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Review

Analysis of the protection afforded by annexin 1 in ischaemia–reperfusion injury: focus on neutrophil recruitment

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Abstract

Ischaemia–reperfusion injury underlies many of the most important cardiovascular diseases such as myocardial infarction, thrombotic stroke, embolic vascular occlusions and peripheral vascular insufficiency. Neutrophils feature prominently in this inflammatory component of post-ischaemic injury. Experimental therapies, shown to reduce neutrophil-mediated ischaemia–reperfusion injury include neutrophil depletion, direct inhibitors of neutrophil activators, antibodies against neutrophil adhesion molecules and the endothelial adhesion molecules. However, aside from these approaches, it is increasingly recognised that glucocorticoids are potent inhibitors of neutrophil-mediated injury. The anti-inflammatory actions of glucocorticoid include the activation of classical cytoplasmic receptors leading to changes in gene transcription as well as the induction of regulatory proteins, such as annexin 1. Annexin 1 is a potent inhibitor of neutrophil extravasation in vivo. Administration of the annexin 1 or peptides derived from its N-terminal domain, reduce neutrophil extravasation in models of acute inflammation. In addition, as reviewed by this article, annexin 1 protects against ischaemia–reperfusion in the heart and mesenteric microcirculation, as well as in multiple organ failure associated with splanchnic ischaemia–reperfusion. Such findings would suggest annexin 1 is a novel anti-inflammatory agent with a potential for the treatment of cardiovascular pathologies associated with neutrophil activation and recruitment.

Keywords: Annexin 1; Myocardial infarct; fMLP (*N*-formyl-Met-Leu-Phe); Mesentery; Intravital microscopy

1. Introduction

Cells of all tissues undergo irreversible injury and death when deprived of oxygen and other nutrients (ischaemia). The restoration of blood flow (reperfusion) to an ischaemic organ or tissue while having the ultimate aim of reducing cell death, comes at a price and introduces a separate series of stresses that exacerbate injury. This phenomenon has been referred to as "reperfusion injury" and is characterised by increased microvascular permeability, oedema and tissue necrosis. It is associated with free radical release, and most importantly, the infiltration of polymorphonuclear leukocyte (also referred to as neutrophil) (Grace, 1994; Granger, 1988). Activated neutrophils contribute to tissue damage through several mechanisms: (i) release of free radicals (O_2^-; OH^-) following respiratory burst from the NADPH oxidase; (ii) release of proteolytic enzymes (elastase, cathepsin G and proteinase), (iii) stimulation of cytokine release from local cells thus promoting further neutrophil recruitment and finally (iv) plugging of capillaries by neutrophil contributing to the no-flow phenomenon (for recent reviews, see Ambrosio and Tritto, 1999; Jordan et al., 1999; Vermeiren et al., 2000). It is clear then, that the neutrophil-mediated inflammatory cascade during reperfusion represents an important target for therapeutic intervention. The major objectives for this review are to briefly examine the (1) role of neutrophils in the inflammatory-component of ischaemia–reperfusion injury and (2) potential therapeutic modulation focussing on the protective role afforded by the glucocorticoid-regulated protein annexin 1.

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2. Biochemical and pathophysiological features of ischaemia

In tissues deprived of oxygen (ischaemia), the subsequent failure of the electron transport system leads to the depletion of adenosine triphosphate (ATP) (Jennings et al., 1983). Anaerobic metabolism will take over leading to a local increase in lactic acid production. The resulting acidosis disrupts normal homeostasis: there is a loss of Ca^{2+} and sodium ion gradients with subsequent cellular leakage of essential enzymes (i.e., creatinine kinases) and proteins (Chaudry et al., 1981). In addition, adenosine monophosphate (AMP) will be converted into adenosine, xanthine and hypoxanthine (DeWall et al., 1971; Van Bilsen et al., 1989), substrates for the enzyme xanthine oxidase. This enzyme normally functions as a xanthine hydrogenase but operates in the reverse direction during ischaemia and as such has been proposed to play a major

