects and their application would be similar to mechanisms that the body uses to reject inflammation [60]. One particular endogenous ligand of interest is the 37-kDa protein AnxA1, whose involvement with the FPR family has been widely studied and which affords protection in a number of I/R models. Initially, cardioprotection by AnxA1 was observed in rat myocardial I/R injury [64]. It was not until 2001, however, that La *et al.* [53] investigated the mechanistic and molecular basis of AnxA1 action and demonstrated that the pharmacophore responsible for cardioprotection lies within amino acids 2–12, with correct alignment being crucial [53]. A clinical application was hypothesised on the basis of cardioprotection being retained by the AnxA1 N-terminal peptide Ac2–26 (also termed $AnxA1_{Ac2-26}$) when administered 60 min into reperfusion [53]. Boc2 administration demonstrated for the first time that FPRs are involved in the cardioprotective properties of AnxA1. Histology demonstrated that neutrophil influx into the

Table 3. Examples of agonists used in different I/R models[a]

Species	I/R model	Agonist	Protective	Identified as ligand for which FPR family member?	Ref.
hBLTR TG mice	Hind-limb I/R-induced second-organ lung injury (3-h I+3-h R)	ATL analogue	Yes	ALX/FPR2	[67]
		LXA4		ALX/FPR2	[67]
Male FVB mice	Hind-limb I/R-induced second-organ lung injury (1-h I+1-h R)	ATL analogue ZK-994	Yes	ALX/FPR2	[69]
		ATL analogue ZK-142	Yes	ALX/FPR2	[69]
		ATLa	No	ALX/FPR2	[69]
NIH Swiss mice	Ischaemic acute renal failure (30-min I+24-h R)	15-Epi-16-(p-fluorophenoxy)-lipoxin A_4-methyl ester	Yes	ALX/FPR2	[68]
Male Sprague-Dawley rats	Myocardial I/R (25-min I + 2-h R)	Human recombinant annexin 1	Yes	FPR1	[60]
		Annexin 1 peptide Ac2–26	Yes	FPR1	[60]
		Annexin 1 peptide Ac2–12	Yes	FPR1	[60]
		Annexin 1 peptide scrambled Ac2–12	No		[60]
		Annexin 1 peptide Ac2–6	No		[60]
		fMLP	Yes	FPR1	[60]
Male Sprague-Dawley rats	Myocardial I/R (25-min I+2-h R)	Annexin 1/5	No		[7]
		Denatured preparation of annexin 1	No		[7]
		Human recombinant annexin 1	Yes	Unknown receptor	[7]
Male C57bl/6 mice	Myocardial I/R (25-min I+1-h R)	Annexin 1 peptide Ac2–26	Yes	FPR3	[65]
		W peptide	Yes	ALX/FPR2, Fpr3	[65]
		ATLa-ME	Yes	ALX/FPR2, Fpr3	[65]
Male Fpr1-null mice	Myocardial I/R (25-min I+1-h R)	Peptide Ac2–26	Yes	Excluded involvement of Fpr1	[65]
		W peptide	Yes	Excluded involvement of Fpr1	[65]
		ATLa-ME	Yes	ALX/FPR2, Fpr3	[65]
Male albino mice	Myocardial I/R (25-min I+2-h R)	CGEN-855A	Yes	ALX/FPR2	[33]
Male Sprague-Dawley rats	Myocardial I/R (30-min I+3-h R)	CGEN-855A	Yes	ALX/FPR2	[33]
Male C57bl/6 mice	Mesenteric I/R (30-min I+45-min R)	Annexin 1 peptide Ac2–26	Yes	Fpr1 and Fpr3	[36]
Male Fpr1-null mice		Annexin 1 peptide Ac2–26	Yes, 50%	Fpr1 and Fpr3	[36]
		ATLa	Yes	Fpr1 and Fpr3	[36]
		fMLP	No		[36]
Male Sprague-Dawley rats	Splanchnic artery I/R	Annexin 1 peptide Ac2–26	Yes	Fpr1	[104]
Male Fpr2-null mice	Mesenteric I/R (30-min I+45-min R)	None	Yes	Fpr2	[71]