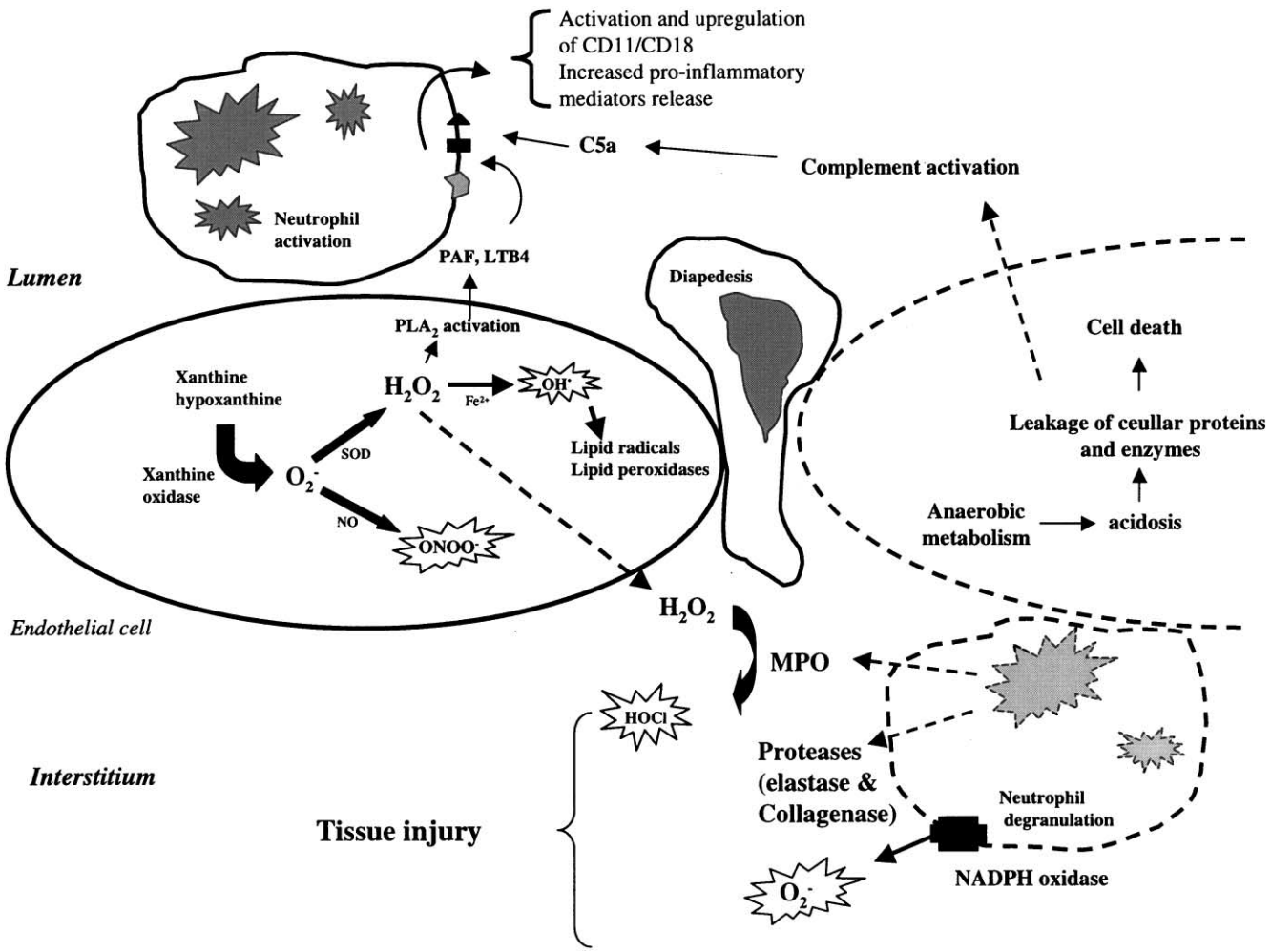

Fig. 1. Schematic representation of free radical production and tissue damage in ischaemia–reperfusion injury. Ischaemia–reperfusion is associated with the release of superoxide (O_2^-) generated from neutrophil NADPH oxidase and the xanthine oxidase system (see Introduction). In the presence of superoxide dismutase (SOD), O_2^- is converted to hydrogen peroxide (H_2O_2) while the highly reactive hydroxyl radical ($OH^{\cdot}$) is formed via the iron catalysed Harber–Weiss (Fenton) reaction. In addition, O_2^- can interact with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), an oxidant that readily nitrates tyrosine residues on cellular proteins (Beckman and Koppenol, 1996). The role of $ONOO^-$ in ischaemia–reperfusion injury is currently ambiguous. Lefer et al. (1997) demonstrated that $ONOO^-$ at nanomolar concentrations inhibits leukocyte–endothelial cell interactions and protects against ischaemia–reperfusion injury in rats. However, $ONOO^-$ has been shown to induce nitration and inactivation of myofibrillar creatine kinase in experimental heart failure (Mihm et al., 2001). Furthermore, increased $ONOO^-$ catalysis has been shown to protect against splanchnic ischaemia–reperfusion injury and reduced paw oedema in a model of acute inflammation (Cuzzocrea et al., 2000; Salvemini et al., 1998). At the same time, the loss of ATP leads to anaerobic metabolism with consequent acidosis and loss of cellular homeostasis. Subsequently, there is a loss of ion gradients across cell membranes causing to Ca^{2+} overloading and sodium movement into cells, hence oedema followed by leakage of enzyme and proteins and finally cell death. The leakage of enzyme and proteins, particularly those of mitochrondrial origin, activate the complement system. In addition, free radical production has been proposed to stimulate the production of platelet activating factor and leukotriene B_4. All together, these chemotactic factors activate neutrophil. Following diapedesis, neutrophil degranulates and releases catalytic and proteolytic enzymes such as myeloperoxidase (MPO) and elastase. Myeloperoxidase catalyses the formation of the highly toxic hypochlorous acid (HOCl), a major determinant of neutrophil oxidative injury. Elastase can degrade key components of the extracellular matrix therefore altering the barrier properties of endothelial cells as well as causing cell lyses.

role in oxygen-free radicals production in ischaemia–reperfusion injury (Chambers et al., 1985; McCord, 1985). The overall consequences of ischaemia–reperfusion injury are Ca^{2+}-overloading, oedema, injury induced by oxygen-free radicals and subsequent cell death (Reimer et al., 1983). Above a certain threshold of cellular leakage, this damage will become irreversible. If reperfusion occurs within certain critical time limits, the cell will be able to recover. Different tissues can withstand ischaemia for different period of time, neuronal cell death occurs within a few minutes (Chervu et al., 1989), irreversible damage to the heart occurs after 20 min (Jennings and Reimer, 1983) whereas skeletal muscle can recover after hours of ischaemia (Chervu et al., 1989). Fig. 1 summaries several pathways that lead to production of oxidant species and release of proteolytic enzymes with subsequent tissue injury. Several reviews have described these aspects in more detail (Carden and Granger, 2000; Granger, 1999). However, another important component of ischaemia–reperfusion injury is the infiltrating neutrophil.

3. The role of neutrophils in ischaemia–reperfusion injury

The damaging role of neutrophil in ischaemia–reperfusion injury was first suggested by the pioneering depletion studies of Romson et al. (1983) who showed that the administration of polyclonal antibodies against neutrophils reduced the size of myocardial infarct in dogs. Similar results were achieved when neutrophils were depleted by anti-neutrophil serum or neutrophil filters (Litt et al., 1989). Subsequently, anti-neutrophil serum or neutrophil filters were also found to significantly reduce the extent of ischaemia–reperfusion injury in skeletal muscle (Korthuis et al., 1988).

The recruitment of neutrophils to a site of inflammation is dependent on the local generation and release of chemotactic factors. The 'classical' chemoattractants include the bacterial product *N*-formyl-Met-Leu-Phe (fMLP), complement products C5a and C5b-9, lipid mediators like platelet activating factor (PAF) and leukotriene B_4 as well as chemokines of the interleukin family. As an example, we will now briefly describe some of these chemoattractants, keeping in mind that this is by no means an exhaustive list.

3.1. Complement factors

The complement system consists of a group of proteins that circulate in the plasma in an inactive form, and its activation leads to the generation of several pro-inflammatory products. Of particular interest are the anaphylatoxins C3a and C5a, as well as the membrane attack complex C5b-9 (Chakraborti et al., 2000; Makrides, 1998; Morgan, 1999). C5b-9 can mediate tissue injury directly by forming pores in lipid bilayer membranes resulting in loss of electrolytes and subsequently cell lysis (Morgan, 1999). In contrast, C5a is a potent neutrophil attractant and mediates many features seen in the acute inflammatory response, including smooth muscle contraction, changes in vascular permeability, histamine release from mast cells, platelet activation and aggregation (Chakraborti et al., 2000; Makrides, 1998; Morgan, 1999), as well as up-regulation of adhesion molecules such as P-selectin and intercellular adhesion molecule 1 (ICAM-1) that play key roles in neutrophil recruitment (Buerke et al., 1998; Foreman et al., 1996). The role of complement in ischaemia–reperfusion injury has been studied in a number of organs including the heart (Hill and Ward, 1971; Ivey et al., 1995; Kilgore et al., 1994a), lung (Eppinger et al., 1997) and skeletal muscle (Rubin et al., 1989; Weiser et al., 1996). An early study (Hill and Ward, 1971) demonstrated that depletion of the complement system with cobra venom factor reduced myocardial ischaemia–reperfusion injury. This finding was confirmed by latter studies showing that the administration of a soluble form of the complement receptor 1 reduced myocardial infarct size by 44% as well as decreasing the accumulation of neutrophils within the infarcted area (Weisman et al., 1990). More recently, the administration of the C5a receptor antagonist was found to decrease local and remote tissue injury in a murine model of intestinal ischaemia–reperfusion (Heller et al., 1999). Interestingly, different complement components appear to be involved in the pathogenesis of ischaemia–reperfusion injury in different organs. The final products of the complements pathway C5a and C5b-9 have been proposed to be the key mediator of ischaemia–reperfusion injury in the myocardium (Vakeva et al., 1998) whereas C5b-9 has been suggested to be the primary mediator of renal ischaemia–reperfusion injury (Zhou et al., 2000).

At present, it is unclear how ischaemic injury leads to the activation of the complement system, but it has been proposed that molecules of mitochondrial origin (released during the ischaemic period) may provide the activating stimuli (Kagiyama et al., 1989). This finding is supported by the demonstration that activation of human complement occurred in vitro when mitochondrial membranes isolated from normal human heart tissue were incubated with normal human serum (Giclas et al., 1979). In addition, it was demonstrated that free radicals such as hydrogen peroxide and hydroxyl radical ($OH^{\cdot}$) could directly activate C5 (Vogt and Hesse, 1992; von Zabern et al., 1987). This theory was further supported by the observation that inhibition of free radical formation decreased complement activation (Collard et al., 1998; Kilgore et al., 1994b).

3.2. Leukotriene B_4

Leukotrienes are the products of the arachidonic acid pathway (for extensive review, see Samuelsson, 2000).

Arachidonic acid is released from phosphatidylcholine by phospholipase A_2. Therefore, the availability of arachidonic acid and phospholipase A_2 is essential for the synthesis of leukotriene B_4. It has been suggested that leukotriene B_4 formation is dependent on the actions of oxygen-free radicals on plasma membrane (Goldman et al., 1992a). Oxygen-free radicals produced at the beginning of reperfusion, attack membrane lipids leading to lipid peroxidation (Rao and Mueller, 1983) resulting in the activation and release of phospholipase A_2 and also the release of arachidonic acid substrate from the membrane (Sevanian and Hochstein, 1985). Leukotriene B_4 has long been known to be a potent chemoattractant for neutrophils and eosinophils (Ford-Hutchinson et al., 1980) and has been implicated in feline splanchnic ischaemia–reperfusion injury (Zimmerman et al., 1990), rodent models of renal (Klausner et al., 1989; Noiri et al., 2000) and hepatic (Hughes et al., 1992) ischaemia–reperfusion injury, as well as ischaemia–reperfusion induced secondary organ injury in mice (Goldman et al., 1992b). These findings are further supported by the observation that inhibitors of arachidonic acid metabolism reduce the extent of canine myocardial infarction (Hoshida et al., 1989) and that leukotriene B_4 antagonists protect against splanchnic (Karasawa et al., 1991), renal (Noiri et al., 2000) and mesenteric (Souza et al., 2000) ischaemia–reperfusion injury in rats. Finally, transgenic mice over-expressing leukotriene B_4 receptors were found to have increased neutrophil trafficking into skin microabscesses and lungs as well as reperfusion-initiated secondary organ injury when compared to non-transgenic animals (Chiang et al., 1999).

3.3. Platelet activating factor (PAF)

PAF is produced by the action of phospholipase A_2 on 1-*O*-alkyl-2-arachidonoyl glycerol-phosphochloline yielding lyso-PAF and free arachidonic acid. Acetylation of the inactive lyso-PAF converts it to PAF, a more stable form (for a recent review, see Montrucchio et al., 2000). Similar to leukotriene B_4, oxygen-free radical generation has been implicated in the induction of PAF synthesis (Hotter et al., 1997). PAF is produced and released by a variety of cells that participate in the development of inflammatory reaction including monocytes/macrophages, neutrophils, eosinophils, basophils, and platelets (Montrucchio et al., 2000). In addition, human endothelial cells were found to express PAF after stimulation by inflammatory mediators including thrombin, angiotensin II, vasopressin, histamine, bradykinin, elastase, cathepsin G, hydrogen peroxide, plasmin, interleukin-8 and interleukin-1, or tumour necrosis factor (TNF)-α (Montrucchio et al., 2000). Cardiomyocytes have also been reported to synthesize PAF under appropriate stimulation (Janero and Burghardt, 1990). In addition, PAF amplifies recruitment signals by promoting the release of further pro-inflammatory mediators from neutrophils such as lipoxygenase products (O'Flaherty et al., 1981) as well as oxygen-free radicals from neutrophils and macrophages (Hartung et al., 1983). In vitro, PAF promotes the aggregation, chemotaxis, granule secretion, and oxygen radical generation from neutrophils and the adherence of neutrophils to the endothelium (Montrucchio et al., 2000). In addition, PAF is known to increase endothelial cell permeability (Bussolino et al., 1987; Evans et al., 1987; Kubes et al., 1990a). Superfusion of the rat mesenteric microcirculation with PAF promotes neutrophil adhesion to post-capillary venules (Zimmerman et al., 1994), whereas PAF receptor antagonists inhibited neutrophil adhesion and migration elicited by ischaemia–reperfusion in the cat mesenteric venule post capillary venules (Kubes et al., 1990b). As for a role for PAF in myocardial ischaemia–reperfusion injury, PAF was detected in the blood of patients with coronary artery disease undergoing atrial pacing to evaluate the severity of ischaemia (Montrucchio et al., 1986). This lipid mediator is also generated in the coronary sinus after occlusion and reperfusion injury in sheep (Ko et al., 1991). In line with these data, PAF receptor antagonists have reported to exert protection in experimental models of ischaemia–reperfusion injury including myocardial (Ioculano et al., 1994) and splanchnic artery occlusion (Canale et al., 1994).

3.4. Interleukins

The interleukins are cytokines with important function in host defence against infection, injury of the acute or chronic inflammation. Several interleukins are potent neutrophil chemoattractants (interleukin-1, interleukin-8 and TNF α) whereas interleukin-10 has been shown to have anti-inflammatory properties (Dinarello, 2000). Interleukin-1 exists in two isoforms, interleukin-1α and interleukin-1β, but the latter is the secreted specy and therefore it is more involved in paracrine and autocrine functions (for review, see Dinarello, 1997). Interleukin-1 is not constitutively expressed but can be synthesised by most cells following activation as result of de novo gene transcription (Dinarello, 1996). Examples of interleukin-1 producing cells are neutrophils, monocytes, macrophages, endothelial cells, T and B-lymphocytes and natural killer cells (Dinarello, 1994). Evidence from experimental models of acute ischaemia indicates that ischaemia–reperfusion can increase the production of interleukin-1β (Ascer et al., 1992; Suzuki and Toledo-Pereyra, 1994). Importantly, interleukin-1β is increased in the serum of patients with coronary heart disease (Hasdai et al., 1996; Marx et al., 1997) and those undergoing right hepatectomy (Clavien et al., 1996). The administration of interleukin-1 receptor antagonists has been found to reduce cerebral (Loddick and Rothwell, 1996; Relton and Rothwell, 1992) and hepatic (Shito et al., 1997) ischaemia–reperfusion injury in rat. Furthermore, interleukin-1 receptor knockout mice have reduced injury associated with neutrophil recruitment fol-

lowing renal ischaemia–reperfusion (Haq et al., 1998). It has been speculated that interleukin-1β formation is induced by ischaemia but the precise mechanism is currently unknown.

Unlike many other cytokines, interleukin-8 has only weak effects on other blood cells but has been found to be the most potent cytokine chemoattractant for neutrophil (Bickel, 1993). Interleukin-8 is produced by a variety of tissue and blood cells. During ischaemia, it is produced by both cardiac myocytes (Kukielka et al., 1995) and endothelial cells (Karakurum et al., 1994). Finally, elevated serum levels of interleukin-8 have been detected in humans with acute myocardial infarction (Abe et al., 1993) and anti-interleukin-8 antibodies reduced myocardial ischaemia–reperfusion injury in the rabbit (Boyle et al., 1998).