[a]ATL, aspirin-triggered lipoxin; ATLa, 15-*epi*-16-(*p*-fluoro)-phenoxy lipoxin A_4; ATLa-ME, ATLa methyl ester; hBLTR, human leukotriene B_4 receptor; ATL analogue, 15-*epi*-16-(*p*-fluoro)-phenoxy-LXA$_4$ [15(*S*)-16-(*p*-fluoro)-phenoxy-LXA$_4$ methyl ester]; peptide Ac2–6, acetyl-AMVSE; peptide Ac2–12, acetyl-AMVSEFLKQAW; peptide scrambled Ac2–12, acetyl-SVEQKMWALFA; peptide Ac2–26, acetyl-AMVSEFLKQAWIENEEQEYVVQTVK; W peptide, WKYMVm (Trp-Lys-Tyr-Met-Val-D-Met).

myocardium induces AnxA1 expression, which was absent in sham and naïve animals.

Although rat models provided novel results, murine models are particularly useful given our current knowledge of the genome and the ease of genetic manipulation (Table 3). The *Fpr1*-null mouse provided evidence that FPR1 does not mediate the cardioprotection afforded by peptide AnxA1$_{Ac2-26}$ [65]. Neutrophil depletion studies did not affect the extent of acute heart injury, but affected protection afforded by peptide AnxA1$_{Ac2-26}$. This study provided evidence of the role of mouse Fpr2 and/or Fpr3 and circulating neutrophils in mediating AnxA1-induced cardioprotection.

Since reports providing theoretical frameworks for considering ALX/FPR2 as a putative target for I/R injury were published [65–68], a number of novel ligands have been identified, such as the peptide and ALX/FPR2 agonist CGEN-855A, which has cardioprotective effects in rat and murine myocardial I/R, reducing both infarct size (by 25% and 36%, respectively) and murine troponin I levels [36]. Thus, CGEN-855A elicits cardioprotection similar to the effect reported for other ALX/FPR2 agonists such as AnxA1 [65]. This study provides further evidence of a beneficial effect of the ALX/FPR2 pathway in inflammation [36].

Other agonists of ALX/FPR2 exhibit protective effects in I/R models, such as15-*epi*-16-(*p*-fluorophenoxy)-LXA$_4$-methyl ester [15-*epi*-16-(FPhO)-LXA$_4$-Me], which is protective in renal I/R in part by modulating cytokine and chemokine expression and neutrophil recruitment [66]. The first evidence of ALX/FPR2 activation by aspirin-triggered lipoxins was provided by Chiang *et al.*, who demonstrated protection from second-organ reperfusion injury [67].

The novel 3-oxa-aspirin-triggered 15-*epi*-lipoxin analogues ZK-994 and ZK-142 have exhibited inhibition of neutrophil accumulation in murine hind-limb I/R-induced second-organ lung injury [69].

Intravital microscopy (IVM) has been used in I/R studies to assess the effect of FPR ligands (Figure 1). IVM is a real-time qualitative and quantitative method for observing cell–cell interactions in the microcirculation of an anaesthetized animal [70]. Our own I/R studies in the mesentery (30 min I+45 min R, followed by mesenteric visualization using IVM) of C57BL/6 and *Fpr1*-null mice demonstrated that more than one FPR family member mediates the effects of peptide AnxA1$_{Ac2-26}$, possibly including mouse Fpr2. These effects are similar to those observed in LPS-induced damage of the mouse mesentery (Hughes *et al.*, unpublished data).

The availability of genetically modified mice has facilitated characterisation and understanding of the FPR family, but differences between species remains an issue. However, very recent studies using *Fpr2*-null mice and IVM have revealed a marked increase in inflammation, as demonstrated by an increase in cell adhesion and emigra-tion following mesenteric I/R [71]. These *Fpr2*-null mice also had an augmented response to carrageenan-induced paw edema in comparison to their wild-type counterparts [71]. These studies, along with studies in my own laboratory looking at the effects of stroke in *Fpr2*-null mice (Gavins, unpublished data), further demonstrate that FPR2/ALX is an anti-inflammatory receptor and serves a function in the host defence response.