4. Adhesion molecules and neutrophil migration

To reach the site of inflammation, circulating neutrophils must first interact with the endothelial cells within the microcirculation. The interaction between the neutrophils and the endothelium represents an early and rate limiting step in neutrophil infiltration and involves three main families of cell adhesion molecules; the selectins, the integrins and the immunoglobulins (Lefer, 1995).

4.1. The selectins

The selectins are involved in the initial weak interaction between neutrophil and the endothelium and are designated as L-, P- and E-selectins. L-selectin (CD62L) is found on neutrophils and it is involved in the initial 'tethering' of neutrophils on the vascular endothelium (Zimmerman et al., 1992). Activation of neutrophils with pro-inflammatory mediators such as interleukin-8 and PAF rapidly down-regulate L-selectin from the plasma membrane following proteolytic cleavage (shedding phenomenon; Kishimoto et al., 1989, 1991). The functional relevance of L-selectin was demonstrated by immuno-neutralisation studies, which reduced the number of spontaneously rolling neutrophils in rabbit mesenteric venules (Fiebig et al., 1991; Von Andrian et al., 1992). More recently, L-selectin knockout mice demonstrated a significant defect in neutrophil recruitment to inflammatory site (Tedder et al., 1995). A role for L-selectin in the late stages of the neutrophil extravasation process has also been proposed (Hickey et al., 2000).

P-selectin (CD62P) is rapidly expressed on the surface of platelet and endothelium, minutes after activation by inflammatory mediators such as histamine and thrombin (Lorant et al., 1991). Functionally, the administration of mAb against P-selectin reduced injury in a feline model of myocardial ischaemia–reperfusion (Weyrich et al., 1993). More recently, it was demonstrated that platelet and neutrophils act synergistically to increase cardiac dysfunction in a canine model of ischaemia–reperfusion injury (Lefer et al., 1998). Finally, E-selectin (CD62E) is not constitutively expressed on endothelial cells, but is up-regulated by pro-inflammatory mediators such as interleukin-1 (Weller et al., 1992), TNF-α (Bevilacqua et al., 1987) or LPS (Fries et al., 1993).

4.2. The integrins

Neutrophil rolling can lead to 'firm adhesion'. This is defined as occurring when the neutrophil remains stationary on the endothelium > 30 s (Granger et al., 1993). 'Firm adhesion' is the result of strong interaction between integrin (namely β_2 integrin) expressed on the surface of the activated neutrophil and their ligand, e.g. ICAM-1. Integrins are heterodimeric proteins consisting of a variable α subunit non-covalently bonded to a common β subunit. At least 15 α and 8 β have been discovered and characterised. Neutrophils can express 13 different integrins, 5 of which belong to the β_1, β_2 β_7 subfamilies with β_2 integrin being the most important in neutrophil-dependent inflammation. The β_2 integrin sub-family consists of a common β unit (CD18) linked to one of four distinct α subunit designated as CD11a, CD11b, CD11c or CD11d. Activation of neutrophils in vitro with pro-inflammatory mediators such as fMLP, PAF (Borregaard et al., 1994; Tonnesen et al., 1989), or interleukin-8 (Carveth et al., 1989; Detmers et al., 1990) causes rapid mobilisation of the heterodimer to the surface (Jones et al., 1988).

4.3. The immunoglobulin super-family

Five members of this family have been implicated in neutrophil and endothelial interaction: intracellular adhesion molecule (ICAM)-1, ICAM-2, vascular adhesion molecule (VCAM)-1, platelet-endothelial cell adhesion molecule (PECAM-1) and the mucosal addressin cell adhesion molecule (MAdCAM-1) (Van de Stolpe and Van der Saag, 1996). In vivo, up-regulation of ICAM-1 is induced by several inflammatory mediators including interleukin-1β, TNF-α and LPS (Luscinskas et al., 1991). Antibodies against ICAM-1 have been shown to attenuate the extent of cell adhesion to the post-capillary venule endothelium (Argenbright et al., 1991), stimulated by PAF (Zimmerman et al., 1994), leukotriene B_4 (Kubes et al., 1990a,b) or interleukin-1β (Tailor et al., 1999). More recently, ICAM-1 knockout mice have been generated and shown to have reduced neutrophils infiltration following myocardial ischaemia–reperfusion (Metzler et al., 2001).

5. Mechanism of neutrophil-mediated injury

Neutrophil and endothelium activators arrest free-flowing white blood cells and to promote their migration into

the site of injury. Once adherent on the endothelium or emigrated in the surrounding tissue, polymorphonuclear cells (mainly neutrophils) can provoke many pro-inflammatory events as listed in Section 1. We will now briefly discuss some effectors of neutrophil-mediated tissue damage.

5.1. Oxygen-free radicals

Activation of neutrophils with pro-inflammatory mediators such as fMLP, C5a, PAF, TNF-α and interleukin-6 produce superoxide (O_2^-) anions, hydrogen peroxide (H_2O_2) and finally hydroxyl radicals ($OH^{\cdot}$) (Babior, 1999; Jordan et al., 1999). O_2^- is generated from the neutrophil-membrane associated NADPH oxidase in a respiratory burst characterised by a marked increased in cellular oxygen consumption (Babior, 1999). Hydrogen peroxide is formed by the dismutation of O_2^-, however, the most toxic radical produced under this type of condition is the hydroxyl radical which is formed via the iron-catalysed Harber–Weiss (Fenton) reaction, and is responsible for most of the cellular damage associated with ischaemia–reperfusion injury. In addition, most stimuli that activate the neutrophil also cause the release of the enzyme myeloperoxidase from the azurophil granule of this cell type. Myeloperoxidase catalyses the formation of the powerful oxidant hypochlorous acid (HOCl) from H_2O_2 and Cl^- (Harrison and Schultz, 1976). Oxygen-free radicals can directly cause tissue injury by oxidising DNA, proteins and lipids (Freeman and Crapo, 1982). In addition, it has been proposed that a sensitive target for oxygen-free radicals is the vascular endothelium where they can promote the release of pro-inflammatory mediators and the up-regulation of adhesion molecules. The resulting damage to the endothelium leads to increases in vascular permeability and neutrophil adhesion.

5.2. Degranulation products

Upon degranulation, the neutrophil also releases several proteolytic enzymes: the serine proteinase elastase, metalloproteases and gelatinase. However, of these, elastase is a major contributor to neutrophil-dependent injury (Carden et al., 1998; Carden and Korthuis, 1996; Inauen et al., 1990). Elastase is a member of the serine protease family (Baugh and Travis, 1976). It is contained in the primary granule of neutrophil and can degrade key component of the extracellular matrix (Weiss and Regiani, 1984; Weiss et al., 1986). The basement membrane represents important functional components of the vascular barrier that limits fluid and electrolytes exchange and restricts neutrophil extravasation. Therefore, elastase contributes to ischaemia–reperfusion injury by degrading basement membrane leading to increase vascular permeability as well as facilitating neutrophil infiltration (Lentsch and Ward, 2000; Zimmerman and Granger, 1990). Elastase inhibitors have been found to reduce ischaemia–reperfusion induced neutrophil accumulation and microvascular dysfunction in skeletal muscle (Carden and Korthuis, 1996) and ischaemia–reperfusion injury induced by liver transplantation in rat (Soejima et al., 1999).

5.3. Pro-inflammatory mediators

Activated neutrophils also release several other pro-inflammatory mediators including leukotriene B_4 and PAF. Both of these are potent stimulators of neutrophil chemotaxis and degranulation and will act to further amplify the destructive action of neutrophil. In addition, activated neutrophil also release phospholipase A_2 into the external fluid and thus can enhance the production of eicosanoids and PAF by other cells.

6. Annexin 1: a new therapeutic target against neutrophil-mediated ischaemia–reperfusion injury

Currently, several experimental therapies have been shown to be successful in reducing neutrophil-mediated ischaemia–reperfusion injury. Based on the understanding of the mechanism of ischaemia–reperfusion injury, it has been possible to devise several treatments for ischaemia–reperfusion injury. These include (1) neutrophil depletion, (2) direct inhibitors of neutrophil activators, (3) antibodies against neutrophil adhesion molecules involved and (4) antibodies against the endothelial adhesion molecules. However, aside from these approaches, it has been known for sometime that glucocorticoids are potent inhibitors of neutrophil dependent tissue damage. In the second part of this review, we will concentrate on the anti-inflammatory activity of the glucocorticoid-regulated protein annexin 1 and we will highlight its potential protective role in ischaemia–reperfusion injury.

Glucocorticoids are among the most effective drugs for the treatment of chronic inflammation. Most of glucocorticoids actions require binding to classical cytoplasmic receptors leading to changes in gene transcription and protein synthesis (Barnes, 1998). In many cases, these effects are mediated through antagonism of nucleus factor κB stimulated gene transcription but other mechanisms operate including the generation by glucocorticoid treated cells of regulatory protein (Wehling, 1997). One of such is the protein called annexin 1.

Annexin 1 (previously referred to as lipocortin 1) belongs to a family of Ca^{2+} and phospholipid binding proteins of which at least 13 distinct members exist (Raynal and Pollard, 1994). Structurally, annexins are characterised by having a core of four or eight conserved repeats each containing about 70 amino acids (Fig. 2). Within each of these cores, there is the sequence responsible for the phospholipid and Ca^{2+} binding properties that is character-

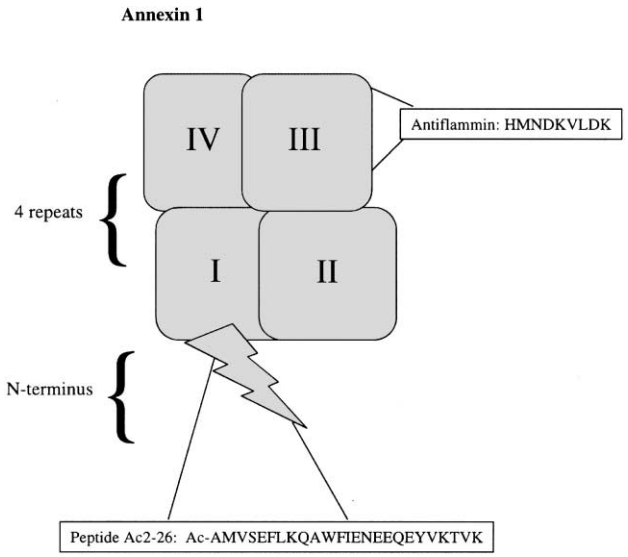

Fig. 2. Scheme of annexin 1 structure. Annexin 1 is formed by four repeats, each of 70–80 amino acids, which are alternatively coupled (repeat I with repeat III, and repeat II with repeat IV) (Weng et al., 1993). Attached to repeat I there is the N-terminus (46 amino acid long), and highlighted is peptide Ac2-26 that has been shown to mimic some of the biological actions of the potent protein. In repeat III, there is the sequence corresponding to antiflammin 2 (region 246–254): this nonapeptide also exerts anti-inflammatory activity.

istic of all members of the annexin family (Kretsinger and Creutz, 1986). The core is attached to an N-terminal segment that is unique for each member of the annexin family and it is thought to be responsible for each annexin's specific biological functions (Fig. 2). In some cases such as of annexin 1, the N-terminus contains phosphorylation sites likely to be important for regulatory purposes. Based on their biochemical characteristics, annexins have been implicated in several biological phenomena ranging from membrane organization and exocytosis, cell growth and differentiation, ion channel formation, inhibition of inflammation (mainly annexin 1) and blood coagulation (annexin 5) (Ahluwalia et al., 1996; Flower and Rothwell, 1994; Kubista et al., 2000).

6.1. Synthesis and secretion

Annexin 1 (37-kDa) is the best characterized of all the annexin family of proteins and has an N-terminal of 46 amino acids. It is expressed constitutively in many tissues and cells including lung, kidney, bone marrow, intestine, spleen, thymus, brain, neutrophils and macrophages (Dreier et al., 1998; Fava et al., 1989). Numerous reports have shown that glucocorticoids promote the synthesis of annexin 1 in vivo (Goulding et al., 1990; Peers et al., 1993) and in vitro (Comera and Russo-Marie, 1995). In isolated cells, at least three pools of annexin 1 can be distinguished: a cytosolic pool, a membrane pool recovered only after washing cells with ethylene diaminetetracetic acid (EDTA), and a membrane pool resistant to EDTA washes and an intracellular pool (Peers et al., 1993; Perretti, 1997). In human neutrophils, annexin 1 accounts for 2–4% of total cytosolic proteins. Under resting condition, > 70% of annexin 1 is found in the *gelatinase granules* (Perretti et al., 2000). Upon neutrophil activation and adhesion to endothelial cell monolayers, annexin 1 is externalised from the cytosol to the plasma membrane by a process of exocytosis such that post-adherent neutrophils are depleted by 50–70% of their total annexin 1 content (Perretti et al., 1996). A recent study performed with immunohistochemistry and electron microscopy analysis of rodent post-capillary venules has confirmed that annexin 1 externalisation occur in parallel with neutrophil adhesion to post-capillary venules in vivo (Oliani et al., 2001).

6.2. Mechanism of action of annexin 1

Despite its well-known anti-inflammatory activity, the cellular mechanism for the action of annexin 1 has so far remained elusive. The existence of specific and saturable binding sites on human and rodent neutrophils and monocytes has been reported (Euzger et al., 1999; Goulding et al., 1996; Perretti et al., 1993a) but these have not fully characterized. In a recent study, Walther et al. (2000) used a series of in vitro studies and reported the existence of a functional interaction between a peptide drawn from the annexin 1 N-terminus and the formyl peptide receptor. This formyl peptide receptor belongs to the super-family of seven transmembrane domain G-protein linked receptors (Prossnitz and Ye, 1997) and its activation by bacterial products like *N*-formyl-Met-Leu-Phe (fMLP) initiates in the neutrophil responses including chemotaxis, superoxide production and cell degranulation (Prossnitz and Ye, 1997). It is not yet clear if annexin 1 and fMLP activate the formyl peptide receptor signalling in a similar fashion (Walther et al., 2000). The suggestion that annexin 1 mediates its anti-inflammatory activity by the activation of the formyl peptide receptor is perplexing as this receptor is often associated with neutrophil activation and chemotaxis in vitro. Nevertheless, it should be noted that G-protein coupled receptors are very susceptible to desensitisation (Ali et al., 1999), and therefore it is feasible that rapid activation of the formyl peptide receptor by annexin 1 and its mimetics would quickly desensitise this receptor and thus ultimately down-regulating not only other receptors for chemoattractant, e.g. for C5a, but also cell activation with a larger meaning. The facts that (i) the formyl peptide receptor is contained in cytoplasmic granules and vesicles of neutrophil and can be exported onto the surface upon adhesion (Gullberg et al., 1997) and (ii) annexin 1 is contained within the gelatinase granules and is also externalised upon neutrophil adhesion (Perretti et al., 2000), lead us to propose that neutrophil adhesion to the endothelium triggers the externalisation of both the formyl peptide

receptor and annexin 1, and that these proteins may be the components of a novel biochemical pathway operating in the host to down-regulate neutrophil infiltration (Perretti, 1997) by altering the fate of adherent neutrophils as shown during inflammation (Lim et al., 1998; Mancuso et al., 1995).