It is clear that I/R elicits an acute inflammatory response that is characterised by inflammatory cell recruitment, oxidative stress and the failure of the endothelial barrier, and contributes to the pathogenesis of many diseases. One area in which targeting of inflammatory markers has failed to yield successful therapeutic drug candidates is stroke. Evidence of FPRs and their agonists as possible novel therapeutic targets for the treatment of cerebral I/R injury is now discussed.

FPRs in cerebral I/R

When cerebral blood flow is restored following ischaemia, several cascades are initiated, leading to activation of
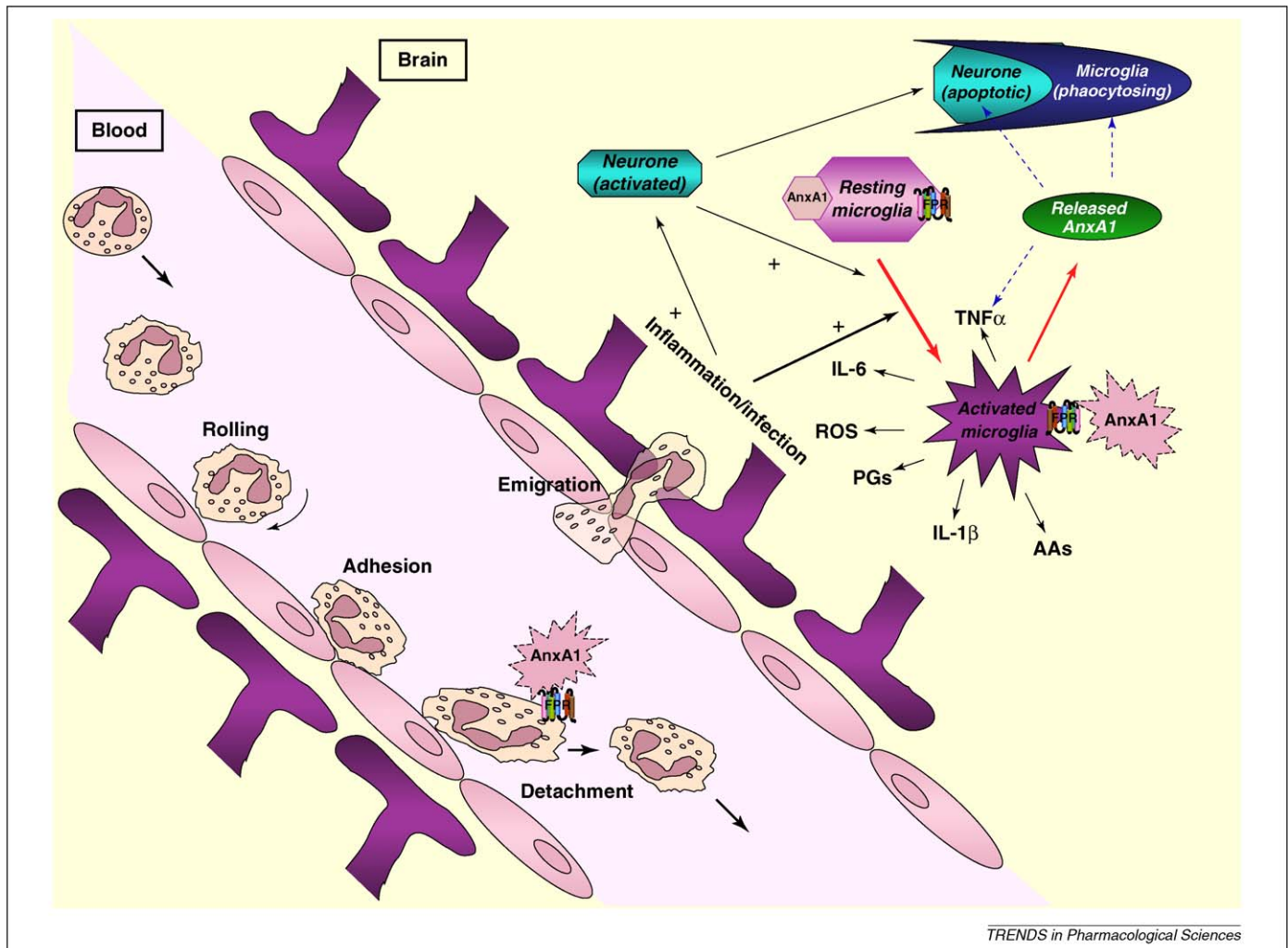