6.3. Catabolism of annexin 1

How are the biological actions of annexin 1 terminated? Annexin 1 induced formyl peptide receptor internalisation it is a possibility (Walther et al., 2000), however, experimental observations in rodents and humans suggest the existence of a specific catabolic process. Smith et al. (1990) showed that annexin 1 from human lung lavage fluids was cleaved and presented as a doublet (37/33 kDa) as analysed by Western blotting. Indeed, we also observed that the *majority* of annexin 1 found in the incubation medium of adherent neutrophil was cleaved (33 kDa form) whereas annexin 1 recovered from the cell surface was mainly intact (37 kDa form) (Perretti et al., 1996). We then proposed that a specific enzyme (a "lipocortinase"?) might exist and regulate annexin 1 actions (Perretti, 1997; Perretti et al., 1996). Annexin 1 cleavage can be seen in experimental models of inflammatory bowel disease (Vergnolle et al., 1995, 1997), as well as in models of heart infarct (La et al., unpublished observation) and peritonitis (Oliani et al., 2001). Furthermore, degradation of annexin 1 to form the 33-kDa fragment is increased in a human model of skin inflammation (Perretti et al., 1999) as well as in the clinic where it has been detected in bronchoalveolar lavage fluids of smokers and patients with cystic fibrosis (Tsao et al., 1998; Vishwanatha et al., 1998). The possible implication of these findings is that chronic inflammation is associated with increased degradation of annexin 1. The identity of the lipocortinase(s) was initially suggested by Tsao et al. (1998) to be the serine proteinase, elastase, since a functional correlation between the appearance of the annexin 1 cleaved form in the bronchoalveolar lavage and the extent of elastase activity was observed. However, this is at variance with the study of Vishwanatha et al. (1998) who could not demonstrate such a link, and suggested that alternative proteases were involved. The identification and characterisation of this novel catabolic process for annexin 1 is an ongoing project in our laboratory. Inhibitors of annexin 1 degradation could be a target for the development of novel anti-inflammatory therapy.

6.4. Anti-inflammatory action of annexin 1 and identification of active peptides

Earlier studies suggested that annexin 1 produced its anti-inflammatory activity by inhibiting phospholipase A_2 activity and thus preventing arachidonic acid mobilisation and prostanoid generation (Cirino et al., 1987). More recently, it has been suggested that annexin 1 possesses other action aside from eicosanoid inhibition. For instance, annexin 1 inhibited neutrophil migration in response to interleukin-1β and interleukin-8 into the murine air pouch, a response that was insensitive to doses of indomethacin that substantially inhibited prostaglandin E_2 formation (Perretti and Flower, 1993, 1994). Similarly, annexin 1 also inhibited neutrophil and monocyte recruitment in zymosan-induced inflammation in the mouse peritoneal cavity (Getting et al., 1997).

The first successful attempt to identify annexin 1 derived peptides endowed with anti-inflammatory activity led to the synthesis and testing of two nonapeptides, antiflammin-1 and antiflammin-2 (Miele et al., 1988). These peptides corresponded to a region of homology between annexin 1 and uteroglobin, another protein exhibiting antiphospholipase A_2 activity in vitro (Miele et al., 1988; Perretti, 1994). Antiflammin-1 was drawn from uteroglobin (39–47 amino acids, MQMKKVLDS) and antiflammin-2 was from annexin 1 (246–254 amino acids, HDMNKVLDK) (Fig. 2). However, data regarding the anti-inflammatory activity of antiflammins remains contradictory. Although initial studies showed that both antiflammins were effective in inhibiting phospholipase A_2 activity and reducing the oedema in response to carrageenin injection into the rat paws (Miele et al., 1988), others have shown them to be ineffective (Marki et al., 1990). More recent studies have confirmed the initial observations (antiflammin anti-inflammatory section), and have implicated the neutrophil as a potential cellular target (Zouki et al., 2000).

As mentioned above, the N-terminal region of each annexin may be responsible for the unique biological activity of individual member of the super-family. Since annexin 1 is clearly found in the extracellular milieu and exerts potent anti-inflammatory actions, smaller fragments drawn from its N-terminus (Fig. 2). A 25-amino acid peptide was synthesised and tested in models of acute inflammation, shown previously to be susceptible to the parent molecule. Intravenous treatment with peptide Ac2-26 (acetyl-AMVSEFLQAWFIENEEQEYVQTVK) resulted in inhibition of neutrophil migration induced by interleukin-1 into a murine air-pouch and carrageenin-induced paw oedema (Cirino et al., 1993), as well as zymosan-mediated oedema and neutrophil influx into the murine peritoneal cavity (Perretti et al., 1993b). Therefore, peptide Ac2-26 was able to inhibit neutrophil migration regardless of the stimulus applied, leading to the speculation that it inhibited a basic step in the neutrophil migration process (Perretti, 1994). Further support for this idea was provided by intravital microscopy studies where peptide Ac2-26 administration mimicked annexin 1 action and inhibited neutrophil extravasation in the hamster cheek pouch preparation by prolonging the time taken for the adherent neutrophil to enter into the process of diapedesis following application of fMLP or substance P (Mancuso et

al., 1995). More recent studies have shown that peptide Ac2-26 and annexin 1 promote the detachment of neutrophils from the activated post-capillary venule endothelium (Lim et al., 1998).

6.5. Effect of annexin 1 in myocardial ischaemia–reperfusion injury

Cardiac ischaemia underlies many of the most important cardiovascular diseases including myocardial infarction, thrombotic stroke, embolic vascular occlusions, angina pectoris, peripheral vascular insufficiency, cardiac surgery and organ transplantation. Neutrophils feature prominently in this inflammatory component of post-ischaemic injury. Ischaemia–reperfusion prompts the release of oxygen-free radicals, cytokines and other pro-inflammatory mediators that activate both the neutrophils and the coronary vascular endothelium. Activation of these cell types promotes the expression of adhesion molecules on both the neutrophils and endothelium, resulting in increased recruitment of neutrophils to the surface of the endothelium and initiate a specific cascade of cell–cell interactions: neutrophil adhesion to the vascular endothelium is followed later by transendothelial migration and direct interaction with the cardiomyocyte. The injurious effect of neutrophils in myocardial ischaemia–reperfusion damage was first suggested by pioneering studies performed in animals depleted of circulating neutrophils (Litt et al., 1989; Romson et al., 1983) and more recently by studies targeting the role of specific adhesion molecules involved in the neutrophil–vascular endothelium interaction (Kubes et al., 1995; McCafferty et al., 2000; Zhao et al., 1997). Interestingly, the β1 integrin, very late antigen 4, seems central in locating the extravasated neutrophil on the cardiomyocyte and therefore in targeting the damaging effects of neutrophil-derived oxygen-free radicals onto the cardiac cell (Poon et al., 2001).

The role played by annexin 1 within the cardiovascular system has remained unexplored. However, we have recently reported that human recombinant annexin 1 exerts a protective action in a model of rat myocardial ischaemia–reperfusion injury (D'Amico et al., 2000). In this experimental setting, administration of annexin 1,5 min post-reperfusion reduced tissue necrosis and preserved the integrity of the myocardium following ischaemia–reperfusion injury. The protective effect of annexin 1 was dose-dependent and this was mirrored by a reduction in neutrophil extravasations as well as a parallel reduction in tissue myeloperoxidase activity (D'Amico et al., 2000). More recently, we have been able to demonstrate that two N-terminal derived peptides, peptides Ac2-26 and Ac2-12, are also effective in this model of ischaemia–reperfusion injury (La et al., unpublished data). Peptide Ac2-26 reduced myocardial infarct, as well as myeloperoxidase values and cytokine levels in the heart tissue. Most significantly, peptide Ac2-26 was still effective when administered 60 min into the reperfusion period. This finding would suggest that peptide Ac2-26 and by extension novel annexin 1 mimetics may have the potential to be applied in clinical situations where drugs can only be administered after the beginning of the reperfusion phase.

In addition, myocardial ischaemia–reperfusion injury is associated with increased annexin 1 immunoreactivity as detected by electron microscopy and Western blotting. Hearts subjected to ischaemia–reperfusion, but not sham or saline treated, were found to express annexin 1 as a doublet (37- and 34-kDa bands). Given that normal cardiac tissue do not express annexin 1, annexin 1 detected in ischaemic heart is likely to derive from infiltrating neutrophils (La et al., unpublished data). This is consistent with a recent study from our laboratory showing annexin 1 expression, detected at the immunocytochemical level, in emigrating neutrophils during an acute inflammatory reaction (Oliani et al., 2001). Interestingly, ischaemic hearts treated with peptide Ac2-26 have reduced annexin 1 catabolism suggesting that protective effect of Ac2-26 may involve reduction in endogenous annexin 1 catabolism. Finally, based on the observation that annexin 1 interacts with formyl peptide receptor, the cardioprotective effect of annexin 1 in the presence of the formyl peptide receptor antagonist was investigated. We found that in this model, formyl peptide receptor antagonists reversed the protective effect of annexin 1 and propose that, annexin 1 and its derived peptide interaction with formyl peptide receptor is instrumental to the cardioprotective properties of these molecules.

A further aspect of this line of research is illustrated in Fig. 3. The glucocorticoid dexamethasone significantly reduced rat myocardial ischaemia–reperfusion injury, when given as pre-treatment, or at the beginning of the reperfusion period (Fig. 3 and D'Amico et al., 2000). A polyclonal anti-annexin 1 neutralising serum was able to revert the protection afforded by dexamethasone, whereas a control rabbit serum was ineffective (Fig. 3). These data, together with the presence of endogenous annexin 1 in infarcted hearts, suggest that the protection afforded by annexin 1 following pharmacological treatment may also be an action produced by the endogenous protein. In essence, the observations made with simple experimental systems of neutrophil migration (Getting et al., 1997; Mancuso et al., 1995; Perretti and Flower, 1993) hold up in this model of pathology, indicating a physio-pathological implication for the role of annexin 1 in the process of neutrophil recruitment.

6.6. Annexin 1 and splanchnic artery occlusion

Splanchnic artery occlusion is a severe form of circulatory shock produced by ischaemia–reperfusion of the splanchnic organs. This type of shock is associated with a large number of pathophysiological changes with possible fatal outcome (Biffl and Moore, 1996; Lefer and Lefer,

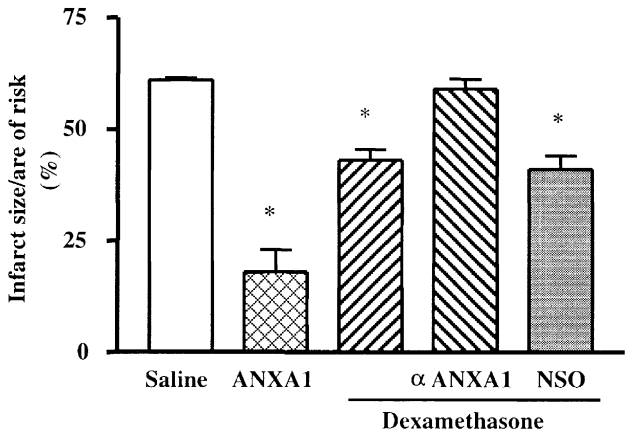

Fig. 3. Effects of endogenous and exogenous annexin 1 on myocardial ischaemia–reperfusion injury. Ischaemia was induced by the occlusion of the left ascending distal coronary artery (LADCA) for 25 min followed by 2 h of reperfusion. The infarcted size is expressed as a function of the area at risk (D'Amico et al., 2000). Animals were treated intravenously with saline (1 ml/kg), annexin 1 (ANXA1; 25 μg/kg) or dexamethasome (Dex; 0.1 mg/kg). Annexin 1 and Dex significantly reduced the infarct size. Note that passive immunisation of animals to annexin 1 (α-ANXA-1) reversed the effect of Dex. The administration of normal sera (NSO) had no effect on the cardioprotective action of Dex. Values are means ± S.E.M. of $n = 5–10$ rats per group. * Indicates $P < 0.05$ vs. saline treatment.

1993). Reperfusion is associated with both local and systemic changes. Local changes include neutrophil recruitment, increased intestinal permeability and vascular reactivity of splanchnic vessels, as well as tissue necrosis and death. Systemic changes include a progressive fall in mean arterial pressure, the release of pro-inflammatory mediators from intestinal tissue into the systemic circulation that can lead to multi organs failure with fatal consequences (Biffl and Moore, 1996). An earlier study (Goldfarb and Glenn, 1976) showed that splanchnic artery occlusion is associated with significant biochemical alterations, such as increased hepatic glucuronidase, adenosine-3′,5′-cyclic monophosphate and guanosine-3′,5′-cyclic monophosphate. These changes were reversed by the administration of dexamethasone. Recently, it was demonstrated that annexin 1 protects against splanchnic artery occlusion injury (Cuzzocrea et al., 1997). In this study, splanchnic artery occlusion was induced in rats by clamping both the superior mesenteric artery and the celiac trunk for 45 min, followed by release of the clamp (60 min reperfusion). Following this reperfusion period, rats developed a fall in mean arterial blood pressure, associated with a significant increase in tissue myeloperoxidase activity in the intestine and a marked histological injury to the distal ileum. Treatment of rats with the annexin 1 mimetic peptide Ac2-26 dose-dependently reduced the progressive fall in blood pressure and prevented the infiltration of neutrophils into the reperfused intestine (by measurement of myeloperoxidase activity). The peptide also reduced the degree of ischaemia–reperfusion injury in the bowel as evaluated by histological examination. Similarly, the glucocorticoid dexamethasone also produced a marked improvement in splanchnic artery occlusion and shock (i.e., maintained mean arterial blood pressure and reduced tissue myeloperoxidase activity), and this was reversed by pre-treatment with two different antisera raised against the annexin 1 derived peptide. These findings suggest that annexin 1 inhibits neutrophil migration and accumulation into reperfused tissues, thereby ameliorating the outcome of splanchnic artery occlusion and reperfusion (Cuzzocrea et al., 1997).