Figure 2. Schematic of how the FPR agonist AnxA1 could reduce inflammation in the brain following I/R injury and exert neuroprotection. Neutrophils are activated after I/R injury. They roll, adhere and migrate into the tissue. AnxA1 is released from neutrophil cytosolic granules to the cell surface, where it interacts with FPRs in an autocrine or paracrine fashion. Administration of AnxA1 (or the N-terminal peptide Ac2–26) causes the leukocyte detachment from endothelial cells. Resident cells, such as microglia, are also activated by I/R injury and, like blood-borne cells, release a plethora of damaging mediators such as reactive oxygen species, cytokines (TNF-α, IL-1β) and leukotrienes. The involvement of AnxA1 in these processes might be similar to that observed in the peripheral microvasculature, namely to promote resolution. The receptors that mediate this process in stroke remain unknown, although evidence suggests that FPR/ALX plays a role.

blood-borne and resident cells (microglia, astrocytes and neurons) [72]. The actual contribution of resident cells versus blood-borne cells has yet to be fully quantified and might explain why clinical trials targeting one specific aspect of the inflammatory cascade have failed. A neuroprotective role for AnxA1, mediated via FPRs, has been demonstrated in studies using a middle cerebral artery occlusion model combined with IVM to quantify cerebral cellular interactions following stroke [54]. Peptide AnxA1$_{Ac2-26}$ decreased infarct volume, leukocyte adhesion and markers of inflammation. Microglia might, in part, contribute to these inflammatory responses [54]. Both blood cells and microglia express mouse Fpr2, which might have mediated the effects of peptide AnxA1$_{Ac2-26}$ in this study. Further studies are being performed to validate this theory, with current data suggesting that microglial cell inhibition reduces brain inflammation post-I/R (Gavins and Perretti, unpublished data). Figure 2 demonstrates how AnxA1 could act in an autocrine and/or paracrine fashion with the members of the FPR family to reduce inflammation by (i) causing detachment of leukocytes within the microvasculature and (ii) inhibiting the production of cytotoxic proinflammatory mediators by activated microglial cells, both of which are thought to play a role in cerebrovascular events such as stroke [54].

Whether the mechanisms that mediate inflammatory responses in the brain are the same as those observed in the periphery remains to be determined. It is likely that similar endogenous pathways are available throughout the host, the mechanism(s) of action of which is to inhibit molecular and cellular responses to (I/R) injury, such as for CGEN-855A and AnxA1 peptides. These bioactive peptides and associated FPRs could represent potential therapeutic targets [33].

The neuroprotective peptide humanin is an agonist of FPR2/ALX and an endogenous ligand of FPR3 [73]. Another endogenous ligand of FPR3 is a peptide isolated from the human haem-binding protein termed F2L (Ac MLGMIKNSLFFGSVETWPWQVLNH2). F2L has weak activity for FPR2/ALX and is not active for FPR1 [74]. However, it is an agonist for FPR3, binding and activating the receptor at concentrations in the low nanomolar range [75].

Concluding remarks

This review has drawn together compelling evidence to suggest that ligands to the FPRs could provide novel anti-inflammatory and pro-resolving therapeutics that reduce the damage associated with I/R injury. The promiscuity of these receptors in binding different ligands, coupled with their presence in different cells and tissue, indicates a diverse role in multiple biological settings. Improved understanding of these fundamental functions would instruct therapeutic target identification and drug development. Thus, further study of endogenous antiinflammatory agonists, such as AnxA1, and the lipoxins could lead to the identification and development of better therapeutics capable of limiting the detrimental effects of I/R injury and promoting resolution, with fewer side effects.

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