6.7. Annexin 1 and mesenteric ischaemia–reperfusion injury

We have recently assessed the effect of exogenous and endogenous annexin 1 as a modulator of neutrophil accumulation into the rat small intestine following ischaemia–reperfusion injury (Cuzzocrea et al., 1997). These data has been recently integrated by a study of intravital microscopy (Fig. 4). The superior mesenteric artery of the rat was occluded for 30 min and this was followed by 45-min reperfusion. Peptide Ac2-26 was administered within the first 2 min of the reperfusion period. Treatment with peptide Ac2-26 had no effect on the number of adherent neutrophil during either the basal period or within 15 min of reperfusion. In contrast, a significant reduction in adherent and migrating cells was observed 40 min post-reperfusion (Fig. 4A). Similarly, there was no change in the effect of the peptide on neutrophil migration during the basal or the 15 min post-reperfusion. Instead, the peptide caused a marked reduction in the number of emigrated neutrophils at 40 min post-reperfusion (Fig. 4B). However, this was only significant within the 50 μm of the vessel wall, indicating that Ac2-26 was altering the fate of adherent neutrophils, such that neutrophils were no longer undergoing the process of trans-endothelial passage through the endothelial vascular system. This is the first direct demonstration, by intravital microscopy, that annexin 1 mimetic can inhibit ischaemia–reperfusion induced neutrophil–endothelium interactions, and therefore reinforces the cellular mechanism discussed above (Sections 6.5 and 6.6). We propose that targeting neutrophil recruitment after ischaemia–reperfusion with annexin 1 itself or with annexin 1 derived peptides can be a novel and effective manner to reduce tissue injury.

6.8. Annexin 1 and cerebral ischaemia

Ischaemic injury to the cerebral tissue can result in cell death within minutes. The mechanisms behind of this neurodegenerative process are not fully understood, but increased extracellular Ca^{2+} level, release of excitatory amino acids, synthesis of arachidonic acid metabolites and cytokines have been implicated. The latter two factors are potent chemoattractors for neutrophil (see above). Annexin

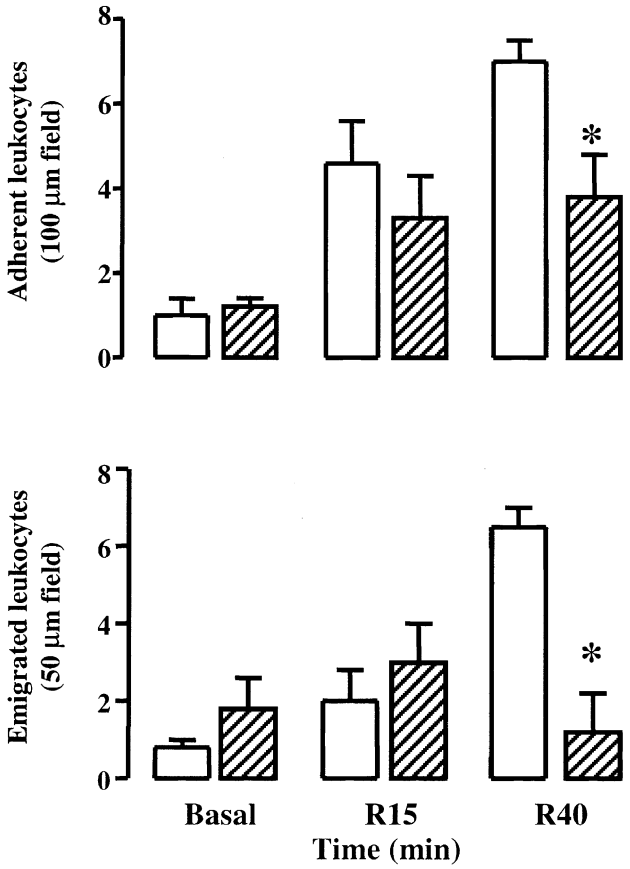

Fig. 4. Effect of peptide Ac2-26 on ischaemia–reperfusion-induced neutrophil adhesion to and emigration through the post-capillary venule endothelium of the rat mesentery. The superior rat mesenteric artery was occluded for 30 min and subjected to a 45-min reperfusion period. Rats were given either saline (0.5 ml i.v., open columns) or peptide Ac2-26 (0.5 mg/kg i.v., hatched columns) within the first 2 min of reperfusion. (A) Number of adherent neutrophils per 100 μM venule length, and (B) number of emigrated neutrophil within the first 50 μm of extravascular tissue in the sub-endothelial space, as quantified at the 15- and 40-min post-reperfusion. Values are reported as mean ± S.E.M. of $n = 6$ rats per group. * Indicates $P < 0.05$ vs. saline treatment.

1 has been proposed to be an endogenous mediator with a protective effect against cerebral ischaemia–reperfusion injury (Relton et al., 1991). Annexin 1 immunoreactivity in certain glial cells and neurons in the rat brain has been detected (Strijbos et al., 1991) and the administration annexin 1 fragment causes marked inhibition of infarct size (60%) and cerebral oedema (46%) measured 2 h after cerebral ischaemia (middle cerebral artery occlusion) in the rat (Relton et al., 1991). In addition, ischaemia caused increased expression of annexin 1 around the area of infarction as demonstrated by immunocytochemistry (Relton et al., 1991). Finally, injection of neutralizing anti-annexin 1 antibodies increased the size of infarct and the development of oedema (Relton et al., 1991). These findings indicate that annexin 1 is an endogenous inhibitor of cerebral ischaemia with considerable therapeutic potential. It is unfortunate that this line of research has not been pursued in more recent years. The molecular mechanism(s) underlying this protective action of annexin 1 in this model have yet to be fully elucidated.

7. Summary and conclusion

Annexin 1 is a potent inhibitor of neutrophil extravasation in vivo, as demonstrated first by our group and then confirmed by several other laboratories. Experimental evidence reviewed by this article showed that an effect on the white blood cell is at the basis of the mechanism of annexin 1 actions against ischaemia–reperfusion in the heart and mesenteric microcirculation, as well as in multiple organ failure associated with splanchnic ischaemia–reperfusion. In addition, annexin 1-derived peptides have been found to produce similar activity to that of the full-length molecule. This finding together with the identification of a receptor that mediates the actions of annexin 1 derived peptides, and possibly also of the full length protein, may suggest that this line of research can lead to the design of novel anti-inflammatory agents with a potential in cardiovascular pathologies characterised by molecular inflammatory mechanisms centered on neutrophil activation and recruitment.

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